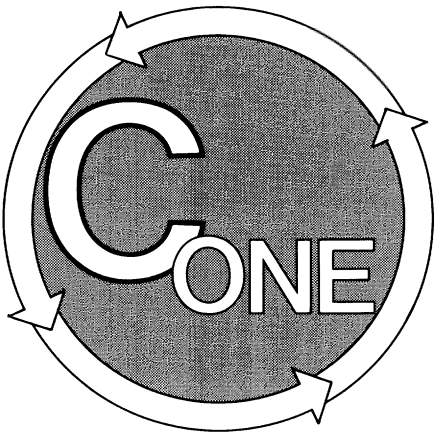
AUTOTROPHIC MICROBIOLOGY AND ONE-CARBON METABOLISM

ADVANCES IN AUTOTROPHIC MICROBIOLOGY AND ONE-CARBON METABOLISM

Vol 1



Autotrophic Microbiology and One-Carbon Metabolism

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Kluwer Academic Publishers
Dordrecht / Boston / London

ISBN-13: 978-94-010-7384-4 e-ISBN-13: 978-94-009-1978-5
DOI: 10.1007/978-94-009-1978-5

Published by Kluwer Academic Publishers,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

Kluwer Academic Publishers incorporates
the publishing programmes of
D. Reidel, Martinus Nijhoff, Dr W. Junk and MTP Press.

Sold and distributed in the U.S.A. and Canada
by Kluwer Academic Publishers.
101 Philip Drive, Norwell, MA 02061, U.SA.

In all other countries, sold and distributed
by Kluwer Academic Publishers Group,
P.O. Box 322, 3300 AH Dordrecht, The Netherlands.

printed on acid-free paper

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Preface

Autotrophic and methylotrophic microorganisms are able to grow at the expense of one-carbon compounds (e.g. carbon dioxide, formaldehyde) as the principal carbon sources for the synthesis of cell material, using light, inorganic compounds or one-carbon compounds as energy sources. The study of the special adaptations required in aerobic and anaerobic microorganisms to sustain an autotrophic or methylotrophic mode of life is a fascinating field of research for scientists from various disciplines. Current research efforts not only focus on fundamental aspects, i.e. metabolic pathways and their regulation, ecology, energy conversion and genetics, but also the possible application of these organisms, in waste water treatment, degradation of xenobiotics, single-cell protein production, as biocatalysts for the production of fine chemicals, draws strong attention.

The aim of this series is to provide annual reviews on the biochemistry, physiology, ecology, genetics, and application of microbial autotrophs and methylotrophs. The scope of the series includes all aspects of the biology of these microbes, and will deal with phototrophic and chemolithotrophic prokaryotic autotrophs, carboxydobacteria, acetogenic-, methanogenic- and methylotrophic bacteria, as well as methylotrophic eukaryotes.

The exciting advances made in recent years in the study of these organisms is reflected in the chapters of this first volume which have been written by experts in the field. We would like to express our sincere thanks to all the contributors for their stimulating and comprehensive chapters.

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1. The Biology of the Prochlorales

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Introduction

The organisms belonging to the Prochlorophyta (Lewin 1976) are oxygenic phototrophic prokaryotes which contain chlorophylls *a* and *b*. Until recently (Lewin 1975) this combination of pigments was thought to be restricted to eukaryotic cells only. Due to this combination of eukaryotic and prokaryotic features their taxonomic position has been subject to discussion for several years. In 1986 Florenzano et al. proposed a new order, the *Prochlorales*, in the class *Photobacteria* (Gibbons and Murray 1978) to accommodate organisms with the aforesaid properties. This prompted Burger-Wiersma et al. (1989) to refer to them as oxychlorobacteria instead of prochlorophytes.

At present, the discovery of three oxychlorobacterial species has been reported, i.e. the marine symbiont *Prochloron didemni* (Lewin 1977), the freshwater planktonic *Prochlorothrix hollandica* (Burger-Wiersma et al. 1986), and a marine picoplanktonic strain (Chisholm et al. 1988). The discovery of these new prokaryotes has stimulated speculation about their phylogeny (e.g. Lewin 1984; Walsby 1986; Cox 1986b) and many studies are focussed on the relatedness of the oxychlorobacteria to cyanobacteria on the one hand and green chloroplasts on the other.

Habitat

The three oxychlorobacteria inhabit fairly different habitats. The oxychlorobacterium described by Chisholm et al. (1988) has been detected in the Pacific and North Atlantic Oceans. Pigment analyses suggested that similar organisms may inhabit the Banda Sea, Indonesia (Gieskes et al. 1988). In all instances maximal abundance appeared to be restricted to the deeper layers of the euphotic zone. However, Gieskes and Kraay (1983) reported an unusual pigment composition in the surface waters of the tropical Atlantic Ocean, which might be associated with oxychlorobacteria also.

Up to now, *Prochlorothrix hollandica* has only been isolated from the

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 1-24.

shallow, highly eutrophic Loosdrecht Lakes system in The Netherlands (Burger-Wiersma et al. 1986, 1989). There is now evidence of significant numbers of this species in several comparable lakes in The Netherlands (L. Van Liere personal communication; J. Van den Does personal communication; T. Burger-Wiersma unpublished). The Loosdrecht Lakes system originated from peat excavation. Due to shallowness of the lake, no thermal stratification develops and the phytoplankton is homogeneously mixed throughout the entire water column. In summer *Prochlorothrix* is one of the dominating species besides several filamentous cyanobacteria (Van Liere et al. submitted). The organism is most abundant in those parts of the lake where the availability of phosphorus is relatively more limiting for phytoplankton growth.

During *Prochlorothrix* blooms the pH varies between 8 and 10, and the water temperature is 15-25 °C (Burger-Wiersma et al. 1989). Based on the optimum temperature curve for growth, these authors proposed that the need for elevated temperatures to exhibit substantial growth might restrict the habitat of *Prochlorothrix* to shallow systems where complete mixing of the entire water column takes place throughout the year. This allows inoculation from the sediments when temperature increases, a situation almost never encountered in deeper lakes.

Thus far *Prochloron didemni* is exclusively found as an extracellular symbiont of marine tropical and subtropical sessile tunicates, the ascidians (e.g. Lewin 1981; Müller et al. 1984; Cox 1986a). It can occupy the external surface, the test or the cloacal cavity of its host, and this may have implications for the nature of the symbiosis. Cox (1986a) discriminates three types of symbiosis based on the aforesaid locations of the *Prochloron* cells: the association with cells inhabiting the external surface of the hosts is clearly not obligate for the host, but appears to be obligate for *Prochloron*. The larvae of the ascidians do not have special modifications to carry the symbiont, infection must be accomplished by *Prochloron* cells carried by seawater. The ascidian species with *Prochloron* embedded in the test are never observed without their symbionts. Therefore, the association is likely to be obligate for both partners. The larvae of this group are especially equipped for transmitting *Prochloron* cells to daughter colonies. The ascidians with cloacal cavity-dwelling *Prochloron* cells may be found without their symbionts, although their larvae have special mechanisms to accommodate the symbiont in order to inoculate the daughter colonies. However, no phylogenetic differences between strains of *Prochloron* isolated from different hosts could be shown using molecular techniques like 16S ribosomal RNA sequence homology (Stackebrandt et al. 1982) or DNA-DNA reassociation (Stam et al. 1985).

The difference in habitats is primarily expressed by the temperature and light climate encountered by the three oxychlorobacteria.

At the depth where the latest discovered oxychlorobacterium occurs most

abundantly, the temperature ranges from 10 to 15 °C (Chisholm et al. 1988). Maximal increase in numbers of *Prochlorothrix* is found when the ambient water temperature is 20 to 25 °C (T. Burger-Wiersma, unpublished results). This coincides nicely with the growth rate vs temperature relationship which was reported for this organism (Burger-Wiersma et al. 1989). *Prochloron* is abundant in tropical waters where seasonal variation in temperature is low and ambient temperatures of approximately 30 °C are customary. However, the organism is also found in areas with a more dynamic seasonality (McCourt et al. 1984, Müller et al. 1984). In these areas a close correlation between water temperature and the number of *Prochloron*-containing colonies was shown (McCourt et al. 1984). This observation agrees well with the sensitivity of photosynthesis at low temperatures (Thinh and Griffiths 1977; Alberte et al. 1986, 1987).

There is only little light attenuation in the tropical waters where *Prochloron* is found; the ambient photon flux density may range from 1000 to 2500 μmol $m^{-2}s^{-1}$ (Thinh and Griffiths 1977; Pardy 1984; Alberte et al. 1986, 1987). However, the real light climate experienced by *Prochloron* may be much lower in the case of the organism being embedded inside its host. Alberte et al. (1986) determined a decrease in photon flux density of 60 to 80% attributable to the animal tissue. Contrasting to *Prochloron*, *Prochlorothrix* is found in rather turbid waters characterized by a steep light gradient. Due to mixing of the entire water column, the organisms are exposed to rapidly changing photon flux densities ranging from complete darkness to levels of incident irradiance. Further studies may elucidate whether these light conditions are advantageous for the abundant presence of *Prochlorothrix*. There is very little information on the light levels encountered by the deep ocean species. The ambient photon flux density reported for this strain is 1 to 10 % of the incident irradiance (Chisholm et al. 1988).

Morphology and Ultrastructure

The morphology of the three oxychlorobacterial species reported so far is rather diverse. *Prochloron didemni* is a spherical unicell with a diameter ranging from 10 to 25 μm (Cox 1986a). The size appears to be independent of the sampling station, host species and location in or on the host, although the larvae may carry smaller cells (Cox 1986a). In contrast, *Prochlorothrix hollandica* is filamentous with long cylindrical cells (Burger-Wiersma et al. 1986). The individual cells are 3 to 10 μm long and 0.5 to 1.5 μm in diameter, but unfavourable growth conditions may increase both cell length and diameter (Burger-Wiersma et al. 1989). The straight, undifferentiated, sheathless trichomes are generally composed of 5 to 25 cells, but trichomes consisting of more than 100 cells have been observed also. The oceanic free-living species is unicellular and coccoid to rod-shaped, but much smaller than *Prochloron*, i.e. 0.6 to 0.8 μm (Chisholm et al. 1988).

Transmission electron micrographs of the oxychlorobacteria reveal their prokaryotic nature (Lewin 1975; Schulz-Baldes and Lewin 1976; Burger-Wiersma et al. 1986, Chisholm et al. 1988).
The electron-dense layer surrounding the cytoplasm of the organisms indicated the presence of peptidoglycan in the cell walls. This was confirmed by cell wall analysis of *Prochloron* (Moriarty 1979; Stackebrandt and Kandler 1982) and *Prochlorothrix* (Jürgens and Burger-Wiersma 1989).

Polyhedral bodies have been observed in all species. Immuno-electron-microscopy has revealed that the polyhedral bodies in *Prochloron* and *Prochlorothrix* are carboxysomes since they contain the CO_2-assimilating enzyme of the Calvin cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase [RuBisCO] (Berhow and McFadden 1983; Codd 1988; Hawthornthwaite and Codd 1988). The carboxysomes in *Prochlorothrix* are generally located in the central cytoplasmic region, though mainly in the vicinity of the thylakoid membranes. In *Prochloron* they usually occur in the peripheral region of the cells, either singly or in small clusters (Griffiths et al. 1984; Cox 1986a). Different opinions have emerged on the presence or absence of membranes surrounding the carboxysomes in *Prochloron*. Schulz-Baldes and Lewin (1976) reported a lack of such a membrane, Griffiths et al. (1984) observed proximity to an adjacent thylakoid membrane and Cox and Dwarte (1981) described a bounding tripartite membrane and suggested it to be a modified thylakoid membrane.

Gas vesicles have been reported to be present in *Prochlorothrix* at the cell poles (Golecki and Jürgens 1989). Pressure nephelometry showed that the constituent gas vesicles had a mean critical pressure of approximately 9 bar, which is within the range encountered in phytoplanktonic cyanobacteria (A.E. Walsby, personal communication).

In general, the thylakoids of the oxychlorobacteria are arranged in parallel layers at the periphery of the cytoplasm around a thylakoid-free central area (Cox 1986a; Burger-Wiersma et al. 1986; Chisholm et al. 1988). In *Prochloron* they may also occupy the central region of the cells (Schulz-Baldes and Lewin 1976). In the latter case the thylakoids are either randomly distributed and separated by so-called thylakoidal sacs (Griffiths et al. 1984; Thinh 1978; Thinh et al. 1985; Cox 1986a), or the cells are packed with thylakoids (Cox 1986a). This morphological difference may be caused by light conditions since the two types of cells were found on the external surface and in the cloacal cavity, respectively. This would agree with the observation, that in *Prochlorothrix* a low growth irradiance induced an increase in stacking and number of thylakoids (H.C.P Matthijs et al., unpublished).

The ultrastructure of *Prochloron* has been studied in greater detail than that of the other two species (Griffiths et al. 1984; Thinh et al. 1985; Cox 1986a). Different results on the location of DNA in *Prochloron* have been reported. Several authors described its presence in the central region of the cells (Schulz-Baldes and Lewin 1976; Whatley 1977; Cox 1986a). This agrees with the position of DNA in the other two oxychlorobacteria (Burger-Wiersma et al.

1986; Chisholm et al. 1988). One report mentioned the uncommon aggregation of DNA in *Prochloron* (Whatley 1977) and Coleman and Lewin (1983) observed its peculiar disposition in areas between the thylakoid membranes. Especially intriguing is the presence of the large crystalline bodies in *Prochloron* obtained from different, but not all, hosts (Griffiths et al. 1984; Thinh et al. 1985). These bodies are significantly larger than the carboxysomes. They are composed of regularly arranged sub-structures and appear to be closely related to the thylakoid membranes, either merging with them or emerging from them. The authors suggest that the production of these large crystalline bodies may represent the response of the organism to less favourable conditions. These conditions have been shown to be accompanied by the formation of excessive amounts of phenolic compounds and coagulation of proteins (Fall et al. 1983; Barclay et al. 1987). The latter process may induce changes in the ultrastructure of the cells. To some extent this may explain unusual features like the formation of large crystalline bodies, thylakoidal sacs and DNA disposition or aggregation.

The oxychlorobacteria demonstrate tight packing of the thylakoid membranes and differ in this respect from cyanobacteria. This may be simply due to the absence of steric restraint of phycobilisomes or merely be a quasi-mechanical consequence of the presence of chlorophyll *b* in these organisms (Walsby 1986). The functional reasons for thylakoid membrane organization into appressed membrane stacks is not very well understood (Miller and Lyon 1985). Although chlorophyll *b* containment by an organism goes hand in hand with thylakoid membrane appression, chloroplasts of barley mutants depleted in chlorophyll *b* nevertheless demonstrate thylakoid membrane stacking and so-called lateral heterogeneity of photosystems 1 and 2 (Miller and Lyon 1985). Such a localization of the 2 types of photosystems in different patches of the thylakoid membrane very interestingly has also been documented for both *Prochloron* (Giddings et al. 1980) and *Prochlorothrix* (Miller et al. 1988). This suggests that, in marked contrast to cyanobacteria, 'true' stacking may occur in these organisms. The presence of chlorophyll *b* in these differently organized thylakoids is of great interest for a better understanding of any additional role for this pigment besides light-harvesting (Barber 1986).

Physiology and Ecology

Growth Characteristics

In contrast to earlier attempts, Chisholm and co-workers have succeeded in growing the deepsea oxychlorobacterium in laboratory cultures (S.W. Chisholm personal communication). Details on optimum growth conditions must await further studies.

Thus far, there has been only one account of successful cultivation of *Prochloron* in laboratory cultures (Patterson and Withers 1982). In this study

the cells were grown in a mineral seawater medium and several organic nitrogen and carbon sources were tested for their growth promoting abilities. Of all the organics tested only L-tryptophan and a combination of indole and serine affected the growth of *Prochloron* in a positive way. The latter two compounds can act as precursors in the synthesis of tryptophan. Further tests revealed that the organism might be deficient in anthranilate synthase. These results might indicate the nature of the obligatory relationship between *Prochloron* and its host. Growth was further maximized by an initial pH of 5.5 (Patterson and Withers 1982). The latter result is rather surprising since several studies emphasize importance of a pH 8-buffered system for isolated cells in order to keep them photosynthetically active (Thinh and Griffiths 1977; Critchley and Andrews 1984; Alberte et al. 1986). In spite of the growth promoting measures applied by Patterson and Withers (1982) less than four doublings could be achieved in these cultures.

Lectins have been detected in the association of *Prochloron* and its host (Müller et al. 1984). Lectins might be fruitful in the propagation of isolated *Prochloron* based on results obtained in the culturing of certain Pseudomonads symbiotic with sponges.

As opposed to *Prochloron*, *Prochlorothrix* can be easily grown in a mineral medium without organic supplements, although all efforts to grow the strain in axenic cultures have failed thus far. Based on these observations, Burger-Wiersma et al. (1989) suggested that growth of *Prochlorothrix* might be dependent on substrates provided by the contaminating heterotrophic bacteria. Optimum growth was found at pH 8.4, significantly different from that found for *Prochloron* (Patterson and Withers 1982). Maximal growth rate occurred at 25 °C, rapidly decreasing at temperatures below 20 °C (Burger-Wiersma et al. 1989). Growth of *Prochlorothrix* was inhibited by NaCl at concentrations exceeding 25 mM. At 100 mM NaCl or its equivalent seawater concentration growth ceased completely. This could be explained by the inability of the strain to synthesize organic osmotica when it was subjected to these osmotic upshocks (Burger-Wiersma et al. 1989; R.H. Reed personal communication).

Carbon Metabolism

A general consensus on the operation of C3-type carbon dioxide fixation has been arrived at by measurements of the early carbon fixation products and detection of appreciable RuBisCO and phosphoribulokinase activity in cell-free extracts of *Prochloron* (Akazawa et al. 1978; Berhow and McFadden 1983; Kremer et al. 1984). The operation of the C3 pathway has also been made very likely for *Prochlorothrix* (Hawthornthwaite and Codd 1988).

Excretion of glycolic acid by isolated cells of *Prochloron* after exposure to light has been reported (Fisher and Trench 1980). This could be indicative for photorespiration to occur in *Prochloron*. Especially with regard to the symbiotic nature of *Prochloron*, initial products of carbon dioxide fixation

have been analyzed (Akazawa et al. 1978, Fisher and Trench 1980, Kremer et al. 1982). These products included 3-phosphoglycerate, sugar-phosphates, polyglucose, maltose, glucose, fructose, glutamate, aspartate and glycolate; sucrose was not detected. None of these products has been earmarked to fulfill a role in the translocation of fixed carbon compounds between *Prochloron* and its host. The photosynthesis products very much point to a type of intermediary metabolism commonly found in prokaryotic cells, illustrated by the lack of sucrose as a primary product of photosynthesis. The finding of substantial amounts of α-1,4- glucan in *Prochloron* may indicate a special type of secondary metabolism possibly occurring because of the interaction with the host (Akazawa et al. 1978). Fredrick (1980, 1981) also speculated about the meaning of the presence of α-1,4 glucan and concluded that this branched polycarbohydrate did not help in further establishing the phylogenetic position of *Prochloron*.

Measurements of enzyme activity in *Prochloron* have frequently been reported to be hampered by the unusual high content of phenolic compounds in isolated cells and the coagulation of proteins. Until now they are limited to the successful assay of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoribulokinase and RuBisCO (Fall et al. 1983, Barclay et al. 1987). Nevertheless, Andrews et al. (1984) and Berhow and McFadden (1983) elegantly succeeded in the characterization of the RuBisCO enzyme from *Prochloron*. The K_m for CO_2 (9μM) was an order of magnitude higher than that normally found for carboxylase from chloroplasts, but corresponds very well to the usually encountered values in cyanobacteria (Berhow and McFadden 1983; Andrews et al. 1984). These observations point to a close relatedness of the *Prochloron* enzyme and cyanobacterial RuBisCO. Berhow and McFadden (1983) obtained active enzyme with a final specific activity of 1.5 units per mg protein (comparable to the activity of enzyme obtained from other organisms) from *Prochloron* cells that were lyophilized directly after harvest. The subunit composition of 57.5 (large, L) and 18.8 (small, S) kDa and the sedimendation position on isopycnic sucrose gradients corresponded to the normally observed molecular mass ranges of occurrence of this enzyme with a L and S subunit stoichiometry of 8 to 8 (L8S8) (Codd 1988). Andrews et al. (1984) succeeded in cross-reconstitution of the *Prochloron* and cyanobacterial enzymes, a result which further demonstrated the close relationship between *Prochloron* and cyanobacteria.

Nitrogen Metabolism

Prochlorothrix can use either ammonium or nitrate ions as nitrogen source (Burger-Wiersma et al. 1989). As in most photoautotrophs, growth is inhibited at higher ammonium levels, probably due to uncoupling of photosynthetic electron transfer.

Parry (1985) showed that *Prochloron* cells in intact associations incorporated ammonium but not nitrate ions. The absence of nitrate uptake

is explained by the ammonium-rich microhabitat of the *Prochloron* cells in the cloacal cavities of their hosts. In such an environment, nitrate reductase, necessary for the assimilation of nitrate ions, is absent and its induction may take several hours. Although intact colonies were incubated, no nitrogen incorporation in the ascidian was detected. Therefore, the author's conclusion that the ascidian acquires usuable nitrogenous compounds is unjustified.

Low but definite nitrogenase activity was shown only in associations of *Prochloron* and its host *Lissoclinum patella* (Paerl 1984). Other associations collected at the same location and at the same period were unable to fix dinitrogen. This may explain why the *Lissoclinum patella/Prochloron* association is most abundant in the oligotrophic regions of its habitat. The capability to fix molecular nitrogen might be restricted to intact colonies since no nitrogenase activity was detected in either isolated *Prochloron* cells or in ascidians free of *Prochloron*. In spite of the extreme oxygen sensitivity of nitrogenase, light enhanced nitrogenase activity. Paerl (1984) assumed a very low oxygen content of the seawater inside the cloacal cavity of the host because of a high oxygen consumption rate of the cloacal tissue. It is however questionable whether the decrease in oxygen in the microhabitat of the cloacal cavity is adequate to guarantee a sufficiently low oxygen level within the *Prochloron* cells.

Parry's assumption (1985) that his results, in which ammonium uptake was demonstrated, conflict with those on the occurrence of dinitrogen fixation (Paerl 1984) is disputable. Dinitrogen-fixing cells switch to ammonium uptake whenever ammonium is available since growth on ammonium is more efficient. Furthermore, omission of direct measurement of the ambient ammonium levels invalidates the claim that the ammonium levels within the cloacal cavity of the host would be too high to allow nitrogen fixation.

Attempts to induce nitrogenase activity in *Prochlorothrix* were unsuccessful indicating that this organism is unable to fix molecular dinitrogen (Burger-Wiersma et al. 1989). A new free amino acid, 3-(N-methylamino)glutaric acid, was identified in extracts of *Prochloron* isolated from different, but not all, hosts and appeared to be present in isolated host material also (Summons 1981). This amino acid was not detected in *Prochlorothrix* (Volkman et al. 1988).

Symbiosis of Prochloron

The relationship between host and symbiont has been subjected to many studies, most of them focussing on the exchange of metabolic intermediates.

Low molecular weight products were shown to be transported following $^{14}CO_2$ fixation in the intact association of host and *Prochloron* (Fisher and Trench 1980; Griffiths and Thinh 1983). The identity of the translocated components was not established. Isolated cells of *Prochloron* were found to excrete glycolic acid (Fisher and Trench 1980), which accounted for seven percent of the CO_2 fixed. This would be insufficient to account for the 50%

photosynthate translocation reported by Griffiths and Thinh (1983), but agrees well with other translocation estimates of 7% (Akazawa et al. 1978) and 15 to 19% (Alberte et al. 1986). Interestingly, Berhow and McFadden (1983) concluded that the presence and activity of RuBisCO relative to the chlorophyll content are in normally encountered ranges. This indicates that factors other than CO_2 fixation must determine the need for symbiosis of *Prochloron*. Addition of extracts from the ascidian host did not improve the excretion of photosynthates from isolated *Prochloron* cells. In this respect the results differ from those obtained in other host/zooxanthellae relationships (Fisher and Trench 1980).

Although the symbiosis between *Prochloron* and its host is obviously obligatory for *Prochloron* (Lewin 1981) there are only a few reports indicating that the organism actually benefits by the association. Kremer et al. (1982) reported that the photosynthetic rates in *Prochloron* were decreased by a factor of three after isolation of the cells. Müller et al. (1984) indicated the presence of a cytostatic compound made by the host, which was active in mouse lyphoma cells but which did not inhibit proliferation of *Prochloron*. Its proposed function is to help in maintaining a unique environment for *Prochloron* and to keep out other bacteria from the host. Otherwise the production of lectins by the host only in presence of *Prochloron* may specifically contribute to a proper environment for the symbiont. The lectins were shown to bind to the glycoproteins of *Prochloron*, as to greatly reduce the tendency for strong aggregation of the *Prochloron* colonies.

In some cases the exchange of metabolites and nutrients may provide a logical explanation for phenomena observed in naturally occurring associations of *Prochloron* and its host. Unfortunately, these speculations are almost never substantiated. Olson (1983) observed that the larvae of *Didemnum molle* can carry low-light adapted cells of *Prochloron*. He suggested that this was necessary to ensure a high photosynthetic activity after settlement of the larvae since the juveniles were dependent on the translocation of photosynthates. In a later study Olson (1986) showed a relationship between light intensity and growth rate of *Prochloron/Didemnum molle* associations. This agrees well with the results of Bachmann et al. (1985), who observed an increasing colony-size with increasing light intensities; the smallest colonies were found at depths over 25 m and were devoid of *Prochloron* cells. The size of the colonies was suggested to depend on the translocation of photosynthates from *Prochloron* to its host. According to these authors, this would be indicated by the fast death of the colonies after being deprived of light. This speculation, however, is in contradiction with their own conclusion that the association is facultative for the host. More probably, the fast death of the association was an artifact since Olson (1986) has described the survival of *Prochloron/Didemnum molle* associations after nine days' exposure to darkness.

Parry (1985) suggested the translocation of ammonium ions from the host to the symbiont. He based this proposal on a combination of observations.

The oxychlorobacterial cells showed ammonium and no nitrate assimilation, whereas the ammonium content of the tropical reef water was very low.

Photosynthesis

The maximal rates for carbon dioxide fixation in the light in *Prochloron*, ranging from 100 to 1000 μmol CO_2/mg chl/h (Fisher and Trench 1980; Berhow and McFadden 1983; Critchley and Andrews 1984; Kremer et al. 1984, Alberte et al. 1986) correspond to the rates commonly observed in cyanobacteria and chloroplasts. Carbon dioxide fixation rates in the dark were less than 5 % (Fisher and Trench 1980) or 1 % (Alberte et al. 1986) of those in the light. From the data of Burger-Wiersma and Post (1989) we calculated a range of 280 to 620 μmol CO_2/mg chl/h. In this study the differences between rates were due to different growth irradiances.

The variation in observed carbon fixation rates in *Prochloron* were far too large to be attributed to the different ambient light conditions. Most likely they were caused by the method of isolating the cells from their host. Usually the cells are isolated by gently squeezing the colonies. In this process however, the acid-containing vesicles of the hosts may be ruptured, thereby liberating sulphuric acid into the isolation medium. Thinh and Griffiths (1977) reported a complete loss of photosynthetic activity after isolation of the cells in a non-buffered system. Collection of the isolated cells into a buffered system (pH 7.5) still resulted in a 50-75 % loss of photosynthesis. Apparently, Alberte et al. (1986, 1987) observed an increase in photosynthetic activity after isolation of the cells. They reported a maximal oxygen evolution rate of 912-1188 μmol O_2/mg chl/h for cells isolated from *Lissoclinum patella* (Alberte et al. 1986); a value high in the range usually found for cyanobacteria and green algae. Whole colonies of the same species showed a maximal oxygen evolution rate of 26.4-40.8 μmol O_2/mg chl/h (Alberte et al. 1987) which is low as compared to those reported in other studies (Thinh and Griffiths 1977; Pardy 1984). Therefore, caution should be taken in interpreting the results of Alberte et al. (1987), the more so as these authors stated that the values of maximal oxygen evolution rate in isolated and *in hospite* cells were roughly the same.

In general, all phototrophic organisms show the same adaptation pattern when grown in different photon flux densities, i.e. increased photosynthetic pigment levels at low photon flux densities in order to sustain optimum photosynthesis. The response of *Prochloron* and *Prochlorothrix* conforms to this general adaptation pattern: Burger-Wiersma and Post (1989) report a fivefold increase in chlorophyll content in *Prochlorothrix* when comparing cells grown at 200 and 8 μmol photons $m^{-2}s^{-1}$. In spite of the pigment increase at low photon flux densities a decrease in light utilization efficiency was found. Self-shading of chlorophyll molecules in the tightly packed thylakoids may have caused this decrease.

Alberte et al. (1986) observed a twofold increase in chlorophyll levels in *Prochloron* cells isolated from *Lissoclinum*-colonies growing at ambient

photon flux densities of 400 and 2200 μmol $m^{-2}s^{-1}$, respectively. The concurrent twofold increase in the efficiency of light utilization in *Prochloron* growing at the low irradiance suggests that the increase in pigments principally benefits antenna function. The enhancement of antenna function was further supported by the larger increase of chlorophyll *b* relative to chlorophyll *a* and by the simultaneous decrease of the number of reaction centres I and II per cell. Apparently, the antenna size is enlarged at the expense of the number of reaction centres.

Two strategies of light-shade adaptation based on the concept of photosynthetic units (PSU) can be distinguished. A PSU is defined as the number of photosynthetic pigment molecules involved in the production of one molecule of oxygen. Either the PSU changes in size due to changes in the amount of light harvesting antennae relative to the reaction centres, or the number of PSU's is altered upon a change in ambient photon flux density. Green algae are known to change both PSU size and number, whereas cyanobacteria show enlarged PSU sizes at low ambient photon flux densities, mainly due to increased phycobiliprotein levels. Similar to green algae, *Prochloron* and *Prochlorothrix* were shown to change both PSU size and number (Alberte et al. 1986; Burger-Wiersma and Post 1989). PSU size can be expressed either as the number of chlorophyll molecules per reaction center (RC) I or as the number of chlorophyll molecules per RC II. Highlight-grown *Prochloron* and *Prochlorothrix* cells had significantly smaller PSU sizes, based both on RC I and RC II values, than lowlight cells. However, the decrease in PSU/RC II in *Prochlorothrix* of 60% was large compared to that in *Prochloron* (25%) and to that of the decrease in PSU/RC I in both organisms (20-30%). From these measurements it can be concluded that *Prochlorothrix* adapts to low light by increasing the ratio RC I/RC II, an adaptation pattern usually observed in cyanobacteria. By contrast, the RC I/RC II ratio remained more or less constant in *Prochloron,* as is commonly found for eukaryotes (c.f. Alberte et al. 1986).

The maximal rate of oxygen evolution is determined by the number of RC II and by the rate at which the RC II can reopen after performing the primary reactions. This is related to the capacity of electron flow and thus by the rate at which electrons are transferred in linear electron transport. From the data of Alberte et al. (1986) we calculate a time constant of 3.4 ms for *Prochloron*, slightly lower than the 4 ms reported for *Prochlorothrix* (Burger-Wiersma and Post 1989). In both cases, this time constant appeared to be independent of ambient photon flux density suggesting that the rate of photochemistry is not affected by light-limited growth. These time constants for photosynthetic electron transport are well in the range found for green algae and diatoms, whereas for cyanobacteria longer time constants (ca. 10 ms) are reported (c.f. Burger-Wiersma and Post 1989).

Molecular Assembly

Pigments

Chlorophylls. The oxychlorobacteria characteristically contain both chlorophylls *a* and *b*. This observation was based on separation by chromatography (TLC and HPLC) followed by identification by spectroscopy (Lewin and Withers 1975; Burger-Wiersma et al. 1986). Additional fluorescence excitation and emission spectroscopy at 77 °K revealed peaks that were in accordance with those reported for isolated chlorophylls *a* and *b*, (chl *a* Exc. 429, Em.678 and chl *b* Exc. 478, Em 658) (Thorne et al. 1978; H.C.P. Matthijs unpublished). Freezing and thawing of the host animals before isolation of *Prochloron* cells was shown to be harmful to the chlorophylls in that an extensive conversion to phaeophytins *a* and *b* was noticed. This relates to the reports on phenolic and acid compounds being present in *Prochloron* under stress conditions (Fall et al. 1983; Barclay et al. 1987).

In the case of the recently described species from the deep ocean, both chlorophyll *a* and *b* appeared to be slightly modified to their bivinyl chlorophyll derivatives (Chisholm et al. 1988; S.W. Chisholm personal communication).

The pathways of chlorophyll synthesis in *Prochlorothrix* have been revealed to involve formation of d-aminolevulic acid from glutamate via a pathway which, in contrast to higher plants, did not depend on RNA as has been found for cyanobacteria (Rieble and Beale 1988).

Chlorophyll *a* is common to all oxygenic phototrophs and is present in both antennae and reaction center pigment beds. Chlorophyll *b* is known to be restricted to the light-harvesting antennae. The stoichiometry in chl *a*/*b* in light harvesting complex 2 of green chloroplasts is about unity, and more than four in the light harvesting complex of photosystem 1. The overall chlorophyll *a* to *b* ratio usually reflects the light conditions in which an organism grows. Low light induces a need for extended antennae, which in the case of green plant chloroplasts results in an increase of chlorophyll *b* relative to *a* and by consequence in a lowered chlorophyll *a* to *b* ratio. In comparison to chloroplasts with a chlorophyll *a* to *b* ratio of about three, the various findings of *Prochloron* demonstrate a ratio of 3 to 20 (Thorne et al. 1977; Paerl et al. 1984; Alberte et al. 1986), the ratio in *Prochlorothrix* amounts from 7 to 18 (Burger-Wiersma and Post 1989). The low light-adapted planktonic oxychlorobacterial species isolated from the deep euphotic zone of the ocean demonstrated a chlorophyll *a* to *b* ratio of 1 (Chisholm et al. 1988). These rather divergent numbers may be species-dependent or could be caused by the light conditions during growth.

Usually, a decrease of the ratio of chlorophyll *a* to accessory pigments is observed upon transfer of phototrophs from high to low photon flux densities due to the increased production of light harvesting pigments relative to

reaction centres. Different observations on whether low light conditions do give rise to a lowered ratio of chlorophyll *a* to *b* in the oxychlorobacteria have emerged. On the one hand, a relative increase in chlorophyll *b* content and a lower *a/b* ratio was reported in low light- adapted cells of *Prochloron* (Olson 1983, Bachmann et al. 1985; Alberte et al. 1986). Olson (1983) even showed a small but significant difference in chlorophyll *a/b* ratio between the top and bottom halves of *Prochloron*/ascidian associations. On the other hand, such a relationship could either not be demonstrated (Paerl et al. 1984) or was found to be even reversed (Thorne et al. 1977). Matthijs et al. (unpublished) have observed a decrease of the chlorophyll *a/b* ratio as a result of low light adaptation in *Prochlorothrix* grown in continuous cultures. However, an opposite adaptive pattern was also reported for *Prochlorothrix* (Burger-Wiersma and Post 1989). In these experiments *Prochlorothrix* increased its chlorophyll *a/b* ratio when grown at low photon flux densities. For *Prochlorothrix*, these controversial results may be explained by the difference in the growth techniques applied: in the experiments of Burger-Wiersma and Post (1989) the cultures were continuously illuminated, whereas Matthijs et al. grew the cultures at 16:8 light/dark cycles. Further experiments should disclose whether the above described differences in adaptative patterns for *Prochlorothrix* are in some way related to a possibly different organization of the photosynthetic apparatus, or to abnormalities in the composition of its constituting chlorophyll protein complexes (Matthijs et al. 1989).

Carotenoids. The carotenoid and xanthophyll type pigments determined in *Prochloron* and *Prochlorothrix* typically resemble those characteristically classified in cyanobacteria. A number of studies have revealed the presence of two major compounds, β,β-carotene and zeaxanthin. These compounds have been shown to make up more than 50 (up to 71) and 20 to 40 % of the total carotenoid pool, respectively (Withers et al. 1978a; Burger-Wiersma et al. 1986; Foss et al. 1987). The cryptoxanthin content amounts to about 5% on average. In addition mutachrome, echinenone, isocryptoxanthin and β,β-carotene monoepoxide have been regularly identified in trace amounts of less than 1 % of the total carotenoid content (Withers et al. 1978b; Foss et al. 1987).

No evident differences in the carotenoid distribution ranges in *Prochloron* and *Prochlorothrix* species have been found. Different carotenoid contents and differences in their relative presence have been determined in *Prochlorothrix* in response to photon flux density during growth (H.C.P. Matthijs unpublished). The carotenoid content of *Prochloron* with regard to the presence of β,β-carotene and zeaxanthin varies widely between different batches. No evident link to differences in irradiant light energy could be arrived at (Hiller and Larkum 1985; Alberte et al. 1986). The carotenoid synthesis pathways clearly differ from the ones found in green plants, i.e. alenic, ϵ-type carotenoids, carotenoid epoxides and glycosidic type carotenoids which are normally encountered synthesis products in chloroplasts were

lacking in *Prochloron* (Foss et al. 1987).

Omata et al. (1985) have separated the cell and thylakoid membranes of *Prochloron*. Zeaxanthin was nearly completely recovered in the cell membrane fraction. The thylakoid membrane contained most of the β,β-carotene, very much like in cyanobacteria. Interestingly however, the β,β- carotene content of the cell membrane fraction from *Prochloron* was distinctly higher than the one encountered in cell membranes from cyanobacteria. It was suggested that the cell membrane of *Prochloron* in this respect resembles the chloroplast envelope which normally contains β,β-carotene (Omata et al. 1985). The physiological function of β,β carotene (additional light-harvesting and/or protection against photooxidation), can be correlated with its linkage to the thylakoid membrane. The association of zeaxanthin with the cell membrane points to a light-shielding function only.

Components of the Photosynthetic Electron Transfer Chain

The light-harvesting complexes of the oxychlorobacteria were thought to be analogous to LHC2 of green chloroplasts, with a chl *a* to *b* ratio of about unity (Withers et al. 1978a). More recently, deviating *a* to *b* ratios of 2.4 for *Prochloron* (Hiller and Larkum 1985) and about 4 for *Prochlorothrix* have been reported (Bullerjahn et al. 1987). A further indication of the difference of the chlorophyll *a/b* complexes from *Prochlorothrix* and the LHC2 from chloroplasts is found in the lack of the typical negative deflection at about 650 nm, normally detected in CD spectra of LHC2 (Matthijs et al. 1989). Furthermore, biochemical analysis of the complexes has indicated a molecular mass of the major polypeptide of the chlorophyll *a/b* complexes of 31 to 34 kDa (Hiller and Larkum 1985; Schuster et al. 1984; Bullerjahn et al. 1987) which is high compared to a similar range of estimates for LHC2 (24 to 29 kDa). A lack of immunological cross-reactivity of the polypeptides from the chlorophyll *a/b*-protein complexes of both *Prochloron* and *Prochlorothrix* with antibodies raised against LHC2 from various plants and green algae was also reported. These data point to major differences between the green plant chloroplast LHC2 complexes and the chlorophyll *a/b* protein complexes of oxychlorobacteria. The observed differences may have an impact on the physiological role of the chlorophyll *a/b* -protein complexes. In the case of green plant chloroplasts, light energy captured by chlorophyll *b* contributes to photosynthesis mostly via photosystem 2. However, a significant contribution to photosystem 1 activity has been shown for *Prochloron* (Hiller and Larkum 1985) and *Prochlorothrix* (Bullerjahn et al. 1987; G.S. Bullerjahn et al. 1990; A.F. Post personal communication). Green plant chloroplast LHC1 also contains a minor amount of chlorophyll *b*. No data on a possible analogy between LHC1 and the chlorophyll protein complexes from the oxychlorobacteria are available at present.

Optimal transfer of electrons requires continuous adjustment of the light energy distribution to photosystems 1 and 2. To achieve this, the model for

green plant chloroplasts predicts that the LHC2 antenna may get closer to or further away from photosystem 2. This process involves interplay between activated phosphorylation and continuous dephosphorylation (Bennett 1984). The question arises as to whether such a regulatory system also operates in oxychlorobacteria. Schuster et al. (1984) have noted that the kinase involved in phosphorylation of the 34 kDa polypeptide of the *Prochloron* chlorophyll *a/b*- protein complex is always activated. This would exclude a regulatory mechanism similar to the one operative in green chloroplasts. In the case of *Prochlorothrix*, light intensity-dependent phosphorylation was absent also. However, some kind of regulatory mechanism is likely to operate since controlled reduction and oxidation of isolated thylakoid membranes clearly switches kinase activity on and off respectively (Van der Staay et al. 1989). Interestingly, Burger-Wiersma and Post (1989) have clearly demonstrated light quality-dependent changes in the variable fluorescence yield in *Prochlorothrix*.

Although the chlorophyll *a/b*- protein complexes of oxychlorobacteria may be quite different from those found in green chloroplasts, it has very recently been concluded from experiments involving antibodies against the isolated complexes of *Prochloron* and *Prochlorothrix* that these complexes are very much alike (G.S. Bullerjahn et al. 1990).

Other chlorophyll protein complexes in *Prochloron* and *Prochlorothrix* have also been characterized. SDS gel electrophoresis and sucrose gradient centrifugation employing thylakoid membranes of *Prochloron* demonstrated that PS1 includes a major polypeptide of 70 kDa and contains considerable amounts of chlorophyll *b*. The chl *a/b* ratio of this PS1 complex was 3.8 and its molecular mass was estimated at 300 kDa with a chlorophyll/P700 ratio of approximately 100 (Hiller and Larkum 1985). Schuster et al. (1985) described the isolation of RC1 preparations from *Prochloron* with chlorophyll *a* to P_{700} ratios of 120 and 40. Information on whether the larger of the two complexes would contain any chlorophyll *b*, such as was reported by Hiller and Larkum (1985) for a similar preparation and which would point to an antenna function for the excess chlorophyll, has not been indicated by Schuster et al. (1985). The isolated RC1 complex contained 4 polypeptides with masses of 70, 16, 10 and 8kDa (as opposed to the 7 usually found in higher plant chloroplasts, but analogous to the number in the green algal chloroplasts from *Dunaliella* and *Chlamydomonas*). The 70 kDa band was found to be immunologically related to the P700-binding polypeptide of spinach, *Dunaliella* and *Chlamydomonas*. The molecular mass of the 16 kDa subunit was slightly lower than the presumed analogue in the other organisms, also including the cyanobacterium *Mastigocladus laminosum* (range of masses 20 to 22 kDa). The other 2 subunits could not be unambiguously related to those of the other organisms. The equimolar presence of P700 and the special chlorophyll molecule (Chl RC1) in a range of oxygenic phototrophic organisms as well as in *Prochloron* indicates a close similarity of their RC1 complexes (Senger et al. 1987).

Analysis of the chlorophyll-protein complexes obtained from the thylakoid membranes of *Prochlorothrix* by non-denaturing gel electrophoresis revealed

five different complexes (Bullerjahn et al. 1987). By spectroscopic and immunological methods two of these complexes could be assigned to PS1 (CP1) and PS2 (CP4), respectively. The complex CP4 contained polypeptides which cross-reacted to antibodies against *Chlamydomonas* PS2 proteins 5 and 6. CP1 contained some chlorophyll *b*, the other three complexes all contained chlorophyll *b* besides of chlorophyll *a* and have been judged to be antennae.

In *Prochlorothrix*, preliminary studies on other components involved in photosynthetic and respiratory electron transfer have indicated the presence of a-, b- and c-type cytochromes (Matthijs et al. 1988). In difference spectra of reduced and oxidized samples of thylakoid membranes from *Prochlorothrix* peaks were observed at 549, 553, 557, 559 and 563 nm. The former three are indicative for c-type cytochromes and the latter two for b-type cytochromes. Plastocyanin could not be detected regardless of the absence or presence of copper in the growth medium. Interestingly, the type cytochrome aa_3 which has been proven to play a functional role in respiratory electron transfer in cyanobacteria was also detected in *Prochlorothrix* and was predominantly found in the cell membrane (Peschek et al. 1989).

Cell Wall

The cell walls of *Prochloron* and *Prochlorothrix* resemble those of cyanobacteria. The peptidoglycans of *Prochloron* (Moriarty 1979; Stackebrandt and Kandler 1982) and *Prochlorothrix* (Jürgens and Burger-Wiersma 1989) are of the A1γ-type like those of several cyanobacteria. The cross-linkage indices for *Prochloron* and *Prochlorothrix* were 40 and 63%, respectively. Only the latter falls within the range of those found for Gram-positive bacteria (46-93%). The cross-linkage index of *Prochloron* is not in the range of that for Gram-positive, but significantly higher than those usually found for Gram-negative bacteria (26-33%). These findings are in good agreement with the thickness of the peptidoglycan layers of both organisms, i.e. for Gram-negative strains significantly thinner layers are reported (c.f. Jürgens and Burger-Wiersma 1989).

Lipid Composition

The lipid composition of phototrophic organisms is often considered a valuable tool in taxonomy. Cyanobacteria are characterized by a limited variety of hydrocarbons with 17:0 often predominating. This hydrocarbon was also abundant in *Prochloron* (Perry et al. 1978) and *Prochlorothrix* (Volkman et al. 1988). In *Prochlorothrix* the most abundant hydrocarbon was n-heptadec-5-ene which is common in cyanobacteria, whereas in green algae the isomer n-heptadec-7-ene may be present, possibly representing a bifurcation between the biosynthetic pathways in the two groups of organisms. The latter alkene was not detected in *Prochlorothrix*. Another important hydrocarbon in *Prochlorothrix* is hop-22(29)-ene (Volkman et al. 1988). Hopanoids are not

found in eukaryotic algae, but are common in most bacteria and cyanobacteria. There are no records of hopanoids being present in *Prochloron*. Poly-unsaturated straight-chain alkenes typical of eukaryotes such as n-$C_{21:6}$ were not detected in *Prochloron* nor in *Prochlorothrix*.

Although Perry et al. (1978) originally detected relatively high levels of sterols in *Prochloron* isolated from *Lissoclinum patella*, a more recent study of Johns et al. (1981) suggested that the sterols in *Prochloron* were contaminants from its ascidian host. No sterols were detected in *Prochlorothrix* (Volkman et al. 1988).

The prokaryotic signature of the lipid composition of *Prochloron* was further established by the presence of monoglucosyl diacylglycerol (Murata and Sato 1983). Cyanobacteria and higher plant chloroplasts differ in the composition and the biosynthetic pathways of their polar lipids. Higher plant chloroplasts are characterized by the presence of phosphatidylcholine in addition to the four common glycerol derivatives, whereas cyanobacteria contain monoglucosyl diacylglycerol (c.f. Murata and Sato 1983).

The overall distribution of total fatty acids in *Prochloron* (Perry et al. 1978; Johns et al. 1981; Murata and Sato 1983; Kenrick et al. 1984) and *Prochlorothrix* (Volkman et al. 1988) is very similar to those reported for cyanobacteria. A predominance of C_{14}-C_{18} saturated and unsaturated fatty acids was observed. Both *Prochloron* and *Prochlorothrix* lacked the *trans*-16:1 ω13 fatty acid, which is restricted almost exclusively to the chloroplast phospholipids of eukaryotic microalgae, and is thought to be closely associated with the stacking of thylakoid membranes. Interestingly, there is a great deal of evidence that 'true' stacking occurs in both *Prochloron* and *Prochlorothrix* (Miller et al 1988). No longer-chain poly-unsaturated fatty acids typical for eukaryotic cells were detected in *Prochlorothrix*. *Prochloron* was shown to contain trace amounts of these compounds (Perry et al. 1978), but the authors question the importance of their observation in terms of phylogeny. Interestingly, *Prochlorothrix* contains considerable amounts of two unusual unsaturated fatty acids. These compounds have not been detected before, neither in prokaryotes nor in eukaryotes. Therefore, they may be of significance in further chemotaxonomic studies of the oxychlorobacteria.

Marked variations occur in the fatty acid composition of the glycolipids isolated from *Prochloron* cells from the same host at different locations or at different times (Kenrick et al. 1984). Thus, although the lipid composition is clearly a useful tool in establishing the taxonomic position of the oxychlorobacteria at the ordinal level, the method appeared not suitable to discriminate at the specific level.

Phylogeny

It has been widely argued that eukaryotic organisms arose from endosymbiosis of prokaryotes, and that in eukaryotic plants the chloroplast originated from

a phototrophic prokaryote, presumably an ancestral cyanobacterium. There was, however, one problem: the cyanobacteria contain phycobiliproteins as accessory pigments, whereas the majority of eukaryotic phototrophic species possess another accessory pigment. Therefore, *Prochloron* (Lewin 1976, 1977) was considered to be a 'missing link' in green chloroplast evolution, moreover, since the organisms were never encountered free-living but observed exclusively as exosymbionts on certain colonial ascidians.

Florenzano et al. (1986) outlined two scenarios hypothesizing the origin of the oxychlorobacteria and their relatedness to cyanobacteria. Here, we extend these scenarios by subsequent endosymbiosis resulting in chloroplast development, and discuss the relatedness of chloroplasts to the oxychlorobacteria.

One scenario is that cyanobacteria and oxychlorobacteria evolved from a common photosynthetic ancestor which exclusively contained chlorophyll *a*. The phycobiliproteins would have developed as accessory pigments for photosystem II in the cyanobacteria, whereas a chlorophyll *a*/*b*-protein complex served this function in the oxychlorobacteria. Combining this line of argument with the endosymbiosis theory, the green chloroplasts could have evolved from an endosymbiotic event involving this hypothesized chlorophyll *a* containing progenitor or an oxychlorobacterium-like organism. Only the latter event might, but does not have to, result in a close relationship between oxychlorobacteria and green chloroplasts.

The second possibility raised by Florenzano et al. (1986) is that the oxychlorobacteria developed from a typical cyanobacterium. The ability to synthesize chlorophyll *b* might have been acquired either by molecular evolution or by transfer of the genetic information from some green alga in a natural transformation process. Further evolutionary steps, like loss of phycobilisomes and modified thylakoid arrangement, might have occurred rapidly as a consequence of the presence of chlorophyll *b*. The first of these possibilities, the molecular evolution, may have occurred once in cyanobacterial evolution resulting in the development of the progenitor of both oxychlorobacteria and chloroplasts which, by consequence, would be closely related. However, it cannot be excluded that molecular evolution occurred more than once, one time leading to oxychlorobacteria and another time to the (progenitors of) green chloroplasts. The second possibility, transfer of genetic information, assumes the presence of green algae already and automatically rules out the possibility that green chloroplasts evolved from oxychlorobacteria. Consequently, following the endosymbiosis theory this outline suggests that the green chloroplasts evolved directly from the cyanobacteria and no specific relationship with oxychlorobacteria is to be expected. Within the line of this second senario a third possibility can be hypothesized, i.e. natural transfer of genetic information from an ancestor of the oxychlorobacteria to a phycobiliprotein-containing 'chloroplast'. The latter hypothesis may find some support in the specific relationship between the green chloroplasts and the cyanelles of *Cyanophora paradoxa*, a photosynthetic

organelle containing phycobilin pigments (Giovannoni et al. 1988). In either of the hypothesized evolution patterns a close relationship between oxychlorobacteria and cyanobacteria is obvious.

From a thorough homology study of 16S ribosomal RNA sequences of 29 cyanobacterial strains and 5 green chloroplasts Giovannoni et al. (1988) have concluded that green chloroplasts arose from within the cyanobacterial line of descent. Based on this result, scenario one has to be rejected.

The genome size of *Prochloron* (3.59×10^9 daltons) was found to be at the upper limit for unicellular prokaryotes, and 10 times larger than that observed for chloroplasts. The similarity in genome size of *Prochloron* and the cyanobacteria advocates a potential relationship (Herdman 1981).

Another indication of a close relationship between *Prochloron* and cyanobacteria came from a homology study of the 5S ribosomal RNA (MacKay et al. 1982). The 5S rRNA of *Prochloron* was much more similar to that of the cyanobacterium than to the 5S rRNA of either the chloroplast or the eubacterial strains tested. No specific relationship between chloroplasts and *Prochloron* could be demonstrated; the nucleotide sequences of *Prochloron* and the cyanobacterium *Anacystis nidulans* were equally different from that of the spinach chloroplast. However, caution should be taken in interpreting these results since only one green alga and one cyanobacterium were involved in this study.

Comparison of the RNase T1-generated 16S ribosomal RNA catalogues of *Prochloron*, seven cyanobacterial strains and four green chloroplasts revealed that *Prochloron* was more closely related to the cyanobacteria *Fischerella* and *Nostoc* than to the green chloroplasts (Seewaldt and Stackebrandt 1982). Van Valen (1982) debated their conclusions for methodological reasons and constructed an alternative phylogeny in which a cyanobacterial line, a green chloroplast line and *Prochloron* diverge from a common ancestor. In his opinion, *Prochloron* might be a comparatively little modified descendant of this common ancestor. However, Bandelt and Von Haeseler (1989) reinvestigated the original data set using more sophisticated methods. They concluded that from a numerical point of view the estimated phylogeny as proposed by Seewaldt and Stackebrandt (1982) is slightly superior over the one proposed by Van Valen (1982).

Like the green chloroplasts (Giovannoni et al. 1988) *Prochlorothrix* was found to fall within the cyanobacterial line of descent (Turner et al. 1989). Interestingly, *Prochlorothrix* was found to be most closely related to two unicellular strains, whereas *Prochloron* showed the highest similarity to two filamentous strains (Seewaldt and Stackebrandt 1982). In contrast to green chloroplasts, *Prochlorothrix* is a deeply branching member of the cyanobacteria, implying that the organism is not specifically related to the green chloroplasts. Their conclusion was based on a 16S ribosomal RNA study including 18 cyanobacterial strains and 5 green chloroplasts using reverse transcriptase elongation from deoxynucleotide primers. As a consequence of the difference in techniques used to determine the sequences, the relatedness

between *Prochloron* and *Prochlorothrix* could not be established from these studies (Turner et al. 1989). Based on the 16S ribosomal RNA studies (Seewaldt and Stackebrandt 1982; Giovannoni et al. 1988; Turner et al. 1989) it is very unlikely that green chloroplasts resulted from an endosymbiotic event involving (a progenitor of) either *Prochloron* or *Prochlorothrix*. This implies that the ability to synthesize chlorophyll *b* arose more than once, or was acquired by natural transfer of genetic information.

A contrasting phylogenetic relationship between cyanobacteria, green chloroplasts and *Prochlorothrix* can be derived from the sequences of the *psb*A genes, which encode the photosystem II thylakoid protein D1 (Morden and Golden 1989a). Interestingly, the number of these genes (two) present in *Prochlorothrix* is different from that found in the genomes of cyanobacteria (three or more) and of green chloroplasts (one). In spite of the presence of two *psb*A genes in *Prochlorothrix* only one D1 polypeptide was predicted, whereas the *psb*A genes in cyanobacteria encode two different polypeptides.

The D1 polypeptide that was predicted from the sequence of the *psb*A genes from *Prochlorothrix* showed, like in chloroplasts, a seven amino acid gap at the C-terminal when compared to those in cyanobacteria. According to the authors, this suggests a more recent common ancestor among green chloroplasts and *Prochlorothrix* than among green chloroplasts and cyanobacteria. Phylogenetic analyses of the amino acid sequences of the predicted D1 proteins of cyanobacteria, chloroplasts and *Prochlorothrix* have resulted in two phylogenetic tree topologies depending on the significance attributed to the missing seven amino acid domain (Morden and Golden 1989b). When treated as a less important factor, for instance as a single binary character, a phylogenetic tree was generated that associated *Prochlorothrix* in a lineage with two *Synechococcus* strains. However, if the presence or absence of residues in this domain is considered to be more significant than one or two amino acid substitutions, *Prochlorothrix* is part of a lineage that led to green chloroplasts after the divergence from the cyanobacteria.

In conclusion, the oxychlorobacteria resemble the cyanobacteria to a great extent. Thus far, the only discrepancy is found in properties directly associated with the photosynthetic apparatus, i.e. pigment composition, light quantity adaptation patterns, composition of the chlorophyl-protein complexes of the thylakoids, the arrangement of the thylakoids and a seven amino-acid domain gap near the C-terminus of the *psb*A genes.

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2. Inorganic Carbon Transport and Accumulation in Cyanobacteria

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Abbreviations

CA, carbonic anhydrase; C_i, inorganic carbon (CO_2 + HCO_3^- + $CO_3^{=}$); DCMU, 3-(3,4-dichlorophenyl) – 1, 1 – dimethylurea; DES, diethylstilbestrol; $K_{0.5}$, concentration required for half maximal response; K_m, Michaelis constant; $P(CO_2)$, permeability coefficient for CO_2; PGA, 3-phosphoglyceric acid; RuPB, ribulose 1,5-bisphosphate; RuBisCo, ribulose bisphosphate carboxylase-oxygenase; $\Delta\Psi$membrane potential difference.

Introduction

The cyanobacteria use ribulose bisphosphate carboxylase/oxygenase (RuBisCo) as the first enzyme of photosynthetic CO_2 fixation (Codd and Marsden 1984; Tabita 1988). This enzyme uses CO_2 as its substrate (Filmer and Cooper 1970) and does so with surprisingly poor efficiency (Andrews and Lorimer 1987). For example, the enzyme from cyanobacteria has a K_m (CO_2) of about 300 μM and a turnover number of only about $12s^{-1}$ (Andrews and Abel 1981; Badger 1980). The ability of RuBisCo to react with CO_2 is further compromised by the fact that O_2 is an alternate and competitive substrate (Andrews and Lorimer 1987). At air saturation, aqueous media will contain about 250 μM O_2 (25°C) and the effective K_m (CO_2) of cyanobacterial RuBisCo in the presence of this O_2 will be about 600 μM (Pierce and Omata 1988). Intact cells of cyanobacteria, however, have a much higher affinity for CO_2 than the isolated RuBisCo. For example, in HCO_3^- – limited chemostats *Synechococuus* was found to grow at half the maximal rate when the total inorganic carbon (C_i) concentration was only 10 to 15 μM (Miller et al. 1984a). The chemostats were closed systems operated at pH 9.6, so that the actual CO_2 concentration in the medium with cells growing at half the maximal rate was only 5.6 to 8.4 nM (Miller et al. 1984a).

Even cells from air-bubbled batch cultures have $K_{0.5}$ (CO_2) values for CO_2 fixation at pH 8 of about 0.5 to 2.0 μM (Abe et al. 1987a; Abe et al. 1988;

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 25-53.

Badger and Andrews 1982; Kaplan 1981a; Kaplan et al. 1980; Miller and Colman 1980b; Shelp and Canvin 1984). Such whole cell $K_{0.5}$ (CO_2) values for CO_2 fixation are well below the K_m (CO_2) for isolated RuBisCo and, given the turnover number and cellular content of the enzyme, it can be easily calculated that there must be an active accumulation of CO_2 within the cells to allow for the observed rates of CO_2 fixation (Espie et al. 1988a; Miller 1985). Considerations such as these led Badger et al. (1978) to search for 'CO_2 – concentrating mechanisms' in green microalgae and cyanobacteria. They found that *Anabaena* could accumulate C_i (HCO_3^- + CO_2) to a concentration of 4 mM within the cells even when the extracellular C_i concentration had dropped to about 3 μM (Badger et al. 1978). Hence, C_i was accumulated in excess of 1000-fold over the extracellular concentration. More significantly, with respect to the CO_2 – using RuBisCo, the CO_2 concentration at the site of RuBisCo can be calculated as about 240 μM, assuming rapid interconversion of CO_2 and HCO_3^- within the cells (Badger and Price 1989; Tu et al. 1987) and an intracellular pH of 7.5 (Coleman and Colman 1981). Thus by virtue of a 'CO_2 – concentrating mechanism' the CO_2 concentration was raised to a value close to the K_m (CO_2) of cyanobacterial RuBisCo from a value (0.05 μM at pH 8) in the extracellular medium that, by itself, would have supported a negligible rate of CO_2 fixation (Miller 1985).

In this review I will outline the techniques that can be used to study the 'CO_2 – concentrating mechanisms' of cyanobacteria, the results that have been obtained and what the results may mean in terms of the mechanisms involved.

The Nature of Inorganic Carbon (C_i)

There are two important points that must be kept in mind when one is studying C_i transport. Firstly, C_i can exist in solution in four very different molecular forms viz. CO_2, H_2CO_3, HCO_3^- and $CO_3^=$. Secondly, atmospheric CO_2 is an ever present source of C_i contamination for experimental solutions. Although CO_2 accounts for only about 0.05% of the volume of the air in most laboratories it is soluble enough in water that an aqueous solution in equilibrium with the air will contain 10-12 μM CO_2. Since dissolved CO_2 exists in equilibrium with HCO_3^-, an exposed buffer at pH 8.0 will have a C_i concentration of about 500 μM, mainly as HCO_3^-. When studying C_i transport one must take this contaminant level into account. Better still one should prepare buffers that are low in C_i (15 μM) (Miller et al. 1984a). The residual C_i is then rapidly removed by the cells themselves as they reach their very low CO_2 – compensation points of about 0.05 μM CO_2 (Birmingham and Colman 1979).

When CO_2 is dissolved in water a predominant feature of CO_2 chemistry becomes evident – the carbon atom is susceptible to nucleophilic attack by both H_2O (reaction 1) and OH^- ions (reaction 2):

1. $CO_2 + H_2O \overset{a}{\rightleftharpoons} H_2CO_3 \overset{b}{\rightleftharpoons} H^+ + HCO_3^- \overset{c}{\rightleftharpoons} CO_3^{=} + 2H^+$
2. $CO_2 + OH^- \overset{a}{\rightleftharpoons} HCO_3^- \overset{b}{\rightleftharpoons} CO_3^{=} 2H^+$

In reaction 1 it is the hydration of CO_2 to form H_2CO_3 that is the slow step ($t_{1/2}$ = 7s at 30°), while dissociation of carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) are essentially instantaneous. At equilibrium the concentration of H_2CO_3 above pH 6 is less than 0.3% of the CO_2 concentration at 25°, so it is usually assumed that H_2CO_3 itself is not a substrate for the C_i transport systems. Reaction 2 starts to become significant above pH 8.0. At pH 10, where many cyanobacteria happily thrive, it is the predominant reaction (Kern 1960). It is essentially this reaction that is catalyzed by CA since a Zn-OH attack on CO_2 is the mechanism of HCO_3^- formation (Silverman and Vincent 1983). The uncatalyzed, or spontaneous, formation of HCO_3^- from CO_2 by both reactions is rather slow, taking about 8s or so to go to half-completion at pH 8.0 at low ionic strength at 30° (Miller et al. 1988a, Price and Badger 1989a). It is clear from Reactions 1 and 2 that as the solution becomes alkaline, greater concentrations of HCO_3- and CO_3 will exist at equilibrium. The pKa for HCO_3^- dissociation to $CO_3^{=}$ is about 10.3 at 30° and low ionic strength (Buch 1960) so above pH 10.3 $CO_3^{=}$ becomes the predominant form of C_i (see Lucas 1983 and Smith 1988). One effect of the pH dependence of Reactions 1 and 2 is that above about pH 7 there is a 10-fold increase in total C_i for each rise in pH of 1 unit. It is very important to note that, because CO_2 solubility is pH-independent, the CO_2 concentration in an open system will remain quite constant as the pH rises. Only in a system closed to atmospheric CO_2 will the CO_2 concentration drop to very low levels as the pH becomes more alkaline. The kinetics and equilibrium conditions of Reactions 1 and 2 are discussed in more detail elsewhere (Asada 1982; Filmer and Cooper 1970; Kern 1960; Lucas 1975; Miller and Colman 1980b; Smith 1988). The mechanistic consequences of Reactions 1 and 2 with respect to C_i transport are significant indeed. A neutral and linear molecule (CO_2) that is quite lipid soluble and that is susceptible to nucleophilic attack is converted to anions (HCO_3^- and $CO_3^{=}$) that differ from CO_2 on all counts (Gutknecht et al. 1977; Palmer and van Eldik 1983; Rubio 1986; Simon and Gutknecht 1980). Considering the very significant differences between CO_2 and HCO_3^- it is not surprising that a given carboxylating enzyme uses only one or the other (Asada 1982, Filmer and Cooper 1970; Rubio 1986).

Techniques for Measuring C_i Transport

A number of quite different techniques for measuring C_i transport by cyanobacteria have been developed.

Silicone Fluid Centrifugation (SFC)

This was the method used by Badger et al. (1978) to obtain the first evidence

for active C_i transport and accumulation by cyanobacteria. In a 400 μL micro-centrifuge tube are placed, successively, 100 μL terminating solution (such as 2N KOH with a 10% methanol), 100 μL silicone fluid and 100 μL cell suspension at the CO_2 compensation point. The tube is placed in an illuminated micro-centrifuge and uptake is initiated by the addition of $H^{14}CO_3^-$ or $^{14}CO_2$. After the desired uptake period the transport is terminated by turning on the centrifuge and spinning the cells through the silicone fluid into the terminating solution. For unicellular species such as *Coccochloris* or *Synechococcus*, about 85% recovery of cells in the terminating solution is achieved after 6s centrifugation (Miller and Colman 1980a; Espie et al. 1988b). The terminating solution is assayed for total ^{14}C activity (fixed + unfixed ^{14}C) and acid-stable ^{14}C activity (fixed ^{14}C). The difference between the total ^{14}C activity and the acid-stable ^{14}C activity represents the accumulated but unmetabolized C_i. Typical results for *Synechococcus* are shown in Fig. 1. Accumulation of C_i by *Synechococcus*, grown with air levels of CO_2 requires the presence of either Na^+ or CA (Fig. 1). A similar Na^+ requirement has been shown for *Anabaena* (Abe et al. 1987b; Kaplan et al. 1984; Reinhold et al. 1984). Transport of C_i is inhibited by darkness (Fig. 1), the electron transport inhibitor DCMU and the uncoupler CCCP (Badger and Andrews 1982, Coleman and Colman 1981, Miller and Colman 1980b, Ogawa and Ogren 1985, Shelp and Canvin 1984). The centrifugation method also shows that C_i transport proceeds unabated when C_i fixation is blocked by D,L – glyceraldehyde (Shelp and Canvin 1984) or iodoacetamide (Ogawa et al. 1985b). The centrifugation method was used by Kaplan et al. (1980) to obtain the first detailed kinetics of C_i accumulation. The extent of C_i accumulation by cyanobacteria can be impressive. When the initial C_i concentration in the medium is low, accumulation factors from 1000 to 2000-fold are common (Raven 1985).

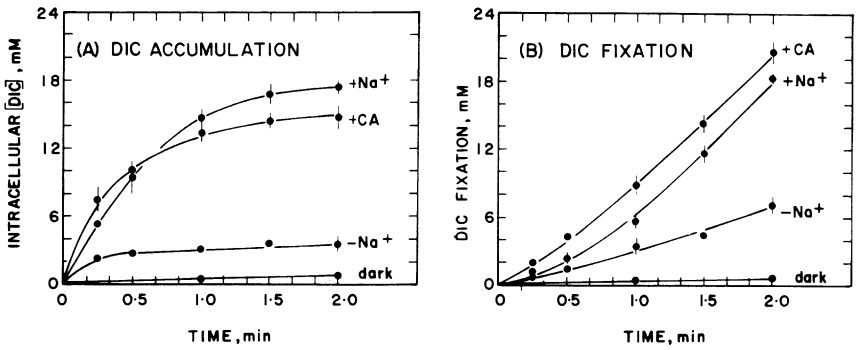

Fig. 1. Measurement of C_i accumulation by the silicone fluid centrifugation method (A) Acid labile ^{14}C activity in the pellet, i.e. unfixed ^{14}C (B) Acid stable ^{14} activity in the pellet, i.e. fixed ^{14}C. Figure taken from Miller and Canvin (1985).

Membrane Filtration

This has undoubtedly been the most commonly used method in studies of bacterial transport. It has not been used in the study of C_i transport by cyanobacteria, probably because of the fear of atmospheric C_i contamination and the fear that leakage of accumulated C_i (mainly as CO_2) during the filtration would be extensive. The method has been used successfully with the chemoautotrophic *Thiobacillus neapolitanus* (Holthuijzen et al. 1987). The uptake of $^{14}C_i$ by the cells was terminated by the addition of 0.1M LiCl and 0.1M NaOH and the samples were then filtered through cellulose acetate filters. *Thiobacillus* can accumulate C_i to levels 1500-fold the external when provided with thiosulphate as an energy source. Transport of C_i was blocked by uncouplers, inhibitors of electron transport and by agents that collapsed $\Delta\Psi$(Holthuijzen et al. 1987).

Infra-Red Gas Analysis (IRGA)

Ogawa et al. (1985a) have described an IRGA system that allows one to monitor the C_i uptake by cells in suspension. A gas stream, containing CO_2, is vigorously bubbled through a cell suspension at pH 7 and the changes in the CO_2 concentration of the effluent gas are monitored by IRGA (Ogawa et al. 1985a). Since H_2O also absorbs in the infra-red region, the effluent gas stream must be dried before analysis. The uptake of C_i by the cells upon illumination results in a transient drop in the CO_2 concentration of the effluent gas stream (Ogawa and Inoue 1983). When the lights are turned off the accumulated C_i leaks back into the medium and a transient rise in the effluent CO_2 concentration is observed (Ogawa and Inoue 1983). Care must be taken that the rates of CO_2 exchange between the gas phases and the cell suspension do not become limiting factors (Ogawa et al. 1987). The method has been successfully used to demonstrate that C_i transport by *Anabaena* can be mediated by cyclic electron flow driven by photosystem I (Ogawa and Inoue 1983; Ogawa et al. 1985b).

Membrane Inlet Mass Spectrometry (MIMS)

This technique was first used to investigate C_i transport in cyanobacteria by Badger and Andrews (1982). It has since been used by a number of other laboratories (Miller et al. 1988a; Tu et al. 1987). Cells are incubated in a cuvette that is sealed from the atmosphere. The capillary inlet of the mass spectrometer is inserted via a gas-tight port into the side of the cuvette. The end of the capillary inlet is covered by a dimethylsilicone rubber membrane supported by a metal grid. Dissolved gases, such as CO_2, leak slowly across the membrane inlet into the MS so that the CO_2 concentration of the medium can be monitored. The results obtained by Badger and Andrews (1982) with the marine *Synechococcus* were startling. They found that upon illumination,

cells at pH 8.2 took up free CO_2 so rapidly that the extracellular CO_2 concentration fell almost to zero. The rapid drop in the CO_2 concentration occurs even when CO_2 fixation is blocked by iodoacetamide (Miller et al. 1988a; Spiller et al. 1988) or by glyceraldehyde or glycolaldehyde (Miller and Canvin, 1989). Hence the rapid drop in the extracellular CO_2 concentration observed upon illumination of the cells is not related to CO_2 fixation by RuBisCo (Badger and Andrews 1982; Miller et al. 1988a). The precipitous drop in the CO_2 concentration is not observed when CA is added to maintain equilibrium between the CO_2 and HCO_3^- in the extracellular medium (Espie et Canvin 1987; Miller et al. 1988a). The species of cyanobacteria so far investigated do not produce their own extracellular CA (see Badger and Price 1989).

The ability of cyanobacteria, in the absence of CA, to remove CO_2 so effectively from the medium that the CO_2/HCO_3^- system is displaced far from equilibrium can only be explained by the presence of a 'pump' that uses CO_2 as a substrate. The displacement of reactions 1a and 2a so far from equilibrium obviously requires the expenditure of metabolic energy in the CO_2 uptake process. In accord with this, CO_2 transport is blocked by the electron transport inhibitor DCMU, by uncouplers, by the ATPase inhibitor DES and by darkness (Badger and Andrews 1982; Miller et al. 1988a; Spiller et al. 1988).

The observation that upon illumination of cells at pH 8.0 the extracellular CO_2 concentration drops almost to zero demonstrates that the rate at which HCO_3^- can be converted spontaneously to CO_2 can limit the rate of CO_2 uptake (Miller 1985). This is the reason why the addition of CA stimulates the rate of C_i accumulation, via CO_2 uptake (Fig. 1) when HCO_3^- transport is blocked by the absence of Na^+ (Fig. 1).

The extent of C_i accumulation by the cells can be estimated, using MIMS, from the amount of C_i that leaks back into the extracellular medium when the lights are turned off (Badger et al. 1985; Miller et al. 1988a; Spiller et al. 1988). This must be done in the presence of CA so that total C_i (CO_2 + HCO_3^-) leakage is measured. Using this technique Badger et al. (1985) estimated intracellular C_i concentrations as high as 130 mM for a marine *Synechococcus* and Miller et al. (1988b) estimated values of about 60 mM for the freshwater *Synechococcus* UTEX 625. To more readily separate the C_i leakage from the slower contribution of C_i from respiration, the cells can be allowed to accumulate ^{13}C-labelled C_i(Miller et al. 1988a, b; Spiller et al. 1988). If the uptake of $^{13}C_i$ takes place in the presence of iodoacetamide or glycolaldehyde, to block CO_2 fixation, then a quantitative leakage of the accumulated C_i back into the extracellular medium occurs within two minutes of turning off the light (Miller et al. 1988b).

Quenching of Chlorophyll a Fluorescence

Recently we have developed a technique for monitoring C_i accumulation that involves the quenching of chlorophyll *a* fluorescence (Miller and Canvin 1987; Miller et al. 1988b). At the CO_2 compensation point the fluorescence yield of chlorophyll *a* in *Synechococcus* UTEX 625 is at, or close to, its maximum. Upon the addition of C_i, as CO_2 or HCO_3^-, there is a rapid drop in the fluorescence yield (Miller and Canvin 1987). A large and rapid drop in fluorescence yield occurs even when CO_2 fixation is blocked by iodoacetamide (Miller et al. 1988b) or glycolaldehyde. When total C_i uptake, in the presence of CA, is monitored in the mass spectrometer at the same time as the chlorophyll *a* fluorescence yield, there is a surprisingly close correlation between the extent of fluorescence quenching and the extent of C_i accumulation (Miller et al. 1988b). Very similar results are obtained when iodoacetamide (Miller et al. 1988b) or glycolaldehyde (Miller and Canvin 1959) are used to block CO_2 fixation. The accumulation of C_i by the cells is correlated with an enhanced rate of water splitting (O_2 evolution) and an enhanced rate of O_2 photoreduction (Miller et al. 1988c). The two rates are very similar so that no net O_2 evolution or uptake are seen when C_i is added in the presence of iodoacetamide (Miller and Canvin 1987) or glycolaldehyde. The enhanced rate of O_2 photoreduction is consistent with the observation that most of the quenching that occurs when C_i is added is q-quenching due to oxidation of the primary acceptor, Q_A, of photosystem II (Miller et al. 1988c; Miller and Canvin, unpublished). How accumulation of C_i stimulates linear electron flow in *Synechococcus* UTEX 625 is not known. It is tempting to suggest that intracellular HCO_3^- stimulates the oxidation of Q_A as it does in isolated chloroplasts (Govindjie and van Rensen 1978). We have found the fluorescence monitoring of C_i accumulation in *Synechococcus* UTEX 625 to be a very simple and rapid technique for testing the effect of putative inhibitors of CO_2 or HCO_3^- transport (Miller et al. 1988b; Miller et al. 1989).

Distinguishing Between CO_2 and HCO_3^- Transport

Using the previous techniques it has been demonstrated that cyanobacteria expend metabolic energy to accumulate C_i to levels greatly above those in the extracellular medium. But which of the radically different forms of C_i are the actual transport substrates – CO_2, H_2CO_3, HCO_3^- or $CO_3^=$? It turns out that cyanobacteria possess transport systems for at least CO_2 and HCO_3^-, and perhaps $CO_3^=$ as well. The possibility of H_2CO_3 transport is usually not considered because of its very low concentration at equilibrium above pH 6. A number of authors have outlined the methods that can be used to distinguish CO_2 from HCO_3^- (or $CO_3^=$) transport (Kaplan et al. 1988; Kerby and Raven 1985; Miller 1985; Raven 1984). These methods are briefly given below.

pH Dependence

Reactions 1 and 2 show that in a *closed* system the CO_2 concentration will drop and the HCO_3^- (or $CO_3^=$) concentration will rise as the pH becomes more alkaline. If the rate of C_i transport at, a limiting C_i concentration, drops drastically as the pH rises it suggests that the cells may only be capable of transporting CO_2. This is not usually the case for cyanobacteria grown at levels of CO_2 at or below those in air (e.g. Badger and Andrews 1982; Coleman and Colman 1981; Shelp and Canvin 1984). Such results do not prove that these cells cannot transport CO_2, only that they must transport HCO_3^- (or $CO_3^=$). This method assumes that the only effect of changing pH is to shift the equilibrium position of reactions 1 and 2. Obviously other changes may also occur, such as a change in the degree of protonation of groups involved in the transport process. Still, it must be admitted that the indications for HCO_3^- transport given by this method have been fully confirmed by all the other methods. The rate of C_i transport by the chemoautotroph *Thiobacillus* did drop drastically as the pH was raised from pH 6.0 (69% CO_2) to pH 7.5 (6.6% CO_2), suggesting that these cells could only transport CO_2 (Holthuijzen et al. 1987).

The pKa for HCO_3^- dissociation to $CO_3^=$ is about 10.3 at 30° (Buch 1960). For *Synechococcus* UTEX 625 the $K_{0.5}$ for CO_2 fixation does not vary much from pH 8.0 (about 98% HCO_3^-) to pH 10.6 (28% HCO_3^-), suggesting, albeit weakly, that this species may be able to transport $CO_3^=$ as well as HCO_3^- (Miller and Canvin, unpublished).

Consideration of the HCO_3^- Dehydration Rate

For a cell capable of transporting only CO_2, its rate of C_i transport will be limited by the rate at which HCO_3^- can be dehydrated according to reactions 1a and 2a. The cyanobacteria so far examined have no extracellular CA capable of catalysing the conversion of HCO_3^- (see Badger and Price 1989). In the absence of CA, the rate of spontaneous HCO_3^- dehydration can be calculated (Lucas 1975; Miller 1985). For air-grown cyanobacteria the rates of C_i transport at alkaline pH (Miller and Colman 1980b; Espie et al. 1988a) are much faster than the rate at which CO_2 can be formed spontaneously from HCO_3^-. In these cases HCO_3^- transport must contribute very substantially to the C_i influx. An advantage of this kinetic approach is that there is no change in the extracellular pH.

'Active Species' Experiments

Reactions 1a and 2a are quite slow, so that when a small volume of solution that contains only CO_2 at pH 4 is added to a buffer at pH 8.0 it takes in the order of 7s (30°) for half of the added CO_2 to be converted to HCO_3^-. Thus, transiently, the CO_2 concentration can be much higher than allowed by the

equilibrium conditions of reactions 1a and 2a. A similar disequilibrium is obtained upon the addition of HCO_3^- at pH 8.0, that is transiently, there is almost no CO_2 present. Filmer and Cooper (1970) made use of these transient disequilibrium situations to determine the form of C_i (the 'active' species) used by various carboxylases. The same method has been used very successfully to reveal that air-grown cyanobacteria possess the ability to actively transport both CO_2 and HCO_3^-, whereas cells grown with 1-5% CO_2 can actively transport only CO_2 (Abe et al. 1987a, b; Badger and Andrews 1982; Espie et al. 1984; Miller and Canvin 1987; Price and Badger 1989a; Volokita et al. 1984).

Stimulation by Added CA

In the absence of Na^+, air-grown cells of *Anabaena* and *Synechococcus* are unable to effectively transport HCO_3^- at pH 8.0 and above (Kaplan et al. 1984; Miller et al. 1984b; Miller and Canvin 1985; Abe et al. 1987 a, c). Under these conditions the cells must rely on the active CO_2 transport system to accumulate C_i and, at pH 8.0 in reasonably dense suspensions, the transport soon becomes limited by the rate at which CO_2 can be produced by reactions 1a and 2a. Under these conditions, the addition of CA greatly stimulates the rate of C_i transport (Fig. 1, Kaplan 1985; Miller and Canvin 1985). The simplest explanation is that CO_2 transport is stimulated by an increase in the CO_2 supply rate. Cells grown on high levels of CO_2 (1-5%) also respond to the addition of carbonic anhydrase (Price and Badger 1989b; Schwarz et al. 1988) demonstrating that these cells possess a CO_2 transport system.

Direct Observation of Disequilibrium

Using MIMS it is possible to determine when the system defined by reactions 1 and 2 is out of equilibrium. It has already been mentioned that when air-grown cells of *Synechococcus* are illuminated the extracellular CO_2 concentration falls far below the equilibrium value, even when CO_2 fixation is blocked. Active CO_2 transport by the cells is the only possible explanation for this observed disequilibrium (Badger and Andrews 1982; Miller et al. 1988a).

The Influence of C_i Concentration During Growth

Kaplan et al. (1980) found that *Anabaena* grown on high levels of CO_2 (5% v/v) had a much reduced capacity to transport C_i, at low C_i concentrations, than air-grown cells. Similar results have since been obtained by other workers with *Anabaena* and several strains of *Synechococcus* (Abe et al. 1987a; Badger and Andrews 1982; Miller and Canvin 1987; Omata and Ogawa 1986; Shiraiwa and Miyachi 1985). Cells grown on high levels of CO_2 lose the capacity for

HCO_3^- transport, but do retain a capacity for active CO_2 transport (Abe et al. 1987a; Badger and Gallagher 1987; Miller and Canvin 1987; Price and Badger 1989a; Schwarz et al. 1988). Transport of CO_2 by high CO_2-grown cells has a 5-10 fold lower CO_2 affinity than that seen in air-grown cells (Miller and Canvin 1987; Price and Badger 1989b). The transport of CO_2 by high CO_2-grown cells requires low levels of Na^+ ($K_{0.5}$ = 19 μM) at pH 8.0, but not at pH 6.1 (Miller and Canvin 1987). A requirement for low levels of Na^+ (<100 μM at pH 8.0) is also observed for CO_2 transport by air-grown cells (Espie et al. 1988b).

Work with *Synechococcus* in C_i-limited chemostats has revealed that the induction of the 'high CO_2 syndrome' does not actually require high CO_2, but only high C_i (Miller et al. 1984a). Cells grown under C_i-limited conditions ($0.28d^{-1}$) had a $K_{0.5}$ (C_i) for CO_2 fixation of 3 μM while cells under C_i-sufficient conditions ($1.72d^{-1}$) had a $K_{0.5}$ (C_i) of 1450 μM. This latter value is at least as high as the $K_{0.5}$ (C_i) seen for 'high CO_2' cells of the same strain (Miller and Canvin 1987). However, the steady-state CO_2 concentration in the chemostat reactor, which was closed to the atmosphere, was only 2.3 μM (Miller et al. 1984a). This CO_2 concentration is even lower than the approximately 12 μM CO_2 expected for solutions in equilibrium with air. The total C_i concentration in the C_i-sufficient reactor was high (2.85 mM) and because the pH was 9.6 almost all of it was as HCO_3^- (Miller et al. 1984a). Calculation of the rate of CO_2 fixation as a function of the extracellular CO_2 concentration showed that even these high C_i cells needed to accumulate C_i to levels 10 to 30-fold in excess of the extracellular C_i in order to grow at the observed rates (Miller et al. 1984a). This sort of accumulation is similar to that seen in high CO_2 - grown cells (Miller and Canvin 1987).

If a buffer at pH 9.0 is bubbled with air the HCO_3^- + $CO_3^=$ concentration should be about 5.45 mM and the CO_2 concentration about 12 μM, as determined by reactions 1 and 2. If a batch culture is bubbled with air vigorously enough at this pH, then it is possible to maintain this equilibrium C_i concentration in spite of the rapid CO_2 fixation by the cells. According to the chemostat studies, cells grown under thse conditions should be high C_i, not low C_i, cells because even though the CO_2 concentration is at air equilibrium levels (12 μM), the HCO_3^- concentration is high. The thorough studies of Badger and Gallagher (1987) and Mayo et al. (1986, 1989) fully confirm this predication. Badger and Gallagher (1987) showed that these high C_i (or high HCO_3^-) cells were repressed in terms of HCO_3^- transport, just like high CO_2 cells, but could actively transport CO_2. Turpin et al. (1985) predicted that for C_i concentrations during growth between about 40 μM and 1500 μM cells with characteristics intermediate between fully adapted low and high C_i cells should be found. As predicted, cells with intermediate $K_{0.5}$ (C_i) for CO_2 fixation were found under these conditions (Mayo et al. 1986). Badger and Gallagher (1987) later obtained very similar results. It is clear from these studies that air-bubbling of cultures will yield high affinity cells only when the rate of bubbling is inadequate. Measurements by MIMS and IRGA

(Ogawa et al. 1985a) show that the CO_2 transport system can reduce the extracellular CO_2 concentration to very low levels. If CO_2 is passed into the growth suspension at too slow a rate, then the CO_2 concentration in the medium will be very low so that very little conversion of CO_2 to HCO_3^- will occur in the medium, and the total C_i concentration will be far below the equilibrium value predicted by reactions 1 and 2.

High C_i cells convert to low C_i cells when the C_i concentration is lowered (Marcus et al. 1982, 1983). In the light the conversion is half-completed in about 2 h, but requires about 20 h for completion (Marcus et al. 1982). The conversion is much slower in the dark and is inhibited by spectinomycin, an inhibitor of protein synthesis (Marcus et al. 1982).

In aquatic ecosystems the C_i concentration is often in the range 40 to 1500 μM where cyanobacteria are expected to have intermediate $K_{0.5}$ (C_i) values, with the $K_{0.5}$ (C_i) being close in value to the actual C_i concentration in the water (Mayo et al. 1986; Turpin et al. 1985). Such a situation allows the cells to readily sense changes in the C_i concentration and respond by altering the kinetics of their C_i uptake (Turpin et al. 1985). The mechanistic basis of the intermediate $K_{0.5}$ (C_i) values is unknown, but may perhaps be a reflection of different proportions of the high affinity CO_2 transporter and the lower affinity HCO_3^- transporter.

Possible Mechanisms for C_i Transport

It is now well established that cyanobacteria possess the ability to couple the transport of CO_2 and HCO_3^- to the expenditure of metabolic energy. How these molecules are actually translocated across the plasmalemma and how the translocation is coupled to energy expenditure remain unknown. For a while it was thought that a 42 kilodalton polypeptide, found only in the membrane of low C_i adapted cells, might be involved in C_i transport (Omata and Ogawa 1986). However, Schwarz et al. (1988) have demonstrated convincingly that a *Synechococcus* mutant unable to synthesize this polypeptide was not impaired in either HCO_3^- or CO_2 transport.

The first point of debate about the mechanism of CO_2 and HCO_3^- transport is whether or not two separate transport systems are involved (cf. Abe et al. 1987a; Espie et al. 1988b; Price and Badger 1989b; Scherer et al. 1988; Volokita et al. 1984). Badger and Andrews (1982) were the first to suggest that CO_2 and HCO_3^- might be substrates for a single complex transport system. Volokita et al. (1984) put forward a more explicit model (Fig. 2A) that involved a 'HCO_3^- porter' as the actual translocating element in the complex. According to this model, active CO_2 transport involves the conversion of CO_2 to HCO_3^- in the membrane by a 'CA-like moiety' (Fig. 2A). The HCO_3^- ions so formed are passed on to the 'HCO_3^- porter' for translocation to the cytoplasmic side of the membrane. A feature of this model is that it is HCO_3^- that arrives in the cytoplasm both during HCO_3^- and CO_2

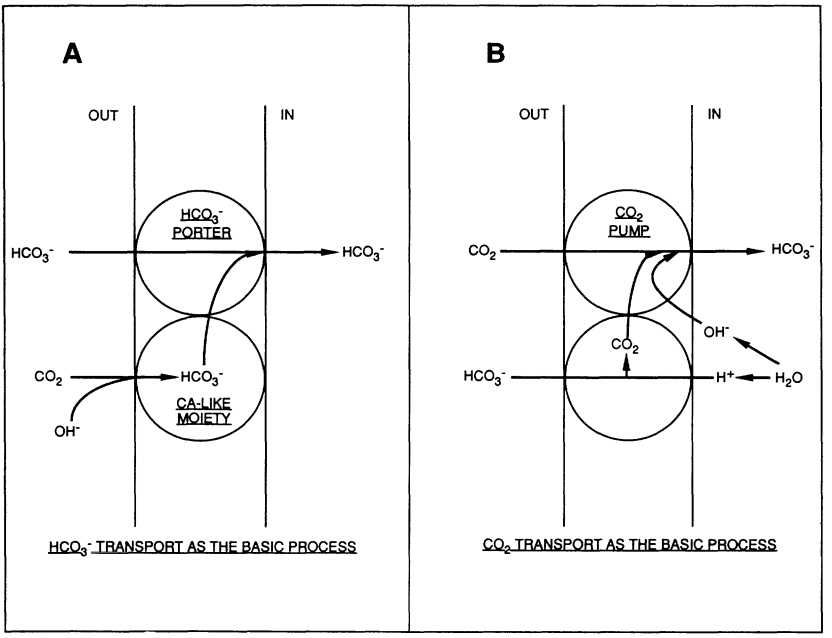

Fig. 2. Two models that have been proposed for a complex system capable of transporting both CO_2 and HCO_3^-. Model (A) is based upon a diagram in Volokita et al. (1984) and Model (B) is based upon a diagram in Price and Badger (1989a).

transport (Volokita et al. 1984). Price and Badger (1989a) have also put forward a model in which CO_2 and HCO_3^- are functionally linked (Fig. 2B). The model is similar to that of Volokita et al. (1984) in that CO_2 is converted to HCO_3^- by a 'CA-like moiety' in the membrane (Fig. 2B). In all other respects, it differs radically. In this model the 'CO_2 porter', not the 'HCO_3^- porter', is the central translocating element of the C_i transport complex (Price and Badger 1989a). The function of the 'CA-like moiety' is now to convert, in the membrane, HCO_3^- to CO_2 which is then passed on to the 'CO_2 porter' (Fig. 2B). Volokita et al. (1984) apparently think the 'CA-like moiety' is only similar to CA in that it catalyses the conversion of CO_2 to HCO_3^- and is apparently inhibited by 10 μM ethoxyzolamide. Abe et al. (1987a) suggests it is actually CA. Price and Badger (1989a) suggest that the catalysis of HCO_3^- to CO_2 may be due to a localized region of low pH in the membrane caused, perhaps, by H^+-ATPase activity. Whatever the 'CA-like moiety' may be, it clearly has no extracellular CA activity (e.g. Badger et al. 1985).

The evidence for a tight functional coupling of CO_2 and HCO_3^- transport (Fig. 2A,B) is weak. Volokita et al. (1984) found a mutual inhibition of CO_2 and HCO_3^- transport, but the kinetics of the inhibition were complex and indicated a non-competitive inhibition (Scherer et al. 1988). We have found that the onset of rapid HCO_3^- transport, caused by the additiion of the

required Na^+, has little or no effect upon the ongoing rate of CO_2 transport at low CO_2 concentrations (Espie et al. 1988a). Also, the uptake of CO_2 measured by MIMS following a pulse of CO_2 is not much affected by increases in the background HCO_3^- concentration (Espie, Miller and Canvin, unpublished). Price and Badger (1989a,b) have found that high concentrations of the CA-inhibitor ethoxyzolamide inhibited CO_2 and HCO_3^- transport equally in low C_i cells of *Synechococcus*. This result is consistent with their model if it is assumed that ethoxyzolamide inhibits the 'CO_2 porter' (Fig. 2B, Price and Badger 1989a). We have found, however, that carbonyl sulphide (COS) and H_2S selectively inhibit CO_2 transport and leave HCO_3^- transport quite unaffected (Miller et al. 1989; Espie et al. submitted).

The model (Fig. 2A) put forward by Volokita et al. (1984) became considerably less economical with the observation that it is CO_2 transport that is constitutive (Abe et al. 1987; Badger and Gallagher 1987; Miller and Canvin 1987). It becomes unclear why high C_i cells should be able to actively transport CO_2 with a 'CO_2 porter' while low C_i cells require a new CA-like moiety to convert the CO_2 to HCO_3^- so it can be passed on to a 'HCO_3^- porter' (Fig 2A). In the model of Price and Badger (1989a) it is HCO_3^- transport that becomes the complex process (Fig. 2B). In animal cells, HCO_3^- is transported straightforwardly across membranes as the anion (Boron 1983). It is too early to rule out this possibility for cyanobacteria.

Other bacteria have multiple systems even for one substrate and separate systems for the transport of substrates as different as CO_2 and HCO_3^- might be expected. In this context, I will now briefly discuss possible separately mechanisms for CO_2 and HCO_3^- transport.

CO_2 Transport

The lack of data allows for a very large degree of freedom in drawing up models for CO_2 transport. Some possibilities are sketched in Fig. 3. A 'CA-like moiety' could be involved in CO_2 transport (Fig. 3A) even if it is not part of a complex that also transports HCO_3^- (Fig. 2). Teleologically, it makes sense to convert the lipid soluble CO_2 to the rather membrane impermeable HCO_3^- (Gutknecht et al. 1977) to reduce the size of the inside-to-outside CO_2 gradient. However, it is experimentally difficult to separate model A (Fig. 3), which involves the 'CA-like moiety' as an integral membrane protein, and perhaps being the 'CO_2 porter' itself, from model B (Fig. 3) in which CO_2 is translocated into the cytoplasm as CO_2 where most of it is converted to HCO_3^- by a peripherally bound CA. Volokita et al. (1984) initially put forward their model involving a 'CA-like moiety' (Fig. 3A) because they found that the rate of CO_2 fixation, in low C_i cells of *Anabaena*, was dependent solely upon the intracellular C_i concentration and it made no difference whether CO_2 or HCO_3^- was the substrate for transport. In the absence of any intracellular CA, as postulated by Volokita et al. (1984), one would have

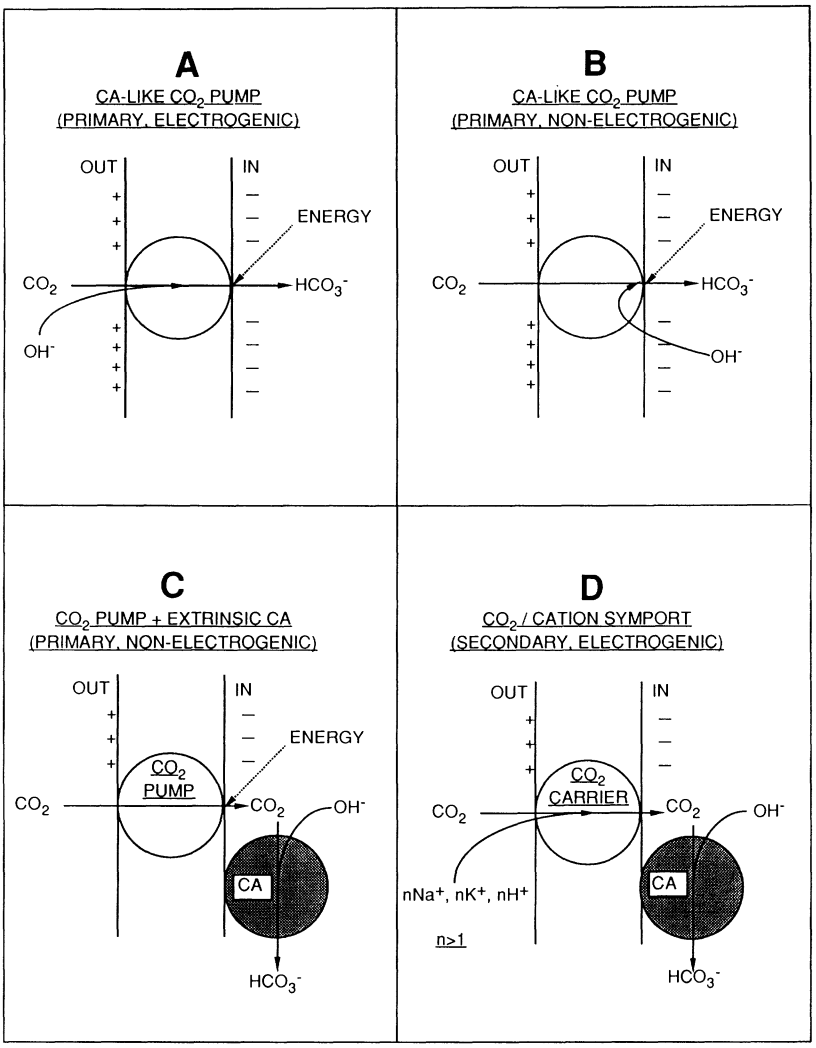

Fig. 3. Models for CO_2 transport.

expected CO_2 transport to have been a better souce of C_i than HCO_3^- transport for the CO_2-using RuBisCo. When Volokita et al. (1984) put forward their model (Fig. 3A) the evidence for the presence of intracellular CA activity in cyanobacteria was conflicting. It is clear now, however, that cyanobacteria must, and do, contain CA in sufficient amounts to rapidly catalyse the conversion of the intracellular HCO_3^- to CO_2 for use by RuBisCo (Badger et al. 1985; Badger and Price 1989). At present, the most convincing evidence for the involvement of a 'CA-like moiety' in CO_2 uptake (Fig. 3A, B) is that uptake is much reduced by ethoxyzolamide, a classical CA inhibitor (Price and Badger 1989a,b; Volokita et al. 1984). Price and Badger (1989a, b)

found that ethoxyzolamide blocked CO_2 transport by both high and low C_i *Synechococcus* without affecting the intracellular CA activity. High concentrations of ethoxyzolamide were required to inhibit CO_2 transport (Price and Badger 1989a). Ethoxyzolamide at 200 μM inhibited CO_2 transport by high C_i by 75% at 100 μM CO_2. Low C_i cells were even less sensitive. Still, several vertebrate isozymes of CA are very resistant to ethoxyzolamide inhibition, with K_i values being in the range of 150 μM (Maren and Sanyal 1983). The high concentrations needed to inhibit CO_2 transport (Price and Badger 1989a) may also have been due to limited access of the inhibitor to the putative 'CA-like moiety'. The degree of inhibition correlated with the lipid solubility of the various sulfonamides with the least soluble, acetazolamide, having very little effect on CO_2 transport even at 400 μM. The important feature of the results of Price and Badger (1989a) is that ethoxyzolamide inhibited CO_2 transport, but had no effect on intracellular CA activity. Volokita et al. (1984) found that CO_2 transport by *Anabaena* was very sensitive to inhibition by ethoxyzolamide, with 10 μM causing 68% inhibition at 100 μM CO_2 even with low C_i cells. They did not study the effect of ethoxyzolamide on the intracellular CA activity. This is unfortunate because at the very high CO_2 concentrations used in the transport assay (100 μM) an inhibition of the peripheral CA of model B (Fig. 3) would allow the intracellular CO_2 concentration to rise very rapidly. Volokita et al. (1984) used the SFC method to measure CO_2 transport and took the first sample nominally at 5s, but, given the spin-down time, it was probably actually closer to 10s (Miller et al. 1988a). In this time very appreciable back leakage of unconverted CO_2 into the medium would be predicted by model B (Fig. 3) if the CA were inhibited. Price and Badger (1989a) found that high C_i *Anabaena* was not substantially inhibited by 10 μM ethoxyzolamide, but was inhibited about 65% by 200 μM.

We have found that H_2S/HS^- is an inhibitor of CO_2 transport, but not of HCO_3^- transport, in low C_i cells of *Synechococcus* (Espie, Miller and Canvin, submitted). The rate of HCO_3^- transport-dependent CO_2 fixation is not severely inhibited, so it is unlikely that the necessary intracellular CA activity (Badger et al. 1985) is inhibited. Hydrogen sulphide is a potent inhibitor of both plant and animal CAs because it, like ethoxyzolamide, binds to the essential Zn atom in the active site (Maren and Sanyal 1983). The transport of CO_2 by *Synechococcus* was more readily inhibited by the lipid soluble H_2S than the less soluble HS^- ion (Espie et al. submitted). It is tempting to suggest that the inhibition is at a 'CA-like moiety' as in model A (Fig. 3).

Carbonyl sulphide, COS, is a structural analogue of CO_2 that inhibits CO_2 transport and is itself transported (Miller et al. 1989). The uptake of COS, presumably by the 'CO_2 porter', is inhibited by H_2S/HS^- and ethoxyzolamide. The concentration of ethoxyzolamide required (50 μM) does not seem to inhibit the intracellular CA activity (Miller et al. unpublished). This is consistent with the involvement of a 'CA-like moiety' in the transport of COS. The uptake of COS by *Synechococcus* results in an almost stoichiometric

conversion of COS to H_2S and CO_2 (Miller et al. 1989). By the use of ^{14}C-labelled COS it may be possible to determine whether COS hydration to H_2S and CO_2 is an obligate process in COS transport, as postulated by the 'CA-like moiety' model (Fig. 3A). If CO_2 transport does occur *via* a 'CA-like moiety' then the source of the required OH^- could be the extracellular medium (Ogawa and Kaplan 1987a) or the cytoplasm (Price and Badger 1989a). In the former case the translocation of CO_2 across the membrane would be electrogenic and in the latter case it would not (Fig. 3). Ogawa and Kaplan (1987a) found a 1:1 stoichiometry between C_i uptake, apparently as CO_2, and proton release into the medium. They suggested that the acidification was due to the uptake of OH^- from the extracellular medium. However, the regulation of intracellular pH would require the rapid efflux of protons from the cells into the medium even if the source of OH^- for the conversion of CO_2 to HCO_3^- was the cytoplasm (Fig. 3A). The pH measurements of Ogawa and Kaplan (1987a) were too slow to determine the source of the OH^- (Fig. 3A, B).

The uptake of C_i, apparently as CO_2, by the chemoautotroph *Thiobacillus neapolitanus* was greatly reduced when the $\Delta\Psi$ across the membrane was abolished by valinomycin (Holthuijzen et al. 1987). Upon the addition of nigericin the $\Delta\Psi$became more negative, and the rate of C_i uptake increased. Thus, a key role for the $\Delta\Psi$ across the plasmalemma is indicated for C_i transport in *T. neapolitanus* (Holthuijzen et al. 1987). The authors suggest that CO_2 transport in this species may involve a cation (H^+, K^+ or Na^+/CO_2 symport system (Fig. 3C). Whether such a system could drive CO_2 transport in cyanobacteria is unclear. Transport of CO_2 is stimulated by low concentrations of Na^+ (Miller and Canvin 1987; Espie et al. 1988b) but in high C_i cells there is no evidence for concommitant Na^+ uptake (Miller and Canvin 1987).

The mode of energy coupling to CO_2 transport is unknown, although in high C_i *Anabaena* cells it does require photosystem I activity (Ogawa and Ogren 1985). It is not known whether CO_2 transport is by primary active transport coupled directly to the expenditure of metabolic energy (e.g. Fig. 3A,B) or whether it is a secondary active transport system driven by the movement of some cation down its electrochemical gradient (Fig. 3C). None of the published models, or those in Fig. 3, fully explain how $\Delta\Psi$ or intracellular pH would be regulated during CO_2 transport. For example, Ogawa and Kaplan (1987b) indicate that during CO_2 transport by an electrogenic system, similar to that in Fig. 3A, there was no influx of Na^+ nor efflux of Cl^-. The experiments were carried out in a K^+-free medium, so no K^+ influx could have occurred. One is left wondering how a reasonable value for $\Delta\Psi$ would be maintained during CO_2 transport according to the model in Fig. 3A.

HCO_3^- Transport

Probably the most noteworthy feature of HCO_3^- transport by cyanobacteria is that it usually requires the presence of Na^+. This is true of unicellular species such as *Synechococcus* UTEX 625 (Espie et al. 1988b; Miller et al. 1984b) and *Synechocystis* PCC6803 (Miller, unpublished) and filamentous species such as *Anabaena variabilis* (Abe et al. 1987b; Kaplan et al. 1984; Reinhold et al. 1984); and *Oscillatoria* sp. (Miller, unpublished). The concentration of Na^+ required for HCO_3^- transport varies with both extracellular pH and C_i concentration (Espie et al. 1988b). At pH 8, about 30 mM Na^+ is required for maximal rates of HCO_3^- transport by both *Synechococcus* (Espie et al. 1988b) and *Anabaena* (Kaplan et al. 1984). Below pH 7, considerable C_i transport takes place without millimolar Na^+ concentrations (Abe et al. 1987). It is difficult to decide whether C_i transport below pH 7 occurs in the absence of Na^+ because of a H^+/HCO_3^- symport (Abe et al. 1987b) or as CO_2 transport. The difficulties of separating HCO_3^- from CO_2 transport when one is analysing the effect of changes in Na^+ and C_i concentration have been outlined by Espie et al. (1988b). For example, it has been considered that the main effect of Na^+ is on the apparent affinity of HCO_3^- for the transporter, without having a significant effect on V_{max} (Reinhold et al. 1984). This conclusion was based on the observations that high rates of C_i transport occur even in the absence of Na^+ when the C_i concentration is raised (Miller et al. 1984; Reinhold et al. 1984). However, it is just as likely that HCO_3^- transport remains inhibited in the absence of Na^+ even when the extracellular C_i concentration is raised to high levels. The increasing rate of C_i transport would be due to increasing rates of CO_2 transport as the C_i concentration is raised (Espie et al. 1988b). As the C_i concentration is increased, CO_2 transport becomes more favourable as both the absolute concentration of CO_2 increases and its rate of formation from HCO_3^- increases (Miller 1985).

The role of Na^+ in HCO_3^- transport cannot be replaced by K^+, Li^+ or Cs^+ (Kaplan et al. 1984; Miller et al. 1984b). In fact, Li^+ is a competitive inhibitor with respect to Na^+ (Espie et al. 1988b; Kaplan 1984). It has been postulated that Na^+ may be involved in a Na^+/HCO_3^- symport system (Abe et al. 1988; Kaplan et al. 1984; Miller et al. 1984b). This possibility will be discussed in more detail later. It is unfortunate that while we have convincing evidence that Na^+ stimulates HCO_3^- transport, we have no information regarding the effect of HCO_3^- on Na^+ transport.

Most experiments on HCO_3^- transport are carried out with cells harvested from rapidly growing cultures. As just described, these cells require Na^+ for HCO_3^- transport. Espie and Canvin (1987), however, found that when cells of *Synechococcus* UTEX 625 were harvested from slow-growing standing cultures they possessed a high level of Na^+-independent HCO_3^- transport. When these cells were inoculated into medium bubbled with air they lost this Na^+-independent HCO_3^- transport within 48 h (Espie and Canvin 1987). The

reverse process, the conversion of Na^+-dependent to Na^+-independent cells, could be carried out but only with a considerable (5-7 days) lag period (Espie and Canvin 1987). Li^+, which inhibits Na^+-dependent HCO_3^- transport (Espie et al. 1988b; Kaplan et al. 1984), had little effect upon the Na^+-independent HCO_3^- transport process (Espie and Canvin 1987). No specific cation or anion requirement could be demonstrated for the Na^+-independent HCO_3^- transport process (Espie and Canvin 1987). It remains to be seen whether the Na^+-dependent and Na^+-independent processes are due to separate HCO_3^- transport systems.

Numerous models for HCO_3^- transport in cyanobacteria could be put forward. Many of them would have some precedence in the extensive work on HCO_3^- transport in giant cell algae, aquatic macrophytes and various animal cells (Boron 1983; Lucas 1983; Smith 1988).

Primary Electrogenic HCO_3^- Transport

Kaplan et al. (1982) found the $\Delta\Psi$ became more negative upon the addition of 1mM $NaHCO_3$, but not NaCl, to cells of *Anabaena* at the CO_2 compensation point. The $\Delta\Psi$ was measured by uptake of the lipophilic cation tetraphenylphosphonium. The $\Delta\Psi$ dropped from about -74mV to about -89mV upon the addition of HCO_3^- (Kaplan et al. 1982). Such a hyperpolarization would occur if HCO_3^- transport across the plasmalemma was unaccompanied by a charge balancing ion (Fig. 4A). This model for a primary HCO_3^- pump was put forward before evidence for active CO_2 transport had accumulated (Kaplan et al. 1982). With hindsight, one can no longer be so sure that only the effects of HCO_3^- transport on $\Delta\Psi$ were being observed, especially since a high concentration of C_i (1mM) was used. At this concentration the rate of CO_2 formation from HCO_3^- would be sufficient to support considerable uptake by the CO_2 transport system (Miller 1985; Espie et al. 1988b). In fact, the $\Delta\Psi$ dropped from about -74mV to about -83mV in high C_i cells (Kaplan et al. 1982) that probably had only CO_2 transport capacity (Abe et al. 1987a). If CO_2 transport involves an obligatory conversion to HCO_3^- (Fig. 3A), it may also be electrogenic. These interesting experiments need to be repeated at lower C_i concentrations, and the degree of hyperpolarization needs to be better correlated with the actual rate of HCO_3^- transport. For primary electrogenic HCO_3^- transport (Fig. 4A) there would have to be an uptake of a cation or efflux of an anion. In the absence of any charge balancing flux the $\Delta\Psi$ would rise by about $9mV.ms^{-1}$ (given a normal membrane capacitance). An electrophoretic uptake of Na^+ would serve this purpose and would explain the Na^+-dependence of HCO_3^- transport in air-grown *Anabaena* and *Synechococcus* (Espie et al. 1988b; Kaplan et al. 1984; Miller et al. 1984b).

Primary Electroneutral Transport of HCO_3^-

Zenvirth et al. (1984) have shown that OH^- efflux (or H^+ influx) down its

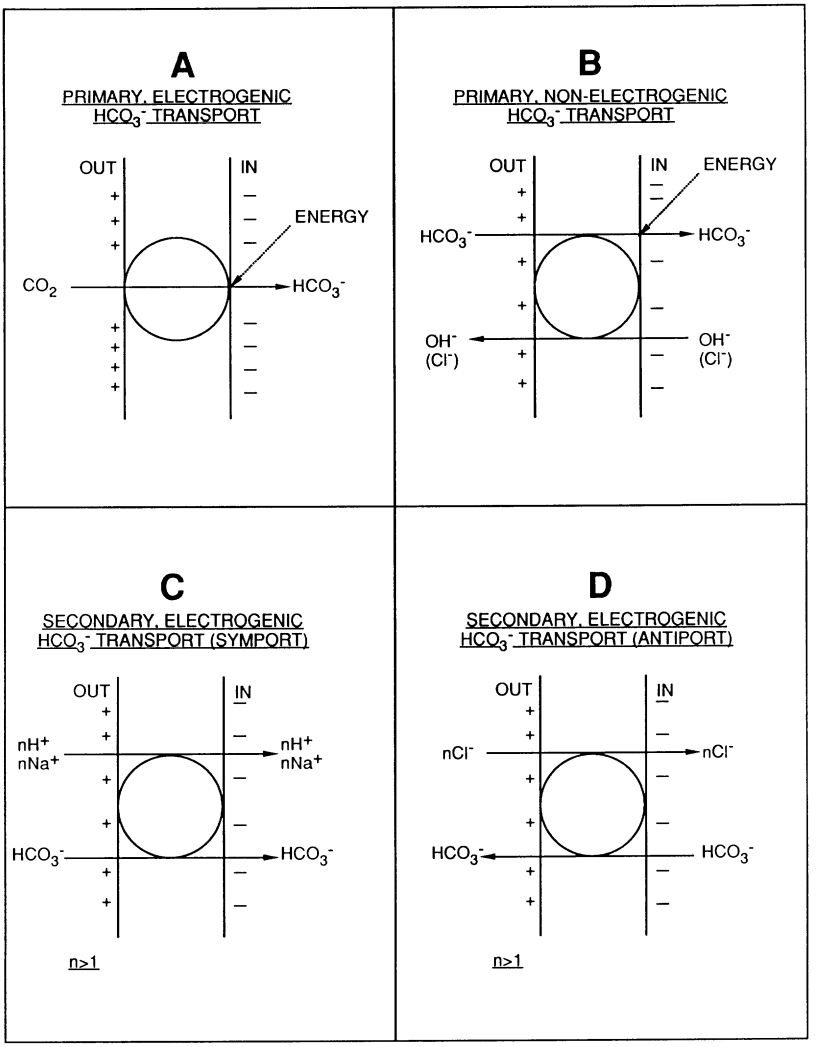

Fig. 4 Models for HCO_3^- transport.

electrochemical gradient could not drive the electroneutral exchange influx of HCO_3^- in *Anabaena*. Given the measurements of intracellular pH (Coleman and Colman 1981), $\Delta\Psi$ (Budd and Kerson 1987) and the C_i accumulation ratio (Shelp and Canvin 1984) the same can be said for *Synechococcus*. Thus, if electroneutral HCO_3^-/OH^- exchange were to be a means of accumulating the usual high levels of C_i, the exchange process would have to be directly coupled to the expenditure of metabolic energy (Fig. 4B). Kaplan (1981a) did not measure significant OH^- efflux during the first 20 to 40s of HCO_3^- transport by air-grown *Anabaena*. This certainly suggests that, in these cells at least, HCO_3^-/OH^- exchange was not occurring (Kaplan 1981a). However,

it is known that the properties of HCO_3^- transport by *Synechococcus* UTEX 625 are different for cells growing rapidly in bubble culture compared to standing culture cells (Espie and Canvin 1987). The former require high levels of Na^+, at pH 8.0, for HCO_3^- transport, whereas the latter do not (Espie and Canvin 1987). Perhaps in the standing culture cells HCO_3^-/OH^- exchange occurs, whereas in rapidly growing cells it does not.

There is no evidence that Cl^- acts as a counter ion for HCO_3^- transport by cyanobacteria, although it does so frequently in animal cells (Boron 1983; Smith 1988).

Secondary Active Transport of HCO_3^-

Active transport of an ion may be either 'primary' (directly coupled to the hydrolysic of ATP or electron transport) as in the case of the two models just discussed (Fig. 4A,B) or it may be 'secondary' (energized by the movement of another solute down its electrochemical gradient) (Fig. 4C). The electrochemical potential difference for HCO_3^- accumulated 1000-fold against a $\Delta\Psi$ of -120mV (Budd and Kerson 1987; Miller et al. 1984), is the sum of $\Delta\Psi$ and 60 log (C_i/C_o) and equals 300 mV. To raise the HCO_3^- ions to this electrochemical potential requires the expenditure of a substantial amount of free energy. For secondary active transport (Fig. 4C) the change in electrochemical potential for the driver ion would be a decrease of about -300mV. At an extracellular pH of 8.0 the intracellular pH, in the light, is about 7.5 (Coleman and Colman 1981) so that with a $\Delta\Psi$ of about -120mV the inward driving force for H^+ is only -90mV. A symport stoichiometry of almost $3H^+$ to I HCO_3^- would be required to drive the secondary active transport of HCO_3^- at pH 8.0 (Fig. 4C). If we assume that the intracellular Na^+ concentration is about 5mM upon harvest (Dewar and Barber 1973) then at $pH_0 = 8$, $\Delta\Psi$ = -120mV and 25mM Na^+ (Espie et al. 1988b) the driving force for Na^+ leakage into the cell will be about -160mV. A symport stoichiometry of slightly more than $2Na^+$ to 1 HCO_3^- could thus drive secondary transport of HCO_3^- (Fig. 4C). Two lines of evidence argue against the routine involvement of H^+ and Na^+ as driver ions. Firstly, secondary active transport is, in itself, electrogenic and should result in a transient, at least, drop in (Fig. 4C). This does not agree with the hyperpolarization observed by Kaplan et al. (1982). Secondly, for the stoichiometries considered, the rate of HCO_3^- transport would be very dependent on the magnitude of $\Delta\Psi$. The $\Delta\Psi$ of *Synechococcus* UTEX 625 is lowered by about -40mV upon the addition of 10m M K^+ (Budd and Kerson 1987), presumably because of electrogenic K^+ transport (Abe et al. 1988). Such a drop in the magnitude of $\Delta\Psi$ should reduce the rate of HCO_3^- transport very considerably for the driver ion stoichiometries we have considered. In fact, transient depolarization of the membrane by K^+ addition had little effect upon the rate of HCO_3^- transport (Miller and Canvin, unpublished). Active transport of HCO_3^- appears more likely to be a primary process (Fig. 4A, B) with other cations and anions acting solely as charge balancing agents.

The Role of Intracellular CA and the Problem of CO_2 Leakage

The raison d'etre for active C_i accumulation in cyanobacteria is to permit CO_2 fixation at CO_2 concentrations where RuBisCo by itself would be ineffective. This means that the CO_2 concentration must be raised to high levels around the active sites of the RuBisCo. The conceptual problem that immediately arises is, given the high lipid solubility of CO_2, how can such a high concentration gradient for CO_2 be maintained anywhere within such small cells without the need for a massive energy expenditure to overcome the rapid leakage of CO_2 (see Raven and Lucas 1985). The answer to the problem is that the leakage rate of CO_2 from the cells is much lower than expected. Thus, the passive permeability of cyanobacterial cells appears to be several orders of magnitude lower than that previously reported for any cell or bilayer lipid membrane (Badger et al. 1985; Gutknecht et al. 1977; Ogawa and Kaplan 1987b). As Badger et al. (1987) have pointed out, the measured resistance to CO_2 efflux from a single cell of *Synechococcus*, with a diameter of less than 2 microns, corresponds to the resistance offered to CO_2 diffusion by an unstirred layer of water, around the cell, 1 cm thick! This low passive permeability of the cells to CO_2 explains how the CO_2 concentration around the RuBisCo can be raised to high levels, without there being a back leakage of CO_2 so high that it would place impossible energetic demands upon the cell (Raven and Lucas 1985). But where in the cell is this barrier, with such an anomalously high resistance to CO_2 diffusion, located? What is its structure?

At first thought it is reasonable to assume that this barrier to CO_2 leakage resides in or near the plasmalemma. Two major objections to this location soon become apparent. Firstly, Walsby (1985) has used an elegant technique, based on the rate of collapse of gas vacuoles under pressure, to measure the permeability of *Anabaena* heterocysts and vegetative cells to gases. He found that the permeability of vegetative cells to N_2 was quite high, with $P(N_2)$ being in the order of 10^{-3} cm.s.$^{-1}$. Values of $P(CO_2)$ in the range of 1 to 6 x 10^{-6} cm.5[1] have been estimated (Badger et al. 1985; Ogawa and Kaplan 1987b). It is difficult to believe that a bilayer lipid membrane could be constructed that would be 1000-fold more permeable to N_2 than to CO_2. There is also a physiological reason why a gas impermeable plasmalemma is unlikely. As Badger et al. (1985) first noted, a plasmalemma with a high resistance to CO_2 diffusion would undoubtedly also restrict O_2 movement. Oxygen is, of course, produced during photosynthesis and Badger et al. (1985) calculated, that for normal rates of photosynthesis, a plasmalemma having the required resistance to CO_2 leakage would cause the intracellular O_2 concentration to be as high as 7 mM, or 5.9 atmospheres of oxygen. Such high intracellular O_2 concentrations would undoubtedly be toxic. Overall, then, it seems unlikely that the resistance to CO_2 leakage is at or near the plasmalemma.

Most of the functional RuBisCo of cyanobacteria, grown under conditions of limiting CO_2 concentration, is contained in polyhedral shaped bodies

called carboxysomes (Codd and Marsden 1984; Coleman et al. 1982; Shively 1988). The carboxysomes are 100-200 nanometers in diameter with a 3-4 nanometer thick membrane or shell surrounding them (Codd and Marsden 1984; Shively 1988). In addition to RuBisCo they also contain CA activity, at least in *Synechococcus* (Badger and Price 1989). They do not appear to contain any enzymes of the Calvin cycle other than RuBisCo (Shively 1988). Beudeker et al. (1981) were the first to suggest that the carboxysomes might be impermeable to gases. They raised the possibility that they might be impermeable to O_2, thus reducing the *in vivo* oxygenase activity of RuBisCo. Coleman et al. (1982) isolated carboxysomes from the cyanobacterium *Coccochloris peniocystis* and reported that the RuBisCo contained in isolated, intact carboxysomes showed no oxygenase activity whereas solubilized RuBisCo did. If cyanobacterial carboxysomes were to be impermeable to O_2 they would probably be also rather impermeable to CO_2. Reinhold et al. (1987) have put forward an intriguing model that does place the diffusion barrier to CO_2 at the carboxysomes. This model postulates that:

1. It is HCO_3^- that arrives in the cytoplasm, even when CO_2 is the transported C_i species (see Fig. 3A,B).
2. CA is found *only* in the carboxysomes, not in the cytoplasm.
3. The resistance to HCO_3^- and RuBP uptake and to PGA and OH^- efflux is reasonably low ($P(HCO_3^-) = 10^{-4}$ cm.s^{-1}).
4. The resistance to efflux of CO_2, generated from CA catalyzed HCO_3^- dehydration within the carboxysomes, is high ($P(CO_2) = 10^{-5}$cm.s^{-1}).

Regarding the first postulate, it remains to be clearly demonstrated that CO_2 is converted to HCO_3^- during its transport, as previously discussed. The cellular location of CA (postulate 2) becomes critical if the carboxysome is to be the site of the CO_2 diffusion barrier. There is now no doubt that cyanobacteria do, in fact, contain intracellular CA activity, although the amount seems to vary from species to species. In some strains or species it can be measured quite readily in cell-free extracts by the classic electrometric, pH change technique (Döhler 1974; Firius et al. 1982; Firius et al. 1985; Ingle and Colman 1975; Komarova et al. 1976; Yagawa et al. 1984). In other strains or species the CA content is lower and is best measured by MIMS to monitor the rate of ^{18}O-exchange from ^{18}O-labelled HCO_3^- (Badger et al. 1985; Badger and Price 1989; Spiller et al. 1988; Tu et al. 1987). This can be done with intact cells as well as with cell-free extracts. While it is now well established that cyanobacteria do contain CA, its location is less certain. Badger and Price (1989) found with *Synechococcus* that much, but not all, of the CA pelletted with the RuBisCo activity. Yagawa et al. (1984) found that some strains of *Anabaena* had only soluble CA whereas other strains had both soluble and insoluble CA. Firius et al. (1985) did not find a good correlation between the distribution of CA and RuBisCo activity on sucrose density gradients when *Synechococcus cedrorum* was examined. Considerable CA activity seemed to be associated with the thylakoid fraction (Firius et al. 1985). Lanaras et al. (1985) presented convincing evidence that CA activity in the filamentous

species *Chlorogloeopsis fritschii* was not specifically associated with the carboxysomes or thylakoids. Much of the CA activity was associated with a membrane fraction that may have included the plasmalemma. It is obvious that determining the exact location, or locations, of CA within the cyanobacterial cell has become an important endeavour if CO_2 retention is to be understood. If CA does exist in locations other than the carboxysome then the HCO_3^-/CO_2 system might be in equilibrium throughout the cell and then the carboxysome could not be the main location of the CO_2 diffusion barrier. Since the location of CA is so important for the mechanism of CO_2 retention it is puzzling that such different intracellular localizations have been reported.

The physical properties of the carboxysome shell that would confer upon it such a low permeability to CO_2, but a reasonably high permeability to the anions HCO_3^-, RuBP, PGA and OH^-, are unknown. It is tempting to postulate that the carboxysome shell might contain proteins similar to the so-called 'band 3' proteins that are so important in the movement of anions across the red blood cell membrane (Boron 1983). An analogy has been made between the carboxysome shell and the gas vesicle of cyanobacteria, with the assumption being made that the latter is impermeable to gases (Coleman et al. 1982). This analogy is erroneous. The gas vesicle is, in fact, highly permeable to gases (Hayes 1988; Walsby 1969). The gas vesicle functions as a bouyancy regulating device not because it is impermeable to gases, but because it is impermeable to liquid water (Walsby 1969). This impermeability to liquid water is more a function of the small size and rigidity of the gas vesicle than any innate permeability properties of the shell itself. The small size and rigidity prevent the inward flow of liquid water because of high surface tension forces (Walsby 1969). If the carboxysome shell is impermeable to CO_2, but permeable to anions it must have a rather interesting physical chemistry. Unfortunately, carboxysomes isolated from cyanobacteria usually do not retain the postulated impermeability to CO_2 and O_2 and the initial results of Coleman et al. (1982) have not yet been reproduced (Badger 1987). It should be noted that some strains and species of *Thiobacillus* do not seem to produce carboxysomes even when they are growing chemoautotrophically (Codd and Marsden 1984). This suggests that the carboxysome is not the only site of CO_2 retention.

Genetic Analysis of C_i Transport

No specific gene or gene product involved in C_i transport has been recognized, but mutants able to grow on high but not low concentrations of C_i have been isolated. They are usually termed high CO_2-requiring mutants (Abe et al. 1988; Marcus et al. 1986; Ogawa et al. 1987c; Schwarz et al. 1988). Mutants were isolated following a sequence of mutagenesis with nitrosoguanidine, penicillin enrichment, formation of colonies by growth on solidified medium under 5% CO_2 and eventual replica plating of these

surviving cells onto plates incubated in air. Colonies able to grow on the plates in 5% CO_2 but unable to grow on the plates in air were selected as putative high – CO_2 requiring mutants. All the published results have concerned mutants of *Synechococcus* PCC 7942 which is a transformable strain (Golden et al. 1989). Most of the mutants are deficient in the ability to effectively use accumulated C_i rather than in the ability to transport C_i (Marcus et al. 1986; Ogawa et al. 1987, c,d; Schwarz et al. 1988). Although the exact nature of the defects is unclear, these mutants have been useful in experiments in which it was useful to have CO_2 fixation blocked (Ogawa and Kaplan 1987a). One served to convincingly demonstrate that the 42kD membrane polypepticle had no obvious role in C_i transport (Schwarz et al. 1988). Price and Badger (1979a) have suggested that the use of the sensitive ^{18}O-exchange assay may reveal that some of these mutants are deficient in intracellular CA. Such mutants would be very useful indeed (see Fig. 3). The first mutants actually deficient in C_i transport were isolated by Abe et al. (1988). They selected cells that were high CO_2-requiring at 40°C, but not at 30°C. These high CO_2 mutants were unable to accumulate C_i from low concentrations of C_i (55 μM) at 40°, whereas the wild-type could (Abe et al. 1988). With the ability to isolate mutants defective in C_i transport and various early steps of CO_2 fixation, and with the ability to genetically transform *Synechococcus* PCC 7942, a genetic analysis of the transport processes is imminent.

Acknowledgements

I sincerely thank Dr. David Canvin in whose laboratory I have had the privilege to work for the past number of years. In addition to financial support Dr. Canvin has provided constant encouragement and intellectual input. I thank my colleague, Dr. George Espie for many helpful discussions as well as technical help. For helpful discussions, I thank Dr. Brendan Birmingham, Dr. Ken Budd, Dr. Brian Colman, Dr. Ron Smith, Dr. David Turpin, and Mr. Harold Weger. I thank Mr. Doug Birch for expert technical assistance with the mass spectrometer and for preparing Fig. 2-4.

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3. Hydrogenases in Lithoautotrophic Bacteria

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Introduction

Hydrogen is a major element of the cell, it plays a key role in the biological energy cycle and thus in the cycle of matter on earth. The ability to consume or to evolve molecular hydrogen (H_2) is widespread among prokaryotes (Schlegel and Schneider 1985) but rare in eukaryotic cells like algae (Stuart and Gaffron 1972), protozoa (Lindmark and Müller 1973) or higher plants. Hydrogen is metabolized in aerobic, facultatively anaerobic, strictly anaerobic or phototrophic eubacteria (both Gram-negative or Gram-positive), or in archaebacteria.

Molecular hydrogen has not accumulated in the atmosphere during the evolution of the earth. It is found at a concentration as low as 0.55 ppmv. A major source of H_2 formation is due to anthropogenic activity, methane or hydrocarbon oxidation (Conrad and Seiler 1981). Hydrogen is produced aerobically in a side reaction during nitrogen fixation by *Rhizobium* bacteroids (Evans et al. 1977) or photoproduced anaerobically from purple bacteria under nitrogen starvation (Gray and Gest 1965) or from algae (Okura 1986) and from anaerobic mineralization of organic matter. However, of the total flux of H_2 formation, nitrogen fixation contributes up to 5 percent and thus exceeds by far the amount released from anaerobic mineralization (Conrad and Seiler 1980). Despite the low mixing ratio of 0.55 ppmv, H_2 is oxidized aerobically from soil by soil microorganisms. Hydrogen-oixidizing bacteria can be easily isolated from soil. However, the results of Conrad and Seiler (1981) suggest that the H_2-oxidizing bacteria examined do not contribute to the observed consumption of H_2 at 0.55 ppmv, due to a threshold of affinity for H_2 (Conrad et al. 1983; Conrad 1988). Moreover, two affinity constants for H_2 uptake have been determined from soil of which the higher one is due to H_2-oxidizing bacteria (Schuler and Conrad, in press). However, a threshold concentration of 10 ppm H_2 was determined from studies on induction of *A. latus* hydrogenase (Doyle and Arp 1987). *In vitro* affinities of hydrogenase from N_2 fixing bacteria are high and have been determined to be about 1 μM H_2.

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 55-92.

Oxidation of H_2 may be an attractive energy yielding process for microorganisms depending on the electron acceptor (Table 1). These lithotrophic reactions have in common the activation of molecular hydrogen which is catalyzed by the enzyme hydrogenase according to the following equation:

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

In principle, this reaction is reversible, and thus hydrogenases may react bidirectionally. In nature, however, the reaction appears to be directed by the physiological function of the enzyme and is determined by the redox potential of the electron acceptors and the concentrations of the reactants involved.

The reader is referred to previous reviews on various aspects of biological H_2 conversion, including the microorganisms and their physiology, aerobic and anaerobic H_2 metabolism, the enzymes catalyzing oxidation or evolution of H_2, and the genetics of H_2-oxidizing bacteria (Bowien and Schlegel 1981; Schlegel and Friedrich 1985; Schlegel and Schneider 1985; Maier 1986; Gogotov 1986; Friedrich et al. 1986; Evans et al. 1987; Fauque et al. 1988). This article deals almost exclusively with the hydrogen-oxidizing ability of the lithoautotrophic bacteria. We here summarize recent physiological, enzymatic and genetic data on the function, expression and composition of hydrogenases from this group of organisms.

Aerobic and Anaerobic Hydrogen Metabolism

Hydrogenobacter thermophilus was the first obligately autotrophic aerobic H_2-oxidizing bacterium isolated. This extremely thermophilic strain was isolated from soil of a hot spring in Japan (Kawasumi et al. 1984). Hydrogen oxidation was not the only lithotrophic ability found in strains assigned to this genus since lithoautotrophic growth with thiosulfate as energy source was also reported from subsequent isolates (Bonjour and Aragno 1986). Unlike the facultatively lithoautotrophic H_2-oxidizing bacteria which generally fix carbon dioxide (CO_2) via the Calvin cycle (Tabita 1988), *H. thermophilus* assimilates CO_2 via the reductive tricarboxylic acid cycle (Shiba et al. 1985).

The ability to utilize H_2 as a facultative energy source is widespread and found among Gram-negative and Gram-positive eubacteria, phototrophic bacteria and in archaebacteria. In Table 1 examples are compiled for the aerobic utilization of inorganic electron donors. Of the facultatively lithoautotrophic bacteria the carbon monoxide (CO)-oxidizing species have been found to also grow, with a few exceptions, with H_2 (Meyer 1989). Several hydrogen bacteria exhibit alternative lithotrophic abilities, for instance using thiosulfate as an energy source like *Paracoccus denitrificans* (Friedrich and Mitrenga 1981). Also, CO oxidation has been found in strains initially isolated as hydrogen bacteria, like *Arthrobacter* sp. or *Bacillus schlegelii* (Meyer 1989).

Table 1. Metabolic versatility of aerobic lithotrophic and of phototrophic bacteria.

	Characteristics				
	H_2	H_2S or $Na_2S_2O_3$	CO	N_2-fixation	Phototrophic growth
Alcaligenes eutrophus	+	−	−	−	−
Alcaligenes latus	+	−	−	+	−
Aquaspirellum autotrophicum	+	+	−	−	−
Arthrobacter sp. strain 11x	+	−	+	−	−
Azomonas sp.	+	ND	+	+	−
Bacillus schlegelii	+	−	+	−	−
Hydrogenobacter sp.	+	+	−	ND	−
Paracoccus denitrificians	+	+	−	−	−
Pseudomonas carboxydovorans	+	−	+	−	−
Bradyrhizobium japonicum	+	−	−	+	−
Rhodobacter capsulatus[a]	+	+	−	+	+
Rhodopseudomonas acidophila	+	−	−	+	+
Rhodocyclus gelatinosus[b]	ND	−	+	+	+
Xanthobacter autotrophicus	+	+	−	+	−

[a] formerly *Rhodopseudomonas capsulata* (Imhoff et al. 1984)
[b] formerly *Rhodopseudomonas gelatinosa* (Imhoff et al. 1984)
ND, no data. This table is based on the following references: Aragno and Schlegel 1981; Friedrich and Mitrenga 1981; Meyer 1989; Siefert and Pfennig 1979; Uffen 1983; Bonjour and Aragno 1986; Trüper 1989.

The phototrophic purple nonsulfur bacteria are most versatile with respect to lithotrophic abilities. For photolithotrophic growth, *Rhodobacter capsulatus* or *Rhodopseudomonas acidophila* utilize H_2 anaerobically. However, H_2 is also utilized for chemolithoautotrophic growth in the dark, albeit at a low oxygen partial pressure (Madigan and Gest 1979; Siefert and Pfennig 1979). Moreover, some strains of *Rhodocyclus gelatinosus*, formerly *Rhodopseudomonas gelatinosa* (Imhoff et al. 1984), grow with CO as carbon and energy source anaerobically in the dark (Uffen 1983).

Hydrogen is liberated by nitrogenase during the nitrogen fixation process in a side reaction. The energy loss due to the transfer of electrons to protons was estimated to be one-third of the energy consumed for N_2 fixation (Lim et al. 1980), according to the generalized equation:

$$8\ [H] + N_2 \rightarrow 2\ NH_3 + H_2$$

Nitrogen-fixing ability is often linked with H_2-oxidizing ability. About 24 percent of the symbiotic N_2-fixing *Rhizobium japonicum* strains exhibit hydrogen uptake (hup) activity (Evans et al. 1987). Lithoautotrophic growth has also been established in aerobic free-living N_2-fixing bacteria (Malik and Schlegel 1981). On the other hand N_2 fixation was observed in strains initially isolated as hydrogen bacteria (Gogotov and Schlegel 1974; Aragno and Schlegel 1981).

Thus, aerobic lithotrophic energy generation, derived from the oxidation of H_2, CO or from reduced inorganic sulfur compounds, is not restricted to specific taxa. In facultatively lithoautotrophic bacteria such metabolic versatility may be of selective advantage since growth with poor energy sources approximates the natural soil, lake or hot springs environment. In this respect, H_2 oxidation is the most common reaction and may add to the metabolic versatility of these bacteria to utilize reduced substrates, presumably produced from anaerobic fermentations or set free from geological sources (Table 1).

The principle reactions of anaerobic H_2 metabolism (Table 2), and the microorganisms involved are only briefly summarized since these topics are the subject of previous reviews (Fauque et al. 1988; Gottschalk 1989; Thauer 1989; Trüper 1989). The ability to grow anaerobically with H_2 as the electron donor and nitrate as electron acceptor is the exception among strictly aerobic hydrogen bacteria; this was reported for *Paracoccus denitrificans* (Aragno and Schlegel 1981). However, heterotrophic anaerobic growth with nitrate is common. This discrepancy is possibly due to a failure to express hydrogenase properly under these conditions, as suggested recently for *A. eutrophus* (G. Eberz, Ph.D. thesis, Free University, Berlin), or may be due to a failure to fix CO_2 autotrophically (Im and Friedrich 1983).

Phototrophic bacteria can use H_2 anaerobically in the light and other inorganic electron donors, e.g. reduced sulfur compounds, for growth with CO_2. This photolithoautotrophic metabolism is found in members of the families of the *Chromatiaceae* and *Ectothiorhodospiraceae* (purple sulfur bacteria), *Chlorobiaceae* (green sulfur bacteria) and in the *Rhodospirillaceae* (purple nonsulfur bacteria). In species of the latter family only a few strains have been shown not to utilize H_2 (Trüper 1989). In *Rhodobacter capsulatus* hydrogenase functions physiologically as a H_2 uptake hydrogenase (Colbeau and Vignais 1983).

Table 2. Hydrogen consumption by bacteria with electron transport phosphorylation[a].

Reaction	Product	ΔG^o (kJ/reaction)	Representative organism
$H_2 + 1/2\ O_2$	H_2O	-237.2	*Alcaligenes eutrophus*
$H_2 + NO_3^-$	$NO_2^- + H_2O$	-163.2	*Paracoccus denitrificans*
$4\ H_2 + HSO_4^-$	$HS^- + 4\ H_2O$	-151.9	*Desulfovibrio vulgaris*
$4\ H_2 + CO_2$	$CH_4 + 2\ H_2O$	-131.0	*Methanobacterium sp.*
$4\ H_2 + 2\ CO_2$	$CH_3COO^- + H^+ + 2\ H_2O$	-95.0	*Acetobacterium woodii*

[a] modified after Schlegel and Schneider 1985

The strictly anaerobic sulfate-reducing bacteria are not autotrophic but can use H_2, lactate, acetate, or alkanes as electron donors for generation of energy. The energy metabolism and the physiology of this group has been reviewed recently (Thauer 1989; Widdel 1988). The metabolism of H_2 and the molecular biology of the hydrogenases of the genus *Desulfovibrio* has been summarized in detail (Fauque et al. 1988).

The acetogenic eubacteria are able to grow with H_2 and CO_2 as sole source of energy and carbon according to the reaction given in Table 1 (Fuchs 1986; Gottschalk 1989). They grow well on hexoses and thus are facultatively lithoautotrophic. The methanogenic archaebacteria also grow with H_2 and CO_2. Their metabolic versatility is limited to few other methanogenic substrates like acetate, methanol or methylamines (Jones et al. 1987). The hydrogenases in these organisms will be discussed in a subsequent section.

Regulation of Hydrogenase Formation

In most of the H_2-oxidizing bacteria, formation of active hydrogenase is regulated in response to environmental stimuli. Of the aerobic bacteria, *Alcaligenes eutrophus* forms a soluble, NAD-linked and a membrane-bound hydrogenase. Both enzymes are expressed under lithoautotrophic and under certain heterotrophic growth conditions. Growth with H_2 and CO_2 of *A. eutrophus* was shown to be poor in a mineral medium in which cells grow well heterotrophically unless nickel ions are provided (Bartha and Ordal 1965). To elucidate the function of nickel in lithoautotrophic metabolism, heterotrophic culture conditions were developed that permitted differential expression of the hydrogenases and the key enzymes of autotrophic CO_2 fixation. In this system nickel was found to be required for active hydrogenase formation and not for CO_2 fixation (Friedrich et al. 1981a). This observation initiated a straightforward search and finally the discovery that nickel is a constituent of the hydrogenases in lithoautotrophic bacteria (Hausinger 1987; Walsh and Orme-Johnson 1987).

In *A. eutrophus* both hydrogenases are expressed coordinately. An inverse relationship exists between the level of activity and the specific growth rate determined by the carbon source (Friedrich et al. 1981c). With glycerol as substrate ($t_d = 20$ h) hydrogenase specific activity was about 4 to 6 U/mg of protein and thus 5- to 10-fold the activity found in cells grown with H_2 and CO_2 ($t_d = 3.5$ h). The combination of glycerol with a fast-growth supporting substrate like fructose ($t_d = 2.5$ h) had several advantages: the medium was easy to handle, permitted hydrogenase expression independent of H_2 and CO_2, and yielded high protein levels (Friedrich et al. 1981a). An organic substrate normally provides the cell with both carbon and energy. The only way to discriminate between these two functions is with a chemostat under lithoautotrophic conditions, where cells are grown under either H_2 or CO_2 limitation. At low dilution rates the hydrogenase activities of *A. eutrophus* were highly expressed (6 U/mg of protein). Low activities were found under CO_2 or O_2 limitation (0.2 to 0.5 U/mg of protein). Thus, formation of the hydrogenases in *A. eutrophus* is derepressed whenever electron donors limit growth, irrespective of whether the hydrogen donor is H_2 or an organic compound (Friedrich 1982). Thus, the environmental signal which controls hydrogenase formation in *A. eutrophus* is the degree of reduction of the redox couples.

This mode of hydrogenase regulation is not unique to *A. eutrophus*. *Pseudomonas facilis* contains only a membrane-bound hydrogenase which is expressed during growth with gluconate or glycerol as well as with H_2 and CO_2 (Table 3; Warrelmann and Friedrich 1986). In the Gram-positive actinomycete *Nocardia opaca* 1b the NAD-linked soluble hydrogenase is considered to be subject to derepression (Sensfuss et al. 1986). About 10 percent of the activity found in cells grown with H_2 and CO_2 was present in pyruvate-grown cells but complete repression occurred with fructose as carbon source (Ecker et al. 1986). Under conditions of N_2 fixation hydrogenase activity in *Bradyrhizobium japonicum* was formed at a higher level than with ammonia. This effect was assigned to the presence of H_2 produced by the nitrogenase reaction (Graham et al. 1984).

Table 3. Growth conditions for hydrogenase formation.

Strain	Hydrogenase[a]	Spec. act. (U/mg of protein) Organic substrate		$H_2/O_2/CO_2$	Reference
Alcaligenes eutrophus	s	Glycerol	7.71	0.91	Friedrich et al. 1981a
	m		5.82	0.39	Friedrich 1982
Alcaligenes hydrogenophilus	s	Fructose	0.005	0.186	Friedrich et al. 1984a
	m		0	0.818	
Alcaligenes latus	m	Malate	0.018[b]	1.30	Doyle and Arp 1987
Aquaspirillum autotrophicum	m	Gluconate	0	2.12	Aragno and Schlegel 1978
Nocardia opaca	s	Pyruvate	0.075	0.769	Ecker et al. 1986
Paracoccus denitrificans ATCC17741	m	Fructose	0	1.22	Nokhal and Schlegel 1980
Paracoccus denitrificans ATCC19367	m	Fructose	1.147	0.889	Nokhal and Schlegel 1980
Pseudomonas facilis	m	Glycerol	0.504	0.731	Warrelmann and Friedrich 1986
Bradyrhizobium japonicum SR		Arabinose + H_2	0.033	0.021	van Berkum 1987
Rhodobacter capsulatus	m	Malate	0.006	ND	Colbeau and Vignais 1983
		Malate + H_2	0.033		

[a] s, Soluble hydrogenase; m, membrane-bound hydrogenase.
[b] Recalculated from 'percent activity'.

In many bacteria, the substrate H_2 is required for hydrogenase synthesis. *Alcaligenes hydrogenophilus* resembles *A. eutrophus* with respect to its physiology and hydrogenase pattern. The regulation, however, is quite different from that in *A. eutrophus* (Friedrich et al. 1984). Also, in *Aquaspirillum autotrophicum* the membrane-bound hydrogenase is exclusively formed in the presence of H_2 (Aragno and Schlegel 1978), as it is in the N_2-fixing strains *Alcaligenes latus* (Doyle and Arp 1987) and *Pseudomonas*

saccharophila (Podzuweit et al. 1983). In the latter strain carbon sources supporting fast growth, repressed hydrogenase synthesis (Barraquio and Knowles 1988).

Controversial results were obtained for *B. japonicum*. H_2 was found to be required for expression of hydrogenase activity (van Berkum 1987), whereas Graham et al. (1984) reported activity in cells grown without H_2. An H_2 requirement is known for the type strain of *Paracoccus denitrificans* 'Stanier'. All other strains of this species formed membrane-bound hydrogenase constitutively (Nokhal and Schlegel 1980). Hydrogenase formation of *Rhodobacter capsulatus* growing in the dark requires H_2 (Colbeau and Vignais 1983). Under photoautotrophic conditions hydrogenase synthesis in this strain was also dependent on the presence and the concentration of H_2 (Gogotov 1984). The level of the enzyme even increased in the stationary phase of growth or in resting cells (Colbeau and Vignais 1983; Gogotov 1984).

The acetogenic bacterium *Acetobacterium woodii* forms hydrogenase constitutively under heterotrophic growth (Braun and Gottschalk 1981). Some methanogenic bacteria produce methane from methanol, trimethylamine, acetate or CO. Evidence has been obtained that H_2 is formed and consumed during growth on these substrates. In *Methanosarcina barkeri* ATP synthesis is coupled to proton translocation during consumption of CO with concomitant production of CO_2 and H_2. Thus, a hydrogenase appears to be involved in electron transport and is apparently indispensable during growth on these substrates (Bott and Thauer 1989; Fiebig and Friedrich 1989; Terlesky and Ferry 1988).

Oxygen is toxic to organisms. From triplett oxygen toxic oxygen species are formed (Morris 1979). The rate of formation of such toxic species correlates with the oxygen partial pressure. Most aerobic microorganisms detoxify such species with catalase or superoxide dismutase. Strictly anaerobic bacteria are not able to detoxify and therefore are oxygen-sensitive. Despite detoxification mechanisms in aerobic bacteria metabolic pathways exist which may be sensitive to high oxygen partial pressure, e.g. the N_2 fixation process. This is due to an irreversible inactivation of the nitrogenase protein by O_2 and an oxygen-sensitive *nif* gene expression (Hennecke et al. 1988).

Hydrogenase proteins are generally oxygen-sensitive during storage (Schlegel and Schneider 1978). The hydrogenases of *A. eutrophus*, however, are considered to be stable under air. On the other hand, in the presence of O_2, the active soluble enzyme was shown to be inactivated *in vivo* (Schlesier and Friedrich 1981), and a transfer of electrons from H_2 to O_2, producing superoxide radicals, was demonstrated *in vitro* (Schneider and Schlegel 1981). The membrane-bound hydrogenase of *A. eutrophus* is competitively inhibited by O_2 ($K_i = 17\ \mu M$; Schink and Probst 1980).

Oxygen also plays a major role in lithoautotrophic growth and the formation of the hydrogenase protein. Exposure of cells growing with H_2 and CO_2 at 20 to 40% (vol) of O_2 resulted in the transition from exponential to linear and finally to the cessation of growth while the heterotrophic growth rate was

almost unaffected under these conditions (Wilde and Schlegel 1982). This suggests that O_2 rather inactivates than inhibits a crucial function in lithotrophic metabolism.

Cangelosi and Wheelis (1984) investigated the sensitivity to O_2 (Ose^+) of lithotrophic growth with H_2 of *A. eutrophus* H1 (ATCC 17707). 4% O_2 was optimal for hydrogenase expression which was repressed at 20% O_2. Mutants resistant to 25% O_2 occurred relatively frequently, they exhibited the identical hydrogenase pattern as the O_2-sensitive parent. The authors concluded that O_2 inactivates a so far unidentified regulatory protein for hydrogenase expression.

For *Pseudomonas saccharophila* 2% of O_2 was found to be optimal for growth with H_2 (Podzuweit et al. 1983), higher O_2 concentrations partially repressed the synthesis of hydrogenase while the activity of the enzyme was not sensitive to O_2 (Barraquio and Knowles 1988). In *A. latus* the rate of induction of hydrogenase by H_2 declined sharply above 12.5% of O_2 as shown from Western blot analysis (Doyle and Arp 1987). In *B. japonicum* a threshold below 1% of O_2 was required for optimum expression of hydrogenase (Maier et al. 1978). So far, the molecular basis for oxygen mediated control has not been identified. The fact that in many hydrogen bacteria the O_2 sensitivity is not as pronounced as in *A. eutrophus* H1, *B. japonicum* or *R. capsulatus* indicates that O_2 tolerant species have adopted to high O_2 concentrations under laboratory cultivation conditions and behave similarly to the O_2 resistant mutants.

Most meso- or thermophilic H_2-oxidizing bacteria exhibit the same temperature optimum for lithotrophic as well as heterotrophic growth. For *A. eutrophus*, however, a temperature optimum for growth with H_2 and CO_2 of 30°C was observed while the optimum for heterotrophic growth was 37°C and above. From immunological studies it was evident that this thermosensitivity results from a failure to form soluble and membrane-bound hydrogenases and other proteins subject to the hydrogenase control circuitry at high temperatures. Both hydrogenases are not subject to inactivation at 37°C (Friedrich and Friedrich 1983; Kärst and Friedrich 1987) which suggests that the temperature control is mediated by a thermosensitive regulatory gene product as postulated for the temperature control of the N_2-fixing system in *Klebsiella pneumoniae* (Hennecke and Shanmugam 1979).

Characteristics of Hydrogenase Proteins

The major characteristics of the hydrogenases from various sources are summarized in Table 4. Two types of hydrogenases are to be differentiated in aerobic lithoautotrophic bacteria with respect to the cellular location, subunit composition and cofactor content: (i) the soluble hydrogenase (HoxS) being composed of four nonidentical subunits (α, β, γ, δ), and (ii) the membrane-bound enzyme (HoxP) consisting of two nonidentical subunits. One of the best characterized enzyme is that of *Nocardia opaca* 1b (subunits of: α, 64

kilodaltons (kDa); β, 56 kDa; γ, 31 kDa; δ, 27 kDa). Its analysis led to the proposition of the following model (Hornhardt et al. 1986): A large and a small heterodimer consisting of the subunits α, γ and β, δ, respectively, catalyze different functions. The small nickel-containing dimer constitutes a hydrogenase protein with H_2 oxidizing and H_2 evolving activity in the presence of artificial electron carriers. The large dimer functions as a diaphorase with NADH-acceptor-oxidoreductase activity. Both dimers form the holoenzyme and transport electrons from H_2 to NAD. The dimers aggregate in the presence of chaotropic agents of which nickel ions are most effective. This nonspecific function of nickel has to be carefully distinguished from its essential function in hydrogenase catalysis. Therefore, the content of nickel per mol of enzyme was 3.8 mol of which 2 were removed by dialysis while 2 remained bound to the enzyme. The NAD linked hydrogenase contains as prosthetic group flavinmononucleotide (FMN), three [4Fe-4S], one [2Fe-2S], and one [3Fe-xS] clusters (Table 4; Schneider et al. 1984b). The soluble hydrogenase of *A. eutrophus* is similarly composed as the enzyme from *N. opca* 1b (Schneider et al. 1979). It contains 2 mol of nickel (Friedrich et al. 1982) and is immunologically closely related to the *N. opaca* HoxS protein (Schneider and Piechulla 1986).

The membrane-bound hydrogenase of aerobic lithotrophic and phototrophic bacteria is unable to transfer electrons to NAD. The natural electron acceptors have not been conclusively identified so far. Nevertheless, a reverse electron transport is required for biosynthetic processes in those organisms that contain a single HoxP protein. One mol of enzyme of *A. eutrophus* (subunits 62 kDa and 31 kDa) contains about 1 mol of nickel and 7 to 9 mol of iron organized in [4Fe-4S] clusters. The enzyme reacts well with a number of artificial electron acceptors like methylene blue (MB), phenazine methosulfate (PMS) or quinones and at a low rate with cytochrome c. When solubilized from the membrane, the enzyme exhibits a pH optimum of 5.5 instead of 8.0 when measured with membrane extracts (Schink and Schlegel 1979; Podzuweit et al. 1986; Schneider et al. 1983).

In previous studies the small subunit of HoxP was not detected from *B. japonicum, Rhodobacter capsulatus* and *Paracoccus denitrificans* (Arp and Burris 1979, Colbeau and Vignais 1983; Sim and Sim 1979). Subsequent investigations revealed that the HoxP proteins of *A. latus, B. japonicum, R. capsulatus* and *P. denitrificans* resemble the enzyme of *A. eutrophus* with respect to subunit composition, molecular weight, nickel and iron content and reactivity with artificial electron acceptors (Harker et al. 1985; Arp 1985; Seefeldt and Arp 1987; Knüttel et al. 1989). Some of these data are summarized in Table 4. For *B. japonicum* selenium was suggested to be part of the hydrogenase since it was copurified with the enzyme (Boursier et al. 1988). This result, however, awaits further confirmation on the genetic level.

Hydrogenases in strains of the *Chromatiaceae, Ectothiorhodospiraceae* and *Rhodospirillaceae* as well as *Chlorobiaceae* and *Chloroflexaceae* have been characterized (Adams et al. 1981; Leclerc et al. 1988; Drutschmann and

Table 4. Molecular properties of purified hydrogenases.

Strain[a]	Holo-enzyme[b]	M_r (kDa) Subunit	Metal/cofactor content (mol/mol enzyme)	Temp. optimum (°C)	K_m H_2 (μM)	Ref.
A. woodii	S 150	75/60/53	ND	ND	ND	1
A. eutrophus	S 205	63/56/30/26	2Ni;16Fe;1FMN	33	37	2
	M 98	62/31	0.65Ni;7-9Fe	45	ND	3
A. latus	M 113	67/34	0.54Ni;1.7Fe	48	25	4
B. schlegelii	M 100	ND	ND	90	ND	5
B. japonicum	M 104	65/33	0.6Ni;6.5Fe;0.56Se	ND	0.97	6
C. limicola	M 66	66	ND	65	10	7
C. vinosum	M 98	50	1.8Ni;7Fe	ND	ND	8
M. formicicum	M 70	ND	1Ni;10Fe;1Zn;2Cu	ND	ND	9
M. thermoautotrophicum	S 800 115	47/31/26	0.7Ni;14Fe;0.9FAD	ND	10	10
M. voltae	ND 745 105	55/45/37/27	0.6Ni;4.5Fe;0.66Se 0.8FAD	37	ND	11
M. barkeri	S 845 198	48/33/30	0.6-0.8Ni;8-10Fe	ND	ND	12
M. hungatei	M 720	1x50/3x30	6-7Ni	ND	ND	13
N. opaca	S 178	64/56/31/27	1.8Ni;13.6Fe;1FMN	30	20	14
P. denitrificans	M 100	64/34	0.6Ni;7.3Fe	ND	ND	15
R. capsulatus	M ND	76/31	1Ni;4Fe	70	ND	16
T. roseopersicina	M 68	47/25	1Ni;4Fe	ND	ND	17

[a] Full names of genera are given in the text.

[b] S, soluble enzyme; M, membrane-bound enzyme; ND, no data.

1, Ragsdale and Ljungdahl 1984; 2, Schneider et al. 1979, Friedrich et al. 1982; Petrov et al. 1989; Schneider and Piechulla 1986; 3, Schink and Schlegel, 1979; Schneider et al. 1983; Podzuweit et al. 1986; 4, Pinkwart et al. 1983a; 5, Pinkwart et al. 1983b; 6, Harker et al. 1985; Arp 1985; Boursier et al. 1988; 7, Serebryakova et al. 1987; 8, Albracht et al. 1983; 9, Baron et al. 1987; 10, Livingston et al. 1987; Fox et al. 1987; 11, Muth et al. 1987, Muth 1988; 12, Fauque et al. 1984; Fiebig and Friedrich 1989; 13, Sprott et al. 1987; 14, Schneider et al. 1984a, Schneider et al. 1984b; 15, Knüttel et al. 1989; 16, Seefeldt et al. 1987; 17, Gogotov 1984.

Klemme 1985; Gogotov 1984; Serebryakova et al. 1987). The best studied example is the enzyme of *R. capsulatus*. Based on recent data it is composed of subunits of 67 kDa and 31 kDa, contains one nickel and four iron atoms in a [4Fe-4S] cluster; it reacts with cytochrome c' and b_{560} (Leclerc et al. 1988; Gogotov 1984). Both subunits are immunologically related to the corresponding polypeptides of *B. japonicum, A. latus* and *A. eutrophus* (Seefeldt et al. 1987; Gogotov 1984). The hydrogenase of *Thiocapsa roseopersicina* (68 kDa) is composed of two subunits (47 kDa and 25 kDa) and thus smaller than the average HoxP proteins (Kondratieva 1989). The holoenzyme of *Chlorobium limicola* and of *Rhodospirillum rubrum* have been described to consist of one polypeptide of 66 kDa (Serebryakova et al. 1987; Adams et al. 1981). From the *Chloroflexus aurantiacus* enzyme (170 kDa) no subunit composition has been reported. It reacts with MV, BV and menadione and requires nickel for catalytic activity (Drutschmann and Klemme 1985).

Although the membrane-bound hydrogenases of aerobic lithotrophic and phototrophic bacteria differ with respect to their catalytic properties, they appear to have three features in common: (i) they constitute [NiFe] proteins, (ii) consist of two subunit species of approximately 60 kDa and 30 kDa and (iii) form a dimeric holoenzyme of approximately 100 kDa.

In acetogenic and methanogenic bacteria the existence of heterotrimeric hydrogenases has been reported from several species. The enzyme of *Acetobacterium woodii* (150 kDa), isolated from the soluble portion of the cell extract, was shown to be composed of three subunits of 75 kDa, 60 kDa, and 53 kDa. It seems exceptional for a hydrogenase from a lithoautotrophic bacterium that this particular enzyme does not require nickel for catalytic activity. The pure enzyme exhibited an extremely high specific activity of 3500 U/mg of protein (Ragsdale and Ljungdahl 1984).

From methanogenic bacteria two hydrogenases have been characterized: (i) a 8-hydroxy-5-deazaflavine (coenzyme F_{420}) – reducing hydrogenase and a methylviologen – reducing enzyme, unable to reduce coenzyme F_{420} (Baron et al. 1987; Kojima et al. 1983). The major proportion of active enzyme from *Methanobacterium thermoautotrophicum* ΔH is a high molecular weight aggregate of 800 kDa. Moreover, a minor form of approximately 115 kDa has been found. Both species are composed of three heterologous subunits (α, 47 kDa; β, 31 kDa; γ, 26 kDa). The enzyme contains 13 to 14 iron atoms, about 1 atom of nickel, and 1 molecule of FAD (Table 4). The exact location of the latter has not been established yet (Fox et al. 1987). From kinetic data a model has been proposed suggesting the transfer of two electrons via FAD to coenzyme F_{420}, while a single electron is transferred from the iron-sulfur clusters to MV (Livingston et al. 1987).

Hydrogenases isolated from other methanogenic bacteria like *Methanosarcina barkeri, Methanococcus voltae,* or *Methanospirillum hungatei* also form high molecular weight aggregates. The enzyme of *M. barkeri* was reported to contain one subunit of 60 kDa, FMN, 1 atom of nickel, and 8 to 10 iron atoms (Fauque et al. 1984). In a recent report, however, a trimeric subunit composition was found for the purified *M. barkeri* hydrogenase which resembled that of *M. thermoautotrophicum* (Fiebig and Friedrich 1989). The enzyme of *M. voltae* was shown to be composed of 745 kDa and 105 kDa aggregates consisting of four nonidentical subunits. This enzyme contains one molecule of FAD and about 0.6 to 0.7 atoms of nickel and of selenium (Muth et al. 1987). Again the selenocystein content awaits approval by molecular studies. A tetrameric composition was suggested for the hydrogenase of *M. hungatei* with one subunit of 50.7 kDa and three identical subunits of 30.7 kDa with a high content of 6 to 7 atoms of nickel per enzyme molecule (Sprott et al. 1987).

Plasmid Encoded Hydrogenases

Plasmids have been found in Gram-positive and Gram-negative hydrogenase-

containing autotrophic microorganisms including aerobic hydrogen-oxidizing bacteria, nitrogen-fixing species, thiobacteria, carboxydotrophic and photosynthetic bacteria (reviewed by Friedrich 1989). In most cases no specific physiological function could be assigned to these extrachromosomal elements. Those plasmids which have conclusively been shown to be involved in hydrogen metabolism (Table 5) are normally characterized by an extremely large size of 450 ± 50 kilobase (kb). The size of plasmid pHG1 from *A. eutrophus* H16 was precisely determined by electron microscopic conture length measurements (Rhode et al. 1986a).

The first evidence for the existence of megaplasmids in lithoautotrophic bacteria was obtained by the application of techniques specifically designed for the identification of large plasmid molecules. The method is based on alkaline sodium dodecylsulfate lysis of cells (Hanson and Olsen 1978; Casse et al. 1979; Kado and Liu 1981), and was further modified for the isolation of megaplasmid DNA at a preparative scale. This permitted the construction of restriction maps (Hogrefe and Friedrich 1984), gene libraries (Eberz et al. 1986) and physical identification of plasmid mutations such as deletions and insertions (Kortlüke et al. 1987). The laborious, one week lasting plasmid isolation protocol was previously simplified and can now be accomplished within one day of experimentation (Nies et al. 1987).

The first indication that plasmids may be involved in hydrogen metabolism arose from the frequent loss of lithoautotrophy in *Nocardia opaca* and its recovery by cell contact with a lithoautotrophically positive donor (Reh and Schlegel 1975). In fact, *N. opaca* 1b was shown to contain two differently sized plasmid molecules, a cryptic 174 kb plasmid and a 104 kb thallium resistance conferring plasmid (Reh and Schlegel 1981; Sensfuss et al. 1986). Very recently, Reh and coworkers made the interesting observation that lithoautotrophy in *N. opaca* 1b is not associated with the circular plasmids mentioned above, but with linear plasmid DNA (J. Kalkus and M. Reh, abstract of the VAAM meeting 1989, Marburg, FRG).

P. facilis ATCC 17695 was reported to be genetically rather unstable in its hydrogen-oxidizing ability (Pootjes 1977) and host of a 270 kb plasmid (Gerstenberg et al. 1982). Analysis of transposon-induced hydrogenase defective mutants, however, suggested a chromosomal rather than a plasmid location of hydrogenase genes in this strain (Warrelmann and Friedrich 1986). Nevertheless, screening of various *P. facilis* wild type strains for the presence of plasmid DNA revealed a heterogeneous pattern with respect to number and sizes of plasmids, and evidence was obtained that one of the two plasmids detected in *P. facilis* DSM 620 (Table 5) in fact, determines hydrogenase genes (Warrelmann and Friedrich 1989).

Although the data of Table 5 suggest the conclusion that hydrogen bacteria of the genus *Alcaligenes* bear hydrogenase genes on megaplasmids, care has to be taken before generalizing such a statement. The heterogeneity is not only restricted to *P. facilis* but also apparent among strains of *Alcaligenes* whose plasmids also differ in numbers and restriction patterns (Hogrefe and Friedrich

Table 5. Plasmids encoding hydrogenase genes.

Strain	Plasmid			Reference[b]
	Designation	Size (kb)	Relevant phenotype[a]	
A. eutrophus				
H16 ATCC 17699	pHG1	450	HoxS HoxP Tra	1 2 3
TF93 ATCC 17697	pHG2	450	HoxS (HoxP) Tra	1 2 3
N9A DSM 5	pHG3	450	HoxS HoxP Tra	1 2 3
H20 ATCC 17700	pHG7	430	HoxS (HoxP) Tra	2 3 4
G27 DSM 516	pHG4	450	HoxS HoxP Tra	1
ATCC 17704	pHG10	500	HoxS HoxP Tra	2 3
ATCC 17706	pRMB1	ND	HoxS HoxP Tra$^-$	5
ATCC 17707	pHG12-b	500	HoxS HoxP Tra	6
4a-2	ND	238	HoxS HoxP$^-$ Tra$^-$	7
A. hydrogenophilus				
M50	pHG21-a	410	HoxS HoxP Tra	8 9
P. facilis				
DSM 620	pHG22-a	440	HoxS$^-$ HoxP Tra$^-$	9 10

ND, no data

[a]HoxS, NAD-linked soluble hydrogenase; HoxP, membrane-bound hydrogenase; (HoxP), the presence of HoxP could only be identified in the soluble fraction of the extract; Tra, self-transmissible by conjugation.

[b]1, Friedrich et al. 1981c; 2, Hogrefe and Friedrich 1984; 3, Hogrefe et al. 1984; 4, Friedrich et al. 1986; 5, Behki et al. 1983; 6, Cangelosi and Wheelis 1984; 7, Timotius and Schlegel 1987; 8, Ohi et al. 1989; 9, Friedrich et al. 1984; 10, Warrelmann and Friedrich 1989.

1984; Table 5). *A. eutrophus* CH34, a typical member of the two hydrogenases-containing bacteria, exhibit two medium-sized plasmids which confer multiple resistance to heavy metal ions but no hydrogen-oxidizing ability (Mergeay et al. 1985; Nies et al. 1987). It is not known yet whether *Alcaligenes ruhlandii* or the nitrogen-fixing *A. latus* carry hydrogenase genes on the indigenous plasmids (Gerstenberg et al. 1982). On the other hand, *Rhizobium leguminosarum* strain 128C53 encodes genes for hydrogenase activity and nodulation ability on large plasmids (Brewin et al. 1980; Kagan and Brewin 1985). DNA-DNA hybridizations with oligonucleotides derived from two subunits of CO dehydrogenase from *Pseudomonas carboxydovorans* OM5 revealed a plasmid location of CO dehydrogenase genes in six plasmid-harbouring carboxydotrophic strains (Kraut et al. 1989), these plasmids may also play a role in hydrogen metabolism (Kraut and Meyer 1988).

Megaplasmid pHG1, indigenous to *A. eutrophus* H16, is one of the best studied hydrogenase gene (*hox*) carrying plasmids. Although hydrogenase was the first phenotypic marker assigned to this extrachromosomal element (Lim et al. 1980; Andersen et al. 1981; Friedrich et al. 1981b) the number of metabolic activities associated with plasmid pHG1 has since been increasing (Table 5). Plasmid pHG1 determines a second copy of the *cfx* genes, genes coding for enzymes of autotrophic CO_2 fixation, which is reiterated on the chromosome

(Bowien et al. 1984; Klintworth et al. 1985). A similar observation was reported for other *A. eutrophus* wild type strains (Behki et al. 1983; Andersen and Wilke-Douglas 1985). The fact that plasmid-cured derivatives of *A. eutrophus* H16 were able to grow with formate autotrophically suggested that at least under these conditions of growth the plasmid copy of *cfx* genes is dispensable (Friedrich et al. 1981b).

A third metabolic gene cluster is located on plasmid pHG1 of *A. eutrophus* H16, encoding denitrification (Römermann and Friedrich 1985). This includes determinants of the reductases for nitrate, nitrite and nitrous oxide (B. Schneider, U. Warnecke, B. Friedrich, unpublished results). Moreover, megaplasmid pHG1 codes for a soluble oxygen-binding flavohemoprotein of unknown physiological function (Weihs et al. 1989).

Genetic Transfer of Hydrogen-Oxidizing Ability

The fact that hydrogenases are abundant in phylogenetically diverse microorganisms and occasionally plasmid-determined, raised the question, do Hox plasmids play a role in the genetic transmission of these proteins? Among Gram-positive bacteria hydrogen autotrophy has been transferred by cell contact from *N. opaca* to even non-lithoautotrophic recipients such as *Rhodococcus erythropolis* and *Corynebacterium hydrocarboclastus* (Table 6; Reh and Schlegel 1975; Reh and Schlegel 1981), and it is an extremely interesting question whether the newly discovered linear plasmids are involved in this process.

There are several reports on naturally occurring genetic transfer within the group of Gram-negative lithoautotrophs. A cell contact dependent transfer of large stretches of chromosomal DNA, including lithoautotrophic determinants, has been described for *Xanthobacter autotrophicus* GZ29 (Wilke 1980) although there is no physical evidence for the presence of plasmid DNA in this strain (Gerstenberg et al. 1982). A phage with general transducing abilities was detected in *X. autotrophicus* which transferred single chromosomal characters at a frequency of 10^{-4} per marker and phage particle (Wilke and Schlegel 1979).

Almost all of the Hox megaplasmids resident in *Alcaligenes* (Table 5), are self-transmissible by conjugation and probably bear *tra* genes. The non-conjugative plasmid pRMP1 (Behki et al. 1983) appears to be an exception to this rule. The acquisition of Tra functions provides an efficient mode of transmitting whole sets of metabolic properties among various strains of *A. eutrophus*. Interstrain-specific transfer of Hox occurred at a frequency up to 10^{-2} per donor (Friedrich et al. 1981b). Moreover, interspecific megaplasmid exchange was observed between *A. eutrophus* and *A. hydrogenophilus* (Table 6). The resulting transconjugants expressed the Hox phenotype of the plasmid parent (Friedrich et al. 1984). Umeda and coworkers (1986) reported the conjugal transfer of hydrogen-oxidizing ability from *A. hydrogenophilus* into strains of the chemoorganoautotrophic bacterium *Pseudomonas oxalaticus*

Table 6. Intra-and intergeneric hydrogenase gene transfer.

Donor	Recipient	Mechanism	Transconj. phenotype	Ref.[b]
N. opaca	*R. erythropolis* *C. hydrocarboclastus*	Cell contact	HoxS	1, 2
A. eutrophus H16	*A. hydrogenophilus*	Conjugation	HoxS HoxP HoxTs Hoxd	3
A. hydrogenophilus	*A. eutrophus* H16	Conjugation	HoxS HoxP HoxTr Hoxi	3
A. hydrogenophilus	*P. oxalaticus* strains OX1 and OX4	Conjugation	HoxS HoxP	4
A. eutrophus H16	*A. eutrophus* JMP134	RP4 enhanced mobilization	HoxS HoxP Cfx	5
P. facilis DSM620	*P. delafieldii* ATCC17506	RP4-pHG22-a cointegrate	HoxP Cfx	6
P. oxalaticus OX1 (pAH3)	*P. oxalaticus* OX1	Recombinant cosmid pYM11	HoxP	7
A. eutrophus (pHG1)	*P. facilis*	Recombinant cosmid pGE15	HoxS	8

[a]HoxS, NAD-linked hydrogenase; HoxP, membrane-bound hydrogenase, HoxTs/Tr, temperature-sensitive/resistant hydrogenase synthesis; Hoxd/i, energy-dependent derepression/ hydrogen-dependent induction of hydrogenase synthesis. Cfx, autotrophic carbon dioxide fixation.

[b]1, Reh and Schlegel 1975; 2, Reh and Schlegel 1981; 3, Friedrich et al. 1984a; 4, Umeda et al. 1986; 5, Schneider et al. 1988; 6, Warrelmann and Friedrich 1989; 7, Yagi et al. 1986; 8. U. Warnecke and B. Friedrich, unpublished results.

(Table 6). The pHG21-a containing transconjugants could grow with hydrogen autotrophically and stably maintained the megaplasmid. But not all of the *P. oxalaticus* strains were suitable as recipients, suggesting a limited host range of the pHG megaplasmids. This is consistent with results of an extensive mating program conducted with *A. eutrophus* H16 as the donor and a variety of Gram-negative bacteria as recipients. A successful transfer and expression of H_2-oxidizing and CO_2-fixing capacities were obtained only with the non-lithoautotrophic bacterium *Alcaligenes eutrophus* JMP134 (Don and Pemberton 1981). The megaplasmid DNA, reisolated from the respective transconjugants, often exhibited deletions and rearrangements (Schneider et al. 1988).

Hydrogen autotrophy was successfully transferred by RP4-mediated mobilization from *Pseudomonas facilis* DSM620 into the heterotrophic bacterium *Pseudomonas delafieldii* (Table 6). Both strains, the donor and the recipient, had been shown to be phylogenetically closely related (Palleroni et al. 1973). Analysis of transconjugants from *P. delafieldii* revealed the presence of a plasmid cointegrate composed of RP4 DNA and a 50 kb insert which derived from the indigenous *P. facilis* plasmid pHG22-a (Table 5). Attempts to convert other *Pseudomonas* strains, such as *P. acetovorans* or *P. testosteroni* into

hydrogen-oxidizing autotrophs failed (Warrelmann and Friedrich 1989). The data show that it is possible to transmit lithoautotrophy en bloc to other bacteria. Nevertheless, this transfer seems to be a rare event and presumably restricted by a narrow host range of Hox encoding plasmids, their genetic instability in a foreign strain and complications attributable to hydrogenase expression.

Cloning of Hydrogenase Genes

The first recombinant cosmids containing the so-called 'hydrogen uptake' (*hup*) genes were isolated from gene libraries of *B. japonicum* genomic DNA (Cantrell et al. 1983, Hom et al. 1985) by complementing mutants impaired in hydrogen dependent methylene blue reduction or the ability to grow lithoautotrophically (Lepo et al. 1981; Maier 1986; Moshiri et al. 1983). Cosmid pHU1 (Table 7) was shown by Tn*5* insertion mutagenesis to contain at least 15-kb of *hup* specific DNA (Haugland et al. 1984). Nevertheless, to complement all

Table 7. Recombinant plasmids with hydrogenase genes.

Source	Recombinant plasmid	DNA insert (kb)	Relevant gene (s)[a]	Ref.[b]
B. japonicum	pHU1	15	*hup*	1, 2
	pHU52	20	*hup*	3
	pMZ610	5.9	*hup*	4
	pSH22	22.7	*nif/hup*	5
R. capsulatus	pAG202	18.1	*hup*	6
	pAC76	3.5	*hup*	7
	pRHP4	8.0	ND	8
	pRHP8	6.7	*hup*	8
	pRHP20	4.5	ND	8
A. eutrophus	pGE15	15	*hoxS*	9
	pGE125	7.1	*hoxP hoxM*	10
	pGE4	10.6	*hoxC*	11
	pCH125	17	*hoxN*	12
A. hydrogenophilus	pYM11	29	*hoxP*	13
P. facilis ATCC176995	pGE61	22	*hoxP*	14
	pGE62	10	*hoxC*	14
Xanthobacter sp.	pLL483	22	*hox*	15
strain H4-14	pLL451	5.1	*hox*	15
	pLM432	3.2	*hox*	15
M. thermoautotrophicum strain ΔH	pET479	4.4	*mvhGAB*	16

[a]ND, no data

[b]1, Cantrell et al. 1983; 2, Haughland et al. 1984; 3, Lambert et al. 1985; 4, Zuber et al. 1986; 5, Hom et al. 1985; 6, Colbeau et al. 1986; 7, Leclerc et al. 1988; 8, Xu et al. 1989; 9, U. Warnecke and B. Friedrich, unpublished results; 10, C. Kortlüke and B. Friedrich, unpublished results; 11, Eberz et al. 1986; 12, Eitinger et al. 1989; 13, Yagi et al. 1986; 14, J. Warrelmann and B. Friedrich, unpublished results; 15, Lehmicke and Lidström 1985; 16, Reeve et al. 1989.

Hup$^-$ strains of *B. japonicum* an additional 5-kb DNA was necessary, present on cosmid pHU52 (Table 7). The latter plasmid also conferred hydrogenase activity to *R. leguminosarum* (Lambert et al. 1985) in which the *hup* specific DNA region closely parallels that of *B. japonicum* (Leyva et al. 1987). The cosmids were shown to contain structural genes for the large and the small hydrogenase peptides which were finally subcloned on plasmid pMZ610 (Table 7; Zuber et al. 1986).

The 5.9 kb *Hin*dIII fragment encoding both subunits of the membrane-bound hydrogenase of *B. japonicum* was used as a probe to isolate hydrogenase genes of *R. capsulatus*. A positive hybridization signal was observed with a 3.5 kb *Hin*dIII fragment (Table 7). The recombinant plasmid pAC76 was able to restore the activity of hydrogenase deficient mutants of *R. capsulatus* (Leclerc et al. 1988). Other cosmids have been isolated and used in complementation assays with different classes of mutants deficient in growth with H_2 and CO_2. The results suggest the involvement of a minimum of five genes in the synthesis of active hydrogenase in *R. capsulatus* (Xu et al. 1989).

Molecular cloning of hydrogenase specific genes, named *hox*, from *A. eutrophus* H16 revealed the presence of at least five independent *hox* loci on megaplasmid pHG1 (Table 7). They are clustered on a DNA stretch of approximately 100 kb (Kortlüke et al. 1987) flanked by two regions, *hoxS* and *hoxP*, which encode the structural genes for the NAD-linked and the membrane-bound hydrogenase, respectively (Fig. 1). The internal *hox* loci encode accessory hydrogenase genes and will be subsequently discussed. The first evidence for pHG1 located structural genes arose from crosses with HoxS$^-$ and HoxP$^-$ mutants as donors and plasmid-free mutants as recipients. The resulting transconjugants inherited the structural gene mutation of the respective donor (Hogrefe et al. 1984).

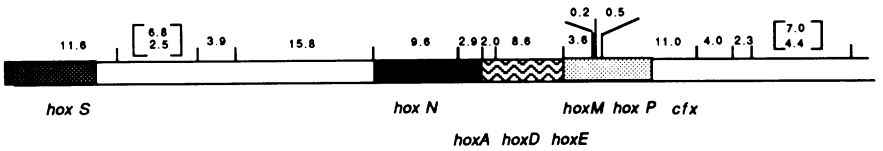

Fig. 1. Genetic map of the hydrogenase gene cluster of megaplasmid pHG1 from *A. eutrophus* H16. *hoxS*, structural genes for NAD-linked hydrogenase; *hoxN*, nickel transport; *hoxA*, regulatory *hox* gene; *hoxD, hoxE*, loci for HoxS and HoxP activities; *hoxM*, membrane association of HoxP; *hoxP*, structural genes for membrane-bound hydrogenase. The numbers above the bar indicate the sizes of *Eco*RI fragments in kilobase (kb); those in brackets indicate the sizes of fragments whose precise arrangement is still unknown.

Hox genes were further identified by screening a megaplasmid gene bank, constructed in *Escherichia coli,* for the ability to restore hydrogenase activity in various *A. eutrophus* Hox$^-$ mutants. Initially only parts of the information required for HoxS and HoxP were detected on *EcoR*I fragments of 11.6 and 11.0 kb, respectively (Eberz et al. 1986). Meanwhile the complete *hoxS* sequence has been cloned on a 15 kb *Hin*dIII fragment (Table 7) which encodes

in addition to the four structural genes, the gene for the B protein (U. Warnecke and B. Friedrich, unpublished results). The B protein is supposed to be specifically related to the soluble hydrogenase, since it has been exclusively found in those hydrogen-oxidizing bacteria which do contain HoxS protein (Kärst et al. 1987). The two HoxP structural genes have been separated from neighbouring DNA with *cfx* encoding functions and assigned to a fragment of 4.4 kb (Table 7).

Another locus, *hoxM*, has been identified adjacent to *hoxP;* its product appears to be involved in the membrane association of the HoxP protein. This assumption is based on the following observation: The transfer of *hoxM* into the wild type strain *A. eutrophus* H20 generated transconjugants which contained after cell disruption HoxP tightly attached to the membrane (C. Kortlüke and B. Friedrich, unpublished results). The same effect had been noticed before, by replacing the H20 megaplasmid pHG7 by plasmid pHG1 from *A. eutrophus* H16. Normally HoxP of disrupted H20 cells could only be identified immunologically in the soluble but not in the membrane fraction of the extract (Friedrich et al. 1986).

Difficulties in expressing hydrogenase genes on recombinant plasmids cloned in *E. coli* were circumvented by using autotrophic bacteria as cloning organisms. Thus genes coding for the membrane-bound hydrogenase of *A. hydrogenophilus* were transferred to *P. oxalaticus* and identified by conferring lithoautotrophic growth ability (Table 6; Yagi et al. 1986). Catalytic and immunological activity of the NAD-linked hydrogenase of *A eutrophus* H16 was expressed in *P. facilis* after introduction of a cosmid carrying a 15 kb DNA insert of megaplasmid pHG1 (Table 6). These transconjugants grew faster with hydrogen than the cosmid-free parental strain (U. Warnecke and B. Friedrich, unpublished results).

Attempts to clone hydrogenase genes from other lithoautotrophic bacteria look promising (Table 7). *hox* genes with mutant-complementing activity were isolated from genomic DNA of *Xanthobacter sp.* (Lehmicke and Lidström 1985). A megaplasmid DNA bank of *A. hydrogenophilus* yielded a recombinant cosmid with a 29 kb insert which conferred HoxP activity (Yagi et al. 1986), and HoxP related genes were isolated recently from *P. facilis* ATCC 17695 (J. Warrelmann and B. Friedrich, unpublished results). The genes *mvhDGA* which encode the polypeptides of methyl viologen-reducing (MV) hydrogenase of *M. thermoautotrophicum* strain ΔH have been cloned in *E. coli*. It is interesting to note that recombinant DNA containing clones were identified immunologically by using antibodies raised against the α-subunit of the F_{420}-reducing hydrogenase although the MV-coupled hydrogenase in this strain is devoid of F_{420}-reducing activity (Reeve et al. 1989).

Most of the cloning experiments described here, have been conducted with broad-host-range plasmids. These vectors have the advantage of being transferable via conjugation from *E. coli* to Gram-negative lithoautotrophs which are subject of this survey. Relevant characteristics and references of the routinely used plasmids are listed in Table 8.

Table 8. Vectors used for cloning of hydrogenase genes.

Plasmid	Relevant characteristics[a]	Reference
pLAFR1	Tc^r*mob* IncP1 containing λ*cos*	Friedman et al. 1982
pRK2013	Km^r *tra* helper plasmid	Ditta et al. 1980
pRK2073	Sm^r *tra* helper plasmid	Ditta et al. 1980
pRK404	Tc^r IncP1 pUC9 linker	Ditta et al. 1985
pVK100	Tc^r Km^r *mob* IncP1	Knauf et al. 1982
pVK102	Tc^r Km^r *mob* IncP1 λ*cos*	Knauf et al. 1982
pSUP202	Tc^r KM^r Ap^r *mob* pBR325	Simon et al. 1983

[a]Abbreviations: Tc^r, tetracycline resistant; Sm^r, streptomycin resistant; Km^r, kanamycin resistant; Ap^r, ampicillin resistant.

Organization and Sequence Comparison of Structural Hydrogenase Genes

Voordouw and Brenner (1985) were the first to report the nucleotide sequence of hydrogenase genes from the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris*. This periplasmic nonheme iron-containing enzyme is composed of two subunits. The large subunit has three iron-sulfur clusters, two of which are probably located in the amino terminal part of the peptide. It contains a duplication of the following cysteinyl-rich sequences (Cys-Ile-Xaa-Cys-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Cys-Pro-Xaa-Xaa-Xaa-Ala-) which exhibit homology to the bacterial [8Fe-8S] ferredoxin and are possibly the coordination of the two electron-transferring ferredoxin-like [4Fe-4S] clusters. The third cluster is supposed to be the catalytic domain. From the sequence data it was further concluded that this hydrogenase operon consists of two genes separated by 14 bp and coding for 46 and 13.5 kDa polypeptides. There was no evidence for a leader sequence in the large subunit (Voordouw and Brenner 1985). The mature small polypeptide of 10 kDa, however, was shown to lack 34 amino acids coded by the β-subunit gene (Prickril et al. 1986). Expression of this [Fe] hydrogenase in *E. coli* suggested a mechanism for export based on the translocation of the dimeric protein and removal of the β-peptide pre-sequence (van Dongen et al. 1988).

The structural genes *mvhDGA* of the methyl viologen-reducing hydrogenase in the archaebacterium *M. thermoautotrophicum* ΔH together with a fourth open reading frame, *mvhB*, were shown to be tightly linked and apparently organized within an operon. From sequence analysis of the *mvhB* gene it is predicted that its product constitutes a bacterial ferredoxin. Unlike the β-subunit of the *D. vulgaris* [Fe] hydrogenase the *mvhB* gene contains not only two, but six tandemly-repeated ferredoxin-like domains, and was therefore termed poly-ferredoxin (Reeve et al. 1989). The authors discuss its participation in hydrogenase associated electron-transport, possibly passing electrons through a membrane or into the complex subcellular structure where methanogenesis occurs. The organization and sequences of the *mvhG* and *mvhA* structural genes coding for the γ- and α-polypeptides of the MV-

reducing hydrogenase from *M. thermoautotrophicum* share homology with the genes encoding the small and large subunits of hydrogenases in eubacterial species (Reeve et al. 1989). Among these organisms are the hydrogen-oxidizing bacteria *B. japonicum* (Sayawedra-Soto et al. 1988) and *R. capsulatus* (Leclerc et al. 1988) and the sulfate-reducers *Desulfovibrio gigas* (Li et al. 1987) and *D. baculatus* (Menon et al. 1987).

Table 9. Molecular comparison of hydrogenases.

Characteristics	Origin of cloned hydrogenase genes				
	B.j.	*R.c.*	*M.t.* MVH	*A.e.*	
				HoxP	HoxS
Gene arrangement[a]	S L	S L	δ γ α	S L	α γ δ β
Leader peptide					
-length (aa)	46	46	0	43	0
-homology (%)	100	53	NR	70	NR
Intergenic region (bp)	32	3	12 (δγ)	41	0 (αγ)
			0 (γα)		0 (γδ)
					20 (δβ)
Cysteinyl residues	13 (S)	13 (S)	6 (δ)	13 (S)	17 (α)
	6 (L)	10 (L)	11 (γ)	9 (L)	12 (γ)
			8 (α)		8 (δ)
					6 (p)
Putative promoters	ND	ND	boxA	-12 -24;	-12 -24
			boxB	box	box
Reference[b]	1	2	3	4	5

Abbreviations: *B.j., Bradyrhizobium japonicum; R.c., Rhodobacter capsulatus; M.t., Methanobacterium thermoautotrophicum* ΔH*; A.e., Alcaligenes eutrophus* H16.
aa, amino acid; ND, no data; NR, not relevant.
[a]S, small subunit; L, large subunit.
[b]1, Sayavedra-Soto et al. 1988; 2, Leclerc et al. 1988; 3, Reeve et al. 1989; 4, C. Kortlüke, K. Horstmann, B. Schütze and B. Friedrich, unpublished results; 5, A. Tran-Betcke, C. Böcker and B. Friedcrich, unpublished results.

Although the nucleotide sequences of the six structural genes of the two hydrogenases in *A. eutrophus* are not yet completely determined, the data available so far are compared with those from other lithoautotrophic bacteria (Table 9). The subunit gene arrangement of the dimeric membrane-bound [NiFe] hydrogenase appears to be identical in all lithoautotrophs investigated so far and reversed to that reported for *D. vulgaris* (Voordouw and Brenner 1985). A more complex situation prevails for the multiple molecular forms of hydrogenases present in *M. thermoautotrophicum* and *A. eutrophus* (Table 9). Nevertheless, the genes corresponding to the small and the large subunit of the prototypic dimeric hydrogenases were shown to be tightly linked, and the small precedes the large one. However, leader sequences were absent in these cases (Reeve et al. 1989; A. Tran-Betcke, C. Böcker, B. Friedrich, unpublished results) as expected for proteins that reside in the soluble fraction (Kojima et al.

1983; Rohde et al. 1986b). All of the hydrogenase structural genes, whose nucleotide sequence is known, proved to be either separated by fairly small intergenic regions or tightly linked (Table 9). Although the data on transcription start sites, promoter activities and transcription terminators are still very limited, it is likely that the structural genes for the hydrogenases described here, constitute single operons. The significance of *nif*-like promoter sequences upstream from *hoxS* and *hoxP* in *A. eutrophus* will be discussed in a subsequent section.

The small subunit gene of the membrane-bound hydrogenases of lithoautotrophs (Table 9) encodes in addition to the mature protein a fairly large hydrophobic leader peptide with extensive sequence homology. Two arginine-rich clusters are located at the amino terminal part of the putative signal peptide from *B. japonicum, R. capsulatus,* and *A. eutrophus* (Fig. 2). This cluster is also conserved in the pre-sequences of the proteins from *D. vulgaris* (Prickril et al. 1986) and *D. baculatus* (Menon et al. 1987).

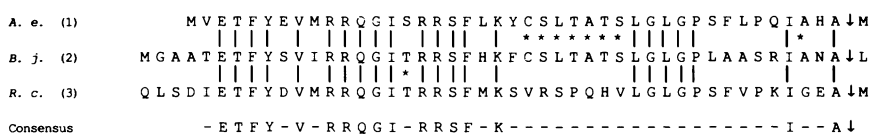

Fig. 2. Alignment of the predicted amino acid sequences of the start of the ORFs assigned to the gene for the small subunit of membrane-bound hydrogenases. The consensus sequence indicates the locations at which the same amino acid residue occurs in all three sequences. Arrows denote the putative cleavage site for leader peptidase. *A.e., A. eutrophus,* (1) C. Kortlüke, K. Horstmann, B. Schütze and B. Friedrich, unpublished results; *B.j., B. japonicum,* (2) Sayavedra-Soto et al. 1988; *R.c., R. capsulatus,* (3) Leclerc et al. 1988.

Alignment of the amino acid sequences of the two mature membrane-bound hydrogenase subunits revealed a high degree of similarity between *B. japonicum* and *R. capsulatus* ranging from 70 to 80 % overall identity (Sayavedra-Soto et al. 1988; Leclerc et al. 1988). Preliminary amino acid sequence data derived from the nucleotide sequence of the HoxP subunits from *A. eutrophus* also indicate an extensive conservation in the amino acid sequence of the two polypeptides (C. Kortlüke, K. Horstmann, B. Schütze, B. Friedrich, unpublished results). These sequence similarities coincide well with the immunological cross-reactivity of these three dimeric hydrogenases (Arp et al. 1985) and explains the positive hybridization signals with the hydrogenase gene probe from *B. japonicum* (Leclerc et al. 1988; Xu et al. 1989). Significant divergence in the overall amino acid sequence can be noticed between the hydrogenases from aerobic lithoautotrophs, the sulfate-reducers (reviewed by Fauque et al. 1988), the γ- and α-polypeptides of the MV-reducing hydrogenase from *M. thermoautotrophicum* (Reeve et al. 1989) and the corresponding δ- and β-polypeptides of the NAD-reducing HoxS protein (A. Tran-Betcke, C. Böcker, B. Friedrich, unpublished results). Well conserved cysteinyl-containing clusters can be identified in all organisms considered in this survey. However, unlike the *D. vulgaris* [Fe] hydrogenase (Voordouw and Brenner 1985) neither

the large nor the small subunits of the lithoautotrophic hydrogenases contain a classical ferredoxin type of iron-sulfur domain. The small subunit, including the γ-polypeptide of *M. thermoautotrophicum,* do all exhibit 8 cysteinyl residues at similar positions and with similar spacings which may play a role in the ligation of metal centers (Sayavedra-Soto et al. 1988; Leclerc et al. 1988; Reeve et al. 1989). Although the number and location of the cysteinyl residues in the large subunit are less well conserved, there are two pairs of clusters present in all lithoautotrophic hydrogenases investigated so far. They are illustrated in Fig. 3. The homology in these regions may reflect their significance in catalytic activity of hydrogenase. Li et al. (1987) proposed that cysteinyl residues and other conserved amino acids may be employed as ligands for nonheme iron clusters and possibly the nickel center.

A striking result arose from sequence comparison of the β-subunit of the HoxS protein from *A. eutrophus* and the α-subunit of the MV-reducing hydrogenase from *M. thermoautotrophicum*. These two polypeptides, which belong to the multiple molecular forms of hydrogenases, are similar in length and exhibit overall sequence homology (partially shown in Fig. 3). This result appears to be particularly interesting with respect to the evolution of hydrogenase proteins.

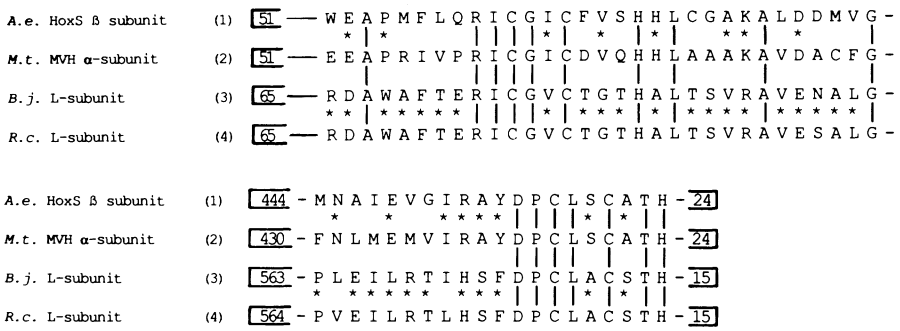

Fig. 3. Comparison of the predicted amino acid sequences of the N-terminal (upper part) and the C-terminal (lower part) end of the large hydrogenase subunits. The sequence of the ORF assigned to the β-subunit of HoxS from *A. eutrophus, A.e.,* (1) A. Tran-Betcke, C. Böcker and B. Friedrich, unpublished results is aligned above the corresponding sequences of the α-subunit of the MV-reducing hydrogenase from *M. thermoautotrophicum, M.t.,* (2) Reeve et al. 1989, and the large hydrogenase (L) subunits from *B. japonicum, B.j.,* (3) Sayavedra-Soto et al. 1988 and *R. capsulatus, R.c.,* (4) Leclerc et al. 1988. The figures in boxes indicate the numbers of amino acids preceding or succeeding the conserved regions where little or no homology can be detected. Identical amino acids are denoted by vertical bars; conserved residues by asterisks.

A role of selenium in [NiFe] hydrogenases of *B. japonicum* (Boursier et al. 1988) and *M. thermoautotrophicum* (Reeve et al. 1989) has been discussed lately. However, the nucleotide sequence of the *B. japonicum* structural genes is devoid of a TGA codon, which is supposed to direct co-translationally the incorporation of a seleno-cysteinyl residue into the [NiFeSe] hydrogenases of

D. baculatus (Menon et al. 1987) as was shown previously for formate dehydrogenase from *E. coli* (Zinoni et al. 1986). Although the DNA sequence of the *mvhG* gene from *M. thermoautotrophicum* did contain a TGA codon, careful re-examination of the nucleotide sequence indicate that this resulted rather from a cloning artefact than from the original genomic DNA sequence (Reeve et al. 1989).

Regulation of Gene Expression in *Alcaligenes Eutrophus*

Our knowledge of the regulation of hydrogenase gene expression is still in its infancy. The major reason for this lack of information is that hydrogenase-containing lithoautotrophs are genetically often difficult to handle. In order to study gene regulation on the molecular level, however, the application of genetic techniques is indispensable. Thus, *A. eutrophus* has proven to be an excellent model organism. It is accessable to various kinds of mutagenesis, genetic transfer via conjugation, gene exchange and molecular cloning (reviewed by Friedrich 1989).

The expression of the structural genes for the HoxS and the HoxP proteins, which supposedly constitute two single operons (Fig. 1) is coordinate and strictly dependent on the presence of two gene products, *hno* and *hoxA*. *hno (rpoN)* is located on the chromosomal DNA of *A. eutrophus* H16 (Römermann et al. 1989), whereas *hoxA* maps on plasmid pHG1 (Fig. 1), at a locus formally denoted as *hoxC* (Eberz et al. 1986).

A mutation in *hno (rpoN)* confers an extremely pleiotropic phenotype. Hno^- mutants are impaired in at least eight metabolic functions including the ability to grow with hydrogen, to fully express some of the enzymes of autotrophic CO_2 fixation, to utilize nitrate as electron acceptor or nitrogen source, respectively, to assimilate urea and to take up substances such as C4-dicarboxylic acids (Römermann et al. 1988). Hno (RpoN) mutants occurred relatively frequently by either chemical or transposon mutagenesis and recovered the wild type phenotype by one-step reversion, suggesting a single site mutation (Hogrefe et al. 1984). A specific class of regulatory hydrogenase mutants isolated from *A. eutrophus* ATCC17707 was also shown to result from a mutation on the chromosome (Cangelosi and Wheelis 1984).

The extent of pleiotropy associated with *hno* suggests that the respective gene encodes a global control element. *hno* was cloned from a genomic DNA library of *A. eutrophus* and identified by mutant complementation on a cosmid insert of 12.3 kb. The recombinant plasmid pCH170, containing a subcloned 6.5 kb fragment, not only was able to complement Hno^- mutants of *A. eutrophus* and *P. facilis* but also restored the $NtrA^-$ phenotype of enteric bacteria (Römermann et al. 1989). $NtrA^-$ ($RpoN^-$) mutants are impaired in a minor sigma factor (σ^{54}) of RNA polymerase which regulates transcription by interacting with core RNA polymerase and thus confers a distinct promoter specificity to the holoenzyme (Hunt and Magasanik 1985, Hirschmann et al.

1985). The consensus sequence of NtrA (RpoN) sensitive promoters has been described as follows: 5'-CTGGYAYR-N_4 TTGCA-3ø (R, purin; Y, pyrimidine; N, no specific base). Important for the promoter recognition are the two nucleotides GG and GC (underlined) at positions –24 and –12, respectively, and a proper spacing of 10 nucleotides (Ausubel 1984).

Two tandemly arranged promoter sequences of this type have been identified upstream of the open reading frame of the first structural gene in the putative *hoxS* operon, whereas a conventional enteric promoter sequence, characterized by a –10 and a –35 region, was absent in this part of the DNA sequence (A. Tran-Betcke and B. Friedrich, unpublished results). Preliminary nucleotide sequence data of the *hno* gene from *A. eutrophus* substantiates the previous observation that its product not only is functionally homologous with NtrA (Römermann et al. 1989) but also structurally related. Alignment of the predicted amino acid sequence of Hno and NtrA from various sources identified significant homology located in the N-terminus of the polypeptide and highly conserved C-terminal sequences, including a potential DNA binding domain (J. Warrelmann, M. Feldotte, B. Friedrich, unpublished results). Some of these RpoN specific motifs are not characteristic of other sigma factors (Merrick et al. 1987).

An *hno* like gene has previously been found in *P. facilis*; it is active in intergeneric complementation of Hno^- mutants from *A. eutrophus* (Römermann et al. 1989). Moreover, Birkmann et al. (1987) reported that at least one of the three hydrogenases in *E. coli* are controlled by the *ntrA* gene product. Taking all of these results into account it appears that the NtrA (RpoN) specific sigma factor of RNA polymerase is implicated not only in nitrogen-regulated gene expression but also in pathways involved in energy metabolism such as hydrogenase.

The second hydrogenase-controlling gene in *A. eutrophus, hoxA,* represents a highly specific regulator which responds to the energy status of the cell and the environmental temperature. Current results clearly classify the *hoxA* gene product as a positively, *in trans*, acting transcriptional regulator. The *hoxA* gene has previously been cloned as part of the *hoxC* region (Eberz et al. 1986), sequenced and expressed in *E. coli*. Its predicted apparent molecular weight is 53500, and an alignment of its amino acid sequence with sequences of transcriptional activators revealed extensive homoloy with NtrC and NifA (G. Eberz and B. Friedrich, unpublished results). Like NtrC and NifA (Drummond et al. 1986; Fischer et al. 1988) the HoxA sequence contains a putative nucleotide binding domain at the N-terminal part and a putative DNA binding domain at the C-terminus of the polypeptide. The central domain of HoxA is similar to that of NifA. A comparison of 219 amino acids yielded 50 % overall homology with NifA from *R. meliloti* (G. Eberz and B. Friedrich, unpublished results).

Transcriptional activators such as NtrC are part of a two component regulatory system composed of a regulatory protein (NtrC), and a signal transducer (NtrB), which responds to environmental signals, such as nitrogen

limitation by modulating the activity of the regulatory protein (Ronson et al. 1987). From the physiological data it is evident that hydrogenase expression in *A. eutrophus* H16 is stimulated by limitation of reducing equivalents (Friedrich 1982). Although the mechanism of signal transmission is still not known, HoxA appears to be involved in this process, since the transfer of *hoxA* into the H_2-inducible strain *A. hydrogenophilus* (Friedrich et al. 1984) converts the *hoxS* expression of the transconjugants redox-sensitive and H_2-independent (G. Eberz and B. Friedrich, unpublished results).

It is also not known, whether HoxA is part of a two component system or the direct sensor of the redox control. NifA of *B. japonicum* is oxygen-sensitive; its amino acid sequence exhibits putative metal binding centers, and its activity is inactivated by chelating agents. On the basis of these results a model was proposed which postulates metal center(s) as redox-sensitive targets of oxygen control (Fischer et al. 1988). Potential metal binding sites can also be assigned to a specific region of the predicted amino acid sequence of the HoxA ORF. Current investigations focus on the question whether metals play a role in the hydrogenase control circuitry in *A. eutrophus*. It is tempting to speculate that nickel may be involved as an effector, since Stults et al. (1986) and Doyle and Arp (1987), reported that hydrogenase expression in *B. japonicum* and *A. latus* respectively, directly correlates with a given nickel concentration in the medium.

Three other aspects have to be considered in future studies on hydrogenase regulation: (i) the temperature sensitivity of *hox* expression in *A. eutrophus* (Friedrich and Friedrich 1983) which is attributable to *hoxA*, since a mutation which confers temperature tolerance maps in *hoxA* (G. Eberz and B. Friedrich, unpublished results); (ii) DNA topology may be an important factor, since hydrogenase synthesis in *B. japonicum* is strongly and specifically inhibited by DNA gyrase inhibitors (Novak and Maier 1987); (iii) the stability of mRNA may be of significance, since DNA-RNA hybridization experiments revealed a half life for *hoxS* mRNA from *A. eutrophus* H16 of approximately 1 h (U. Oelmüller and C.G. Friedrich, unpublished results). This would be one of the most stable prokaryotic mRNAs ever reported (Belasco and Higgins 1988). In this respect it is interesting to note that *nif* specific mRNA from *Klebsiella pneumoniae* was reported to be also rather stable (Collins et al. 1986).

Accessory Genes Involved in the Formation of Active Hydrogenase

The high complexity of hydrogenases implicates the possibility that the formation of catalytically active enzyme requires further proteins. Nickel is an essential constituent of enzymatically active hydrogenase in all lithoautotrophs investigated so far. In order to provide the cells with sufficient amounts of the trace element, nickel ions have to be actively transported across the cytoplasmic membrane. Two energy-dependent processes are known (reviewed by Hausinger 1987). Nickel ions may be taken up by a nonspecific magnesium transport and/or a high affinity, nickel specific carrier. The transport of nickel

appears to be mediated by the magnesium uptake system in many bacteria (Kaltwasser and Frings 1980). The data presented in Table 10, however, demonstrate that most of the hydrogenase-containing autotrophic bacteria exhibit a nickel-specific transporter with a high affinity for the metal ion and a coupling to proton movement.

A. eutrophus is characterized by the possession of two nickel transport systems one of which is genetically linked to megaplasmid pHG1. Mutants defective in *hoxN* require an excess of nickel ions for autotrophic growth with hydrogen. This nickel deficiency was shown to be enhanced by increasing the concentration of magnesium in the medium and restored by a recombinant plasmid carrying the insert *hoxN*, which is derived from the internal region of the *hox* gene cluster (Fig. 1). From the low nickel transport rates in HoxN$^-$ mutants, their sensitivity towards Mg^{2+} ions and a reduced activity of nickel containing urease, it was concluded that *hoxN* encodes the high affinity nickel carrier (Eberz et al. 1989).

Table 10. Nickel transport in autotrophic bacteria.

Organism	Mg^{2+}-specific system		Ni^{2+}-specific system		Ref.
	Presence	K_m	Presence	K_m	
Alcaligenes eutrophus	+	17 μM	+	20 nM	1
Anabena cylindrica	ND	ND	+	17 nM	2
Bradyrhizobium japonicum	−	ND	+	26 μM	3
Methanobacterium bryantii	−	ND	+	3.1 μM	4
Rhodobacter capsulatus	+	5.5 μM	−	ND	5

+, present; −, absent; ND, no data

1, Tabillion and Kaltwasser 1977; Lohmeyer and Friedrich 1987; Eberz et al. 1989; 2, Campbell and Smith 1986; 3, Stults et al. 1987; 4, Jarrell and Sprott 1982; 5, Takaguwa 1987.

Phenotypically similar nickel suppressible hydrogenase mutants were isolated from *E. coli* and assigned to mutations in the loci *hydC* and *hydE* (Waugh and Boxer 1986; Chaudhury and Krasna 1987). A role of these gene products in nickel metabolism was discussed. In a recent communication Stoker et al. (1989) raised the possibility that *hydC* may represent a regulatory component.

Nickel and iron incorporation into the soluble hydrogenase of *A. eutrophus* was shown to depend on protein synthesis. Addition of metal salts in the presence of chloramphenicol did not permit active hydrogenase formation (Friedrich et al. 1984). Two classes of mutants, HoxD$^-$ and HoxE$^-$, may contribute to resolve this complex process. The two mutations map in the neighbouring DNA of the *hoxA* gene (Fig. 1). Mutants defective in the loci *hoxD* and *hoxE*, respectively, failed to grow with hydrogen, due to the loss of HoxS and HoxP activities. Only traces of HoxS and HoxP were detectable immunologically. Nevertheless, the amount of cross-reacting material was significantly higher in transconjugants of these mutants carrying multiple copies of the regulatory gene *hoxA*. The catalytic hydrogenase activity, however, was not restored. These results were substantiated by testing the effect

of *hoxE* and *hoxD* mutations on the expression of β-galactosidase activity in an *A. eutrophus* strain carrying a *hoxS'-lacZ'* fusion (Table 11). The results clearly show that the products of *hoxD* and *hoxE* are essential for catalytic hydrogenase activity and even appear to participate in *hoxS* and *hoxP* expression. The latter function can be suppressed by increasing the gene dosage of *hoxA,* thus we assume that HoxD and HoxE stimulate HoxS and HoxP expression through interaction with HoxA (G. Eberz and B. Friedrich, unpublished results).

Table 11. Effect of *hox* mutations on the expression of HoxS and β-galactosidase activities in *Alcaligenes eutrophus.*

Plasmids[a]	Activity (%)[b]	
	HoxS	β-Gal
pHG1	100 [++]	0
pHG1 + pGE44 (*hoxS'-lacZ'*)	100 [++]	100
pHG1 (*hoxA*⁻) + pGE44 (*hoxS'-lacZ'*)	0 [–]	0
pHG1 (*hoxD*⁻) + pGE44 (*hoxS'-lacZ'*)	0 [+]	40
pHG1 (*hoxE*⁻) + pGE44 (*hoxS'-lacZ'*	0 [+]	10
pHG1 (*hoxD*⁻::*hoxS-lacZ'*) + pGE4 (*hoxA*⁺)	0 [++]	100
pHG1 (*hoxE*⁻)::*hoxS-lacZ'* + pGE4 (*hoxA*⁺)	0 [++]	100

[a]pGE plasmids are pVK100 derivatives and present at an approximate copy number of six.
[b]Symbols in parenthesis refer to the amount of immunologically detectable HoxS protein. ++, wild type level; +, reduced by 50-90 %; –, no cross-reacting material. The cells were cultivated in fructose-glycerol-containing mineral medium as described by Friedrich et al. (1981a).

In a previous study it had been demonstrated that at least five polypeptides of unknown function are formed under the hydrogenase control system in *A. eutrophus* (Kärst and Friedrich 1987). It is the subject of current investigations to analyse the protein pattern of $HoxD^-$ and $HoxE^-$ mutants for alterations in these specific polypeptides. A rather complex genetic system for the production of three active hydrogenase isoenzymes has also evolved in *E. coli.* Besides the nickel linked loci *hydC* and *hydE,* at least four additional genes *hydA, hydB, hydD* and *hydF* are needed for the production of active hydrogenases (Wu and Mandrand-Berthelot 1986; Sankar and Shanmugam 1988).

Acknowledgements

We thank our colleagues, students, postdoctoral fellows, and coworkers, too many to name, who provided strains, antisera, reprints, preprints, unpublished results, helpful comments and contributions over the years.

Original research in our laboratories was supported by grants from the Deutsche Forschungsgemeinschaft.

Summary

The main physiological function of hydrogenases in lithoautotrophic bacteria is the consumption of molecular hydrogen as energy source. Three types of hydrogenases can be differentiated on the basis of their metal content, namely [Fe], [NiFe] and [NiFeSe] hydrogenases. The enzymes present in lithoautotrophs almost exclusively belong to the class of [NiFe] hydrogenases. The majority of organisms, including aerobic hydrogen bacteria, phototrophs and nitrogen-fixing lithoautotrophs, contain a heterodimeric membrane-bound hydrogenase which is coupled to the electron transport chain. Methanogenic bacteria and species of *Alcaligenes* and *Nocardia* are characterized by the possession of soluble hydrogenases which differ in subunit composition, cofactor content and physicochemical properties. Three modes of hydrogenase expression exist: (i) Constitutive synthesis (e.g. *Paracococcus denitrificans*), (ii) induction by H_2 (e.g. *Alcaligenes hydrogenophilus*) and (iii) derepression under growth with poor energy supply (e.g. *Alcaligenes eutrophus*).

From the gene organization and nucleotide sequence analyses polypeptides were identified in the heterotrimeric methyl viologen-reducing hydrogenase of *Methanobacterium thermoautotrophicum* and the heterotetrameric NAD-linked hydrogenase of *A. eutrophus* which indicate significant sequence identity with specific domains in the large and the small subunit of the membrane-bound enzymes, although a leader peptide is missing. The latter is characteristic for the coding region of the small subunit of the membrane-bound hydrogenases. There is a remarkable homology in the primary amino acid sequence of the putative nickel-ligating subunit of *M. thermoautotrophicum* and *A. eutrophus*, suggesting that the eubacterial and the archaebacterial enzymes have envolved from a common ancestral sequence. Especially the cysteinyl residues in the hydrogenases discussed here, appear to be well conserved, which may indicate that they act as potential iron-binding centers. Their sequences, however, are distinct from the iron-binding site of ferredoxin.

The formation of catalytically active hydrogenases requires accessory gene products which might function in hydrogenase processing, membrane incorporation, nickel uptake, subunit assembly, liganding of metal centers and the regulation of hydrogenase gene expression. In *A. eutrophus* most of these genes appear to be clustered in a 100-kb DNA region of megaplasmid pHG1. The expression of the *A. eutrophus* hydrogenase structural genes depends on the product of a chromosomal gene, *hno (rpoN)* and a plasmid-located gene, *hoxA*. Nucleotide sequence analysis and mutant complementing activity indicate that *hno* encodes a minor sigma factor (σ^{54}) of RNA polymerase which is homologous to the sigma factor NtrA, involved in the regulation of nitrogen controlled operons. In all lithoautotrophs investigated so far, the hydrogenase structural genes are tightly linked and appear to be organized in a single operon. The expression of the genes coding for the NAD-linked and the membrane-bound hydrogenases in *A. eutrophus* is coordinate, and responds to environ-

mental stimuli, energy deprivation and temperature. The regulatory *hoxA* gene is involved in this process. Its molecular structure exhibits extended homology with transcriptional activators such as NtrC and NifA.

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4. Denitrification by Obligate and Facultative Autotrophs

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Introduction

The Organisms

Denitrification is the use of nitrate, nitrite or the other nitrogen oxides as terminal electron acceptors in bacterial respiration. The end product is generally N_2, although a few species appear to terminate at N_2O (Gayon & Dupetit 1882;. Payne 1981; Stouthamer 1988a; 1988b). Some organisms can only convert nitrate to nitrite, a process known as nitrate respiration, although many (e.g. *Escherichia coli* and *Proteus mirablis*) can then reduce the nitrite to ammonia (Cole 1987; Cole & Brown 1980). This last is generally termed dissimilatory nitrate reduction in order to distinguish it from denitrification, where nitrogen is actually lost. In many organisms, the primary role of dissimilatory nitrate reduction is to serve as an additional sink for electrons during fermentative metabolism.

The denitrifiers form one of the most diverse groups of bacteria, with representatives of most physiological types being included. For example, there are obligate autotrophs and heterotrophs, methylotrophs and phototrophs, extreme halophiles and even nitrogen-fixing strains which denitrify. One of the most unusual denitrifying bacteria may be *Vibrio succinogenes* which reduces nitrate and nitrite to ammonia (as might be expected with a species from this genus) but which can also grow anaerobically with N_2O as the terminal electron acceptor, producing N_2. The resemblance of the responsible enzyme in this fermentative species with that of the true denitrifiers was emphasized by the fact that N_2O reduction by *V. succinogenes* was also inhibited by acetylene (Yoshinari 1980; Stouthamer 1988a). For a broad overview of most known denitrifying species, the reader should consult Payne (1981).

Recent work in our laboratory has concentrated on a denitrifier, *Thiosphaera pantotropha*, which combines a number of unusual properties. As will be discussed below, this organism is a constitutive denitrifier which is able to denitrify under fully aerobic conditions. It is also a heterotrophic nitrifier, being able to oxidize ammonia to nitrite in the presence of an organic

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 93-115.

substrate (for a review of heterotrophic nitrification, see Verstraete 1975). In addition, *Tsa. pantotropha* is a facultative autotroph capable of growth on hydrogen and reduced sulphur compounds under both aerobic and anaerobic (denitrifying) conditions. The combination of all of these properties prompted us to survey the literature on denitrifying autotrophs, and this is the subject of the present review. It was, of course, not a surprise that this cross-section through the microbial world involves a very heterogenous group of organisms, encompassing both obligately and facultatively autotrophic organisms. As will be discussed below, some facultative autotrophs appear to become obligately heterotrophic while denitrifying. Table 1 shows examples of obligate and facultative autotrophs which denitrify. Those species which only reduce nitrate to nitrite (e.g. *Thiobacillus thioparus* or *T. tepidarius*) or which use the dissimilatory nitrate reduction pathway to ammonium (e.g. *Escherichia coli* and *Proteus mirablis*) have been omitted. This paper concentrates on the consequences, some of them dramatic, which a change in electron acceptors (i.e. from oxygen to nitrate, nitrite or N_2O) can have on the physiology of this group of organisms, particularly with reference to our recent findings with *Tsa. pantotropha*.

Table 1. Examples of bacteria capable of chemolithotrophic growth which can also denitrify. obl = obligately autotrophic; fac = facultatively autotrophic.

Genus	Species	Obl/fac	End product	Substrates which support denitrification
Thiobacillus	*denitrificans*	obl	N_2	S^{2-} $S_2O_3^{2-}$ S^0
	versutus	fac	N_2	organic compounds
Thiomicrospira	*denitrificans*	obl	N_2	S^{2-} $S_2O_3^{2-}$ S^0
Thiosphaera	*pantotropha*	fac	N_2	S^{2-} $S_2O_3^{2-}$ H_2 organics
Paracoccus	*denitrificans*	fac	N_2	H_2 organics
Thermothrix	*thiopara*	fac	N_2	organic compounds
Alcaligenes	*eutrophus*	fac	N_2	H_2 organics
Pseudomonas	*saccharophilia*	fac	N_2	H_2 organics
	pseudoflava	fac	N_2	H_2 organics
Nitrosomonas	*europaea*	obl	N_2O	NH_4^+
Nitrosomonas	sp.	obl	N_2	NH_4^+
Nitrobacter	*hamburgensis*	fac	N_2	organics

Of the many inorganic compounds which support aerobic growth, only hydrogen, reduced sulphur compounds and (recently) ammonia have been shown to support denitrification. As can be seen below, the standard free energy charges ($\Delta G'$) per mol of substrate for denitrification are, in many cases, not much lower than those for oxygen respiration.

	$\Delta G_0'$ (kj per mol)
$2H_2 + O_2 \rightarrow 2H_2O$	-237
$5H_2 + 2H^+ + 2NO_3^- \rightarrow N_2 + 6H_2O$	-224
$S^{2-} + 2O_2 \rightarrow SO_4^{2-}$	-831
$5S^{2-} + 8NO_3^- + 8H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O$	-781

Of course, $\Delta G'$ values are extremely useful for showing how much energy can be derived from a given reaction from a thermodynamic point of view, and thus pin-pointing which reactions are possible. However, they can only give a very rough indication of the amount of ATP which may be generated in a physiological reaction because this is determined by a range of factors including the enzymes and cytochromes involved, as well as environmental parameters.

The Denitrifying Pathway and Enzymes

The enzymes and energetics of denitrification have been recently reviewed (Stouthamer 1988a; 1988b) and will not be dealt with in any great detail here. Consideration of the enzymes involved, however, reveals that despite the wide range of energy generating and carbon assimilating systems, those denitrifying enzymes which have been studied appear to be similar, even though they were isolated from widely different species. Indeed, even the nitrate reductase involved in dissimilatory nitrate reduction appears to be similar to that found in various denitrifying bacteria. Two different types of denitrifying nitrite reductase have been observed in different strains. One of these, known as cytochrome cd, has been identified in a number of bacteria (e.g. *Paracoccus denitrificans, Thiobacillus denitrificans*) and has been relatively well studied (e.g. Boogerd 1984; Alefounder & Ferguson 1981; Stouthamer 1980). A second nitrite reductase is copper-based rather than a cytochrome-based enzyme, and has been found in strains such as *Rhodopseudomonas sphaeroides var denitrificans* and *Achromobacter cycloclastes* (Michalski & Nicholas 1985; Iwasaki et al. 1975). Perhaps because of the limited number of strains in which it has thus far been found, this second nitrite reductase has not yet been studied to the same extent as cytochrome cd. The next step in the denitrification pathway, from nitrite to NO, remains a subject of debate. NO may be a free intermediate (with a separate reductase) in some strains, but not in others. Four different pathways which aim to describe the involvement of NO and N_2O have been proposed (Stouthamer 1988b). At least one of these postulates that NO remains enzyme-bound before being reduced to N_2. This might explain the apparent lack of free NO production by most intact bacteria under 'normal' growth conditions, although isolated enzymes do so (e.g. that of *Ps. perfectomarinus;* Payne 1981). The examination of N_2O reductase has been hampered by its instability. However, it appears, like one of the nitrite

reductases, to be a copper protein (Zumft & Matsubara 1982; Zumft et al. 1987; Michalski et al. 1986).

Autotrophy and Denitrification

The first evidence that chemolithotrophs could denitrify was published by Beijerinck in 1904. He used full, tightly-stoppered bottles with sulphur, carbonate and nitrate, and showed that a bacterial population which oxidized the sulphur to sulphate appeared. This was the first mention of *Thiobacillus denitrificans*. In 1910, Beijerink & Minkman described the growth of bacteria on hydrogen and N_2O. By means of tall glass columns filled with a mineral salts/thiosulphate medium and with an oxygen gradient from top to bottom, Lieske (1912) was able to obtain bands of bacterial growth at different redox levels. From the lowest band, he obtained a cultures of a denitrifying autotroph with a requirement for reduced sulphur compounds. In contrast to the strain of *T. denitrificans* described by Beijerinck, this isolate could not tolerate dissolved oxygen concentrations above 20% air and is thus more reminiscent of *Thiomicrosopira denitrificans*, which will be discussed below. Table 2 aims to place these discoveries related to autotrophy and denitrification within their historical context.

Table 2. Autotrophy and autotrophic denitrification in their historical context. Data from van Iterson (1902), Beijerinck & Minkman (1910), Lieske (1912) and Payne (1981).

Phenomenon	Authors/Year	Comment
'Knallgas' oxidation (H_2/air)	de Saussure (1939) Niklewski (1907)	Small, motile, slime-producing rods *B. saussurei*
Nitrate disappearance during putrefaction of animal tissue and from soil.	Davy (1814) Pelouse (1857) Boussingault (1858) Schloesing (1873)	
Microbial responsibility for nitrate disappearance noted	Gayon & Dupetit (1882) Gayon & Dupetit (1886)	'Denitrification' used to describe nitrate reduction
Oxygen-dependent ammonia oxidation	Schloesing (1873) Winogradsky (1890)	*Nitrosomonas* and *Nitrobacter*
Oxidation of $S_2O_3^{2-}$ and S^{2-}	Natanssohn (1902)	Small motile rods
Oxidation of tetrathionate	Beijerinck & Minkman (1902)	*T. thioparus*
Oxidation of sulphur	Jacobsen (1908)	
Denitrification with $S_2O_3^{2-}$	Beijerinck & Minkman (1902) Lieske (1912)	*T. denitrificans*
H_2 oxidation with N_2O	Beijerinck & Minkman (1910)	
CH_4 production from H_2 and CO_2	Söhngen (1906)	

As already mentioned, even when the field is limited to bacteria able to grow autotrophically, the denitrifiers are a diverse group. Some species have been studied in greater detail than others, and different aspects (e.g. energetics, genetics) of denitrification have been emphasized in the various studies. This review will concentrate on representative species from the various physiological groups, and will aim to highlight some of the, sometimes unexpected, consequences of changing electron acceptor.

The Obligate Autotrophs

a. The Hydrogen Oxidizing Bacteria

It is not clear whether the dominance of facultatively autotrophic, hydrogen-utilizing bacteria in the literature is a true reflection of nature, or due to problems associated with the growth of obligately autotrophic species. Two obligately autotrophic strains, *Calderobacterium hydrogenophimum* and *Hydrogenobacter thermophilus* have been described (Kryukov et al. 1983; Kawasumi et al. 1984). In the descriptions of these new species, *H. thermophilus* is described as being 'nitrate reduction positive' and *C. hydrogenophilum* is reported as being able to reduce nitrate to nitrite, but in neither case is it clear whether this nitrate reduction is associated with the assimilatory or dissimilatory pathways. The question of denitrification by obligately autotrophic hydrogen bacteria must therefore remain open.

Among the autotrophic denitrifiers, the most extensively studied are probably the colourless sulphur bacteria and, very recently, the nitrifiers.

b. The Colourless Sulphur Bacteria

Two obligately autotrophic members of this group can grow anaerobically while reducing nitrate or nitrite to nitrogen. These are *Thiobacillus (T.) denitrificans* and *Thiomicrospira (Tms.) denitrificans* (Kelly 1989a; 1989b; Kuenen & Tuovinen 1981; Kuenen & Robertson 1989a). Although superficially similar in that they both grow autotrophically on reduced sulphur compounds, they are morphologically different. *T. denitrificans* is a rod which may be motile by means of a polar flagellum, whereas currently available strains of the spiral-shaped *Tms. denitrificans* are non-motile. Chemostat cultures of *T. denitrificans* can be grown at a range of dissolved oxygen concentrations from air saturation (211 μM) to anaerobic (Justin & Kelly 1978a). The growth of *Tms. denitrificans* is inhibited by oxygen (even at μmolar concentrations), and aerobic growth has only been achieved under oxygen limitation (Timmer ten Hoor 1975; 1977). Unlike *T. denitrificans, Tms. denitrificans* resembles the facultatively autotrophic *Thiosphaera (Tsa.) pantrotropha* (which will be discussed below), in that its denitrifying enzymes are constitutive.

As with other denitrifiers, both *T. denitrificans* and *Tms. denitrificans* gave

lower yields when denitrifying than when using oxygen (Table 3). The aerobic biomass yields reported for *T. denitrificans* are, like those of *T. tepidarius*, approximately double those found for other autotrophic sulphur bacteria such as *T. thioparus* or *T. neopolitanus*. Kelly (1989b) has suggested that these different yields are related to the coupling of reduced sulphur compound oxidation to either cytochrome b (higher yields) or cytochrome c (lower yields). If the oxidation of reduced sulphur compounds is also coupled to cytochrome c in *Tms. denitrificans* and *Tsa. pantotropha* (both of which give 'low' yields) their cytochrome chains must be substantially different from those published (e.g. Stouthamer 1988a) for either *T. denitrificans* or *Paracoccus denitrificans* (another strain which gives 'low' aerobic yields on thiosulphate), otherwise *Tms. denitrificans* and *Tsa. pantotropha* would not be able to denitrify on thiosulphate and nitrate. It is believed that in strains such as *Pa. denitrificans*, the branch point by which electrons flow to nitrate occurs 'upstream' of cytochrome c, and thus before the electrons from thiosulphate enter the cytochrome chain (Stouthamer 1988a; 1988b).

Table 3. Comparison of aerobic and anaerobic yields obtained with obligately autotrophic, colourless sulphur bacteria (data from Timmer ten Hoor 1977). Yields expressed as g dry weight per mol electron donor.

Species	Electron donor	Electron acceptor	Yield
T. denitrificans	$S_2O_3^{2-}$	NO_3^-	9.3
T. denitrificans	$S_2O_3^{2-}$	O_2	13.2
Tms. denitrificans	$S_2O_3^{2-}$	NO_3^-	5.2
Tms. denitrificans	$S_2O_3^{2-}$	O_2	7.7

T. denitrificans cultures grown at 12 μM O_2 transiently accumulated nitrite when switched to anaerobic conditions, but had fully adjusted after 4 hours. It has been reported that *T. denitrificans* can be switched between aerobic and denitrifying growth with relative ease (Justin & Kelly 1978a). Interestingly, it was shown that *T. denitrificans* gave higher biomass yields at low dissolved oxygen concentrations (12μM). The yield decreased as the dissolved oxygen increased, indicating that at higher dissolved oxygen concentrations, oxygen can act as a metabolic inhibitor (Justin & Kelly 1978b).

At first glance, the existence of physiologically similar species in the same habitat seems puzzling. However, research by Timmer ten Hoor (1975; 1977) shed some light on the probable ecological niches of the two species when she found that the presence (or absence) of even very low concentrations of dissolved oxygen determined which species occurred. In sulphide-dependent enrichment cultures grown in chemostats from which oxygen had been rigorously excluded, *Tms denitrificans* tended to dominate. However, if oxygen was not completely excluded, *T. denitrificans* appeared. It was psoposed by the author that the predominant selective pressure determining the outcome of these experiments was the redox of the cultures. Thus *Tms. denitrificans* would

tend to be favoured in deep, low-redox, sulphide-rich sediments, whereas *T. denitrificans* would fit an ecological niche nearer the surface, where conditions might fluctuate between aerobiosis, oxygen limitation and anaerobiosis.

c. The Nitrifiers

Broda (1977) proposed that denitrifying bacteria which obtain their energy for growth from the oxidation of ammonium to nitrite ($\Delta G' = -360$ kj with nitrite as electron acceptor and N_2 as the end product) should exist, and termed them one of the 'lithotrophs missing in nature'. However, *Nitrosomonas* species have long been known to be able to produce N_2O during aerobic growth with ammonium as their energy source. Many papers about N_2O production by nitrifiers have been published, and it is not possible to cover more than a representative selection here. For a more extensive overview, the reader is referred to Bremner & Blackmer (1981). Figure 1 shows a compilation of the aerobic and anaerobic reactions which have been proposed by various authors.

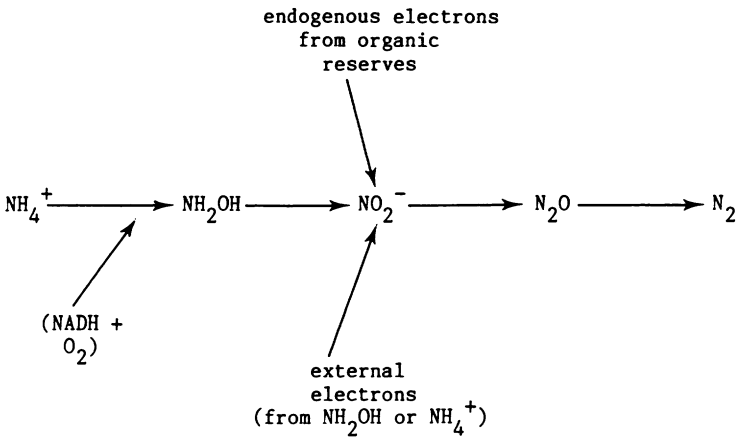

Fig. 1. Hypothetical scheme compiling the postulated pathways for electron transport during nitrite production and reduction by ammonia oxidizers.

Hooper (1968) described the isolation of a strictly aerobic enzyme from *N. europaea* which reduced NO_2^- to N_2O and NO with NH_2OH as the electron donor. Since then, it has become widely accepted that *N. europaea* can produce N_2O during nitrification, but it was assumed that this was a result of the breakdown of an unstable nitrification intermediate (Poth & Focht 1985). However, Ritchie & Nicholas (1972) showed that under anaerobic conditions, resting *N. europaea* cells provided with NH_4^+ and NO_2^-, or NO_2^- alone, produced twice as much N_2O as aerobic cells with NH_4^+ (83-84 and 38 nmol per mg protein per hour, respectively). Subsequent experiments with $^{15}NH_4^+$ and $^{15}NO_2^-$ confirmed that the bulk of the N_2O was being derived from NO_2^-.

Hynes & Knowles (1984) also showed that the rate of N_2O production by *N. europaea* was five times higher under anaerobic conditions than aerobically. The highest anaerobic rate of N_2O production (36 nmol per hour per culture flask) was reached with a mixture of NH_4^+ and NO_2^-, but rates approximately half this were found with NO_2^- alone. Acetylene inhibited (aerobically incubated) or partially inhibited (anaerobically incubated) N_2O production from NH_4^+, but not from NO_2^- (Fig. 1). Aerobic acetylene-treated cells supplied with NH_4^+ and NO_2^- gave N_2O production rates similar to those achieved with only NO_2^-. Aerobic NO_2^- production from NH_2OH was not affected by acetylene. As acetylene inhibits ammonia monooxygenase as well as nitrous oxide reductase, this appears to indicate that although NH_4^+ can contribute to NO_2^- reduction, it is not essential and the reaction can proceed on the basis of endogenous energy. That N_2O could be generated from NO_2^- in the absence of NH_4^+ indicates that the enzyme responsible is not hydroxylamine oxidoreductase, which requires NH_2OH as well as NO_2^- (Hooper 1984). Poth & Focht (1985) used ^{15}N-labelled compounds to confirm that NO_2^- (but not NO_3^-) is reduced to N_2O under oxygen stress. They postulated that by using NO_2^- as terminal electron acceptor, the organism was able to conserve the limited amount of available oxygen for nitrification. Poth (1986) followed this work with a later paper in which a *Nitrosomonas* sp. which could reduce NO_2^- to N_2 was described. Again, ^{15}N-labelled compounds were used to confirm the source of the N_2O, and the reactions occurred under oxygen limitation while the strain derived energy for growth from NH_4^+ oxidation. Poth observed in this paper that the demonstration of anaerobic, autotrophic denitrification by this strain would have to await the finding of a suitable artificial energy source because ammonia monooxygenase requires O_2 and NH_2OH is toxic, even in aerobic cultures. However, if substrate-limited chemostat cultures are used, rather than batch cultures, it is relatively easy to grow bacteria on toxic substrates, e.g. sulphide (Timmer ten Hoor 1975), methyl sulphides (Suylen 1988) and NH_2OH (Robertson 1988). If it is physiologically possible, it should therefore be feasible to show anaerobic, hydroxylamine-dependent denitrification by this strain in chemostat cultures. Finally, *Nitrosomonas* cells rich in organic reserve materials should be able to denitrify anaerobically.

The denitrifying reactions carried out by autotrophic, ammonia-oxidizing bacteria may not be so much a way of generating energy for growth, since the rates are very low, but may rather be a mechanism for surviving periods of anaerobiosis at the expense of internal (organic) energy sources. However, resolution of the role of denitrification by these bacteria must await experiments to determine whether or not they gain energy from the reactions, probably in chemostat studies. Bremner & Blackmer (1981) have observed that it is likely that most, of not all, ammonia-oxidizing bacteria are able to carry out this reaction under suitable conditions.

The Facultative Autotrophs

Membership of the physiological group known as the facultative autotrophs is steadily rising, and it appears that many strains are unrecognised as belonging to this group for lack of testing, rather than for lack of physiological ability. This is especially true for the ability to grow at the expense of reduced sulphur compounds as this property is not generally included in routine taxonomic tests (Kelly 1989a; 1989b; Mason & Kelly 1988; Robertson et al. 1989a). Friedrich & Mitrenga (1981) showed that many strains known to be facultatively autotrophic on hydrogen (including *Pa. denitrificans* and *Hydrogenobacter* strains) were also capable of autotrophic growth on thiosulphate, and Suylen & Kuenen (1986) were able to grow a pink methylotroph on thiosulphate. It is clear that the ability to grow autotrophically on a given class of inorganic substrates is an insufficiently precise criterion for taxonomic classification among the facultative autotrophs (Kuenen & Tuovinen 1981; Kelly 1989a).

In order to complete the picture, the chemolithoheterotrophs and 'incidental lithotrophs' should be briefly considered. The chemolithoheterotrophs are capable of generating energy from the oxidation of inorganic compounds, but cannot fix CO_2. In this group are many hydrogen- and sulphur-oxidizing bacteria (see, for example, Suylen, 1988). The 'incidental lithotrophs' are heterotrophs which can oxidize inorganic compounds but seem to be unable to gain metabolically useful energy from the reaction. Some examples are able to oxidize reduced sulphur compounds (Kelly 1989a), and it might be considered that the heterotrophic nitrifiers also fall within this category. As yet, facultatively autotrophic ammonia oxidizers have not been found, and ammonia oxidation by those heterotrophs known to be capable of doing it requires an organic substrate to drive the reaction (Verstraete 1975). As heterotrophic nitrification is frequently found in combination with denitrification, it will be discussed in more detail below.

Because many of the facultative autotrophs are capable of autotrophic growth on more than one type of compound (e.g. hydrogen, thiosulphate, etc), they cannot be so clearly separated in terms of substrate as most of the obligate autotrophs, and many strains cannot be allocated to either (e.g. *Tsa. pantotropha, Pa. denitrificans*). Thus, although the strains are discussed below in terms of their classically-recognised groups, for the sake of convenience, the reader should bear in mind that many strains have only been tested on either hydrogen or reduced sulphur compounds, and have been named on the basis of incomplete taxonomic tests. Where screening for the use of both has been done, for example in the study of thiosulphate metabolism by hydrogen-utilizing bacteria carried out by Friedrich & Mitrenga (1981), it rapidly becomes clear that the division is artificial. The nitrite-oxidizing strains form, perhaps, the only well-defined group among the facultative autotrophs.

As seen in Table 1, most strains which are capable of mixotrophic and autotrophic growth under aerobic conditions lose this ability when they denitrify. Among these are most strains of *T. versutus* (Wood & Kelly 1983), the

thermophilic colourless sulphur bacterium, *Thermothrix (Tx.) thiopara* (Brannan & Caldwell 1980) and *Nitrobacter hamburgensis* (Bock et al. 1986). *Paracoccus denitrificans* loses its ability to oxidize reduced sulphur compounds when denitrifying, but is able to grow anaerobically on hydrogen. In contrast, *Tsa. pantotropha* (Robertson & Kuenen 1983a; Kuenen & Robertson 1989b) retains its autotrophic potential on reduced sulphur compounds as well as on hydrogen while denitrifying. However, its μ_{max} while denitrifying on thiosulphate is at least a factor 10 lower than that of aerobic cultures.

For obvious reasons, most of the studies on denitrification by facultative autotrophs have been undertaken with heterotrophically-grown cultures. *Pa. denitrificans* has been one of the favourite experimental species for denitrification experiments in various laboratories (e.g. Kucera & Dadak 1983; Kucera et al. 1984; Alefounder et al 1983; 1984; Stouthamer 1980), and the work has been extensively reviewed (Stouthamer 1988a; 1988b). This section will therefore concentrate on facets of (generally heterotrophic) denitrification by other species which can oxidize hydrogen or reduced sulphur compounds, or which nitrify.

a. The Hydrogen-Oxidizing Bacteria

Hydrogen-oxidizing heterotrophs include species from many genera including *Pseudomonas, Alcaligenes* (including the former *Hydrogenomonas*), *Bacillus, Xanthobacter* and *Nocardia*. Their aerobic hydrogen metabolism was extensively reviewed by Bowien & Schlegel in 1981. Of course, not all of these species denitrify. Of those which do, perhaps the best known are *Paracoccus denitrificans* and *Alcaligenes eutrophus*. Some *Alcaligenes* strains are remarkable in that they lack a denitrifying nitrate reductase and can generally only denitrify if supplied with nitrite or if they are grown in ammonium-deficient medium when the assimilatory nitrate reductase can generate nitrite for denitrification (Pfitzner & Schlegel 1973). Much of the work on hydrogen metabolism by these organisms has been on their genetics, and is thus not appropriately treated here.

N_2-fixing bacteria which also denitrify embody both extremes of the nitrogen cycle. Species from various genera, including *Azospirillum, Bradyrhizobium, Rhizobium* and *Rhodopseudomonas (Rhodobacter)* are able to do this. Batch culture experiments on such an organism, which can also oxidize hydrogen, were described by Chan (1985). *Pseudomonas sp.* H8 grew in complex media at the expense of nitrate, nitrite and N_2O. Moreover, denitrifying growth was observed with hydrogen as the sole source of energy. Hydrogen oxidation was slower while denitrifying than when 10% or 21% oxygen was supplied (50%, 100% and 66% of the initial hydrogen after 22 hours incubation, respectively). This change in substrate oxidation rates is reflected in the lower growth rates shown by hydrogen-oxidizing *Paracoccus denitrificans* when denitrifying (Nokhal & Schlegel 1983).

b. The Colourless Sulphur Bacteria

Thiobacillus versutus presents an excellent example of a species which undergoes drastic alterations in its metabolism as a result of changing from aerobic to anaerobic, denitrifying growth. Firstly, *T. versutus* can only denitrify heterotrophically. Cultures grown aerobically in the presence of thiosulphate (and which therefore have the appropriate enzymes induced) have been shown to be capable of thiosulphate-dependent N_2 production in short-term anaerobic experiments with resting cells (Robertson & Kuenen 1983a), but there is no evidence that this reaction occurs in anaerobically growing mixotrophic cultures, or that it provides energy. It is more likely fortuitous. This failure to denitrify with reduced sulphur compounds as electron donors is probably due to the electrons from thiosulphate being fed into the cytochrome chain at cytochrome c. Electron flow to the denitrifying enzymes (to nitrate reductase 'upstream' of cytochrome c in the electron transport chain, and to nitrite and nitrous oxide reductase directly from cytochrome c) would therefore not generate any energy.

In addition to the loss of its ability to generate energy from the oxidation of reduced sulphur compounds on transfer to denitrifying conditions, the heterotrophic enzymology of *T. versutus* presents another example of the dramatic alterations in microbial physiology which can occur when the electron acceptor is changed. *T. versutus* has, for a long time, been studied as a member of a group of baceria which grow on acetate by means of an unknown pathway which does not involve the first enzyme of the glyoxylate cycle, isocitrate lyase (Gottschal & Kuenen 1980; Claassen et al. 1986). However, when denitrifying cells were examined, a significant isocitrate lyase activity was found (Table 4). This activity was not present in aerobically-grown cells, or in biomass grown aerobically or anaerobically on succinate (Claassen & Zehnder 1986). The authors used a range of techniques including enzyme assays, ^{13}C NMR spectroscopy and mass spectrometry to confirm that the enzyme indeed converted isocitrate to glyoxylate and succinate (i.e. it really was isocitrate lyase). The isocitrate lyase-negative pathway used during aerobic growth remains, as yet, unknown.

Table 4. The appearance of isocitrate lyase in denitrifying *T. versutus* cultures (data from Claassen & Zehnder, 1986).

Substrate	Electron acceptor	Isocitrate lyase
	nmol min^{-1} mg^{-1}	
Acetate	oxygen	0
Acetate	nitrate	52
Succinate	oxygen	1
Succinate	nitrate	1

c. The Ammonia-Oxidizers

Thiosphaera pantotropha is especially appropriate for inclusion in this review as it is capable of aerobic and denitrifying growth on thiosulphate, sulphide and hydrogen as well as a range of organic compounds including acetone (Robertson & Kuenen 1983; Bonnet-Smits et al. 1988). As will be discussed below, it can also oxidize ammonia, a phenomenon which, combined with its other properties, earns it a place in all three of the groups delineated by the definitions used among the obligate autotrophs. Ammonia oxidation by means of heterotrophic nitrification appears to be inseparable from denitrification in *Tsa. pantotropha*, and the two phenomena will therefore be discussed together.

During the comparison of its denitrifying potential with those of related organisms such as *T. versutus,* it was found that the denitrifying enzymes of *Ts. pantotropha* appeared to be constitutive. Aerobically grown biomass was able to produce nitrogen immediately it was supplied with nitrate and substrate (Robertsen & Kuenen 1984a), in contrast to *T. versutus* and *Pa. denitrification*, both of which required 3-4 hours induction period before they were able to denitrify. As the existence of aerobic denitrification was, at the time, somewhat controversial, experiments were carried out to discover whether or not the constitutive denitrification enzymes of *Tsa. pantotropha* were active under aerobic conditions.

Aerobic (>80% air saturation) batch cultures of *Tsa. pantotropha* grew more rapidly on organic substrates when provided with two electron acceptors

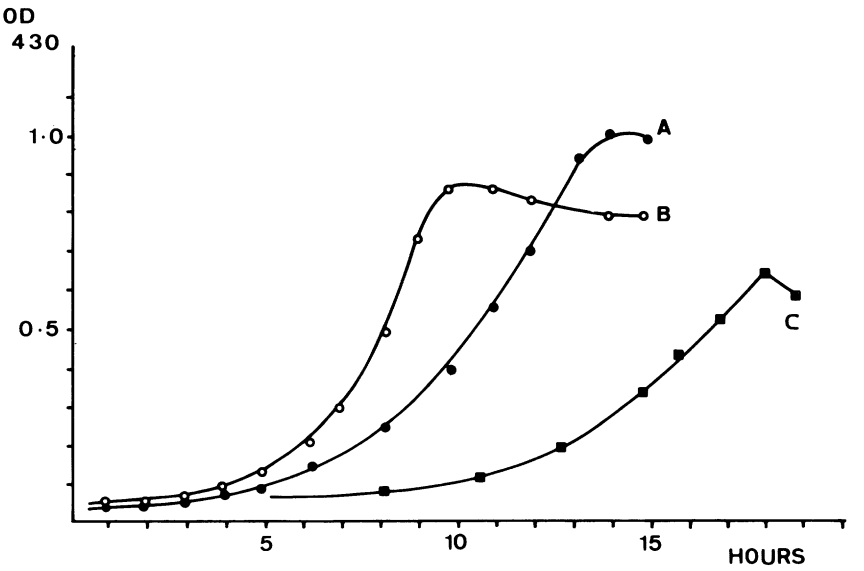

Fig. 2. Growth curves obtained with batch cultures of *Tsa. pantotropha* provided with acetate and ammonium. A: aerobic (>80% air). B: aerobic (>80% air) with nitrate. C: anaerobic with nitrate (Robertson & Kuenen 1984a).

(O_2 and NO_3^-) than with only one (Fig. 2). Analysis showed that about half of the respiration of the culture was taking place via denitrification. Protein measurements confirmed the impression give by the optical density readings that the yields were, as might be expected, intermediate between those obtained with either of the single electron acceptors (Robertson & Kuenen 1984a; 1984b). Oxygen and nitrate electrodes were used to show that aerobically-grown cell suspensions simultaneously utilized nitrate and oxygen, even when the only nitrogen compound supplied in the original growth medium was ammonium (Robertson et al. 1986). During experiments to test whether nitrite had the same effect on the aerobic growth rate as nitrate, it was found that the nitrite concentration in the cultures increased for a time, before disappearing (Fig. 3). This only happened in aerobic cultures in the presence of an organic substrate such as acetate, and is therefore heterotrophic rather than autotrophic nitrification (Verstraete 1975).

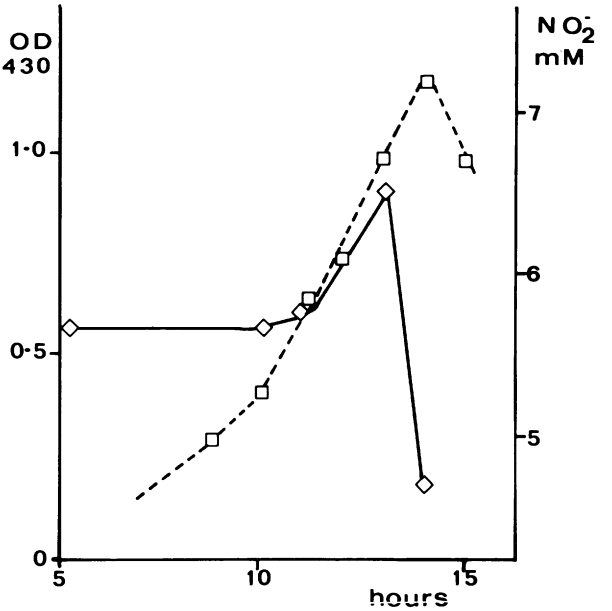

Fig. 3. Growth and nitrite concentration in aerobic (>80% air) batch cultures of *Tsa. pantotropha* provided with acetate, nitrite and ammonium. solid line = optical density at 430 nm, broken line = nitrite concentration.

Chemostat studies showed that aerobic denitrification and heterotrophic nitrification are intimately linked in *Tsa. pantotropha* (Robertson et al. 1988). As nitrite is common to both pathways (Fig. 4) and inhibits heterotrophic nitrification (Robertson & Kuenen 1988), it is not unlikely that the concentration of nitrite and nitrite reductase are controlling factors in determining the relative raltes of nitrification and denitrification in these cultures.

The nitrification and denitrification rates found in acetate-limited chemostat

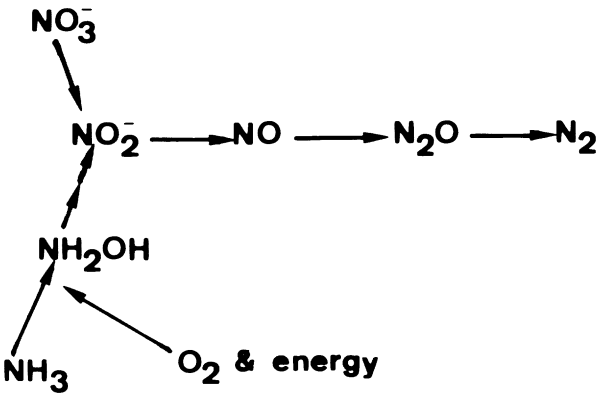

Fig. 4. Pathways of nitrification and denitrification as they appear to occur in *Tsa. pantotropha*.

cultures increased as the growth rate (dilution rate) increased, and also as the dissolved oxygen fell (Robertson et al. 1988). The provision of nitrate or nitrite in the medium resulted in lower nitrification rates (Table 5). Moreover, cultures grown mixotrophically on acetate and thiosulphate showed steadily decreasing nitrification and aerobic denitrification rates as the amount of thiosulphate in the medium increased (Robertson et al. 1988). Neither phenomenon occurred in mixotrophic cultures where the thiosulphate and acetate concentrations were roughly equivalent, or where the thiosulphate concentration exceeded that of the acetate (Nanninga et al. 1988).

Table 5. Correlation of chemostat yields (mg/L) with nitrification and denitrification rates (nmol^{-1} min^{-1} mg protein^{-1}). All cultures were substrate limited, and supplied with NH_4^+. Dissolved $O_2 = 80\%$ air saturation. $D = 0.04\ h^{-1}$. * indicates yield lower than expected. (Data from Robertson et al., 1988).

Additive	Nitrification	Denitrification	Yield
–	43	43	81*
NO_3^-	12	107	103
NO_2^- (limiting)	48	85	80*
NO_2^- (saturating)	25	98	115
NH_2OH	45	45	75*
$S_2O_2^{2-}$	21	21	145
$S_2O_3^- / NO_3^-$	6	36	120

It has always been believed that heterotrophic nitrifiers do not gain energy from nitrification, in contrast to the autotrophic nitrifiers. The results obtained with the chemostat experiments indicated that not only is energy not gained, but that it actually appears to be lost during heterotrophic nitrification by *Tsa. pantotropha*, despite the resemblance of its nitrifying pathway to that found in autotrophic ammonia oxidizers (Robertson & Kuenen 1988). Cultures exhibiting high nitrification rates gave protein yields which were only about

60% of those expected. Denitrifying and mixotrophic cultures (with lower nitrification rates) both gave biomass yields in the range expected (Table 5).

A model based on physiological data and preliminary cytochrome experiments has been developed in an effort to explain the appearance of heterotrophic nitrification and aerobic denitrification in *Tsa. pantotropha*, and their relationship to other physiological phenomena such as autotrophy and NO_x assimilation. The basic assumption is that there is a bottleneck on the flow of electrons along the cytochrome chain to oxygen via cytochrome aa_3. Allowing electrons to flow to the denitrification pathway and oxygen simultaneously would thus permit a faster flow of electrons through the main part of the cytochrome chain, and therefore a faster reoxidation of NAD(P)H. If nitrogen oxides are not available, and denitrification cannot proceed, NAD(P)H can be reoxidized during nitrification as it is required by the ammonia monooxygenase (Robertson & Kuenen 1988). This hypothetical flow is outlined in Fig. 5. During the transition from heterotrophic metabolism to mixotrophic and then autotrophic growth, additional cytochromes are induced (Robertson 1988), and these would also be a means of overcoming any bottleneck.

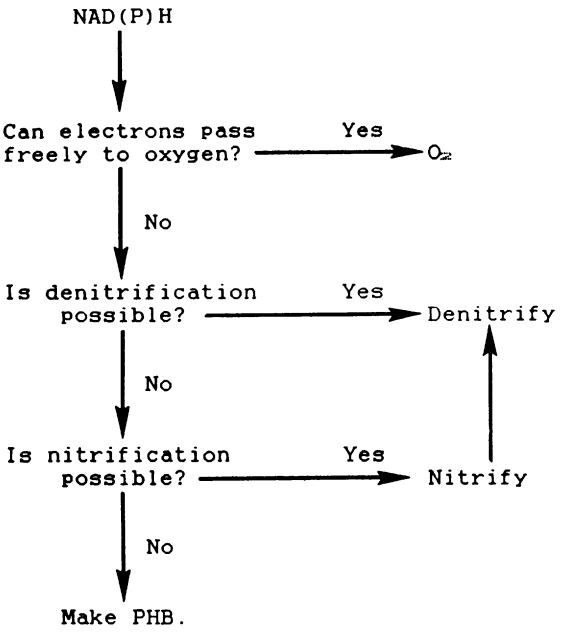

Fig. 5. Flow chart to show, schematically, the working hypothesis developed to explain the control of heterotrophic nitrification and aerobic denitrification in *Tsa. pantotropha* (Robertson et al. 1988).

Having established that *Tsa. pantotropha* was simultaneously nitrifying and denitrifying, and that it would not have been identified as a heterotrophic nitrifier by the classically-employed methods (which involve the measurement

of nitrite accumulation), the next interesting question was whether other strains also possessed the combined pathway. One example from this screening programme is a strain which was formerly known, together with other, unrelated strains, as *'Pseudomonas denitrificans'* (Douderoff et al. 1974; JCSB, 1982). Aerobic, heterotrophic chemostat cultures of this strain also nitrified and denitrified simultaneously (Robertson et al. 1989b), but they differed from *Tsa. pantotropha* in that the denitrifying nitrate reductase was not constitutive, although the rest of the denitrification pathway was. As thiosulphate inhibited nitrification by *Tsa. pantotropha* (Kuenen & Robertson 1987; Robertson & Kuenen 1988; Robertson et al. 1988; Nanninga et al. 1988), it seemed useful to test its effect on heterotrophic nitrification using a strain which was believed to be an obligate heterotroph. During acetate-limited chemostat experiments in which 5 mM thiosulphate was added to the medium, the nitrification rate fell by almost half, and the yield rose by almost a third. That this increase in yield was, however, not an indication of the amount of energy 'lost' during nitrification became clear when it was found that the strain had induced a thiosulphate-oxidizing capacity (615 nmol min^{-1} mg protein $^{-1}$). CO_2 was, of course, being fixed by means of the Calvin cycle (Robertson et al. 1989a; 1989b). Although the strain resembled *Tsa. pantotropha* in its simultaneous nitrification and denitrification and in its facultative autotrophy, it proved unable to use reduced sulphur compounds anaerobically. It has not yet been tested for the ability to oxidize hydrogen.

Having shown that at least two species were simultaneously nitrifying and denitrifying, it became important to establish how widespread this combination of the two phenomena is. Castignetti & Hollocher (1984) described their finding that many common denitrifying bacteria from soil were able to nitrify oximes or hydroxylamine heterotrophically. However, as with other nitrification studies, the nitrification rates were evaluated in terms of nitrite accumulation and it seemed that the nitrification rates were relatively insignificant. As *Tsa. pantotropha* and *'Ps. denitrificans'* would not have been detected as nitrifiers by this method because they simultaneously denitrify the nitrite produced, other bacterial strains were screened for the combined heterotrophic nitrification and aerobic denitrification pathway. Table 6 shows some of the results obtained with batch cultures. It was found that all of the heterotrophic nitrifiers tested were also capable of aerobic denitrification, and their growth rates were stimulated by the provision of nitrate in the medium. However, the dissolved oxygen concentration above which denitrification began to be inhibited was different in the various strains. For example, chemostat cultures of an *Alcaligenes* species nitrified and denitrified efficiently at dissolved oxygen concentrations below 50% air, but began to accumulate intermediates such as nitrite and hydroxylamine of the dissolved oxygen was above this level (Kuenen & Robertson 1987; van Niel et al. 1988).

The combination of heterotrophic nitrification and aerobic denitrification thus appears to be fairly widespread, and the physiological and ecological implications of a pathway which is apparently so wasteful of energy pose

Table 6. Comparison of the maximum specific growth rates (μ_{max}), protein concentrations and nitrate reduction obtained from aerobic or anaerobic batch cultures of bacteria known to be capable of heterotrophic nitrification. All of the media contained ammonia as the nitrogen source. The cultures were maintained at a dissolved oxygen concentration above 80% of air saturation. The growth rate and yield of a strain of *Pa. denitrificans* (which does not nitrify) were unaffected by the presence of nitrite. Adapted from Robertson et al., 1989A.

Organism	μ_{max} (h^{-1})			Protein (mg/l)		Delta $NO3^-$
	O_2	O_2/NO_3^-	NO_3^-	O_2	O_2/NO_3^-	mM
Pseudomonas sp. LMD 84.60 (ex. *Ps. denitrificans*)	0.1	0.41	0.15	78	60	5.0
A. faecalis LMD 84.59	0.17	0.25	0.07	30	14	4.1
Ps. aureofaciens LMD 37.26	0.19	0.21	0.07	66	66	5.0
T. pantotropha LMD 82.5	0.28	0.34	0.25	81	60	5.5
Pa. denitrificans LMD 22.21	0.28	0.28	nd	92	88	<1.0

nd = not determined

interesting questions. Even if it has evolved as a means of overcoming redox problems in the cytochrome chain, the possession of a constitutively active denitrifying system has obvious advantages in situations where the dissolved oxygen concentration fluctuates. This is clearly shown by a comparison of the results from experiments where the response of chemostat cultures of *Tsa. pantotropha* and a *Pa. denitrificians* strain (which is not an aerobic denitrifier) to a sudden shift from steady-state aerobic conditions to anaerobiosis was monitored (Fig. 6). The *Tsa. pantotropha* culture showed a small drop in optical density, which might be expected as the additional energy obtainable from oxygen respiration was lost, but soon stabilized under the new conditions. In contrast, *Pa. denitrificans* washed out at a rate in line with the dilution rate. Moreover, because of the amount of nitrite produced during its anaerobic phase (>10mM), *Pa. denitrificans* failed to recover when the air supply was

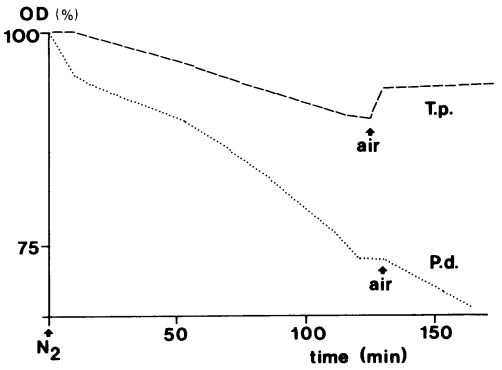

Fig. 6. The outcome of switching steady-state, acetate-limited chemostat cultures of *Tsa. pantotropha* (T.p.) and *Pa. denitrificans* (P.d.) from aerobiosis (80% air saturation) to anaerobiosis (Robertson 1988).

restored, and continued to wash out (Robertson & Kuenen, in preparation).

Situations where the dissolved oxygen is either low, or fluctuating occur at aerobic/anaerobic interfaces where the products of anaerobic metabolic activity such as reduced sulphur compounds and ammonia can also be expected to occur. With the results obtained with *'Ps. denitrificans'* in mind, other heterotrophic nitrifiers (including *Pseudomonas* and *Alcaligenes* strains) were also screened for the ability to oxidize reduced sulphur compounds. Of 7 strains tested, only 2 failed to induce a thiosulphate-oxidizing capacity when grown in batch cultures with 5 mM acetate and 10 mM thiosulphate (Robertson et al. 1989a). Whether or not these nitrifier/denitrifiers are able to grow autotrophically (aerobically or anaerobically) on reduced sulphur compounds remains to be tested. However, the fact that these strains combine the abilities to oxidize reduced sulphur compounds and ammonia indicates that a wider screening programme which tests putative heterotrophs for the ability to oxidize reduced sulphur compounds and ammonia in combination with denitrification may reveal whether a link between these properties is only superficial, or deserves further investigation.

d. The Nitrite-Oxidizing Bacteria

Recent work has shown that members of the genus *Nitrobacter* are facultative autotrophs. The pattern of mixotrophic growth in three species described by Bock et al. (1986) differs from that of the colourless sulphur bacteria in that *N. hamburgensis* and *N. winogradskyi* utilized organic compounds and nitrite simultaneously, even in batch culture when diauxy might be expected. Another *Nitrobacter* species exhibited diauxic growth, but still differs from the colourless sulphur bacteria in that it consumed the inorganic substrate first, rather than the organic one (Bock et al. 1986).

Recent studies (Freitag et al. 1987; Bock et al. 1988) have shown that various strains of *Nitrobacter* are able to reduce nitrate to nitrite, ammonia and to nitrogen gases, especially N_2O. However, in common with *Pa. denitrificans* and *Tx. thiopara*, they required the presence of an organic substrate (e.g. pyruvate) in order to denitrify. Approximately 40% of the nitrogen content of the medium was lost from the anaerobic cultures. In addition to their production of ammonia (generally associated with dissimilatory nitrate reduction) as well as N_2O, these organisms differed from all of the other species discussed in this review in one major respect – their protein yield after anaerobic growth (56 mg/l) in a mixotrophic (pyruvate and nitrite) medium was approximately 3 times higher than that obtained with similar, aerobic cultures (Freitag et al. 1987), but the large amounts of poly β-hydroxybutyrate (PHB) synthesised under mixotrophic and heterotrophic conditions should, perhaps, be taken into account.

Anaerobically-grown *Nitrobacter* cells are morphologically very different from aerobic, autotrophically grown cells (Freitag et al. 1987). They lose most, or all of their carboxysomes and synthesize substantial amounts of poly

β-hydroxybutyrate. This was clearly shown in experiments where an oxygen-permeable silicon tube was suspended in the culture medium and a biofilm formed on the surface of the silicon (Freitag et al. 1987). Examination of the biolayer with an electron microscope revealed that two morphologically distinct forms were appearing. At the bottom of the biolayer, near the silicon surface, the cells contained carboxysomes and were probably growing autotrophically on nitrite and oxygen. Closer to the surface of the biofilm, as the oxygen became depleted, the cells contained poly β-hydroxybutyrate and were morphologically similar to those grown in suspension at the expense of pyruvate and nitrate.

Concluding Remarks

If the hydrogen or sulphur-oxidizing denitrifiers and the denitrifying nitrifiers are indeed especially suited to life at aerobic/anaerobic interfaces where sulphide and nitrate might both be expected to occur, is this not also true of methanotrophs? Denitrification on methanol is known to occur in a limited number of strains, notably the Hyphomicrobia, but although there have been claims for methane-dependent denitrification by consortia (Hamer & Meschner 1984), a methane-oxidizing denitrifier remains to isolated. As with the obligately autotrophic hydrogen oxidizing bacteria mentioned above, it is not clear whether this lack is due to the difficulty of growing the bacteria, or reflects the true picture, perhaps because of the requirement for methane monooxygenase. It should be remembered that bacteria utilizing other substrates which were previously believed to require the use of a monooxygenase (e.g. acetone) have now been shown to be capable of denitrification.

At first glance, denitrification is a good illustration of the 'Unity in diversity' concept first voiced by Kluyver in 1924. As mentioned in the introduction, the pathway and enzymes involved are remarkably similar when the wide range of physiological types which denitrify are considered. However, on closer examination, 'Diversity in unity' might be a more appropriate viewpoint. One of the most unifying factors among the denitrifiers is also the most divisive, that the switch from O_2 to NO_3^- or NO_2^- as electron acceptor causes dramatic changes in other physiological factors. These range from the drop in biomass yield under anaerobic conditions, resulting from alterations in the path of electron flow along the cytochrome chain, to the gain and/or loss of pathways, as evinced by the loss of autotrophic potential in some of the facultative autotrophs and the acquisition of isocitrate lyase by anaerobically grown *T. versutus*. As the recent work with the autotrophic nitrifiers has shown, much remains to be discovered before we can claim that we fully understand denitrification.

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5. Formate Dehydrogenase: Microbiology, Biochemistry and Genetics

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Introduction

Formate is produced or consumed in various reactions essential for metabolic pathways of diverse organisms. It is commonly formed as a byproduct of anabolism where it is further oxidized by formate dehydrogenase to carbon dioxide or activated to formyltetrahydrofolate as a one-carbon substrate for biosynthetic reactions (Thauer et al. 1977a). A diversity of microorganisms produce or consume formate in energy yielding pathways. This chapter focuses on the reversible two-electron oxidation of formate ($HCOO^- = CO_2 + H^+ + 2e^-$) catalyzed by formate dehydrogenases from eubacteria, archaeobacteria and eucaryotic yeasts.

Microbiology, Physiology, and Ecology

Aerobic Bacteria

Formate Oxidation

Formate supports the growth of a diversity of aerobic, facultative, chemolithotrophic bacteria. *Alcaligenes eutrophus*, when cultured with formate, synthesizes both a membrane-bound and a soluble formate dehydrogenase (Friedrich et al. 1979). The organism also synthesizes a reversible formic hydrogenlyase system (Klibinov et al. 1982). A method is described for growth of *Thiobacillus* strain A2 by automatic titration with formic acid to maintain a constant pH and substrate concentration (Kelly et al. 1979). Calculations based on the growth yields indicate two ATP coupling sites during formate oxidation. *Escherichia coli*, aerobically grown on formate, synthesizes the same formate dehydrogenase that participates in the formate-nitrate system (Ingeldew and Poole 1984). *Campylobacter sputorum* is a microaerophile that grows aerobically with formate under low oxygen tension and synthesizes a membrane bound formate dehydrogenase (Niekus et al. 1980). Methylotrophic bacteria also utilize formate as a sole source of energy utilizing NAD^+-linked

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 117-141.

formate dehydrogenases (Anthony 1982). *Pseudomonas oxalaticus* grows with either oxalate or formate and synthesize both NAD^+-independent (Dijkhuizen et al. 1979) and NAD^+-dependent (Muller et al. 1978) enzymes.

Pathways in which Formate is an Intermediate

P. oxalaticus grows with oxalate as the sole energy source. Conversion to oxalyl-CoA is catalyzed by a CoA transferase using formyl-CoA as the donor (Dijkhuizen et al. 1980); formate produced in this reaction is oxidized to CO_2 by a NAD^+-dependent formate dehydrogenase. Formyl-CoA is regenerated by decarboxylation of oxalyl-CoA.

Methylotrophic eubacteria convert methane to methanol catalyzed by a NADH-dependent monooxygenase followed by oxidation of the methanol to formaldehyde (Anthony 1982). Organisms without the ribulose-5-phosphate cycle for carbon assimilation oxidize formaldehyde to free formate and therefore synthesize NAD^+-dependent formate dehydrogenases. The pathway for utilization of methanol by yeasts is similar to the eubacteria except in some strains the product of formaldehyde oxidation is S-formylglutathione which is either utilized directly by formate dehydrogenase or enzymatically hydrolyzed to formate. Interestingly, the formate dehydrogenase from the eubacterium *Achromobacter parvulus* is reported to oxidize S-formylglutathione (Egorov et al. 1982a).

Anaerobic Microorganisms

The conversion of complex organic matter in anaerobic habitats involves anaerobic microbial food chains comprised of interacting metabolic groups of organisms (Fig. 1). The fermentative bacteria degrade polymers to H_2, formate, acetate and higher volatile fatty acids. Among the intermediates shown in Fig. 1, formate and hydrogen are the most versatile electron donors. In marine sediments, the sulfate reducing bacteria outcompete methanogens for these substrates and sulfide is the predominant end product. In freshwater environments, the only substrates utilized by the methane-producing bacteria are H_2, formate and acetate; the higher fatty acids are converted to methanogenic substrates by the acetogens. The acetogens rely on the methanogens to lower the concentration of H_2 since the oxidations of fatty acids are thermodynamically unfavorable (McInerney and Bryant 1981). Historically, this syntrophy is termed 'interspecies H_2 transfer' but a major involvement for formate is proposed (Thiele et al. 1988; Thiele and Zeikus 1988). Interspecies formate transfer is reported for the amino acid degrading *Eubacterium acidaminophilum* when grown in coculture with a formate-utilizing (hydrogenase minus) *Desulfovibrio* species (Zindel et al. 1988). The ability of acetogenic bacteria to metabolize formate (Reddy et al. 1972) further supports the proposal that formate is an important intermediate in anaerobic microbial food chains. Many anaerobic bacteria also contain a formic hydrogenlyase system that interconverts formate and H_2 plus CO_2.

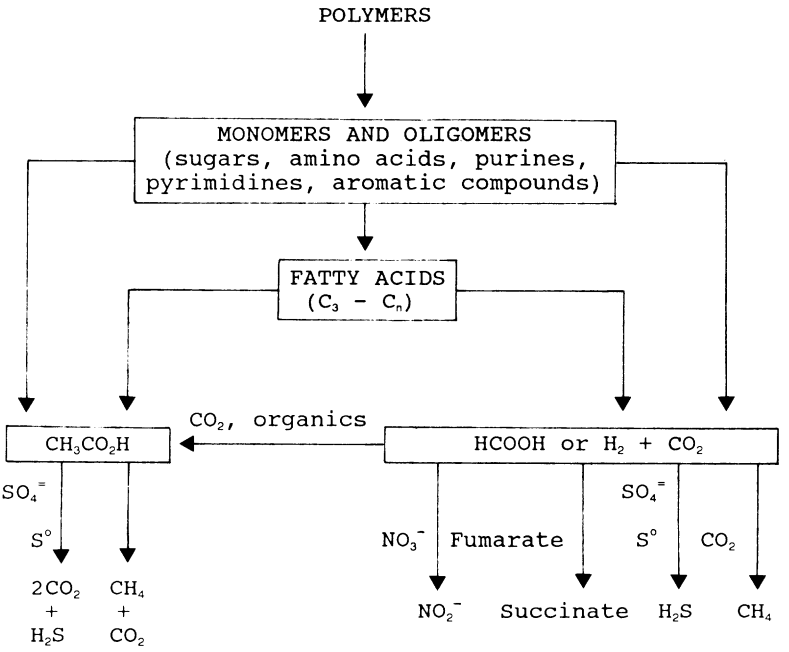

Fig. 1. Principle reactions representative of carbon and electron flow in anaerobic microbial food chains from freshwater and marine sediments. The boxed compounds are intermediates.

Formate Production

Formate is produced during the fermentation of sugars by the pyruvate formate-lyase catalyzed conversion of pyruvate to acetyl-CoA. *Ruminococcus albus*, and many other intestinal nonsporing anaerobes, produce formate during the fermentation of sugars but do not contain pyruvate formate-lyase activity (Miller and Wolin 1979). These results imply that formate is synthesized by the reduction of carbon dioxide (Miller and Wolin 1973; Wolin and Miller 1980), although no direct enzymatic evidence has been reported.

Formate is a product of nonsaccharolytic fermentations. It is produced from formyltetrahydrofolate in the fermentation of purines. This formyltetrahydrofolate synthetase catalyzed reaction is coupled to a substrate level phosphorylation of ADP. Formate is also produced by reduction of carbon dioxide during fermentation of hypoxanthine (Gottschalk 1985). Oxalate, a two-carbon dicarboxylic acid found in many plant and algal species, supports the growth of freshwater and rumen anaerobes and is degraded to formate and carbon dioxide (Smith et al. 1985; Allison et al. 1985). L-(+)-tartaric acid is fermented to acetate, carbon dioxide, and formate, supporting growth of freshwater and marine anaerobes (Schink 1984). *Eubacterium acidaminophilum* degrades amino acids only when cocultured with formate-utilizing organisms indicating that formate is a product of the fermentation (Zindel et al. 1988).

Formate is also a product of the fermentation of aromatic compounds. It is an intermediate in the methanogenic fermentation of benzoic acid (Ferry and Wolfe 1976). An organism is described that cleaves the phenylether bonds of methoxylated aromatic compounds to yield the corresponding hydroxy aromatic derivatives and mixed volatile fatty acids including formate (Mountfort and Asher 1986).

Formate Oxidation

Many facultative and strictly anaerobic bacteria obtain energy for growth by electron transport phosphorylation coupled to the oxidation of formate and reduction of exogenous electron acceptors (Table 1). The most extensively studied are the formate-nitrate and formate-fumarate systems in *Escherichia coli* and *Wolinella succinogenes*. In the *E. coli* formate-nitrate system, formate oxidation is coupled to the transfer of two electrons to a cytochrome b (Ingeldew and Poole 1984) catalyzed by the transmembranous formate dehydrogenase (Graham and Boxer 1981). Ubiquinone links the transfer of electrons from the cytochrome to the nitrate reductase complex. Two protons are translocated to the periplasmic side (on reduction of quinone) and two are consumed on the cytoplasmic side (upon reduction of nitrate) which generates

Table 1. Standard free energy of reactions with formate as reductant.

Equation	Substrates	Products	$\Delta G°'$	References
			(kJ/formate)	
1	$HCOO^- + H_2O$	$HCO_3^- + H_2$	+ 1.3	Thauer et al. 1977b
2	$4HCOO^- + H^+$	$2HCO_3^- + CH_3CO_2^-$	- 25.0	Tschech and Pfennig 1984
3	$4HCOO^- + H_2O + H^+$	$3HCO_3^- + CH_4$	- 32.6	Schauer and Ferry 1980
4	$HCOO^- + H^+ + S°$	$CO_2 + H_2S$	- 37.0	Macy et al. 1986
5	$4HCOO^- + SO_4^=$	$4HCO_3^- + S^=$	- 57.7	Postgate 1979
6	$HCOO^- + H_2O^+$ $^-O_2CCH=CHCO_2$	HCO_3^- + $^-O_2CCH_2CH_2CO_2^-$	- 85.4	Tschech and Pfennig 1084
7	$HCOO^- + NO_3^-$	$HCO_3^- + NO_2^-$	- 161.9[a]	Thauer et al. 1977b
8	$HCOO^- + H^+ + MnO_2$	$H_2O + MnCO_3$	- 209.0	Lovley et al. 1989
9	$HCOO^- + H_2O + 2Fe^{3+}$	$HCO_3^- + 2H^+ + 2Fe^{2+}$	- 227.0	Lovley et al. 1989
10	$HCOO^- + ½O_2$	HCO_3^-	- 235.9[a]	Gottschalk 1985

[a] Calculated using reaction 1 (see reference).

a proton gradient. A similar electron transport chain functions in the formate-fumarate system involving menaquinone. In the *W. succinogenes* formate-fumarate system (Kroger et al. 1980), the formate dehydrogenase faces the periplasmic side of the membrane where formate is oxidized with transfer of electrons to a low potential cytochrome b ($E^{o'}$ = -200 mV). Menaquinone links the low potential cytochrome with a high potential cytochrome b ($E^{o'}$ = -20 mV) which donates electrons to the fumarate reductase complex located on the cytoplasmic side of the membrane. No vectorial translocation of protons occurs but the liberation of protons by the formate dehydrogenase and uptake of protons by the fumarate reductase occurs on opposite sides of the membrane which results in a proton gradient. Like many anaerobic organisms, *E. coli* synthesizes a formic hydrogenlyase system (reaction 1, Table 1). Two carriers mediating electron transport between formate dehydrogenase and hydrogenase are postulated (Grey and Gest 1965) but the system has not been reconstituted *in vitro*. The formate dehydrogenase of the formic hydrogenlyase system is distinct from the enzyme of the formate-nitrate system (Giordano et al. 1983).

The sulfate-reducing bacteria are strict anaerobes that obtain energy for growth by the dissimilatory reduction of sulfate to sulfide (Table 1) (Widdel 1988). The electron donors are diverse and include H_2 and formate (Badziong and Thauer 1978; Magee et al. 1978). Yields of *Desulfovibrio vulgaris* grown on formate indicate the formation of two ATP coupled to the reduction of adenosine phosphosulfate to sulfite, and one ATP formed on reduction of sulfite to sulfide (Magee et al. 1978). The immediate electron acceptor for the formate dehydrogenase from *D. vulgaris* is cytochrome c-553 (Yagi 1979); other electron transport components are not known. Sulfate-reducing bacteria contain an active formic hydrogenlyase system presenting the possibility that formate may first be converted to H_2. A significant portion of the formate dehydrogenase from *Desulfovibrio gigas* (Odom and Peck 1981) is located in the periplasmic space.

In addition to sulfate, several strictly anaerobic bacteria have been isolated that reduce elemental sulfur, or oxides of sulfur, but not sulfate (Widdel 1988). In addition to nitrate, *W. succinogenes* is also able to grow with sulfur as the electron acceptor for formate oxidation (Macy et al. 1986). *Spirillum* 5175 is able to grow by the oxidation of formate coupled to the reduction of sulfur, sulfite or thiosulfate (Wolfe and Pfennig 1977). Interestingly, formate is required for the syntrophic growth of *Spirillum* 5175 and *Chlorobium limicola* in the presence of light and limiting amounts of sulfur. *Chlorobium* is a photosynthetic organism that oxidizes sulfide to elemental sulfur but does not utilize formate as an assessory electron donor.

The strictly anaerobic methane-producing archaeobacteria obtain energy for growth by the cleavage of acetate or the reduction of carbon dioxide (Rouviere and Wolfe 1988). The eight electrons that are required for the reduction of carbon dioxide originate from H_2 or formate (Table 1). The first one-carbon

derivative in the pathway is formylmethanofuran (Rouviere and Wolfe 1988) but free formate is not an intermediate (Sparling and Daniels 1986). *Methanobacterium formicicum* utilizes formate and H_2/CO_2 simultaneously when both are present at saturating concentrations (Schauer and Ferry 1982). Although the free energy change for growth on formate or H_2 is essentially equivalent, molar growth yields with formate are significantly greater than with H_2/CO_2 (Schauer and Ferry 1980). Biochemical and immunocytochemical studies show that the formate dehydrogenase is loosely associated with the membrane (Baron and Ferry 1987; Baron et al. 1989) in agreement with a chemiosmotic mechanism for ATP synthesis (Blaut and Gottschalk 1985). The formate dehydrogenase from *Methanococcus vannielii* (Jones and Stadtman 1981) and *M. formicicum* (Schauer and Ferry 1986) reduces the 5-deazaflavin coenzyme F_{420} (F_{420}) which functions as a low potential electron donor for at least two steps in the pathway of carbon dioxide reduction (Hartzell et al. 1985; Rouviere and Wolfe 1988). Other methanogenic bacteria are described that contain formate dehydrogenases linked to F_{420} (Tzeng et al. 1975; Ferry and Wolfe 1977). The *M. formicicum* enzyme also participates in a F_{420}- mediated formic hydrogenlyase system (Baron and Ferry 1989a) which has been reconstituted with purified components (Baron and Ferry 1989b).

Recently, it was reported that *Alteromonas putrefaciens* obtains energy for growth by the oxidation of formate and reduction of Fe(III) or Mn(IV) (Table 1). The organism is able to metabolize formate to concentrations below 100 μM.

In addition to anaerobic respirations, anaerobes are described that require H_2 or formate as an assessory reductant for the fermentation of organic substrates. *Eubacterium oxidoreducens* utilizes formate as a source of electrons for the reduction of phloroglucinol to dihydrophloroglucinol, intermediates in the pathway of gallic acid fermentation to acetate and butyrate (Krumholz et al. 1987). The phloroglucinol reductase (Haddock and Ferry 1989) and formate dehydrogenase are linked by $NADP^+$. *Eubacterium acidaminophilum* utilizes formate as an electron donor for the fermentation of betaine, sarcosine or creatine to acetate (Zindel et al. 1988). *Acetobacterium woodii* reduces caffeate to hydrocaffeate with formate as the electron donor (Tschech and Pfennig 1984) resulting in increased growth yields, implying an electron transport phosphorylation coupled to electron transport from formate.

Pathways in Which Formate Is an Intermediate

Many anaerobic bacteria utilize the Wood pathway for the synthesis of acetate from carbon dioxide (Ljungdahl 1986) which has been extensively studied in *Clostridium thermoaceticum*. The methyl group is synthesized by reduction of CO_2 to formate followed by the formation of formyltetrahydrofolate and reduction to methyltetrahydrofolate. The formate dehydrogenase from *C. thermoaceticum* is the only enzyme known to catalyze reduction of CO_2 with NADPH, a thermodynamically unfavorable reaction under standard

conditions. The six electrons required for the reduction of CO_2 to the methyl level ultimately derive from the oxidation of carbohydrate or H_2. The methyl group of methyltetrahydrofolate is transferred to a site on carbon monoxide dehydrogenase where it condenses with a carbonyl group originating from CO_2, CO, or the carboxyl group of pyruvate. *Syntrophococcus sucromutans* (Krumholz and Bryant 1986) reduces formate to acetate utilizing the Wood pathway and electrons derived from the oxidation of various carbohydrates, but the organism is unable to reduce CO_2 resulting in a requirement for formate.

Formate is produced from formyltetrahydrofolate during the fermentation of glycine and purines; the formyltetrahydrofolate synthetase catalyzed reaction is coupled to a substrate level phosphorylation (Gottschalk 1985). The formate is further oxidized by formate dehydrogenase to CO_2.

Biochemistry

Aerobic Microorganisms

General Properties

Table 2 lists the properties of extensively purified formate dehydrogenases. Selected properties of partially purified enzymes are also described (Deyhle and Barton 1977; Dijkhuizen et al. 1979; Kato et al. 1979). The enzyme from *P. oxalaticus* also reduces CO_2 (not bicarbonate) to formate (Ruschig et al. 1976). This enzyme is distinct from all others purified from aerobic organisms in that it is a heterodimeric, oxygen-sensitive, iron-sulfur, flavoprotein (Muller et al. 1978). The remaining formate dehydrogenases are homodimeric with no known metals or cofactors. The absence of iron and acid-labile sulfide in the *P. oxalaticus* enzyme may be responsible for insensitivity to oxygen. All of the enzymes reduce NAD^+ and have high K_m values for formate. Despite the similarities, polyclonal antibodies to the yeast enzyme do not cross react with formate dehydrogenases from the facultative and obligately methylotrophic eubacteria (Hou et al. 1982). The formate dehydrogenase from *A. parvulus* also oxidizes the formyl group of S-formylglutathione (Egorov et al. 1982a); thus, the true physiological substrate for formate dehydrogenases in methanol utilizing eubacteria is a matter of controversy.

Enzyme Mechanism

All of the formate dehydrogenases described from aerobic microorganisms are inhibited by azide and sulfhydryl reagents. Azide binds tightly to transition metals but the apparent absence of metals in these enzymes implies a different mechanism. It is noted that azide and nitrate are linear triatomic anions analogous to the proposed transition state of formate during catalysis (Blanchard and Cleland 1980). The tight binding behavior of azide and nitrate supports the hypothesis that these inhibitors may be transition-state analogs

Table 2. Formate dehydrogenases extensively purified from aerobic microorganisms.

Organism	Native molecular weight	Subunit molecular weight	Metals					Acid-labile sulfide
			Mo	W	Se	Zn	Fe	
	(M_r)	(M_r)	(mol/mol native)					(mol/mol native)
Eubacteria								
Pseudomonas oxalaticus	315,000	α_2(100,000) β_2(59,000)	ND[a]	ND	ND[b]	ND	18-25	15-20
Achromobacter parvulus	80,000	α_2(46,000)	ND	ND	ND	ND	ND	ND
Moraxella strain C-1	98,000	α_2(48,000)	ND	ND	ND	ND	ND[f]	ND
Eucaryotic yeast								
Pichia pastoris	94,000	α_2(47,000)	NS[h]	NS	NS	NS	NS	ND
Candida methylica	70,000	α_2(46,000)	ND	ND	ND	ND	ND	ND

[a] Not determined
[b] Selenium not required for growth
[c] p-Hydroxymercuribenzoate
[d] No absorbance in visible spectrum
[e] Dinitrobenzoate
[f] Activity not effected by iron chelating agents
[g] N-ethylmaleimide
[h] Not significant

of formate. Inhibition of the *P. oxalaticus* enzyme by azide and nitrate is partly overcome by formate (Muller et al. 1978). Inhibition of all aerobic formate dehydrogenases by sulfhydryl reagents implies that a cysteine residue is involved at the active site or is essential in maintaining an active conformation of the enzymes.

Flavins are generally required by dehydrogenases to transfer electrons from one-electron centers to the obligate two-electron accepting pyridine nucleotide. Thus, flavins are not a requirement for the metal-free NAD-dependent formate dehydrogenases (Table 2).

The flavin in the iron-containing *P. oxalaticus* enzyme is noncovalently bound and dissociates from the enzyme under reducing conditions yielding an inactive deflavoprotein (Muller et al. 1978). The deflavoprotein is reactivated by incorporation of oxidized FMN but not FAD. The FAD-containing F_{420}-dependent formate dehydrogenase and the F_{420}-dependent hydrogenase from *M. formicicum* (Schauer and Ferry 1983; Baron and Ferry 1989b), and the NAD-

Cofactors	Physiological electron acceptor	K_m Formate	Inhibitors	References
(mol/mol native)		(mM)		
2 FMN	NAD	13.5	O_2, N_3^-, CN^-, NO_3^-, NADH, Hg^{++}, p-HMB[c]	Muller et al. 1978; Ruschig et al. 1976
none[d]	NAD	15	N_3^-, CN^-, p-HMB, DTNB[e], iodoacetamide, pyridoxal, 2,3-butanedione	Egorov et al. 1979; Egorov et al. 1981; Egorov et al. 1982a Egorov et al. 1982b
none[d]	NAD	13	N_3^-, CN^-, NO_3^-, Hg^{++}, DTNB, NEM[g]	Asano et al. 1988
none[d]	NAD	16	N_3^-, Cu^{++}, Hg^{++}, p-HMB, DTNB, 5-nitro-8-hydroxy-quinoline	Hou et al. 1982
ND	NAD	13	N_3^-, CN^-, DTNB iodoacetamide	Avilova et al. 1985

dependant formate dehydrogenase from *Rhodopseudomonas palustris* (Yoch and Lindstrom 1969), behave similarly to the *P. oxalaticus* enzyme. Flavins have marked conformational differences between oxidized and reduced forms, and the affinity of these deflavoproteins for the reduced and oxidized cofactor is different. Thus, the activity of formate dehydrogenases with dissociable flavins could conceivably be controlled by the redox potential of the cell.

A study of yeast formate dehydrogenase indicates an ordered kinetic mechanism with NAD^+ adding before formate (Blanchard and Cleland 1980). Only an intrinsic ^{13}C-formate isotope effect is observed indicating a weak commitment to catalysis. A group with pK = 6.4 must be ionized for binding of formate and another group with pK = 5.9 must be ionized for catalysis, but the roles of these ionizable groups are not clear. Modification of an arginine residue inactivates the *A. parvulus* formate dehydrogenase; protection by NAD^+ suggests an involvement of this amino acid in the active site of this enzyme (Egorov et al. 1981). Modification of a lysine by pyridoxal also inhibits

Table 3. Formate dehydrogenases extensively purified from an aerobic archaeobacteria and eubacteria.

Organism	Native molecular weight	Subunit molecular weight	Metals					Acid-labile sulfide
			Mo	W	Se	Zn	Fe	
	(M_r)	(M_r)	(mol/mol native)					(mol/mol native)
Archaeobacteria								
Methanococcus vannielii	105,000	ND[a]	1	NS[b]	NS	ND	4.8-10.1	10-20
Methanobacterium formicicum	177,000	α_1(85,000) β_1(53,000)	1	NS	NS	2	21-24	25-29
Eubacteria								
Escherichia[e] *coli*	590,000	α_4(110,000) β_4(32,000) γ_4(20,000)	4	ND	4	ND	56	52
Clostridium thermoaceticum	340,000	α_2(96,000) β_2(76,000)	NS	2	2	NS	36	50
Clostridium[h] *pasteurianum*	118,000	α_1(76,000) β_1(34,000)	2	NS	NS	ND	24	28
Vibrio[i] *succinogenes*	263,000	α_2(110,000)	1	ND	NS	ND	19	18

[a] ND, not determined
[b] NS, not significant
[c] F_{420}, 5-deazaflavin
[d] Tiron, 4,5-dihydroxy-m-benzene disulfonate
[e] Formate dehydrogenase coupled to nitrate reduction

the enzyme; protection by formate indicates involvement of this residue in substrate binding (Egorov et al. 1982b).

Anaerobic Enzymes

General Properties

Table 3 lists the properties of formate dehydrogenases purified from anaerobic archaeobacteria and eubacteria. Other, less purified preparations, are also described (Kearney and Sagers 1972; Yagi 1979). All have in common extreme

Cofactors	Physiological electron acceptor	K_m Formate	Inhibitors	References
(mol/mol native)		(mM)		
ND	F_{420}[c]	ND	O_2, EDTA iodoacetamide Tiron[d] α,αdipyridyl 1,10-phenanthroline	Jones and Stadtman 1981
pterin n(Fe_4-S_4) 1 (FAD)	F_{420}	0.6	O_2, N_3^-, NO_3^-, α,α-dipyridyl, 1,10-phenanthroline	Schauer and Ferry 1982; Schauer and Ferry 1983;
4 (cyt b) pterin	Quinone	0.03	O_2, N_3^-, CN^-, iodoacetamide, p-HMB[f], HQNO[g]	Enoch and Lester 1975; Enoch and Lester 1982
pterin 2(Fe_4-S_4) 2(Fe_2-S_2)	NADP	0.2	O_2, N_3^-, CN^-, hypophosphite	Li et al. 1966; Andreesen and Ljungdahl 1974; Yamamoto et al. 1983
pterin n(Fe_4-S_4) n(Fe_2-S_2)	Ferredoxin	1.7	O_2, N_3^-, CN^-, NO_3^-	Thauer et al. 1975; Scherer and Thauer 1978; Thauer et al. 1973; Liu and Mortensen 1984; Prince et al. 1985
ND	Quinone	1.5	O_2, N_3^-, CN^-, Hg^{++}	Kroger et al. 1979

[f] p-hydroxymercuribenzoate
[g] n-heptyl hydroxyquinoline-N-oxide
[h] Formate dehydrogenase coupled to carbon dioxide reduction
[i] Formate dehydrogenase coupled to fumarate reductase.

sensitivity to oxygen. Like the formate dehydrogenases from aerobes, azide is a potent inhibitor of the enzymes from anaerobes; the only exception being the formate dehydrogenase from *M. vannielii* (Jones and Stadtman 1981). Inhibition of the enzymes from anaerobic microorganisms is competitive with formate (Thauer et al. 1975; Kroger et al. 1979; Schauer and Ferry 1982; Liu and Mortenson 1984) consistent with the possibility that azide is a transition-state analogue of formate as proposed for the aerobic formate dehydrogenases (Blanchard and Cleland 1980). The enzymes from anaerobic organisms contain an array of redox centers and reduce a diversity of physiological electron

acceptors, a striking departure from the enzymes of aerobic eubacteria and eucaryotes (Table 2).

Enzyme Mechanism

Molybdenum and Tungsten Centers. In addition to those listed in Table 3, several other molybdenum- and tungsten-requiring formate dehydrogenases are known (Andreesen and Ljungdahl 1974; Leonhardt and Andreesen 1977; Wagner and Andreesen 1977). Many of the purified enzymes are reported to contain a cofactor with spectral properties similar to the 6-substituted pterin (molybdopterin) present in all molybdoenzymes other than nitrogenase (Kramer et al. 1987). The cofactors from all molybdoenzymes studied have in common the ability to reconstitute nitrate reductase activity in extracts of *Neurospora crassa nit-1*, a mutant unable to synthesize a functional pterin cofactor (Ketchum et al. 1970). The cofactor from the tungsten-containing *C. thermoaceticum* formate dehydrogenase reconstitutes activity when molybdate is added but not when tungsten is added (Deaton et al. 1984). The pterin cofactor from the archaeobacterium *M. formicicum* is unable to reconstitute activity of the *N. crassa* mutant (May et al. 1986). The archaeobacterial cofactor is a 6-substituted pterin but additional studies on the structure of the side chain are necessary to determine the basis for the inability to reconstitute activity in the *N. crassa* mutant. Recently it was shown that the side chain of eubacterial molybdopterin (bactopterin) is structurally distinct from the eucaryotic cofactor (Kruger and Meyer 1987).

The formic hydrogenlyase-linked formate dehydrogenase from *E. coli* (formate dehydrogenase-H) also contains molybdenum. This enzyme reduces benzyl viologen ($E^{o'}$ = -360 mV) but not phenazine methosulfate ($E^{o'}$ = -145 mV), and is immunologically distinct from the nitrate reductase-linked formate dehydrogenase (formate dehydrogenase-N) which reduces the latter, but not the former, artificial electron acceptor (Giordano et al. 1983). Comparison of the deduced amino acid sequences of the large subunits of the *E. coli* formate dehydrogenase-H and the archaeobacterial enzyme reveals four regions of high identity (Fig. 2). High similarity in isofunctional enzymes from radically divergent organisms may indicate conservation of structure essential for functions which are common to both enzymes. Interestingly, the boxed sequence in region IV (Fig. 2) from the *E. coli* enzyme is suggested as a potential molybdopterin binding site (Bilous et al. 1988).

The molybdenum center of the *M. formicicum* formate dehydrogenase has been studied by EPR spectroscopy (Barber et al. 1983). The molybdenum signal is the only example of a molybdoenzyme with a g_{ave} of greater than 2.0. The signal is observed in formate-reduced whole cells supporting a catalytic function for the molybdenum center. The redox couples are -330 mV for Mo(VI)/Mo(V) and -470 mV for Mo(V)/Mo(IV). Treatment of the oxidized enzyme with cyanide releases thiocyanate and results in a loss of activity accompanied by a shift in the molybdenum redox couples to less negative values

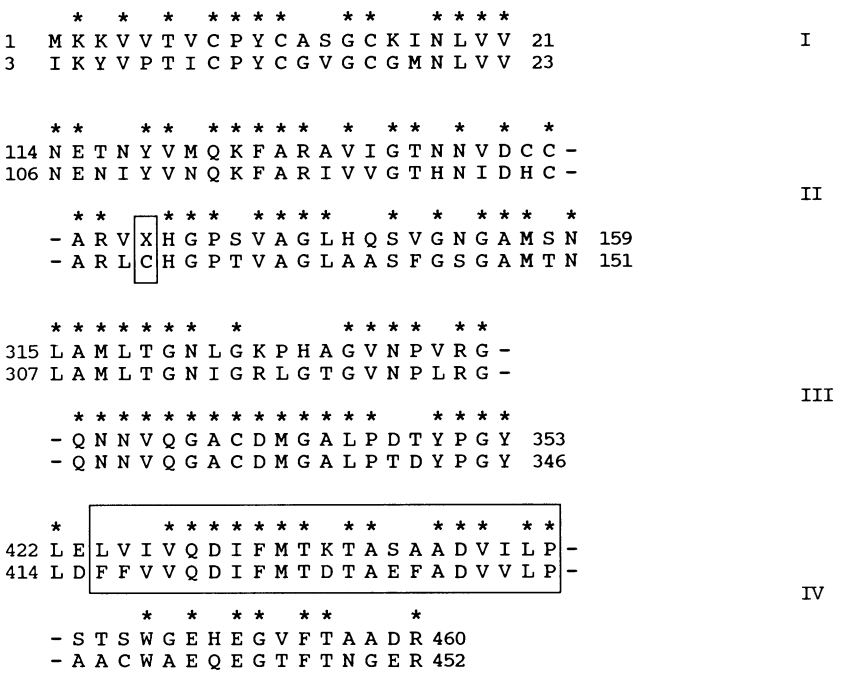

Fig. 2. Comparison of regions of amino acid sequences deduced from the DNA sequences of the genes encoding the large subunits of the formate dehydrogenases from *Escherichia coli* (Zinoni et al. 1986) (top lines) and *Methanobacterium formicicum* (bottom lines) (Schuber et al. 1986). Residue 'X' in region II is selenocysteine. The box in region IV includes sequences implicated as a potential molybdopterin binding site in the formate dehydrogenase from *E. coli* (Bilous et al. 1988).

(Barber et al. 1986). Thus, the archaeobacterial cofactor appears to contain a terminal sulfur ligand to molybdenum which is similar to the oxidized forms of the eucaryotic xanthine-utilizing molybdenum hydroxylases (Wahl and Rajagopalan 1982). Molybdenum EXAFS indicates a combination of Mo-O(N) and Mo-S with bond distances of 2.08 Å and 2.45 Å and with coordination numbers of two and three (N.R. Bastian, unpublished results).

Unlike the methanogen enzyme, the formate dehydrogenases from the clostridia (Table 3) function to reduce CO_2 to formate (Thauer et al. 1975). EXAFS of the *C. pasteurianum* formate dehydrogenase indicates three Mo = O groups with an average bond length of 1.74 Å, but no Mo-S bonds; in contrast, the *C. thermoaceticum* enzyme showed no oxo groups but did exhibit a coordination sphere containing two or more W-O(N) and W-S bonds with average lengths of 2.13 and 2.39 Å respectively (Cramer et al. 1985). Although the purified formate dehydrogenase from *C. thermoaceticum* contains predominantly tungsten (Table 3), growth of the organism in the presence of molybdate stimulates formate dehydrogenase activity (Andreesen and Ljungdahl 1973). An EPR signal (g = 1.97) from the formate-reduced enzyme is attributed to molybdenum but no experiments with ^{95}Mo-enriched enzyme

are reported which demonstrate hyperfine interactions with the molybdenum nucleus (Ljungdahl 1980). A high temperature EPR signal is also reported for the *C. thermoaceticum* formate dehydrogenase with g = 2.101, 1.980 and 1.950 which is tentatively attributed to tungsten but no experiments with ^{183}W-enriched (I = ½) enzyme were performed (Deaton et al. 1987).

Selenium. Selenium is required for the synthesis of formate dehydrogenases not reported in Table 3 (Andreesen et al. 1974; Leonhardt and Andreesen 1977; Wagner and Andreesen 1977) but is not an absolute requirement for all. In addition to the selenium-independent (M_r = 105,000) enzyme, *M. vannielii* synthesizes a high molecular weight selenocysteine-containing formate dehydrogenase complex when grown in the presence of selenium and tungsten (Jones and Stadtman 1981). Selenium is present in the largest subunit of the *C. thermoaceticum* enzyme (Yamamoto et al. 1983). The metal is also present in a selenocysteine residue of the largest subunit (M_r = 110,000) of the formate dehydrogenase-N, and the M_r = 80,000 polypeptide of formate dehydrogenase-H from *E. coli* (Cox et al. 1981) (Figure 2). The use of localized mutagenesis to replace selenocysteine with serine, abolished activity of formate dehydrogenase-H; replacement with cysteine reduced the activity by 90% (Zinoni et al. 1987). The selenium- independent formate dehydrogenase from *M. formicicum* shares striking identity with the *E. coli* formate dehydrogenase-H in the regions flanking the selenocysteine; particularly interesting is the substitution of a cysteine for the selenocysteine (Figure 2). The conservation of this structure in widely divergent organisms implies that it supports a common function. Selenium (selenocysteine) is a component of several enzymes other than formate dehydrogenase (Stadtman 1980). Recently, it was reported that selenocysteine coordinates to the active site nickel in the [NiFeSe] hydrogenase from *Desulfovibrio baculatus* (Eidsness et al. 1989).

Iron-Sulfur Centers. The visible spectrum of all anaerobic formate dehydrogenases examined show absorbance characteristic of iron-sulfur centers. Most of the iron atoms from the *C. pasteurianum* enzyme are reported to be extruded as Fe_4-S_4 centers (Liu and Mortenson 1984). Redox potentiometry and EPR analysis reveal two spectrally and thermodynamically distinct iron-sulfur clusters with g = 1.87, 1.95 and 2.05 (-318 mV) and g = 1.92 and 2.05 (-372 mV), both present at approximately 0.3 spins per native enzyme (Prince et al. 1985). Similar studies suggest two Fe_2-S_2 and two Fe_4-S_4 centers in the formate dehydrogenase from *C. thermoaceticum* (Deaton et al. 1987). The low temperature EPR spectrum of the *M. formicicum* enzyme (Barber et al. 1983) is characteristic of iron-sulfur clusters (g = 2.0465, 1.9482 and 1.9111); EXAFS indicate the centers are of the Fe_4-S_4 type (N. Bastian, unpublished results). The deduced amino acid sequence in the C-terminus of the smaller subunit contains two four-cysteine clusters with spacing reminiscent of clostridial Fe_4-S_4 centers (Schuber et al. 1986).

Cofactors. Other than molybdopterin and cytochrome b, the FAD from the *M. formicicum* enzyme is the only other cofactor reported for formate dehydrogenases purified from anaerobic bacteria (Schauer and Ferry 1986). The flavin is noncovalently bound and dissociates under reducing conditions resulting in a loss of F_{420}-reducing activity (Schauer and Ferry 1983). It is postulated that the flavin accepts electrons from one-electron redox centers becoming fully reduced and then donating a hydride ion to the obligate two-electron acceptor F_{420}. Reconstitution of the deflavo enzyme with FAD analogues indicates that the enzyme is sensitive to alterations in the 6-, 7-, and 8-loci of the benzinoid ring of FAD (Schauer et al. 1986). This formate dehydrogenase is similar to other F_{420}-dependent dehydrogenases in methanogenic archaeobacteria in that it is specific for the *si* face hydride transfer to C_5 of F_{420} suggesting a common substrate binding site.

Regulation and Genetics

Aerobic Organisms

The regulation of formate dehydrogenases in *P. oxalaticus* OX1 grown on mixtures of formate and heterotrophic substrates has been investigated (Dijkhuizen et al. 1978). The organism synthesizes two formate dehydrogenases when growing on the mixtures; the soluble NAD-linked enzyme discussed above and a membrane-bound cytochrome b-linked enzyme (Dijkhuizen et al. 1979). Growth on formate and oxalate is diauxic with formate utilized first. The activities of both formate dehydrogenases increase during both growth phases. The mechanism for repression of formate utilization is unknown, but one possibility is an effect of formate on the internal pool of oxalyl-CoA (Dijkhuizen et al. 1980). Growth of *P. oxalaticus* on formate and acetate is also biphasic but formate is utilized last. Formate oxidizing activity, and the activities of both formate dehydrogenases, increase in the change-over period. Formate is utilized simultaneously with glyoxylate or glycolate but the cells contain low activities of the NAD-linked formate dehydrogenase suggesting the membrane-bound enzyme oxidizes formate during growth on these mixtures. The expression of a soluble NAD-linked formate dehydrogenase from methanol-grown *Paracoccus denitrificans* is dependent on the concentration of molybdenum in the growth medium (Burke et al. 1980). This is the first indication that methylotrophic organisms may synthesize a molybdenum-containing formate dehydrogenase.

A study on the regulation of synthesis of methanol-utilizing enzymes in *Kloeckera* (Egli et al. 1982) shows that formate dehydrogenase is catabolite repressed during the metabolism of glucose in batch cultures. The authors concluded that this repression is separate from the repression by ethanol since the cultures do not produce alcohol during the fermentation. Nitrogen limitation also represses the synthesis of formate dehydrogenase and other

methanol-utilizing enzymes in *Kloeckera* (Egli 1982). The regulation of formate dehydrogenase activity was also studied in *Candida methylica* (Avilova et al. 1985). Interestingly, enzyme activity in this organism increases several fold at the end of the growth on methanol and is induced by including sodium formate in the growth medium. The activity of formate dehydrogenase from *Candida boidinii* is inhibited by NADH and ATP (Kato et al. 1979). The intracellular concentrations of NADH and ATP are near the K_i suggesting the activity of formate dehydrogenase may be regulated by these metabolites.

Anaerobic Organisms

Escherichia Coli

Transfer of *E. coli* from aerobic to anaerobic growth on glucose results in the synthesis of an active *fnr* gene product. The fnr protein is the transcriptional regulator required for expression of anaerobically inducible genes which encode enzymes catalyzing the formate-dependent reduction of fumarate, trimethylamine-N-oxide, or nitrate (Lin and Kuritzkes 1987). The *fnr* gene product is also necessary for full expression of the *pfl* gene which encodes pyruvate formate-lyase (Sawers and Bock 1988). Addition of formate or pyruvate to the growth medium further enhances *pfl* expression (Sawers and Bock 1988); however, the role of formate as an inducer may be indirect due to a shift in the equilibrium towards pyruvate accumulation. The formate-nitrate couple (Table 1) is energetically more efficient than other anaerobic respirations; thus, the presence of nitrate results in the synthesis of an active *narL* gene product which overcomes the effect of the *fnr* regulatory protein and represses transcription of the fumarate reductase and trimethylamine-N-oxide operons (Iuchi and Lin 1987; and Kalman and Gunsalus 1988). Transcription of pyruvate formate-lyase is partially repressed by nitrate, also mediated by the activated *narL* gene product (Sawers and Bock 1988).

Trimethylamine-N-oxide, nitrate, or oxygen inhibits the synthesis of an active formic hydrogenlyase system which directs electron flow from formate towards reduction of the electron acceptors (Lin and Kuritzkes 1987). In the absence of exogenous electron acceptors, formic hydrogenlyase synthesis is induced which converts the fermentation acid to the gaseous end products H_2 and CO_2. Formate induces the synthesis of the hydrogenase-3 isozyme, a component of the formic hydrogenlyase system (Zinoni et al. 1984; Sawers et al. 1985). A mutant, defective in pyruvate formate-lyase, requires exogenously supplied formate for transcription of at least two genes encoding components of the formic hydrogenlyase system (Birkmann et al. 1987b); *fdhF* encoding the M_r = 80,000 subunit of formate dehydrogenase-H (Pecher et al. 1985), and *hyd-17* required for hydrogenase-3 expression. Although molybdopterin is required for synthesis of active formate dehydrogenase-H, mutations blocking the synthesis of functional molybdopterin cofactor have no influence on the transcription of *fdhF* and *hyd-17* indicating that the cofactor is not involved in regulation (Birkmann et al. 1987b; Wu and Mandrand-Berthelot 1987).

Translation of *fdhF* is dependent on *fdhA*, *fdhB*, and *fdhC* gene products which are necessary for incorporation of selenocysteine (Wu and Mandrand-Berthelot 1987; Birkmann et al. 1986).

Anaerobic expression and induction of formate dehydrogenase-H by formate depends on a region of 185 nucleotides upstream of the translational start of the *fdhF* gene (Birkmann et al. 1987b). The *fnr* gene product does not interact with these regulatory sequences; instead, *fdhF* is under transcriptional control by *ntrA* which encodes σ^{54} (Birkmann et al. 1987a). Previously, it was shown that the σ^{54} protein alters the core RNA polymerase to allow recognition of *nif* and *ntr* promoters involved in regulation of nitrogen metabolism. These promoters do not contain the -10 and -35 consensus sequences common to many *E. coli* operons; instead, they have consensus sequences -12 and -24 relative to the transcriptional start. Recently, a *cis*-acting DNA element has been identified that is required for the regulation of this promotor. This regulatory sequence of about 25 base pairs is located 110 base pairs upstream of the transcription start site of *fdhF* (Birkmann and Bock 1989).

Other genes and loci are reported to influence synthesis of an active formic hydrogenlyase system in *E. coli*. Anaerobic growth induces expression of the *ant* locus required for formate-dependent reduction of benzyl viologen suggesting involvement in the synthesis of formate dehydrogenase-H (Yerkes et al. 1984). Recently, it was reported that synthesis of formate dehydrogenase-H, and the formic hydrogenlyase-linked hydrogenase-3, also requires the *fhlA* gene product (Sankar et al. 1988). Expression of *fdhF* is also enhanced by inhibitors of DNA gyrase (Axley and Stadtman 1988). The enhancement is observed for the chromosomal gene as well as with translational fusions. Inhibition of *fdhF* expression due to nitrate was partially overcome by gyrase inhibition. Recently, it was reported that the *narK* gene product is involved in nitrate repression of *fdhF* and *hyd-17* (Stewart and Berg 1988).

Incorporation of selenocysteine into the formate dehydrogenase-H of *E. coli* is through cotranslational insertion directed by an in-frame UGA stop codon (Zinoni et al. 1986; Zinoni et al. 1987). Translation of the UGA codon requires the presence of a functional pathway for incorporation of selenocysteine (Zinoni et al. 1987); mutants unable to incorporate selenocysteine produce a truncated polypeptide indicating that translation stops prematurely at the UGA codon directing the incorporation. Four genes required for incorporation have been described (Leinfelder et al. 1988a). The *selA* and *selB* genes (previously in the *fdhA* locus) code for polypeptides of M_r = 50,000 and M_r = 70,000 but their function is unknown. The *selC* gene (previously *fdhC*) codes for the functional tRNA which inserts selenocysteine into protein (Leinfelder et al. 1988b). The tRNA is charged with serine, not selenocysteine, suggesting a post charge modification of the serine residue at the hydroxyl group and exchange of this group with selenium. Mutations in *selD* (previously *fdhB*) are blocked in selenium incorporation into protein or tRNA.

The genetics of formate dehydrogenase-N of *E. coli* have been studied in parallel with nitrate reductase. Genes involved in the synthesis of active

molybdopterin for both enzymes are designated *chlA*, *chlB*, *chlD*, *chlE* and *chlG*. The *chl* designation stems from chlorate resistant mutations in *E. coli* defective in the expression of nitrate reductase which, in addition to nitrate, also reduces chlorate to toxic chlorite. With the exception of *chlG* (Haddock and Mandrand-Berthelot 1982), mutations in the *chl* genes also effect the synthesis of an active formate dehydrogenase-N and formate dehydrogenase-H (Giordano et al. 1980; Terriere et al. 1981; Haddock and Mandrand-Berthelot 1982). Mutations in *ChlA* and *chlE* result in no measurable cofactor, but low amounts of inactive formate dehydrogenase-N are produced (Terriere et al. 1981). Mixing of extracts from these mutants restores membrane-bound formate dehydrogenase activity. The *chlB* gene product (FA factor) facilitates assembly of active nitrate reductase which implies a similar function for the formate dehydrogenase, possibly molybdopterin insertion into apoenzymes. The *chlD* gene is involved in molybdenum processing when cells are grown with low concentrations of molybdate (Miller et al. 1987). Formate dehydrogenase-N is not synthesized in a *chlD* background unless the mutant cells are supplemented with high concentrations of molybdate to circumvent the *chlD* requirement (Giordano et al. 1980).

The fnr regulatory protein appears to exert a negative control on formate dehydrogenase-N synthesis (Shaw and Guest 1982; Lambden and Guest 1976), possibly to allow derepression for maximal formate oxidation during aerobic growth. The mechanism by which nitrate derepresses formate dehydrogenase-N synthesis is unknown but is likely to involve *narL*.

Methanobacterium Formicicum

The genes encoding the two subunits of the formate dehydrogenase from *M. formicicum* have been cloned and sequenced (Schuber et al. 1986). The genes overlap by one base pair and appear to be cotranscribed as part of a large transcript (Patel and Ferry 1988). Analysis of sequences upstream of the transcriptional start site reveal considerable identity with sequences upstream of the *E. coli fdhF* gene (Birkmann et al. 1987b). A putative promoter sequence for structural genes of methanogenic archaeobacteria is proposed based on comparison of the upstream sequences of other methanogen operons. Decreasing concentrations of molybdenum in the growth medium results in decreased amounts of formate dehydrogenase protein synthesized; however, the amount of *fdh*-specific mRNA increases (May et al. 1988). Cells grown in the presence of tungstate synthesize high levels of the inactive formate dehydrogenase and contain *fdh*-specific mRNA in amounts similar to that of cells grown with sufficient molybdate. The results are consistent with autoregulation by formate dehydrogenase protein.

Summary

Formate metabolism is distributed across the entire spectrum of microorganisms and participates in a diversity of energy yielding pathways. Studies of these organisms have revealed general principles of ecology, physiology and regulation. The diversity of formate metabolism is reflected in the biochemical properties of formate dehydrogenases characterized from aerobic and anaerobic microorganisms. Biochemical and molecular genetic studies of formate dehydrogenases have contributed to a substantial understanding of the selenium, molybdenum, and iron domains.

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6. The Biochemistry and Genetics of C_1 Metabolism in the Pink Pigmented Facultative Methylotrophs

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Introduction

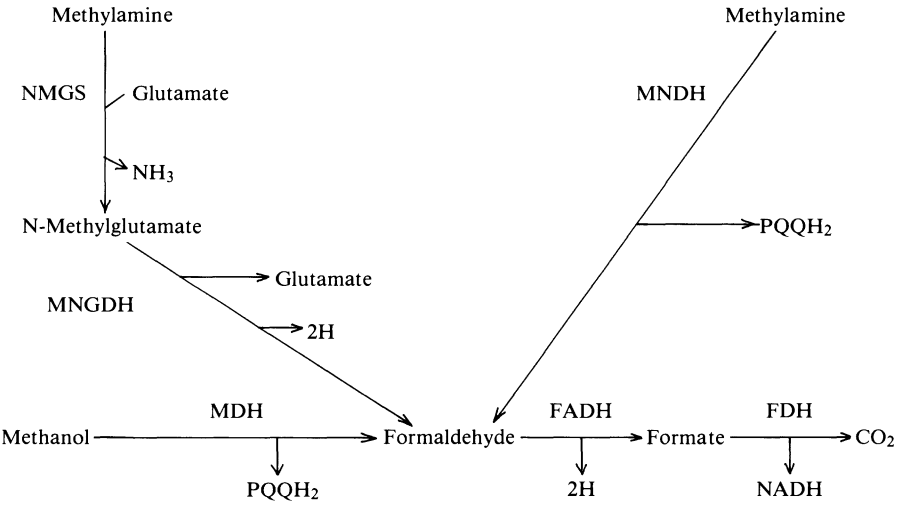
Pink pigmented facultative methylotrophs (PPFMs) are capable of growth on reduced C_1 compounds (methanol and usually also methylated amines and formate). Energy for growth is obtained from the oxidation of these substrates to CO_2 and carbon is assimilated by the *icl*$^-$ variant of the serine pathway. Many isolates were originally considered to be pseudomonads, but now they have all been classified in the genus *Methylobacterium* (Green and Bousfield 1983; Green et al. 1988). Two of the PPFMs – *Methylobacterium extorquens* AM1 (NCIB9133; formerly *Pseudomonas* AM1 or *Methylobacterium* AM1) and *Methylobacterium organophilum* – have been used to develop methods for the investigation of the genetics of methylotrophs. The aim of this review is to summarise this work and to demonstrate how it has enhanced our understanding not only of the genetics but also of the biochemistry of these organisms. There is some confusion concerning variants of *Mb. organophilum*; the strain deposited with The National Collection of Industrial Bacteria was designated *Methylobacterium organophilum* 11278 (XX ATCC27886 DSM760), but Allen and Hanson (1985) and Machlin et al. (1988) imply that they used two variants, namely XX and DSM760, in their work. These two strains appear to differ in some respects and in this review they will be distinguished when possible.

The Biochemistry of C_1 Metabolism in The PPFMS

Current understanding of the biochemistry of C_1 metabolism has been described by Anthony (1982; 1986; 1988) and details of the original experimental work can be obtained from these reviews. The pathways found in the PPFMs are summarised in Figs 1 to 3. Methanol is oxidised by a periplasmic quinoprotein, methanol dehydrogenase, which was initially thought to be composed of two identical subunits (M_r approximately 60,000 Da). However, it has now been shown (Anderson and Lidstrom 1988; Nunn

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 143-162.

Fig. 1. Oxidation of C_1 compounds by the PPFMs



MDH, methanol dehydrogenase; MNDH, methylamine dehydrogenase; NMGS, N-methylglutamate synthase; NMGDH, N-methylglutamate dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

and Anthony 1989) that there is a second subunit (M_r approximately 8,500 Da), the complete enzyme consisting of the two types of subunit arranged in an alpha2:beta2 configuration. The prosthetic group, pyrroloquinoline quinone (PQQ), is non-covalently associated with the enzyme, but it is not known at present which subunit is involved in this binding. *In vitro* methanol dehydrogenase activity is dependent on the presence of ammonium ions. A compound of low M_r which can replace this artificial activator has recently been isolated from *Hyphomicrobium* X (Dijkstra et al. 1988) and a similar factor may be present in the PPFMs. Electrons are transferred from methanol dehydrogenase to an unusual c type cytochrome, designated cytochrome c_L and then to the terminal oxidase, probably via a second c cytochrome, c_H. The subscripts L and H indicate the low (pH 4.2) and high (pH 8.8) isoelectric points of these cytochromes.

Fig. 2. Probable pathways of electron transport during growth of the PPFMs on C_1 compounds. (See Anthony 1988).

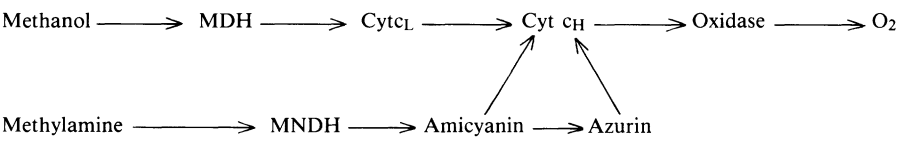
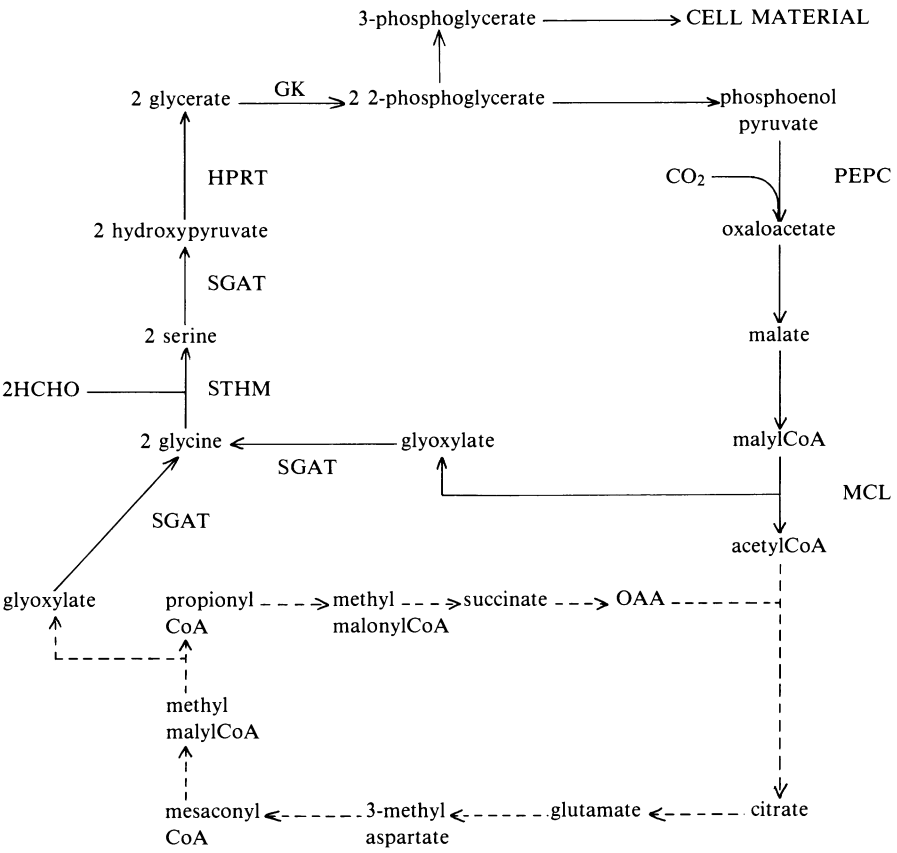


Fig. 3. The *icl*⁻ variant of the serine pathway, showing the key enzymes of the pathway



STHM, serine transhydroxymethylase; SGAT, serine-glyoxylate aminotransferase; HPRT, hydroxypyruvate reductase; GK, glycerate kinase; PEPC, phosphoenolpyruvate carboxylase; MCL, malyl CoA lyase.
Intermediates in the conversion of acetyl CoA to glyoxylate are as proposed by Shimizu et al (1984); this route has yet to be verified in *Mb. extorquens* AM1 and *Mb. organophilum*.

Mb. extorquens AM1 oxidises methylamine by another quinoprotein, methylamine dehydrogenase. This enzyme is a tetramer composed of two large subunits (M_r approximately 40,000 Da) and two small subunits (M_r approximately 13,000 Da) and contains PQQ (or a closely related derivative) covalently bound to the small subunit. A blue copper protein, amicyanin, is usually the primary electron acceptor and electrons are transferred to the terminal oxidase via cytochrome c_H and/or another blue copper protein, azurin. Methylamine dehydrogenase activity has also been observed in extracts of a mutant of *Mb. organophilum* XX (Stone and Goodwin 1989) but Biville et al. (1988a) did not detect this enzyme in *Mb. organophilum* DSM760. They

demonstrated that extracts of this organism contained N-methylglutamate dehydrogenase activity and claimed that methylamine is oxidised to formaldehyde via N-methylglutamate. Further evidence that methylamine dehydrogenase is not involved in methylamine oxidation in *Mb. organophilum* DSM760 was provided by the observation that mutants which were unable to synthesise PQQ could utilise methylamine as their sole carbon and energy source. It is somewhat unexpected that two such closely related bacteria should metabolise methylamine in different ways, but similar observations have been made with different strains of *Methylophaga marina* (Janvier et al. (1985).

Formaldehyde is the product of the oxidation of both methanol and methylamine and can then either be further oxidised or assimilated. The enzyme(s) involved in the oxidation of formaldehyde to formate has not been definitely identified. Methanol dehydrogenase can catalyse this reaction, but there is evidence that it is not responsible for formaldehyde oxidation *in vivo* since mutants lacking this enzyme oxidise formaldehyde at the same rate as wild type cells (Heptinstall and Quayle 1970). Indeed, it is important that methanol dehydrogenase does not convert methanol to formate during growth since formaldehyde must be made available for assimilation by the serine pathway. A 'modifier protein' (M-protein) ensures this by lowering the affinity of methanol dehydrogenase for formaldehyde (Page and Anthony 1986). *Mb. extorquens* AM1 contains at least two dye dependent aldehyde dehydrogenases which oxidise formaldehyde; one of these is induced by methanol (Weaver and Lidstrom 1985) and is probably responsible for formaldehyde oxidation *in vivo*. The resulting formate is converted to CO_2 by an NAD-dependent formate dehydrogenase.

The enzyme responsible for the fixation of reduced C_1 compounds is a C_1 inducible serine transhydroxymethylase, which catalyses the condensation of glycine with a C_1 tetrahydrofolate derivative formed from formaldehyde, to give serine. This enzyme is activated by glyoxylate and is distinct from the isoenzyme which is required for the conversion of serine to glycine during growth on multicarbon compounds. Three key enzymes of the serine pathway (serine glyoxylate aminotransferase, hydroxypyruvate reductase and glycerate kinase) then convert the serine to 2-phosphoglycerate which can be assimilated into cell material. Regeneration of the C_1 acceptor, glycine, involves two other key enzymes – a C_1-inducible, acetyl CoA-independent isoenzyme of phosphoenolpyruvate carboxylase and malyl CoA lyase, which cleaves malyl CoA to acetyl CoA and glyoxylate. The acetyl CoA is then converted to a second molecule of glyoxylate which is transaminated to glycine. In some methylotrophs isocitrate lyase is involved in this part of the pathway but it has not been detected in the PPFMs (Dunstan et al. 1972a; Bellion and Spain 1976) and they are thus said to contain the *icl*$^-$ variant of the serine pathway. Shimizu et al. (1984) proposed that in another species of *Mb. extorquens* (NCIB 2879; formerly *Protaminobacter ruber*), acetyl CoA is converted to glyoxylate via mesaconyl CoA. To confirm that this route operates during growth on C_1 compounds it is necessary to demonstrate that mutants lacking

the relevant enzymes are unable to grow on methanol. A similar pathway may be present in *Mb. extorquens* AM1 and *Mb. organophilum* but this has yet to be verified. Acetyl CoA is converted to glyoxylate by the same route during growth of *Mb. extorquens* AM1 on ethanol, pyruvate, lactate and 3-hydroxybutyrate (see Anthony 1982).

In *Mb. extorquens* AM1 and *Mb. organophilum* the activities of most of the key enzymes of C_1 metabolism are low in succinate grown cells and are induced (or derepressed) following transfer to medium containing methanol or methylamine as sole carbon and energy source. There is evidence suggesting that in the PPFMs methanol dehydrogenase is derepressed when the bacteria are growing slowly (Roitsch and Stolp 1986). However, growth rate cannot be the only factor determining synthesis of this enzyme; both *Mb. extorquens* AM1 and *Mb. organophilum* grow at similar rates on succinate and on methanol (Roitsch and Stolp 1986) yet the activity of methanol dehydrogenase increases when these organisms are transferred from succinate to methanol medium (O'Connor and Hanson 1977; McNerney and O'Connor 1980). This effect is unlikely to be due to removal of a catabolite repressor formed from succinate because when cells were grown on a mixture of methanol and succinate there was no evidence of diauxie and the specific activity of methanol dehydrogenase was similar to that in cells grown on methanol (O'Connor 1981). Thus methanol dehydrogenase is probably induced during growth on methanol. Methanol itself may be the inducer or it may have to be metabolised to formaldehyde before induction occurs, as has been suggested in *Paracoccus denitrificans* (de Vries et al. 1988).

There is evidence that serine glyoxylate aminotransferase, hydroxypyruvate reductase, glycerate kinase and malyl CoA lyase, are coordinately regulated in *Mb. extorquens* AM1; they are all induced immediately when cells are transferred from succinate to methanol medium and are repressed during growth on a mixture of succinate and methanol (Dunstan et al. 1972b; McNerney and O'Connor 1980; O'Connor 1981). However, the C_1-inducible isoenzymes of phosphoenolpyruvate carboxylase and serine transhydroxymethylase are not repressed during growth on succinate plus methanol, and are induced only after a lag when cells are transferred from succinate to methanol medium. A product of methanol oxidation, rather than methanol itself, is probably the inducer of the serine pathway enzymes in *Mb. extorquens* AM1 (O'Connor 1981). In *Mb. organophilum* the serine pathway enzymes are also induced on transfer from succinate to methanol medium, but in this organism they are not strongly repressed during growth on succinate plus methanol (O'Connor and Hanson 1977).

Isolation of Mutants

When investigating the genetics of an organism one of the first priorities is usually to isolate mutants carrying genetic markers. Considerable difficulties

have been experienced in isolating mutants of many methylotrophs, particularly the obligate methylotrophs (de Vries 1986). However, the PPFMs have proved to be amenable to mutagenesis by a variety of agents, and this is one of the reasons why they have been used to study the genetics of methylotrophy.

Mutants Defective in C_1 Metabolism

A summary of the reported properties of mutants of *Mb. extorquens* AM1 and *Mb. organophilum* which are defective in C_1 metabolism is given in Tables 1 to 3. C_1-negative mutants of *Mb. extorquens* AM1 were first isolated by Heptinstall and Quayle (1970), using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as the mutagen. Ultraviolet light (UV), nitrous acid (NA) and ethyl methane sulphonate (EMS) have also been successfully used to obtain mutants unable to utilise C_1 compounds (Tables 1 to 3). Elucidation of the metabolic lesions in these mutants has provided valuable information about the biochemistry of methylotrophic growth (see Anthony 1982).

More recently insertion mutagenesis has been applied to the PPFMs. Whitta *et al.* (1985) used a suicide vector, pMO75, to introduce random Tn5 insertions into *Mb. extorquens* AM1 and isolated 6 C_1-negative mutants. Insertion mutants of *Mb. organophilum* DSM760 were obtained using a different approach, namely 'marker exchange' (Mazodier *et al.* 1988). DNA encoding kanamycin resistance (either on Tn5 or on a 'kan cartridge') was inserted into several different sites on cloned fragments of *Mb. organophilum* DSM760 DNA which complemented methanol oxidation mutants. The mutagenised DNA was then introduced into the wild type strain using a suicide vector. Exconjugants expressing the kanamycin resistance marker, which had arisen by recombination of the cloned DNA fragment with the homologous region on the chromosome, were isolated. Three were unable to grow on methanol, and two of the insertions mapped in previously unidentified C_1 genes. Since several C_1 regions of the chromosome of both *Mb. extorquens* AM1 and *Mb. organophilum* have now been cloned (see below) this approach holds much promise for the future with respect to the isolation of new methanol negative mutants and the identification of new C_1 genes. Furthermore, site directed mutagenesis of cloned DNA fragments encoding C_1 genes, followed by marker exchange, will enable the introduction of specific mutations into methylotrophs.

Auxotrophs

Harder and Quayle (1971a) obtained mutants of *Mb. extorquens* AM1 which required serine or glycine for growth, using MNNG as mutagen and a selection procedure designed to specifically isolate these types of auxotroph. However, attempts to obtain a wide range of auxotrophs of *Mb. extorquens* AM1 have not been very successful. Tatra and Goodwin (1985) made 14 independent

attempts to isolate such mutants using MNNG as mutagen, but only obtained three classes of stable auxotrophs which required adenine, thiamine or methionine for growth. Whitta et al. (1985) also isolated three thiamine auxotrophs, all non-siblings, using Tn5 mutagenesis; in addition they obtained 2 tyrosine auxotrophs and a sixth whose growth requirements could not be identified. The reason for the failure to obtain a wide range of auxotrophs is unclear and requires further investigation.

Antibiotic Resistant Mutants

Spontaneous mutants of *Mb. extorquens* AM1 which are resistant to streptomycin, cycloserine, phosphonomycin and rifamycin can easily be isolated (Tatra and Goodwin 1983, 1985; Goodwin et al. 1988) and these mutations provide useful selective markers. However, there is evidence that the rifamycin resistant mutants are pleiotropic since they are more sensitive to a variety of antimicrobial agents than the wild type strain (Goodwin et al. 1988). The most likely explanation for this hypersensitivity is that the cell envelope of these mutants is altered, making them more permeable than the wild type. Rifamycin resistance usually results from a mutation in the gene encoding the beta subunit of RNA polymerase and it is possible that in *Mb. extorquens* AM1 this mutation results in altered expression of some gene(s) encoding components of the cell envelope with the result that the cell is slightly 'leaky' to a variety of compounds. The mutation to rifamycin resistance may have other effects on the phenotype of *Mb. extorquens* AM1 and such mutants should therefore be used with caution in biochemical, physiological and genetic studies of this organism.

Gene Transfer Systems

An important prerequisite for the investigation of the genetics of a bacterial species is the availability of an efficient means of introducing DNA into the organism in question. Transformation, transduction and conjugation are the traditional methods used for gene transfer between different strains of bacteria. Information about the linkage of genes on the chromosome can then be obtained by investigating the frequencies of cotransfer of donor markers to an appropriate recipient. For the PPFMs the method of choice is conjugation since no transducing phages have yet been described for these bacteria and transformation has had only limited success.

Transformation

O'Connor et al. (1977) demonstrated that *Mb. organophipum* can be transformed with exogenous chromosomal DNA, the organism becoming competent near the end of the exponential phase of growth. Transformation

frequencies of about 10^{-3} were obtained but transformation only occurred if rather high concentrations of DNA were used. Attempts to transform *Mb. extorquens* AM1 with chromosomal DNA have been unsuccessful (P K Tatra and P M Goodwin, unpublished observations).

For gene cloning it is useful to be able to transform bacteria with recombinant DNA derived from plasmid or cosmid vectors. Unfortunately attempts to transform PPFMs efficiently with plasmid DNA have been generally unsuccessful (Gautier and Bonewald 1980; Haber et al. 1983; Fulton et al. 1984). An alternative means of introducing exogenous DNA into cells is by electroporation; this has recently been used successfully with a number of different bacteria (Chassy et al. 1988) and there is evidence that it is applicable to methylotrophs (F Biville, personal communication).

Conjugation

Transfer of chromosomal DNA from a donor to a recipient by conjugation depends on the availability of suitable plasmids which can either mobilise the chromosome or form prime derivatives. Although *Mb. extorquens* AM1 contains three endogenous plasmids, of M_r 15,200, 21,500 and 26,700 Da (Warner et al. 1977), no phenotypes have been ascribed to them and there is no evidence that they are conjugative or can mobilise chromosomal genes. However, broad host range plasmids of the IncP1 group are readily transferred to the PPFMs (Jayaseelan and Guest 1979; Tatra and Goodwin 1983; Haber et al. 1983; Al-Taho and Warner 1987) and have provided an effective means of transferring DNA between different strains.

The Inc P1 plasmid R68.45 can mobilise the chromosome of a variety of Gram negative bacteria, including *Mb. extorquens* AM1 (Holloway 1986; Tatra and Goodwin 1983). On the basis of the data from extensive three and four factor crosses, Tatra and Goodwin (1985) constructed a linkage map of the *Mb. extorquens* AM1 chromosome showing the relative positions of three antibiotic resistance markers and four C_1 genes. Three of the C_1 markers, namely *mtd-1* (also designated *mox*A1), *mcl*-1 and *mmf*-2, which are required for the production of methanol dehydrogenase, malyl CoA lyase and glycerate kinase respectively, showed high frequencies of cotransfer with cycloserine and phosphonomycin resistance markers. However, since the size of the DNA fragment mobilised by R68.45 is unknown, these frequencies of co-transfer could not be equated with distances in kbp. The fourth C_1 gene (*mmf*-1, which is required for the conversion of acetyl CoA to glyoxylate) was not very closely linked with the other three. The genes defective in adenine and thiamine auxotrophs were not linked to any of the C_1 genes, nor to each other.

Inc P1 plasmids can also form R′ derivatives, which may contain very large inserts of host chromosomal DNA and thus enable investigation of gene linkage over a relatively large segment of the chromosome. Haber et al. (1983) used R68.45 to isolate R′ plasmids containing inserts of the *Mb. organophilum*

XX chromosome. Although they obtained evidence of the formation of primes which complemented a methanol negative mutant, these plasmids proved unstable and difficult to work with. In contrast, stable R' plasmids carrying C_1 genes of *Mb. organophilum* DSM760 have been isolated by Mazodier et al. (1988), using a derivative of R68.45. One of the primes had an insert of about 30 kbp and contained the *mox*F gene, which encodes the alpha subunit of methanol dehydrogenase, and *pqq*A, a gene required for PQQ synthesis.

Al-Taho and Warner (1987) attempted to isolate R' plasmids carrying auxotrophic markers of *Mb. extorquens* AM1 using pULB113, a derivative of the IncP1 plasmid RP4 which contains part of the Mu genome. They were unable to demonstrate any prime formation in *Mb. extorquens* AM1, but the closely related organism, *Mb. extorquens* NCIB9399 (originally *Pseudomonas extorquens*) did form R's which complemented *E. coli* auxotrophs. These primes were smaller than the original plasmid and must have undergone rearrangement and deletion. It is unclear why R' plasmids were isolated in this organism but not in *Mb. extorquens* AM1.

Cloning PPFM Genes

In view of the difficulties experienced in transforming the PPFMs with plasmid DNA, conjugation is the only efficient way of introducing cloned DNA into these organisms and the application of recombinant DNA technology to the identification and investigation of C_1 genes had to await the development of suitable broad host range mobilisable vectors. Since these have become available gene libraries of *Mb. extorquens* AM1 and both variants of *Mb. organophilum* have been constructed in *E. coli* hosts and clones carrying C_1 genes have been identified by screening for their ability to complement C_1 negative mutants.

Choice of Vector

Several broad host range mobilisable vectors belonging to the IncP1 and IncQ groups of plasmids are now available, and both types have been used for cloning methylotroph DNA. In the first example of cloning of a C_1 gene a mobilisable IncQ plasmid, R1162 (which is thought to be identical to RSF1010), was employed as the vector (Gautier and Bonewald 1980). An *EcaI* clone bank was constructed and used to complement a methanol oxidation mutant of *Mb. extorquens* AM1. However, derivatives of RSF1010 are not always stable in PPFMs, as shown by Mazodier et al. (1988) when they constructed a *Sau*3A gene library of *Mb. organophilum* DSM760 DNA in pSUP106, an RSF1010 derivative. This library could not be used to obtain stable complementation of methanol negative mutants, but pSUP106 was employed as a suicide vector to introduce segments of cloned chromosomal DNA into the chromosome of the recipient by marker exchange, as described above.

Two Inc P1 cosmid vectors have been used to make gene libraries of PPFM DNA; a *Hin*dIII library of *Mb. extorquens* AM1 DNA in pVK100 was constructed by Fulton et al. (1984) and has been used successfully to complement a number of mutants of this organism (Nunn and Lidstrom 1986a; Stone and Goodwin 1989). A similar cosmid vector, pLA2917, was employed by Allen and Hanson (1985) and Machlin et al. (1988) to make a gene library of *Mb. organophilum* XX DNA. Several other broad host range vectors e.g. pRK310, pRK290, have been used for subcloning fragments of DNA isolated from the gene libraries (Nunn and Lidstrom 1986a; Machlin et al. 1988).

Cloned PPFM genes are not expressed from their own promoters in *E. coli* (Nunn and Lidstrom 1986a; Machlin et al. 1988). This may be because essential positive regulatory proteins are not present in *E. coli*, so that the methylotroph promoters are not switched on; or *E. coli* RNA polymerase may not recognise the methylotroph promoters, and/or may terminate transciption prematurely. However, these genes can be expressed in *E. coli* if they are cloned in a beta-galactosidase translational fusion vector (Nunn and Lidstrom 1986a; Machlin et al. 1988) or if a T7 RNA polymerase/promoter gene expression system is used (Anderson and Lidstrom 1988).

Use of Gene Libraries to Complement Mutants Defective in C_1 Metabolism

C_1 negative mutants of *Mb. extorquens* AM1 and both strains of *Mb. organophilum* have been complemented using one or more of the clone banks (Tables 1 to 3). In some cases the locations of the genes involved have been mapped in detail by subcloning and insertion mutagenesis and this work is summarised in Fig 4. It is evident from this information that C_1 genes are located in several different clusters on the chromosomes of the PPFMs. The most detailed data have been provided by Lidstrom and coworkers who have shown that there are at least 12 genes (*mox* genes) involved in methanol oxidation in *Mb. extorquens* AM1 (Nunn and Lidstrom 1986a; Lidstrom et al. 1987; Anderson and Lidstrom 1988). Four (*mox*A1, A2, A3, B) are located on a *Hin*dIII fragment (*Hin*dAB) of 19.5 kbp, which is adjacent to a second *Hin*dIII fragment (*Hin*dFG) of 8.6 kbp encoding four more *mox* genes – F,J, G and I; this in turn is adjacent to *Hin*dE, a 7.5 kbp *Hin*dIII fragment containing *mox*E. Two other *mox* genes, C and D are situated on adjacent *Hin*dIII fragments of 8.4 and 14.8 kbp and the last, *mox*H, spans the junction of two *Hin*dIII fragments of 15.8 and 4.0 kbp.

' The functions of some of the *mox* gene products have been deduced from a consideration of the properties of the Mox mutants and investigation of the expression of cloned *mox* genes. *mox*G is the cytochrome c_L structural gene while *mox*F and *mox*I encode the alpha and beta subunits of methanol dehydrogenase (Nunn and Lidstrom 1986a; Anderson and Lidstrom 1988; Nunn and Anthony 1989); the latter was discovered when the cloned *Hin*dFG fragment was expressed in *E. coli*. All three classes of *mox*A mutants make an inactive methanol dehydrogenase protein. The wild type enzyme has a

Table 1. Mutants of *Mb. extorquens* AM1 which are defective in C_1 oxidation

Mutant[1]	Reported properties			Complementation group		Mutagen
	MDH activity	MDH 60kDa subunit	cyt c_L	AM1	XX	
PG1	–	+	+	*mox*A1 (*mtd*-1)	V-B1	MNNG
UV21	–	+	+	*mox*A2		UV
M15A	–	+	+	*mox*A3	V-A2	MNNG
UV4	–	–	–	*mox*B		UV
AA18	–	low	low	*mox*C		NA
UV9	–	–	+[2]	*mox*D		UV
AA31	–	–	low[2]	*mox*E		NA
UV26	–	–	low	*mox*F	V-C	UV
UV10	+	+	–	moxG		UV
UV48	–	low	low	*mox*H		UV
PCT76	low		–[3]			MNNG
PG5	low		–[3]		III-B	MNNG

+/–; property present/absent. [1]; One mutant representative of each class is shown. [2]; no normal cyt c_L but a 23 kDa c cytochrome is present. [3]; no soluble cytochrome c and MNDH activity also absent.

Information is taken from Heptinstall and Quayle (1970), Anthony (1975), Allen and Hanson (1985), Tatra and Goodwin (1985), Nunn and Lidstrom (1986a, b), Machlin et al. (1988).

characteristic absorption peak at 345nm, due to the presence of the prosthetic group, PQQ; in *mox*A1 mutants this peak is absent, while in *mox*A2 and A3 mutants it is altered. Since these mutants are able to make another quinoprotein, methylamine dehydrogenase, they cannot be defective in the synthesis of the PQQ skeleton and Nunn and Lidstrom (1986b) suggested that they are unable to catalyse association of PQQ with the methanol dehydrogenase apoenzyme. The *mox*D class of mutants do not synthesise the alpha subunit of methanol dehydrogenase, or cytochrome c_L, although they do contain large amounts of a 23 kDa c-type cytochrome. Nunn and Lidstrom (1986b) suggested that this was a precursor of cytochrome c_L, and that *mox*D mutants are defective in a gene required for transport and/or processing of methanol dehydrogenase and cytochrome c_L. However, there is now evidence (Day et al. (1990)) that the 23 kDa cytochrome and cytochrome c_L are the products of two different genes. Trace amounts of the 23kDa cytochrome are found in wild type cells, and the lesion in *mox*D mutants thus appears to have affected the regulation of the synthesis of methanol dehydrogenase and these two c cytochromes. Further work is required to elucidate the role of the 23kDa cytochrome. *Mox*B, C, E and H mutants are also probably regulatory mutants since they produce little or no methanol dehydrogenase alpha subunit or cytochrome c_L (Table 1). The *mox*J gene encodes a 30 kDa protein of unknown function (Anderson and Lidstrom 1988).

*Hin*dCD is adjacent to an 11.3 kbp *Hin*dIII fragment encoding two serine pathway genes, *mcl*-1 and *ppc*, which are required for the production of an active malyl CoA lyase and phosphoenol pyruvate carboxylase respectively (Lidstrom et al. 1987). Two other serine pathway genes, *mmf*-1 and *mmf*-2, which are required for the conversion of acetyl CoA to glyoxylate and for synthesis of an active glycerate kinase respectively, are located on separate *Hin*dIII fragments (Stone and Goodwin 1989). The application of chromosome walking is required to order these fragments further, but the high frequencies of cotransfer obtained in R68.45 linkage mapping suggest that *mmf*-2, *mcl*-1 and *mtd*-1 (*mox*A1) are situated relatively close together on the chromosome (Tatra and Goodwin 1985).

In *Mb. organophilum* XX 11 *mox* genes have been identified; they are located in three different clusters, and each is separated from the other two by at least 40 kbp (Allen and Hanson 1985; Machlin et al. 1988). As yet most of the Mox mutants of *Mb. organophilum* XX have not been as well characterised as those of *Mb. extorquens* AM1, so it is difficult to compare the functions and organisation of the *mox* genes in the two bacteria. However, it does appear that

Table 2. Mutants of *Mb. organophilum* which are defective in C_1 oxidation

Mutant[1]	Reported properties				Complementation[2] by clone bank of			Mutagen (if known)
	MDH activity	60kDa subunit	$cytc_L$	PQQ	AM1	XX	DSM760	
Mb. organophilum XX								
SM13	–	–				V-A1		
SM29	–	+			*mox*A1	V-B1		
SM35	–	+				V-B2		
SM8	–	–			*mox*F	V-C		
SM19	–	–				VI-A		
SM2	–	–				VI-B		
SM3	–	–				VI-C		
PT34	–	+				VI-D		
SM4	–	–				VII-A		
SM16	–	–				VII-B		
17M	–	–						
Mb. organophilum DSM760								
2111I	–	low	+	+	*mox*F	V-C	+/*	EMS
MD4	–	+	+	+			+/*	Tn5
MD14	–	low	–	+			+/*	Tn5
MTM1	–	low	+	–	*pqq*A		*	EMS
MDQ2	–	low	+	–	*pqq*A		*	Kan cartridge

+/-; property present/absent. [1]; One mutant representative of each class is shown. [2]; complementation groups are shown where known;
* complementation by R' plasmid.
Information is taken from O'Connor and Hanson (1978), Allen and Hanson (1985), Biville et al (1988a), Machlin et al. (1988), Mazodier et al. (1988).

Table 3. Mutants which are defective in C_1 assimilation

Mutant[1] (genotype)	Reported properties	Complementation by clone bank of AM1	Complementation by clone bank of XX	Mutagen
Mb. extorquens AM1				
PG50	$SGAT^-$			MNNG
20BL	$HPRT^-$			MNNG
PT1001 (*mmf*-2)	GK^-	+	I	MNNG
PCT57 (*mcl*-1)	MCL^-	+	IIIa	MNNG
PCT48 (*mmf*-1)	ICL^-	+	IV	MNNG
*cou*4	ICL^-			Tn5
20ST-1	$SGAT^-$ PSP^-			MNNG
82GT-1	$SGAT^-$ $STHM^{-2}$			MNNG
Mb. organophilum XX				
8Z[3]	ICL^-	+	IV	UV
Mb. organophilum DSM760				
2121M	GK^-	+	I	EMS
2111B	$Methanol^{-4}$		II	

Icl^-; unable to convert acetyl CoA to glyoxylate. PSP; phosphoserine phosphatase. [1]: one mutant representative of each class is shown. [2]; isoenzyme required for growth on multicarbon compounds is missing. [3]; Mutant 8Z, isolated by O'Connor and Hanson (1977) was reported to be deficient in glycerate kinase. However, the mutant used by Allen and Hanson (1985) and Stone and Goodwin (1989), which was thought to be 8Z, was GK^+ and had the properties of an Icl^- mutant. [4] 2111B was reported to be cytochrome c negative by Allen and Hanson (1985), but has since been shown to contain cytochromes c_H and c_L (F. Gasser, personal communication).
Information is taken from Heptinstall and Quayle (1970), Harder and Quayle (1971b), Dunstan et al. (1972b), Salem et al. (1974), Fulton et al. (1984), Allen and Hanson (1985), Whitta et al. (1985), Stone and Goodwin (1989).

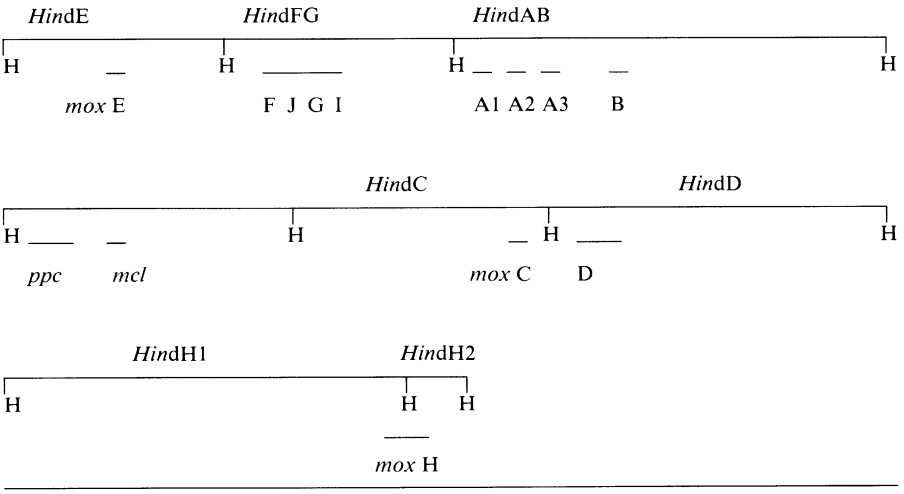
there are some similarities. The gene encoding the 60 kDa subunit of methanol dehydrogenase (*mox*F in *Mb. extorquens* AM1 and V-C in *Mb. organophilum* XX) has been identified in both organisms and two of the *Mb. extorquens* AM1 mutants (PG1, a *mox*A1 mutant and M15A, a *mox*A3 mutant) have been complemented by both banks. In both organisms these genes are arranged in the order A3, A1, F. In *Mb. extorquens* AM1 *mox*A2 separates A1 and A3; in *Mb. organophilum* these genes appear to be about 1 kbp apart and it is possible that this region encodes an equivalent of the *mox*A2 gene of *Mb. extorquens* AM1.

A *Sau*3A clone bank of *Mb. organophilum* XX DNA has also been used to complement mutants of *Mb. organophilum* and *Mb. extorquens* AM1 which are unable to grow on methanol or methylamine (Allen and Hanson 1985). This work demonstrated that three serine pathway genes, which are equivalent to *mmf*-1, *mmf*-2 and *mcl*-1 in *Mb. extorquens* AM1, are complemented by different clones and each is separated from the others by more than 30kbp of DNA; however, the *Mb. extorquens* AM1 mutant PG5, which has no detectable soluble cytochrome c nor any methylamine dehydrogenase activity, was complemented by one of the clones which complemented PCT57 (*mcl*-1),

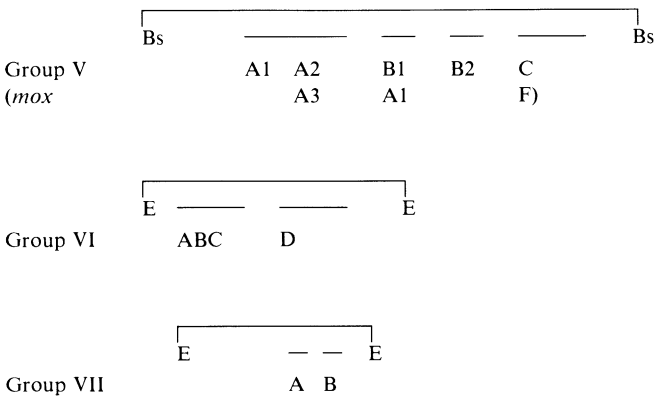
indicating that the C_1 genes defective in these two mutants are close to each other. A methanol negative mutant of *Mb. organophilum* DSM760 (2111B) was complemented by a different clone.

Fig. 4. Linkage groups of some genes involved in C_1 metabolism

(a) *Mb. extorquens* AM1 (see Nunn and Lidstrom (1986a), Lidstrom et al. (1987) and Anderson and Lidstrom (1988)).



(b) Mb. organophilum XX; complementation groups as defined by Machlin et al. (1988) are shown. Where appropriate equivalent *mox* genes as defined by Nunn and Lidstrom (1986a) in *Mb. extorquens* AM1 are shown in brackets.



H; *Hin*dIII. Bs; *Bst*EII. E; *Eco*R1. <---> 2 kbp

Some serine pathway mutants have been complemented using both the *Mb. extorquens* AM1 and the *Mb. organophilum* XX banks. However, when DNA fragments which encode similar functions in the two organisms were digested with *Hin*dIII and *Eco*RI in single and double digests no similarity in the resulting restriction fragment patterns was observed (Stone and Goodwin 1989). Furthermore, DNA:DNA hybridisation studies showed that these two species of *Methylobacterium* are in different homology groups (Hood et al. 1987). Thus, although genes from the two organisms are interchangeable in terms of function, they show considerable divergence at the DNA level.

There is less information available about the organisation of the C_1 genes in *Mb. organophilum* DSM760, although Mazodier et al. (1988) have cloned a 17 kbp fragment containing the methanol dehydrogenase structural gene (*mox*F) and at least two other genes involved in methanol oxidation. These were identified by complementation of chromosomal Tn5 insertion mutants. One (MD14) lacks cytochrome c_L and has only trace amounts of the alpha subunit of methanol dehydrogenase. The other (MD4), has a similar phenotype to the MoxA mutants of *Mb. extorquens* AM1 in that it synthesises an inactive methanol dehydrogenase and has normal levels of cytochrome c_L. These *mox* genes are within 30 kbp of *pqq*A, a gene required for PQQ synthesis and there are at least two other regions of the *Mb. organophilum* DSM760 chromosome encoding genes necessary for the synthesis of PQQ (Biville et al. 1988b).

Sequencing of the moxF, I and G Genes

The *mox*F gene of *Mb. organophilum* XX, which encodes the 60kDa subunit of methanol dehydrogenase, has been sequenced by Machlin and Hanson (1988). The transcription start site has been identified and the probable -35 and -10 promoter regions have the sequences AAGACA and TAGAAA respectively. In comparison the consensus sequence for -35 and -10 regions of *E. coli* promoters are TTGACA and TATAAT; (the most conserved bases are underlined). Thus the -35 sequence of the methylotroph promoter differs from the consensus *E. coli* sequence in two of the most conserved bases and this may be one of the reasons why the *mox*F gene is not expressed from its own promoter in *E. coli*. Situated 8bp downstream from the translation stop codon is a potential transcription termination site, indicating that the *mox*F gene of *Mb. organophilum* XX is unlikely to make up part of an operon. In contrast, Nunn and Lidstrom (1986a) suggested that, in *Mb. extorquens* AM1, *mox*F and *mox*G are cotranscribed (presumably with *mox*J, which is now known to separate them). Further work is needed to confirm this and to define more precisely the promoter and transcription termination sites in these methylotrophs.

Two transcripts of the *mox*F gene of *Mb. organophilum* XX have been identified in wild type cells, a major one of 2.1kb and a minor one of 1.55kb. The mRNA has a leader sequence of 170 nucleotides containing a typical Shine-Dalgarno sequence seven base pairs upstream of the translation start site. The

predicted amino acid sequence of the *mox*F gene product indicates that the first 27 amino acids make up a typical signal peptide which is cleaved on transport of the protein into the periplasm. The protein coding sequence has a high degree of homology (up to 82% in some regions) with the sequence of the *mox*F gene of an autotrophic methylotroph, *Paracoccus denitrificans* (Harms et al. 1987). Overall these proteins are very hydrophilic but there is a hydrophobic region near the C-terminal end and it has been suggested (Machlin and Hanson 1988) that this may be involved in the loose association of methanol dehydrogenase with the outer face of the inner membrane. The predicted secondary structure of the alpha subunit is dominated by alternating alpha helices and beta sheets, some of which are separated by beta turns.

The *mox*I and G genes of *Mb. extorquens* AM1 (which encode the beta subunit of methanol dehydrogenase and cytochrome c_L respectively) have been sequenced by Nunn and Anthony (1988a, 1988b, 1989) and both have typical Shine-Dalgarno and signal peptide sequences. The beta subunit has a high lysine content and its predicted secondary structure suggests that it contains amphipathic helices with lysyl residues on one side and hydrophobic regions on the other. The predicted amino acid sequence of the *moxG* gene has confirmed that cytochrome c_L is unusual. It contains a haem binding site, but otherwise has no homology with the sequences of other c cytochromes, which are usually highly conserved. For example, the typical arrangement of lysine residues around the haem binding site which usually occurs in c type cytochromes is absent. This is not surprising, however, since these lysine residues are thought to be involved in the reaction of the typical c cytochromes with the terminal oxidase, and the work of Anthony and colleagues has shown that cytochrome c_L transfers electrons to cytochrome c_H (Anthony 1988) and not to the terminal oxidase.

The DNA of the PPFMs has a high GC ratio (Green and Bousfield 1982) and as expected, in all three genes which have been sequenced there is a clear bias against triplets ending in A or U, with over 90% of codons ending in G or C. It has been suggested that the PPFMs may be members of the alpha-3 division of the eubacterial kingdom, together with other bacteria which have DNA containing a high percentage of codons ending in G or C, such as *Rhodobacter* and *Paracoccus* (Nunn and Anthony 1988b).

Looking to the Future

The isolation and characterisation of mutants and investigation of the genetics of methanol oxidation has demonstrated the complexity of this system. In addition it has already provided unexpected and important information about the biochemistry of this process, for example, the discovery of the beta subunit of methanol dehydrogenase and the 23 kDa c-type cytochrome. Sequencing and site directed mutagenesis of the *mox* genes will yield further information about their functions. The application of genetic techniques to the study of other

aspects of C_1 metabolism e.g. methylamine oxidation, formaldehyde oxidation and the role of M protein, PQQ biosynthesis, and the regulation of dissimilatory and assimilatory pathways, promises to yield equally significant and exciting information.

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Note added in proof

Since this manuscript was prepared it has been shown that a further 5 genes are involved in methanol oxidation in the PPFMs, bringing the total number of known *mox* genes to 17 (Bastien et al. 1989; Lidstrom 1989). *Mox*A1, A2 and A3 have been redesignated *mox*A, K and L respectively.

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7. C_1-Metabolism in Anaerobic Non-Methanogenic Bacteria

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Introduction

C_1-compounds contain only one carbon atom, or at least one carbon atom that is not bound to any other carbon atom. A multitude of such C_1-compounds is available as potential substrates for microbial growth. There has been a tremendous increase in the interest in *anaerobic* C_1-utilization during the past decades, as illustrated by the number of papers dealing with anaerobes presented at the three-yearly International Symposia on Microbial Growth on C_1-compounds (Crawford and Hanson 1984; Van Verseveld and Duine 1987).

Three major types of metabolism on the basis of anaerobic C_1-substrate conversions are known, namely methanogenesis, acidogenesis and sulfidogenesis. We will not review anaerobic growth on C_1-compounds with nitrate as electron acceptor by facultatively anaerobic nitrate-reducing bacteria because of the many parallels between aerobic C_1-metabolism and that in nitrate reducers. Most methanogenic substrates are C_1-compounds: H_2/CO_2, formate, methanol, methylamines and dimethylsulfide. The only important non-C_1-substrate from which methane is formed is acetate. The awareness that methanogens are archaebacteria that possess unique novel coenzymes and the practical importance of methanogenesis in anaerobic waste water purification have strongly stimulated research on methanogens. This chapter, however, will not deal with methanogenesis because several recent reviews were published on this topic (e.g. Jones et al. 1987; Vogels et al. 1988; Oremland 1988; Gottschalk 1989). Thus, this review will be restricted to a discussion of anaerobic non-methanogenic bacterial growth on C_1-substrates in the presence (or absence) of CO_2, or sulfate, as electron acceptors.

There are several potential C_1-substrates in anaerobic environments. Methane, CO_2, and formate are products of the anaerobic breakdown of various compounds of biological origin. Carbon monoxide (CO) is not only produced by anthropogenic sources but also in biological reactions, for instance as a by-product of methanogenesis (Uffen 1981; Eikmanns et al. 1985). The methoxylated lignin 'monomers' (Fig. 1) and related compounds form another major source of C_1-substrates (Young and Frazer 1987). Pectin,

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 163-191.

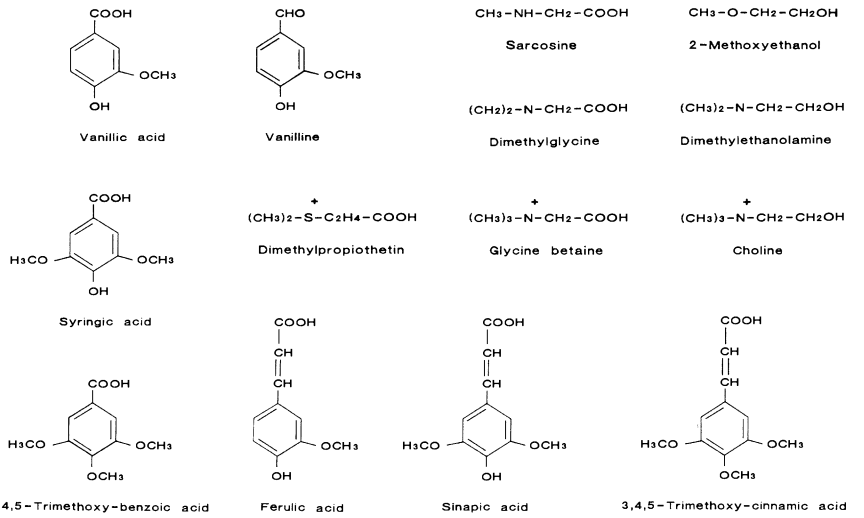

Fig. 1. Structures of various C1-compounds mentioned in the text.

an important component of plant material, is a polygalacturonate containing carboxylic groups esterified with methanol. Methanol is liberated but not degraded by anaerobic bacteria that ferment the polygalacturonate. In the rumen, *Lachnospira multiparus* is an active pectin fermenter (Rode et al. 1981). *Clostridium butyricum* was found to be the prevalent pectin-degrading, methanol-producing anaerobe in wetwood of living trees and in anoxic lake sediments (Schink et al. 1981; Schink and Zeikus 1982). Betaine (*N,N,N*-trimethylglycine) and choline (*N,N,N*-trimethylethanolamine) are osmotic solutes (Fig. 1) of many plants, animals and bacteria and a component of phospholipids, respectively (Yancey et al. 1982; Le Rudulier et al. 1984; Imhoff and Rodriguez-Valera 1984). These compounds can give rise to the formation of methylamines as a result of anaerobic microbial degradation processes (e.g. Fiebig and Gottschalk 1983; Naumann et al. 1983; Möller et al. 1984; Heijthuijsen and Hansen 1989a). Dimethylsulfoniopropionate (also called dimethylpropiothetin; Fig. 1) occurs in high concentrations in marine algae where it plays a role in osmoregulation (Vairavamurthy et al. 1985; Dacey and Wakeham 1986). Dimethylsulfoniopropionate itself can be converted into three other C1-compounds, namely dimethyl sulfide, methanethiol and 3-methiolpropionate (Kiene and Taylor 1988; Oremland et al. 1989). Methanethiol can also be formed from methionine (Zeikus 1983).

Anaerobic non-methanogenic growth of a pure bacterial culture on C1-compounds was first demonstrated by Wieringa (1940) who isolated *Clostridium aceticum*, an organism converting H_2/CO_2 into acetate. The process of acetate formation is called acetogenesis and the organisms are acetogens (or acetogenic). Unfortunately, these terms are also used to describe the process (and the organisms involved) in which e.g. propionate or butyrate

is converted to acetate (and CO_2) and protons are reduced to hydrogen. Here, we will use the term acetogenesis only in the first sense, namely a synthesis of acetate from or involving C_1-compounds. Some bacteria not only form acetate but also other short-chain fatty acids such as butyrate. This type of acidogenesis from C_1-substrates is thought to proceed via the level of acetate (acetyl CoA) and therefore we will usually include such bacteria when using the term acetogens.

Utilization of C_1-Compounds

Organisms Involved in the Anaerobic Degradation of C_1-Compounds

The first acetogenic bacteria were described in the early 1940's, namely *Clostridium aceticum* (Wieringa 1940), *Clostridium thermoaceticum* (Fontaine et al. 1942) and *Eubacterium limosum* (*Butyribacterium rettgeri*) (Barker and Haas 1944). *C. thermoaceticum* produced only acetic acid during growth on glucose and Barker and Kamen (1945) demonstrated that after addition of $^{14}CO_2$ the label was recovered in both the methyl group and the carboxyl group of the acetic acid produced. These early observations already indicated that in *C. thermoaceticum* glucose fermentation involves a partial oxidation of the substrate to two molecules of acetic acid and CO_2, followed by a reduction and condensation of the carbon dioxide to a third molecule of acetic acid (Fig. 2). Similar results were obtained with *E. limosum* (Barker et al. 1945) during growth on lactate and this organism was considered to possess the same pathway as *C. thermoaceticum*, although it produced also butyric and caproic acid probably by condensation of acetic acid.

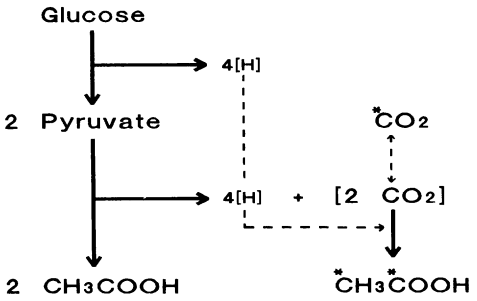

Fig. 2. Formation of the third molecule of acetate in the homoacetogenic fermentation of glucose.

C. aceticum originally isolated on H_2/CO_2 by Wieringa (1940), forms only acetic acid as fermentation product (see Tables 1 and 3) during chemolithotrophic and chemoorganotrophic growth (Karlsson et al. 1948). Although it was the first acetogen recognized to grow on a C_1-compound, not much work was carried out with *C. aceticum*. The organism was considered

Table 1. Utilization of C_1-substrates by non-methanogenic anaerobic bacteria.

Species	Growth substrates: H_2/CO_2	CO	HCOOH	CH_3OH	other C_1-substrates
Acidogens					
Acetobacterium					
carbinolicum	+	NR	+	+	TMB, TMC, betaine
malicum	+	NR	+	–	TMB, TMC, 2-ME, betaine, choline
wieringae	+	NR	+	–	betaine
woodii	+	+	+	+	TMB, TMC, betaine
Acetoanaerobium					
noterae	+	NR	–	–	
Acetogenium					
kivui	+	+	+	–	
Butyribacterium					
methylotrophicum	+	+	+	+	vanill, syring, TMB, betaine
Clostridium					
aceticum	+	NR	+	–	
formicoaceticum	–	NR	–	+	methoxylated aromatics
pfennigii	–	+	–	–	vanilline, ferul, TMB vanill, syring
thermoaceticum	+	+	NR	+	vanill, syring
thermoautotrophicum	+	NR	NR	+	vanill, syring
Eubacterium					
limosum	+	+	+	+	betaine, choline, vanill, syring, ferul
callanderi	–	–	–	–	ferul, sinapate, TMC vanill, syring
Peptostreptococcus					
productus	+	+	–	–	
Sporomusa					
acidovorans	+	NR	+	+	
ovata	+	NR	+	+	sarcos, DMG, betaine
paucivorans	+	NR	+	+	2-DMAE, TMA, betaine
sphaeroides	+	NR	+	+	betaine, TMA, DMG, 2-DMAE, (sarcosine)
termitida	+	+	+	+	sarcos, betaine, TMB

Table 1. Further information.

Species	Products	References
Acidogens		
Acetobacterium		
carbinolicum	acetate	Eichler and Schink (1984)
malicum	acetate	Tanaka and Pfennig (1988)
wieringae	acetate	Braun and Gottschalk (1982) Tanaka and Pfennig (1988)
woodii	acetate	Bache and Pfennig (1981)
Acetoanaerobium		
noterae	acetate	Sleat et al. (1985)
Acetogenium		
kivui	acetate	Leigh and Wolfe (1983)
Butyribacterium		
methylotrophicum	acetate, butyrate	Zeikus et al. (1980) Lynd et al. (1982) Kerby and Zeikus (1987a) Heijthuijsen (unpublished)
Clostridium		
aceticum	acetate	Braun et al. (1981)
formicoaceticum	acetate	Andreesen et al. (1970) Braun et al. (1981) Wu et al. (1988)
pfennigii	acetate, butyrate	Krumholz and Bryant (1985)
thermoaceticum	acetate	Daniel et al. (1988)
thermoautotrophicum	acetate	Daniel et al. (1988)
Eubacterium		
limosum	acetate, butyrate	Genthner et al. (1981) Müller et al. (1981) Genthner and Bryant (1982) Genthner and Bryant (1987)
callanderi	acetate, butyrate, formate	Mountfort et al. (1988)
Peptostreptococcus		
productus	acetate	Lorowitz and Bryant (1984)
Sporomusa		
acidovorans	acetate	Ollivier et al. (1985)
ovata	acetate	Möller et al. (1984)
paucivorans	acetate	Hermann et al. (1987)
sphaeroides	acetate	Möller et al. (1984)
termitida	acetate	Breznak et al. (1988)

Table 1. (Continued)

Species	Growth substrates: H_2/CO_2	CO	HCOOH	CH_3OH	other C_1-substrates
Syntrophococcus sucromutans	–	–	+	–	vanilline, vanill, ferul, syring, TMB
unnamed					
strain TH-001	–	NR	NR	–	vanill, syring, ferul
strain AOR	+	+	+	–	betaine
Sulfidogens					
Desulfovibrio					
'classical' strains	+[a]	NR	+	–	
carbinolicus	+[a]	NR	+	+	
baarsii	–	–	+	NR	
Desulfobacterium					
autotrophicum	+	NR	+	–	betaine
catecholicum	+	NR	+	+	
indolicum	–	NR	+	NR	
sp. PM4	–	NR	+	–	betaine
vacuolatum	+	NR	+	NR	
Desulfotomaculum					
orientis	+	+	+	+	TMB
Miscellaneous[b]					
Rhodocyclus					
gelatinosus	–	+	–	–	

vanill, vanillate; syring, syringate; ferul, ferulate; TMB, 3,4,5-tri- methoxybenzoate; TMC, 3,4,5-trimethoxycinnamate; 2-ME, 2-methoxyethanol; sarcos, sarcosine; DMG, dimethylglycine; TMA, trimethylamine; 2-DMAE, 2-dimethylaminoethanol; NR, not reported.

[a] only H_2 energy source

[b] not exhaustive

lost until a spore preparation of the original strain was found and revived by Braun et al. (1981). In 1967 a sporeforming anaerobic bacterium was isolated with several properties similar to the original strain of *C. aceticum* (El Ghazzawi 1967). However, this strain did not grow on H_2/CO_2 and because of its ability to form small amounts of formate in addition to acetate it was named *Clostridium formicoaceticum*. Over the years it has become clear that inability to grow with H_2/CO_2 is rather exceptional amongst acetogens (Table

Table 1. Further information.

Species	Products	References
Syntrophococcus		
sucromutans	acetate	Krumholz and Bryant (1986)
unnamed		
strain TH-001	acetate	Frazer and Young (1985)
strain AOR	acetate	Lee and Zinder (1988a)
Sulfidogens		
Desulfovibrio		
'classical' strains		Widdel (1988)
carbinolicus		Nanninga and Gottschal (1987)
baarsii		Jansen et al. (1984)
		Widdel (1988)
Desulfobacterium		
autotrophicum	DMG from betaine	Brysch et al. (1987)
		Heijthuijsen and Hansen (1989b)
catecholicum		Szewzyk and Pfennig (1987)
indolicum		Bak and Widdel (1986)
sp. PM4	DMG from betaine	Heijthuijsen and Hansen (1989b)
vacuolatum		Widdel (1988)
Desulfotomaculum		
orientis	acetate	Klemps et al. (1985)
		Cypionka and Pfennig (1986)
Miscellaneous		
Rhodocyclus		
gelatinosus	H_2/CO_2	Uffen (1976)
		Champine and Uffen (1987)

1). Most of the strains, including *C. thermoaceticum* (Kerby and Zeikus 1983) and *E. limosum* (Genthner et al. 1981), can grow on H_2/CO_2 with doubling times ranging from 14 to 19 h.

Most acetogens that can use H_2/CO_2 also grow on formate, except *Acetoanaerobium noterae* and *Peptostreptococcus productus* (Tables 1 and 2). Growth of acetogens with methanol was first described for *Butyribacterium methylotrophicum* (t_d 9-10 h), an organism which is physiologically similar to

E. limosum. The latter also appeared to grow on methanol (t_d 7 h) and other C_1-compounds (Genthner et al. 1981). Methylotrophic growth of both species requires the presence of bicarbonate (see Table 2). The presence of acetate at the onset of growth influences the amount of butyrate produced by *B. methylotrophicum* (Datta and Ogeltree 1983). Another acetogen able to grow on methanol/CO_2, a property only discovered after its original description, is *Acetobacterium woodii* (Balch et al. 1977). Bache and Pfennig (1981) demonstrated methylotrophic growth of several strains of this species, following their selective isolation on methoxylated aromatic compounds such as vanillic acid (Fig. 1). The molar growth yield on these compounds is proportional to the number of methyl groups per molecule. In addition, *A. woodii* is able to reduce the double bond in the acrylic side chain of some of these methoxyethers (ferulic and 3,4,5-trimethoxy-cinnamic acid) and caffeic acid and energy is conserved in this reduction reaction. The double bonds of these acrylic side chains function as electron acceptors and are an alternative to CO_2 (Tschech and Pfennig 1984). An example of such a reduction reaction is shown in Fig. 3. In addition to *A. woodii*, several other bacteria appeared to grow on these phenyl-methyl-ethers (e.g. *E. limosum*, t_d 8.2 h) and recently even growth on methoxyethanol was demonstrated (Table 1). Utilization of the methyl groups is stoichiometrically comparable to the utilization of methanol by organisms like *A. woodii* or *E. limosum*, but methanol is not an intermediate. An example of a reaction equation is given below.

2 syringic acid + 2 CO_2 + 2 H_2O $\rightarrow$ 2 3,4,5-trihydroxybenzoic acid + 3 acetate

Certain organisms grow on methoxylated compounds but not on methanol. The oxygen atom of the methoxyl group is recovered in the hydroxy compound which is formed as a product (DeWeerd et al. 1988).

Betaine, dimethylglycine, sarcosine and choline can also serve as methyl donors for acetogens (Table 1; Fig. 1). The first acetogenic organism described to grow (t_d 6 h) on betaine was *E. limosum*, which demethylates betaine to dimethylglycine and utilizes the methyl group in a similar way as methanol to produce acetate and butyrate (Müller et al. 1981). *Sporomusa ovata* and

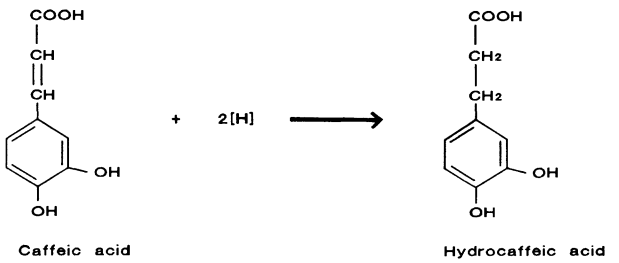

Fig. 3. Reduction of the acrylic side chain of caffeic acid.

Table 2. Reactions involved in C_1-transformations
[Thermodynamic data taken from Thauer et al. (1977)].

Reaction	$\Delta G^{0'}$ (kJ/reaction)
Acidogenic	
$2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow$ acetate$^-$ $+ 4\ H_2O$	-104.6
$4\ CO + 4\ H_2O \rightarrow$ acetate$^-$ $+ 2\ HCO_3^- + 3\ H^+$	- 45.7
4 formate$^-$ $+ H^+ \rightarrow$ acetate$^-$ $+ 2\ HCO_3^-$	-139.0
$4\ CH_3OH + 2\ HCO_3^- \rightarrow 3$ acetate$^-$ $+ 4\ H_2O + H^+$	-221.1
$10\ CH_3OH + 2\ HCO_3^- \rightarrow 3$ butyrate$^-$ $+ 10\ H_2O + H^+$	-541.9
7 betaine $+ 2\ HCO_3^- \rightarrow 7$ dimethylglycine$^-$ $+ 1.5$ acetate$^-$ $+$ 1.5 butyrate$^-$ $+ 8H^+$	
Sulfidogenic	
4 betaine $+ 3\ SO_4^{2-} \rightarrow 4$ dimethylglycine$^-$ $+ 4\ HCO_3^- +$ $5\ H^+ + 3\ HS^-$	
4 formate$^-$ $+ SO_4^{2-} + H^+ \rightarrow 4\ HCO_3^- + HS^-$	-186.6
$4\ CH_3OH + 3\ SO_4^{2-} \rightarrow 4\ HCO_3^- + 3\ HS^- + H^+ + 4\ H_2O$	-324.5

Sporomusa sphaeroides were enriched and isolated with betaine as the substrate (Möller et al. 1984). Growth of these spore-forming gram-negative bacteria on betaine (t_d 2-3 h) is more complex than that of *E. limosum* because in *S. ovata* not only dimethylglycine and acetate were formed as end products, but also sarcosine, ammonia and CO_2. Betaine and sarcosine are reductively cleaved with the reducing equivalents released in the oxidation of methyl groups to CO_2. Dimethylglycine is probably not cleaved, but demethylated to sarcosine and part of the methyl groups released are used to methylate this compound to betaine. Recently, several other *Sporomusa* species were described and one of these isolates, *S. termitida,* is able to grow on methoxylated compounds (Table 1).

The discovery of the utilization of CO as an energy source by acetogens was preceeded by studies focusing on the enzyme CO dehydrogenase, which oxidizes CO to CO_2. Diekert and Thauer (1978) showed that cells of *C. formicoaceticum* and *C. thermoaceticum* growing on hexoses were able to oxidize CO to CO_2 and that the reaction was coupled with the reduction of CO_2 to acetate. Under these conditions cell extracts of both organisms contained relatively high activities of a CO dehydrogenase. Growth with CO as the sole energy source was described for *E. limosum* (Genthner and Bryant 1982) and *B. methylotrophicum* (Lynd et al. 1982; Tables 1 and 2). Both organisms had to be adapted to CO by gradually increasing its level in the gas phase to a maximum of 50 % for *E. limosum* (t_d 7 h) and 100 % for *B. methylotrophicum* (t_d 13.8 h). The same adaptation method was successfully used for growth of *C. thermoaceticum* under a 100 % atmosphere of CO (Kerby and Zeikus 1983). Once growth of acetogenic bacteria with CO as the energy source had been established, Lorowitz and Bryant (1984) used CO in

enrichments and isolated the very rapidly CO metabolizing organism *Peptostreptococcus productus* (t_d less than 2 h). It remains to be established why earlier enrichments with CO led to development of *Rhodocyclus gelatinosus* (formerly *Rhodopseudomonas gelatinosa*), a facultative phototrophic purple bacterium (Uffen 1976). This species was found to grow anaerobically in the dark (t_d 6.7 h) on the basis of the following equation:

$$CO + H_2O \rightarrow CO_2 + H_2$$

The growth of almost all acetogens is stimulated by small amounts of yeast extract, which is often added to media used in isolation procedures. Many acetogens can grow chemolithotrophically on H_2/CO_2, producing acetate. This does not necessarily imply that they should be able to grow autotrophically in the absence of organic supplements other than vitamins. Yet, several species were shown to grow slowly (t_d 25-33 h) on H_2/CO_2 in vitamin-supplemented mineral media (Balch et al. 1977; Genthner et al. 1981; Braun and Gottschalk 1982; Moench and Zeikus 1983; Savage and Drake 1986). A list of essential vitamins has been given by Savage and Drake (1986).

C_1-utilizing acetogens are strict anaerobes which are cultivated in oxygen-free media reduced with sodium sulfide, or a combination of sodium sulfide and cysteine. Sulfide and cysteine are not only reductants but also sulfur sources for acidogenic growth. Sulfide becomes toxic for the growth of acetogens such as *B. methylotrophicum* even at moderate concentrations; several other sulfur sources, like sulfite, thiosulfate, sulfur and dithionite can be used by this species and by *A. woodii*. Both thiosulfate and dithionite are more convenient sulfur sources for *B. methylotrophicum* than sulfide, but dithionite is more versatile because of its reductive properties and its ability to sustain faster growth (Heijthuijsen and Hansen 1989c).

Another important group of bacteria growing on C_1-compounds are the sulfate-reducing bacteria. Several *Desulfotomaculum* species and most of the so called classical *Desulfovibrio* strains are not only able to grow with H_2 as an energy source, but most of these bacteria can also grow on formate (Tables 1 and 2; Klemps et al. 1985; Widdel 1988). Growth on the basis of the oxidation of methanol to CO_2 is rather exceptional but was recently demonstrated for *Desulfovibrio carbinolicus* (Nanninga and Gottschal 1987) and certain *Desulfotomaculum strains* (Klemps et al. 1985). Growth on these C_1-substrates requires addition of yeast extract or acetate for cell synthesis. However, *Desulfovibrio baarsii* can grow on formate/CO_2 (t_d ± 20 h) as the only carbon and energy source (Jansen et al. 1984) and *Desulfotomaculum orientis* grows autotrophically on H_2/CO_2 (t_d ± 13 h; Cypionka and Pfennig 1986). Several other C_1-substrates also can serve as carbon and electron donors (Table 1). *Desulfotomaculum orientis* can grow slowly on formate, methanol and 3,4,5-trimethoxy-benzoate in the absence of sulfate and produces acetate under these conditions, indicating that CO_2 is used as electron acceptor, a growth strategy comparable to the acetogens. H_2 and

CO_2 were converted to acetate but growth yields of sulfate-limited cultures showed that no energy was conserved.

Some species of the recently described genus *Desulfobacterium* grow chemolithoautotrophically on H_2/CO_2 (e.g. *D. autotrophicum*; t_d ± 16 h) and also on formate (Brysch et al. 1987). Recently, Heijthuijsen and Hansen (1989b) reported growth of *D. autotrophicum* and *Desulfobacterium* PM4 on betaine (t_d ± 20 h).

The Acetyl CoA/CO Dehydrogenase Pathway of CO_2 Fixation

As mentioned before, Barker and his coworkers recognized as early as 1945 that during the fermentation of hexoses CO_2 is fixed by the acetogenic bacteria *C. thermoaceticum* and *E. limosum* (Fig. 2). These bacteria, which were the only known acetogens for a period of about twenty years, were considered at that time to be only heterotrophic. The elucidation of the pathway of CO_2 fixation was therefore mainly restricted to studies of *C. thermoaceticum* grown on hexoses and not on C_1-compounds. Although the discovery and elucidation of this pathway is a fascinating story, we refer to several excellent reviews on this topic (Ljungdahl 1986; Fuchs 1986; Wood 1989) and only a general outline is given here. A key role in the study of this new route ('acetyl CoA/CO dehydrogenase pathway') was played by H.G. Wood and for this reason Ljungdahl (1986) proposed the name 'Wood pathway' for it.

Outline of the Acetyl CoA/CO Dehydrogenase Pathway

As shown by labelling experiments and enzyme measurements (e.g. Eden and Fuchs 1982, 1983), acetate formation from CO_2 does not involve a cyclic mechanism such as the Calvin cycle or the reductive tricarboxylic acid cycle. Instead acetyl CoA synthesis proceeds by reducing two molecules of CO_2 with 8[H] to a methyl group and a carboxyl group (Fig. 4). Both groups are joined together with CoA to yield acetyl CoA, which is used as a precursor for cell synthesis or acetate production.

The acetyl CoA/CO dehydrogenase pathway and the various enzymes involved in the transformations of its intermediates are shown in Fig. 4. The reduction of CO_2 to acetate with H_2 requires the additional presence of hydrogenase(s). Hydrogenases have been demonstrated in *C. thermoaceticum* (Drake 1982), *C. thermoautotrophicum* (Clark et al. 1982), *C. aceticum* (Braun and Gottschalk 1981) and *A. woodii* (Braun and Gottschalk 1981; Ragsdale and Ljungdahl 1984). The first step in the formation of the methyl group from CO_2 is the reduction of CO_2 to formate by formate dehydrogenase. *C. thermoaceticum* possesses an NADP-dependent formate dehydrogenase, which catalyzes the reversible reaction and contains tungsten, selenium and iron (Yamamoto et al. 1983). The activity of this enzyme was shown to be dependent on the presence of iron, selenium and either tungsten or molybdenum in the growth medium (Andreesen et al. 1973). Similar effects

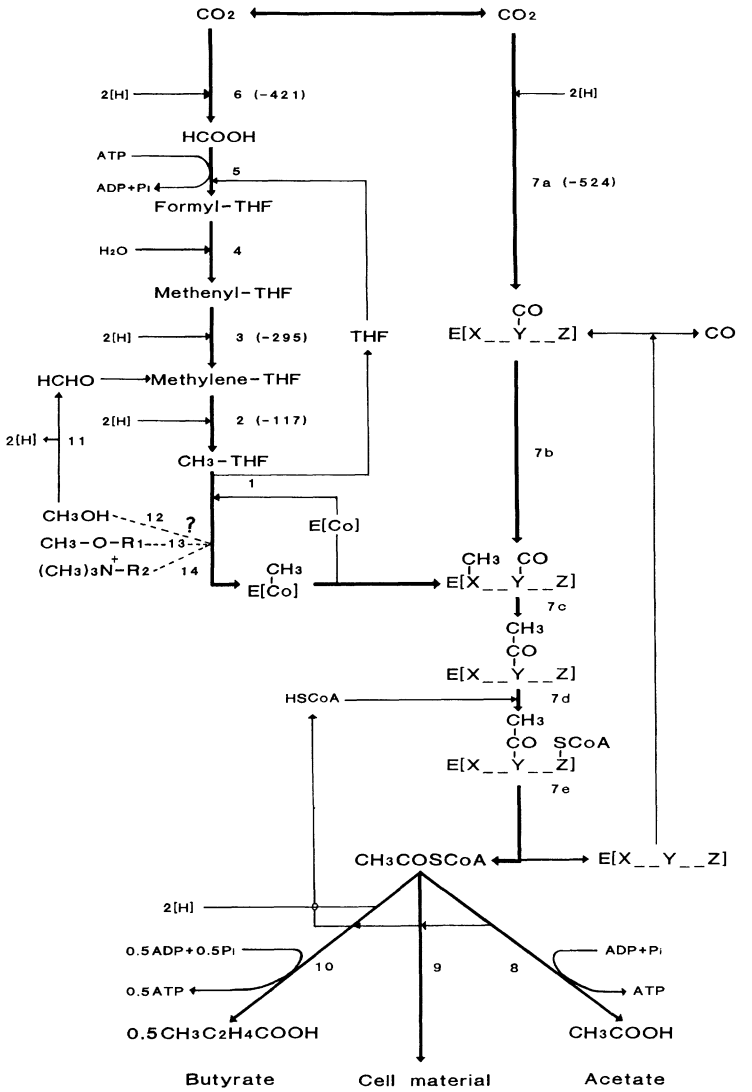

Fig. 4. Acetyl CoA/CO dehydrogenase pathway for growth on C_1-compounds. THF, tetrahydrofolate; R_1, ethanol or aromatic compounds (see Fig. 1); R_2, ethanol or acetate residue; E[Co], corrinoid enzymes; E[X____Y____Z], CO dehydrogenase (acetyl CoA synthase) with 3 sites for binding of CO/CO_2, CHH_3, and HSCoA; (1), methyltransferase; (2), methylene-H4,folate reductase; (3), methylene-H4folate dehydrogenase; (4), methenyl-H4folate cyclohydrolase; (5), formyl-H4folate synthetase; (6), formate dehydrogenase; (7a-e), CO dehydrogenase (acetyl CoA synthase); (8), phosphotransacetylase and acetate kinase; (9), enzymes of anabolic reactions; (10), enzymes of butyrate synthesis from acetyl CoA; (11) methanol dehydrogenase; (12, 13 and 14), methyltransferases. Figures in brackets are values of standard oxidation reduction potentials.

Table 3. Activities of enzymes of the acetyl CoA/CO dehydrogenase pathway in extracts of acetogenic bacteria grown on C_1-substrates (μmol min^{-1} mg^{-1} protein).

Organism	Substrates	FDH[a]	FTHF synthetase[b]	Methenyl-THF-CH[c]
A. woodii	H_2/CO_2	0.11-0.17	9.0-12.7	0.6-1.1
C. thermoautotrophicum	H_2/CO_2	0.04	10.8	0.49
C. thermoautotrophicum	Methanol/CO_2	0.07	7.7	0.94
B. methylotrophicum	Formate	1.87	8.76	1.16
B. methylotrophicum	Methanol/CO	1.3	2.6	0.6
strain AOR	H_2/CO_2	29.7	< 0.01	ND
Acetogenium kivui	H_2/CO_2	ND	7.5	ND

[a] formate dehydrogenase
[b] formyl-H_4folate synthetase
[c] methenyl-H_4folate cyclohydrolase
[d] methylene-H_4folate dehydrogenase
[e] methylene-H_4folate reductase
[f] carbon monoxide dehydrogenase
ND not determined

of tungsten and selenium on the growth have been reported for *C. formicoaceticum* (Andreesen et al. 1974) and *A. woodii* (Schoberth 1977). The first attempts to purify the enzyme from *C. thermoaceticum* were hampered by its oxygen sensitivity (Yamamoto et al. 1983). The enzyme consists of 2 α (M_r 96,000) and 2 β (M_r 76,000) subunits. Ferredoxin is probably the physiological electron acceptor in other acetogens (Fuchs 1986).

Formate is reduced to a methyl group via intermediates of the tetra hydrofolate (H_4folate) pathway, involving four enzymes (Fig. 4). All four have been purified from *C. thermoaceticum* and *C. formicoaceticum*; only methylene-H_4folate dehydrogenase has been purified from *A. woodii*. The properties of these enzymes have been reviewed by Fuchs (1986). The electron donor in the methenyl-H_4folate reduction reaction is NADPH in *C. thermoaceticum* and *C. thermoautotrophicum* and NADH in *C. formicoaceticum* and *A. woodii*. The methylene-H_4folate dehydrogenase in *C. thermoaceticum* catalyzes also the cyclohydrolase reaction. In contrast, *C. formicoaceticum* and *A. woodii* contain two different enzymes. The methylene-H_4folate reductase in *C. formicoaceticum* is an oxygen sensitive 237 kD octamer containing approximately 2 FAD, 15-20 iron, acid-labile sulfur and approximately 2 zinc (Clark and Ljungdahl 1984). Several artificial electron carriers are used in vitro, but not pyridine nucleotides. The methyl group is subsequently transferred to a corrinoid protein by a methyl transferase. Two different corrinoids proteins have been purified from *C. thermoaceticum* (Hu et al. 1984). The function of one of these (27 kD) is still obscure, but the other one with a molecular weight of 89 kD ($\alpha\beta$ dimer) is

Table 3. Further information.

Organism	Methylene-THF-DH[d]	Methylene-THF-Red[e]	CODH[f]	Reference
A. woodii	0.6-1.3	ND	ND	Tanner et al. (1978)
C. thermoautotrophicum	2.03	ND	10.7	Clark et al. (1982)
C. thermoautotrophicum	3.1	ND	7.8	Clark et al. (1982)
B. methylotrophicum	7.1	9.3	23.3	Kerby and Zeikus (1987b)
B. methylotrophicum	2.2	2.7	4.1	Kerby and Zeikus (1987b)
strain AOR	ND	ND	6.4	Lee and Zinder (1988b)
Acetogenium kivui	ND	ND	56.4	Lee and Zinder (1988b)

involved in acetyl CoA synthesis. The cobalt in the corrinoid protein of *C. thermoaceticum* must be reduced to accept the methyl group from 5-methyl-H_4folate.

The most characteristic but also the most complex enzyme of the pathway is the condensing enzyme, which forms acetyl CoA from the methyl group at the corrinoid protein, CO or CO_2 (and a suitable reductant) and CoA. Originally the enzyme was found in *C. thermoaceticum* and *C. formicoaceticum* and thought to function only as a carbon monoxide dehydrogenase, since it catalyzed the reversible reduction of CO_2 to bound CO (Diekert and Thauer 1978). The presence of nickel in the growth medium was essential for the expression of the enzyme in both *Clostridium* species (Diekert and Thauer 1980) and in *A. woodii* (Diekert and Ritter 1982). *C. thermoaceticum* possesses an energy-dependent high-affinity transport system for nickel (Lundie et al. 1988). CO dehydrogenase has been purified from *C. thermoaceticum* and *A. woodii* (Ragsdale et al. 1983a; Ragsdale et al. 1983b) and the enzymes were found to be very similar in both species. The enzymes are extremely oxygen-sensitive, probably possess a subunit composition of $\alpha_3\beta_3$ (60 and 70 kD, respectively) and contain 6 Ni, 1-3 Zn and several iron sulfur clusters. The purified CO dehydrogenase also catalyzes an exchange reaction between 1-^{14}C acetyl CoA and CO (Ragsdale and Wood 1985). The methyl, carboxyl, and CoA moieties have different sites on the enzyme, represented by X, Y and Z in Fig. 4 (Wood et al. 1986). The methyl group from the methyl corrinoid protein is transferred to the X-site of the CO dehydrogenase and coupled to the carbonyl group at the Y-site. The CoA is

added to the Z-site by action of the enzyme CO dehydrogenase disulfide reductase and is coupled to the acetyl group at the Y-site by the CO dehydrogenase to yield acetyl CoA, which is released from the enzyme. The CO dehydrogenase can be considered as the key enzyme of the pathway and it was suggested to rename it as acetyl CoA synthase (Wood et al. 1986). During growth with CO as carbon source and electron donor, the CO molecule is bound directly to the Y-site of the acetyl CoA synthase and either used as the carbonyl precursor or oxidized to CO_2 to provide the electrons for the formation of a methyl group.

In addition to the organisms referred to above, several other acetogenic bacteria and some sulfate-reducing bacteria use the acetyl CoA/CO dehydrogenase pathway, although the evidence is not always complete. Measurements of the C_1-transforming enzymes during growth of acetogens on C_1-substrates show activities high enough to explain their proposed role in the pathway (Table 3). The concentrations of H_4folates and cobamides are extremely high in these bacteria; the corrinoid content of acetogens is 1000-fold higher than in *Escherichia coli* (Fuchs 1986). Further evidence for the functioning was provided by studies on the distribution of ^{13}C-label in the products formed by *B. methylotrophicum* and *A. woodii* during growth on several labeled C_1-compounds and by demonstration of enzymes of the acetyl CoA/CO dehydrogenase pathway (Kerby et al. 1983; Kerby and Zeikus 1987b). *Desulfovibrio baarsii* can grow with formate and sulfate as the energy source and CO_2 as the source of carbon but cannot use H_2 as electron donor. Evidence for the involvement of the acetyl CoA/CO dehydrogenase pathway in the growth of *Desulfovibrio baarsii* on formate/CO_2 and sulfate is based on the fate of ^{14}C-labeled C_1-substrates and the presence of CO dehydrogenase (Jansen et al. 1984, 1985). Under these conditions the pathway functions as a biosynthetic route, in contrast to its role in acetogens. *Desulfovibrio baarsii* and *Desulfobacterium* species do not only grow on certain C_1-substrates (formate or betaine) but also on multi-carbon substrates which are oxidized completely to CO_2. The oxidation of acetyl CoA to CO_2 proceeds via a reverse type of acetyl CoA/CO dehydrogenase pathway (Schauder et al. 1986; Thauer 1988). CO dehydrogenase, formate dehydrogenase and C_1-tetrahydropterin enzymes were present at relatively high levels; tetrahydropterins containing 4 glutamate rather than one (i.e. tetrahydrofolates) are most active in these organisms. During growth of *D. autotrophicum* on H_2/CO_2 and sulfate the acetyl CoA/CO dehydrogenase pathway is used for biosynthetic purposes (Länge et al. 1989; Schauder et al. 1989). It has been speculated that the oxidation of betaine to dimethylglycine by *Desulfobacterium* strains involves the oxidation of the methyl group to CO_2 via the methyl branch of the oxidative acetyl CoA/CO dehydrogenase pathway (Heijthuijsen and Hansen 1989b).

Growth on methanol by acetogenic bacteria is dependent on the presence of either CO_2, formate or CO. In the fermentation of methanol/CO_2 to acetate one out of four methanol molecules is used for the synthesis of a

carboxyl group of the acetate formed and passes through the level of CO_2 (Kerby et al. 1983; Ljungdahl 1986). However, there is no certainty about the level of entrance of methanol in the acetyl CoA/CO dehydrogenase pathway. Van der Meijden et al. (1984) reported evidence for the involvement of a methanol:cobalamin methyl tranferase in the first reaction step of methanol to acetate and butyrate in *E. limosum*. Although the nature of the methyl accepting site in the pathway is still obscure the methyl group is probably coupled to the corrinoid protein of the acetyl CoA/CO dehydrogenase pathway and directly incorporated into acetate. Methanol oxidation might occur via a reversed tetrahydrofolate cycle, but recently some evidence has been presented for the presence of a pyrroloquinoline quinone (PQQ) dependent methanol dehydrogenase. Methanol was oxidized by this enzyme to formaldehyde and formate (Winters-Ivey and Ljungdahl 1988).

It has not yet been established in detail how and where the methyl group of methoxylated compounds enters the acetyl CoA/CO dehydrogenase pathway. In strain TH-001 grown in the presence of O-[methyl-^{14}C]vanillate, the methyl group was preferentially incorporated into the methyl group of acetate (Frazer and Young 1986). Wu et al. (1988) showed some evidence for an O-demethylating enzyme which catalyzed the conversion of syringate to gallate. The levels of this enzyme were repressed by glucose and methanol in glucose-syringate and methanol-syringate cultures, respectively. The released methyl groups served as a methyl donor and the enzyme system required CO, pyruvate or H_2/CO_2 as a carboxyl precursor to form acetyl CoA, from which either cell material or acetate was produced. These results also show that methanol is not an intermediate in the O-demethylating reaction.

Energetic Aspects of Growth on C_1-Compounds

The reduction of CO_2 with H_2 to acetate is exergonic under standard conditions (Table 2). It is, however, not obvious how bacteria are able to derive biochemically useful energy from this process. No net ATP synthesis occurs by substrate level phosphorylation since the gain of one ATP in the conversion of acetyl CoA to acetate is counterbalanced by the investment of one ATP in formyl-H_4folate synthesis. Thus, in principle only electron transport phosphorylation or other membrane-associated processes can be mechanisms for a net ATP synthesis. Growth of *A. woodii* on H_2/CO_2 yields 5.4 g (dry mass) per mol acetate produced, which corresponds with approximately 0.5-1 ATP produced per acetate formed, assuming that the Y_{ATP} in autotrophically grown cells is 5-10 g (dry mass) per mol ATP (Fuchs 1986). Considering the differences between the standard redox potentials of the CO_2 reduction reaction couples and the $H_2/2H^+$ couple, the methylene-H_4folate reductase reaction can be coupled to the synthesis of approximately 1 ATP by electron transport phosphorylation and it cannot be excluded that some energy gain occurs in the reduction of methenyl-H_4folate to methylene-H_4folate. The methylene-H_4folate reductase is membrane-associated in *C.*

thermoautotrophicum and it has been suggested that methylene-H_4folate can accept electrons from hydrogen via electron carriers mediated by a hydrogenase which is present at the outside of the membrane (Hugenholtz et al. 1987; Hugenholtz and Ljungdahl 1989). A proton-motive force generated by this process and the presence of an F_1-ATP-ase in *C. thermoautotrophicum* and *C. thermoaceticum* can explain energy conservation during growth on H_2/CO_2 (Mack Ivey and Ljungdahl 1986; Hugenholtz and Ljungdahl 1989). However, the reduction of CO_2 to the level of CO is an energy requiring process as demonstrated by Diekert et al. (1986) and as illustrated by the fact that the reverse reaction, namely the conversion of CO to H_2 and CO_2, provides biochemically useful energy (Uffen 1976). The CO dehydrogenase is membrane-associated in *C. thermoautotrophicum* and mediates the CO-dependent reduction of membrane components (Hugenholtz et al. 1987). A similar location of the enzyme was found in *Rhodocyclus gelatinosus* (Champine and Uffen 1987). It has been speculated that acetate excretion may contribute to energy conservation in acetogenic bacteria. Experiments of Emde and Schink (1987) on the fermentation of glycerol triacetylester and glycerol by *Acetobacterium*, however, gave no indication for energy transduction by acetate excretion.

Acetate formation from CO/CO_2 and H_2/CO_2 was Na^+-stimulated in *Peptostreptococcus productus* (Geerligs et al. 1989); growth of *A. woodii* on H_2/CO_2 or methanol/CO_2 was Na^+-dependent (Heise, VAAM-DGHM-NVvM meeting March 1989, University of Marburg, Federal Republic of Germany). In cultures of *Peptostreptococcus productus* formate accumulated in the absence of sodium which suggested that Na^+ was involved in the reduction of CO_2 to the methyl group, either by stimulation of the activity of enzyme(s) or by electrogenic transport across the cytoplasmic membrane by a membrane-bound enzyme. A sodium gradient can be coupled to ATP synthesis via a chemiosmotic mechanism (Dimroth 1987). Most likely the methylene-H_4folate reduction is coupled to Na^+ translocation and the sodium gradient is either directly used for ATP synthesis or indirectly via an Na^+/H^+ antiporter which is present in *C. thermoaceticum* (Terraciano et al. 1987); the sodium gradient may also be involved in the reduction of CO_2 to CO (Gottschalk 1989).

A bioenergetic analysis of the fermentation of methanol/CO_2 to acetate or butyrate (see Table 2) is even more difficult than that of the acetogenic fermentation of H_2/CO_2. With several assumptions, Heijthuijsen and Hansen (1986) estimated an overall ATP yield of approximately 2.5 per 4 methanol fermented to acetate and 6-6.5 ATP per 10 methanol fermented to butyrate.

In *C. thermoaceticum* hexoses are fermented via the glycolytic pathway (Wood 1952; Ljungdahl and Wood 1965) to 2 acetate, 2 CO_2, 8[H] and 4 ATP; a third acetate is formed from 8[H] and 2 CO_2 via the acetyl CoA/CO dehydrogenase pathway. Although the formation of the third acetate is formally equivalent to the synthesis of acetate from 4 H_2 and 2 CO_2, it is energetically slightly less favourable (2 NADH instead of 2 H_2) and it is not known whether the organism derives additional energy from this process.

Molar growth yields on glucose are in agreement with the synthesis of at least 4 ATP (Fuchs 1986).

The reduction of C=C double bonds in the acrylic side chain of aromatic compounds, e.g. caffeate, in *A. woodii* leads to the synthesis of ATP as evidenced by molar growth yield data (Tschech and Pfennig 1984; Hansen et al. 1988).

Role of Nonmethanogenic, C_1-Utilizing Anaerobic Bacteria in Natural Habitats

Anaerobic C_1-transformations occur mainly in the sediments of aquatic ecosystems such as oceans, marshes, rivers, etc., specific parts of the gastrointestinal tract of animals and in anaerobic waste water purification systems. Mineralization in anaerobic environments is the result of a sequence of degradation processes carried out by different groups of microorganisms with specific catabolic pathways (Laanbroek and Veldkamp 1982). In a simplified scheme, the first group in this sequence are the fermentative bacteria that hydrolyse polymers and ferment the monomers. The second group consists of hydrogen-producing acetogenic bacteria, which ferment alcohols other than methanol and organic acids other than acetate to CO_2, acetate and H_2. The last group consists of bacteria which consume and compete for the end products of all previous processes, namely hydrogen, one-carbon compounds (e.g. formate or methanol) and acetate. Among these bacteria we find the methanogens, which are metabolically virtually restricted to the above substrates and the acetogens and the sulfate-reducing bacteria, which also play a role in the degradation of larger organic molecules. The removal of H_2 by this group is of utmost importance in the breakdown of the organic end products of fermentative bacteria, because several compounds can only be oxidized at the low H_2-pressures maintained by these hydrogenotrophs, e.g. the oxidation of propionate to acetate, CO_2 and H_2. This process of interspecies hydrogen transfer also changes fermentations in the direction of the production of more oxidized products.

The dominant hydrogenotrophs in freshwater sediments are the methanogens, but in sulfate-sufficient sediments such as intertidal mud flats most of the H_2 is oxidized by sulfate reducers (Ward and Winfrey 1986). This is in agreement with the more negative $\Delta G^{o'}$ value of hydrogen oxidation linked to sulfate reduction as compared to CO_2 reduction (-151.9 kJ *vs* -135.6 kJ per 4 mol H_2). Marine environments are rich in sulfate, but in freshwater sediments sulfate is limiting and in general methanogenesis is the main terminal proces. Acetogenesis from H_2/CO_2 is the thermodynamically least favourable process (see Table 2), which in principle would limit its role in natural environments. Viable counts of acetogens in sludge and lake sediments were only 1 % of the methanogens counted (Braun et al. 1979) and in a hypereutrophic lake, Lovley and Klug (1983) estimated that acetogens account

for only 5 % of total H_2 consumption. The situation seems to be different in certain eutrophic lakes where a limited input of organic matter in the late summer resulted in an increased acetogenic H_2 consumption to a level of up to 50 % of H_2 utilization by methanogens (Jones and Simon 1985). In anoxic sediments of a mildly acidic lake (pH 6.2) the carbon mineralization was different from neutral freshwater and marine environments (Phelps and Zeikus 1984). These conditions were of selective advantage to acetogenic bacteria, which were able to compete with methanogens and obligate proton reducing acetogens for common substrates (H_2, lactate, ethanol, formate and methanol). In media with C_1-substrates (H_2/CO_2, CH_3OH, HCOOH) which are suitable substrates for both methanogens and homoacetogens, 10-100 fold higher counts of acetogens were found in these lake sediments. The prevalent methanol-degrading anaerobe isolated was similar to *Butyribacterium methylotrophicum*.

In termites CO_2 reduction to acetate, rather than to CH_4, was found to represent the main electron sink reaction of the hindgut fermentation (Breznak and Switzer 1986). The new species *Sporomusa termitida* was isolated from these wood-eating insects. Factors that may explain the successful competition for H_2 of this type of organism are the fact that the pH in the hindgut may be as low as 5.5 and utilization of other substrates such as methanol and lactate, and of methoxylated aromatics for which methanogens cannot compete (Breznak et al. 1988). H_2/CO_2-supported acetogenesis was also found to be important in the ceca of various rodents (Prins and Lankhorst 1977) and in the colon of humans that do not harbour methanogens (Lajoie et al. 1988). Unlike the situation in cattle fed with a usual diet, the acetogen *Eubacterium limosum* was the most numerous methanol-consuming anaerobe in the rumen of sheep fed with a diet in which molasses was a major component (Genthner et al. 1981). Molasses contains 2-7 % pectin and pectin-degrading organisms are numerous in the rumen and liberate methanol. In cocultures of the rumen pectin degrader *Lachnospira multiparus* and *E. limosum* not only the methanol was consumed by *E. limosum* but there was also a shift in the fermentation products from pectin due to interspecies hydrogen transfer (Rode et al. 1981). We speculate that the presence of betaine in the molasses may have been another important factor for the abundance of *E. limosum*.

The above examples concerned H_2 comsumption by acetogens in natural habitats. These bacteria, however, do not only use H_2 but can also be active H_2 producers when the pH_2 is low. In continuous cocultures of *A. woodii* and *Methanobrevibacter arboriphilus* AZ fructose was not converted to 3 acetate, but to 2 acetate, 1 CO_2 and 1 methane, which shows that interspecies hydrogen transfer occurred (Winter and Wolfe 1980). During growth of similar cocultures of an acetogen (*A. woodii*, *Sporomusa* strains or *B. methylotrophicum*) and a hydrogenotrophic methanogen or sulfate reducer on methanol, a major part of the electrons generated were channelled to either methanogenesis or sulfate reduction (Cord-Ruwisch and Ollivier 1986; Heijthuijsen and Hansen 1986; Cord-Ruwisch et al. 1988).

Although there is an example of an anaerobic O-demethylation of a methoxylated aromatic compound by a non-acetogen, namely the anaerobic growth of an *Enterobacter* species on ferulate (Grbić-Galić 1985), virtually all known cultures that carry out such an anaerobic O-demethylation are acetogens. As in the case of *Sporomusa termitida* discussed above, this ability is likely to be of significant competitive advantage for acetogens in nature.

Apart from the very slow growth of *Desulfovibrio carbinolicus* on methanol (Nanninga and Gottschal 1987), utilization of C_1-substrates other than formate by sulfate reducers seems to be restricted to organisms with the acetyl CoA/CO dehydrogenase pathway. This is illustrated by the utilization of betaine by marine *Desulfobacterium* strains, and of methanol and 3,4,5-trimethoxybenzoate by certain *Desulfotomaculum* strains (Table 1). Although it has been speculated that energetically the tricarboxylic acid cycle for the oxidation of acetyl CoA in *Desulfobacter* is certainly not by far superior to the oxidative acetyl CoA/CO dehydrogenase pathway (Thauer 1988), a real advantage of the latter pathway may be that it can be used for the oxidation of C_1-compounds. From measurements in sediment slurries there is evidence for an important role of sulfate reducers in the oxidation of dimethylsulfide (Kiene 1988). We speculate that also these sulfate reducers will have the oxidative acetyl CoA/CO dehydrogenase pathway.

The sulfate-reducing bacterium *D. autotrophicum* can also transform tetrachloromethane to trichloromethane, dichloromethane and CO_2, and the acetogens *A. woodii* and *C. thermoaceticum* completely degraded tetrachloromethane to dichloromethane and traces of chloromethane and CO_2 (Egli et al. 1988). *D. autotrophicum* also converted 1,1,1-trichloroethane to 1,1-dichloroethane (Egli et al. 1987).

A C_1-oxidation process that has been claimed to occur in marine anaerobic sediments is the oxidation of methane (Reeburgh and Heggie 1977; Alperin and Reeburgh 1984). The nature of the organisms that slowly oxidize methane to CO_2 in anoxic sediments is not yet known (Alperin and Reeburgh 1985).

Applied Aspects of One-Carbon Metabolism

Microorganisms can be used as single-cell protein or for the production of interesting chemicals like amino acids, organic acids, exopolysaccharides, enzymes, etc. The feedstocks used for their production are mainly carbohydrates, but there is a wide interest in their replacement by one-carbon compounds (Zeikus 1983; Dijkhuizen et al. 1985; Linton and Niekus 1987). The alternatives are methane, methanol and synthesis gas (H_2/CO), which can be used by microorganisms as the sole source of carbon and energy. Methanol has several advantages over methane and synthesis gas. It is a commodity chemical of high purity, it is low in price, miscible with water and much easier to store, transport and handle than the explosive gases. In general, methanol is considered to be the most suitable one-carbon feedstock for aerobic and

anaerobic fermentations and there is still a large overcapacity for its production (Linton and Niekus 1987). Replacement of traditional feedstocks by methanol depends on the inverse relation between the price of the substrate and the product volume. The difference in the feedstock price for the production of high value chemicals is less important than for the production of bulk chemicals and processes in which methanol could become important are probably the production of alcohols and organic acids. Application of anaerobes for production of organic acids has the additional advantage of not requiring investments for oxygen mixing devices. Moreover, the lower cell yields of anaerobes results in relatively higher product yields.

C_1-utilizing acidogens have potential applications for instance in the production of acetate and butyrate. Acetate, however is already produced efficiently from methanol and CO via the 'Monsanto' process (Drent 1987) and the fermentation process has to be greatly improved in a number of ways to become competitive. Linton and Niekus (1987) calculated that for this purpose production values should be as high as 25-80 g/L·h. However, *Clostridium thermoaceticum* has a low tolerance to the end-product (Wang and Wang 1984), which acts as an uncoupler of the proton motive force at low pH values (Baronofsky et al. 1984). In a pH controlled chemostat culture of *C. thermoaceticum* Wang and Wang (1983) measured a productivity of 6.9 g acetate/L·h with glucose as the substrate. In a patent (Reed 1985), productivities as high as 14.3 g acetic acid/L·h were reported for the same organism and substrate. Reed et al. (1987) selected mutants of *C. thermoaceticum* with high tolerance to acetate. A shift towards butyrate production with increasing acetate concentrations was reported for *Butyribacterium methylotrophicum* (Zeikus et al. 1980; Datta and Ogeltree 1983) and *Eubacterium limosum* (Pacaud et al. 1986a) during growth on methanol/CO_2. The butyrate yield of both organisms could be increased by further limitation of bicarbonate (Datta and Ogeltree 1983; Pacaud et al. 1985). In general, addition of lower fatty acids (C_2, C_3 and C_4) to the medium during growth of *E. limosum* on methanol/CO_2 (Pacaud et al. 1986b) resulted in a shift towards higher fatty acids (C_4, C_5 and C_6). Besides an increase in the product yield it is also necessary to improve the recovery of the acids from fermentation liquors. Butyrate can be esterified to yield methylbutyrate which enhances end-product recovery (Zeikus 1983).

Another interesting process (Zeikus et al. 1980) is the production of vitamin B_{12}. Cobalamine is essential for many microbial and animal species and is produced on an industrial scale by microorganisms because chemical synthesis (requiring 70 steps) is difficult (Florent and Ninet 1978). For this purpose, two *Propionibacterium* strains and one *Pseudomonas* strain are cultivated on large scale with carbohydrates as the feedstock. The vitamin B_{12} levels increase drastically in *B. methylotrophicum* during growth on methanol and are as high as those of the *Propionibacterium* strains commercially used. Recently, Hatanaka et al. (1988) reported higher vitamin B_{12} recoveries with immobilized cells of *B. methylotrophicum* during growth on methanol than with *Propionibacterium shermanii*. Other potential vitamin B_{12} producing

organisms are the acetogens *E. limosum*, *A. woodii* and *S. ovata* which also contain relatively high corrinoid concentrations during growth on one-carbon compounds (Vogt and Renz 1988; Stupperich et al. 1988).

Regulatory and genetic manipulation of methylotrophic acidogenic bacteria could not only increase the production of fatty acids and vitamin B_{12}, but also production of important chemicals such as ethanol, acetone and butanol might become feasible in the future. The methylotrophic potential of acidogenic bacteria has been known for less than a decade and it is obvious that more studies will be required to identify further applications of these microbes.

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8. Biochemistry and Applications of Alcohol Oxidase from Methylotrophic Yeasts

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Introduction

The oxidation of methanol by yeasts has been reported by numerous authors and excellent reviews of the biochemistry of methanol assimilation have been written (Veenhuis et al. 1983; Large & Bamforth 1988). The first step in assimilation of methanol by methylotrophic yeasts is its oxidation to formaldehyde with concomitant production of hydrogen peroxide.

$$CH_3OH + O_2 \xrightarrow[\text{oxidase}]{\text{Alcohol}} CHOH + H_2O_2$$

In a reaction which seems positively perverse the yeasts produce two first class anti-microbial compounds! Thus the yeasts contain a vast excess of catalase activity, usually 1000 units per unit of alcohol oxidase activity, and a very effective formaldehyde assimilation pathway (Veenhuis et al. 1983; Large & Bamforth 1988) to avoid the toxic effects of the activity of alcohol oxidase.

Alcohol oxidase has been reported as being produced in the yeasts *Hansenula*. (Kato et al. 1983; Guissepin 1988), *Candida* (Kato et al. 1983; Fuji & Tonomura 1972; Sahm & Wagner 1973; Yamada et al. 1979)) *Torulopsis* (Unichika Co Ltd 1986) and *Pichia* (Patel et al. 1981; Couderc & Barati 1980; Hopkins & Muller 1987; Hopkins 1980). There is also a report of the enzyme being produced in *Saccharomyces* strain H1 (Fuji & Tonomura 1972). The enzyme is generally found to have a relative molecular weight of around 600 000 with eight subunits. All enzymes reported to date have a flavin adenine dinucleotide (FAD) prosthetic group. It is generally considered that one FAD group is bound to each subunit and that the molecule loses activity if FAD is stripped from the subunits (Sahm & Wagner 1973; Bruinenberg et al. 1982).

G.A. Codd et al. (ed.), Autotrophic Microbiology and One-Carbon Metabolism, 193-225.

Spectral Properties of the Enzyme

Alcohol oxidases purified from *H. polymorpha, P. pastoris* and *Torulopsis* show characteristic absorption spectra of flavoproteins. Under oxidised conditions absorbance maxima are at 212nm, 274nm, 375nm and 460 nm with a sharp peak at 396nm for alcohol oxidases from *H. polymorpha* and *P. pastoris*. Alcohol oxidase from *Torulopsis* gives a similar spectrum except that the 375nm peak is shifted to 391nm and the 396nm peak is either absent or is incorporated within the board 391nm peak. Substrate (methanol) reduction of alcohol oxidase (Fig. 1) results in partial decreased absorbance of the 375nm, 396nm and 460nm peaks. This, and the ratio of absorbances at 375nm and 460nm of oxidised spectra, suggests that a 'red' flavin semiquinone is present in addition to oxidised flavin. The difference in absorbance at 460nm between oxidised and substrate reduced forms of the enzyme can be used to determine the oxidised flavin content, assuming $\Sigma_{460} = 11.3 \times 10^3$ litre M^{-1} cm^{-1}. The semiquinone content can be determined from the absorbance at 375nm and 460nm after reduction with substrate assuming: $\Sigma_{375} = 13.3 \times 10^3$ litre $M^{-1}cm^{-1}$; $\Sigma_{460} = 3.9 \times 10^3$ M^{-1} cm^{-1}. These values being derived from the semiquinone form of D-amino acid oxidase (Massey & Palmer 1966). It was found that for alcohol oxidase from *H. polymorpha* only 40% of the total flavin was in the oxidised form and 60% in the semiquinone form (Hill, Woodward, unpublished results). The semiquinone content could not be reduced by the substrate methanol or by sodium sulphite or sodium dithionite.

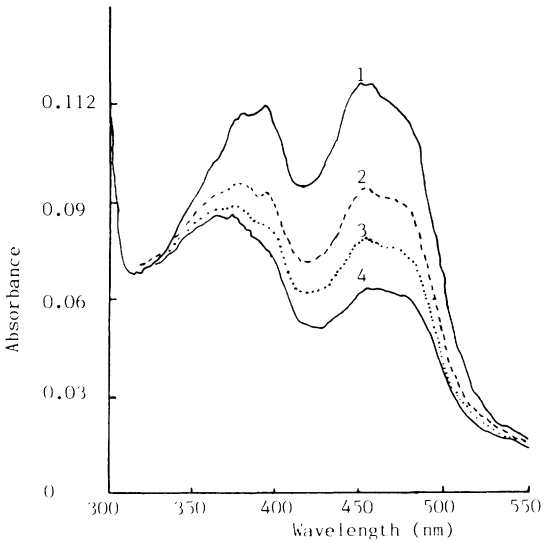


Fig. 1. The solution contained 1.38 mg/ml alcohol oxidase (*H. polymorpha*), 100mM potassium phosphate buffer (pH7.0) at 25°C with the following additions: spectrum 1. No methanol; spectrum 2. +33mM methanol, t = 15 secs; spectrum 3. +33mM methanol, t = 14 mins; spectrum 4. +660mM methanol, t = 7 mins. Scan speed = 500nm/min.

Oxygenation of the enzyme by forced aeration with pure oxygen for several hours did not alter the observed oxidised spectrum.

Similar spectra for alcohol oxidases have already been published (Mincey et al. 1980; Geissler & Hemmerich 1981; Sherry & Abeles 1985).

The effect of sodium sulphite on alcohol oxidase (*H. polymorpha*) spectrum is shown in Fig. 2. The reaction with sulphite is characterised by partial bleaching of the visible absorption spectrum of the enzyme resulting in the production of a new absorption band in the vicinity of 330nm with a spectrum similar to that of substrate reduced enzyme. The reaction with sulphite is slow and at 25°C, pH 7.0, the reaction takes 30 minutes to reach a level where no apparent decrease was observed. Further incubation ($\approx$ 24h) did however result in a further decrease in absorbance at 460nm at the lower sulphite concentrations.

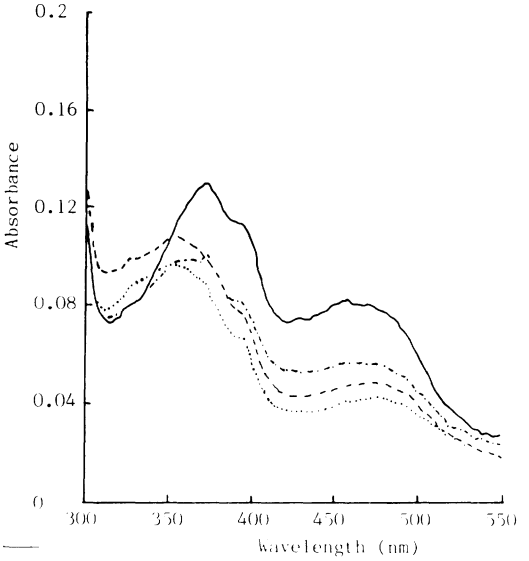


Fig. 2. Effect of sodium sulphite on the absorbance spectrum. The solution contained 1.0mg/ml alcohol oxidase, 100mM potassium phosphate buffer (pH 7.0), at 25°C, with the following sodium sulphite considerations: ____0mM; -.-.-.0.1mM; -------- 0.75mM; ..... 20mM. spectra were recorded after 45 mins incubation. Scan speed = 500nm/min.

Addition of azide to the enzyme causes the colour of the enzyme to turn from yellow to red (Hopkins & Muller 1987). Addition of methanol to the red form of the enzyme bleaches it yellow and aeration of this enzyme sample re-oxidises the flavin to its red colour.

Biochemical and Kinetic Properties

Recent work at Leeds has shown that enzymes from *Hansenula polymorpha, Candida boidinii, Torulopsis* and *Pichia pastoris* have almost identical N

terminal amino acid sequences (Table 1) and similar structural patterns when examined by circular dichroism (Table 2). These enzymes also have similar kinetics. However reports of substrate specificity for enzymes from different yeasts show some variation. Whereas all agree that in general alcohol oxidase attacks N-alcohols up to C_4 or C6 in length and that it has very poor activity on secondary or tertiary alcohols, the relative rates of enzyme activity vary between enzymes. Even within one species, Sahm and Wagner (1973) report a different activity profile for alcohol oxidase from *Candida boidinii* than that from the same species, but an unknown strain, reported by Fuji and Tonomura (1972). Table 3, shows the substrate specificity for a number of alcohol oxidases. As can be seen relative activity against various substrates remain fairly similar (Table 4). It can also be seen (Fig. 3) that the effect of pH on activity varies according to the source of the enzyme.

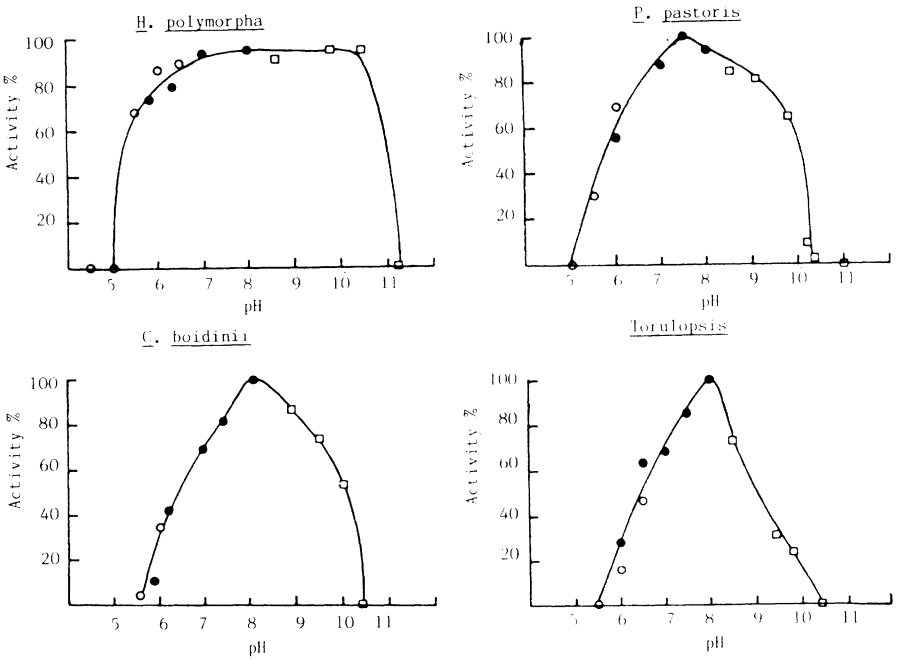


Fig. 3. Activity pH curves for alcohol oxidases from yeasts.
Buffers ○ 100mM Sodium citrate
● 100mM Potassium phosphate
□ 100mM Sorensens glycine
Alcohol oxidase activity was determined using a Clark oxygen electrode at air saturation and at 30°C for alcohol oxidase from *H. polymorpha*.

Table 1. N-terminal amino-acid sequences of alcohol oxidases.

Source	1									10										20									
H. polymorpha :	A	I	P	D	E	F	D	I	I	V	V	G	G	G	S	T	G	C	C	I	A	G	R	L	A	N	L	D
P. pastoris :	A	I	P	F	E	F	D	I	L	V	V	G	G	G	S	K	G	K	A	A	A	G	R	L	A	N	L	D
Torulopsis :	A	I	P	D	E	F	D	I	I	V	V	G	G	G	S	T	G	K	T	I	A	G	R	L	G	N	L	D
C. boidinii :	A	I	P	G	G	F	D	V	I	V	?	G	G	G	S	T	?	?	V	I	A	G	?	L				

H. polymorpha :	D	Q	N	L	T	V
P. pastoris :	H	S				
Torulopsis :	P	D	V	T	?	A

The boxed regions above indicate regions of identity between at least three of the alcohol oxidases.

Table 2. Analysis of circular dichoism spectra of alcohol oxidases.

Source	Alpha	Beta	Turn	Coil
H. polymorpha:	31.9%	22.9%	17.9%	27.2%
P. pastoris:	25.6	27.1	18.8	28.4
Torulopsis:	28.6	31.4	18.6	21.4
C. boidinni:	30.4	29.6	11.4	28.4

Activity of various alcohol oxidases from a number of sources was measured using an oxygen electrode. Activities are given as percentage of that for methanol for which the enzyme has the greatest affinity. The sources of these data are as follows: 1. Fuji & Tonomura (1972); 2. Sahm & Wagner (1973); 3. Kato et al. (1976); 4. Yamada et al. (1979); 5. Patel et al. (1981); 6. Hopkins & Muller (1987); 7. Couderc & Baratti (1980); 8. Hopkins (1980) and Phillips Petroleum Co (1983); 9. Unichika (1984); 10. Kato et al. (1976); 11. J R Woodward (unpublished data). See Table 3.

Table 3. Specificity and activity of various alcohol oxidases.

	Candida				Torulopsis						
					Pichia				H. polymorpha		
Ref:	1	2	3	4	5	6	7	8	9	1	11
Substrate	Relative enzyme activity										
Methanol	100	100	100	100	100	100	100	100	100	100	100
Ethanol	98	75	106	82	92	36	82	100	35	50	45
1-propanol	75	25	79	38	74	20	43	73	60	44	25
2-propen-I-ol	–	65	89	82	–	30	–	4	–	49	–
1-butanol	58	15	69	27	52	10	20	45	53	32	13
1-pentanol	40	5	–	21	30	–	–	5	–	–	3
2-propanol	8	5	–	–	0	2	2	–	–	–	–
2-amyl alcohol	3	0	–	–	0	–	–	–	–	–	–
2-chloroethanol	–	–	71	–	70	10	–	–	–	38	–
Formaldehyde	–	–	–	23	15	33	–	–	–	13	–
2-mercaptoethanol	–	–	–	–	25	7	–	–	–	–	–

Table 4. Kinetic properties of alcohol oxidases with primary alcohols as substrates.

	Substrate			
Source	Methanol	Ethanol	Propanol	Butanol
	Km(mM)			
H. polymorpha	0.712	2.7	27.3	54.6
P. pastoris	0.845	3.68	20.26	18.06
Torulopsis	0.715	1.47	16.74	21.86
C. boidinii	0.417	1.78	6.06	10.56

The effect of temperature on activity is shown in Fig. 4. *Hansenula polymorpha* has activity at a higher temperature range which may reflect its thermophilic growth characteristics. This is illustrated in Fig. 5 where the thermal stability of *H. polymorpha* enzyme clearly contrasts with the instability of the *Pichia* enzyme. We have also observed an effect of temperature on the Km of the enzyme (Fig. 6). With increasing the temperature a dramatic reduction in Km is noted for all the substrates of the enzyme except methanol. Furthermore whereas with methanol the increase in Km, marking thermal stress and the commencement of denaturation, occurs just above 50°C, the same phenomenon does not occur until over 60°C when butanol is the substrate. It is possible that the presence of an aliphatic carbon chain in the active site leads to hydrogen bonding with surrounding amino acids and thus prevents unfolding of the enzyme.

We have also studied the effecs of sodium chloride on the activity of the *Pichia* and *Hansenula* enzymes (Fig. 7). As can be seen *Hansenula* alcohol oxidase

is particularly resistant to chloride inhibition whereas the *Pichia* enzyme is very sensitive.

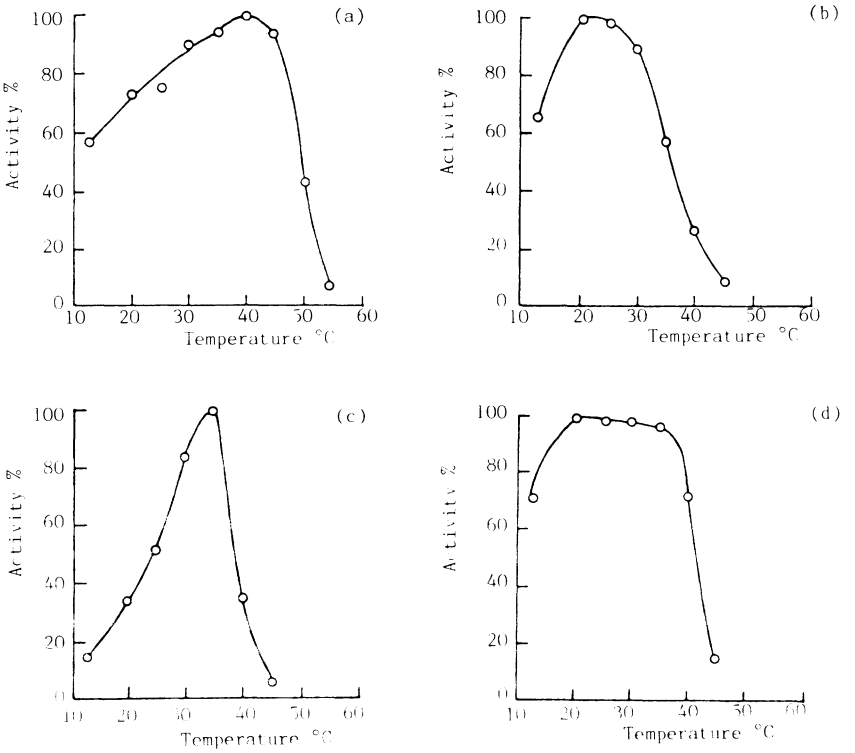


Fig. 4. Effect of temperature on the activity of alcohol oxidase from four species of yeast. Activity was measured in a water-jacked oxygen electrode and adjusted for the effect of temperature on oxygen solubility in buffer. (a) *Hansenula polymorpha* (b) *Pichia pastoris* (c) *Torulopsis* R14 (d) *Candida boidinii.*

So although there is much evidence to show that alcohol oxidases from different yeast sources are structurally similar and generally have similar activities, the enzymes are not identical. Subtle differences are seen in a number of physical and biochemical phenomena.

Professor Harder of the University of Groningen has recently observed that *Pichia pastoris* has two distinct forms of FAD which can be separated by HPLC (personal communication), a point also noted by Bystrykh et al. (1989). Also it is well known that *Pichia* alcohol oxidase forms crystals at low ionic strengths, a property not shared by other alcohol oxidases (Hopkins & Muller 1987). The remarkable difference in activity towards pH shown by the *Hansenula* enzyme (Fig. 3) also suggests that even if the general structure of

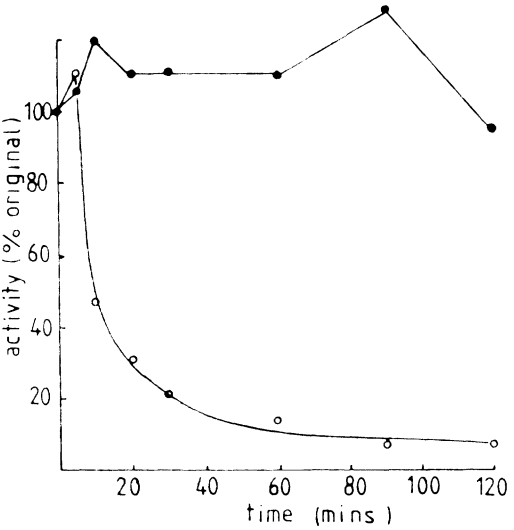


Fig. 5. Effect of temperature on enzyme stability. Alcohol oxidase from *Hansenula polymorpha* (●) and *Pichia pastoris* (○), was incubated at 50°C, samples removed at intervals and enzyme activity determined using the Trinder (1969) colorimetric assay.

the enzyme is similar there are subtle differences at the active site and within the other domains of the oxidase molecule which control these variable activities of the enzyme.

Incorporation of Alcohol Oxidase into Peroxisomal Lattices

Alcohol oxidase is produced in large quantities within the yeast cell. Its poor affinity for methanol requires this high level of enzyme protein. Up to 37% of the cell protein can be in the form of alcohol oxidase and this is entirely contained within the methylotrophic yeasts by incorporation into peroxisomes. The enzyme once within the peroxisome forms a complex, extremely regular three dimensional lattice structure (Plate 1). The structure leads to the production of peroxisomes of various shapes, according to the culture conditions of the yeast. In shake flask cultures, round or square peroxisomes with rounded corners are observed. Triangular peroxisomes can also be observed during batch cultivation (Woodward unpublished results). However, during continuous cultivation, when maximal production of the enzyme occurs, cells can fill with cuboid peroxisomes having sharp, square corners (Plate 2) which can be visualised using electron microscopy and various preparatory techniques. In all these peroxisomes the lattice structure is very clear and is best exemplified in the work of van Dijken et al. (1982).

Veenhuis et al. (1979) have shown that shifting *Hansenula polymorpha* cells from glucose as a carbon source, to methanol, brings about the expansion of

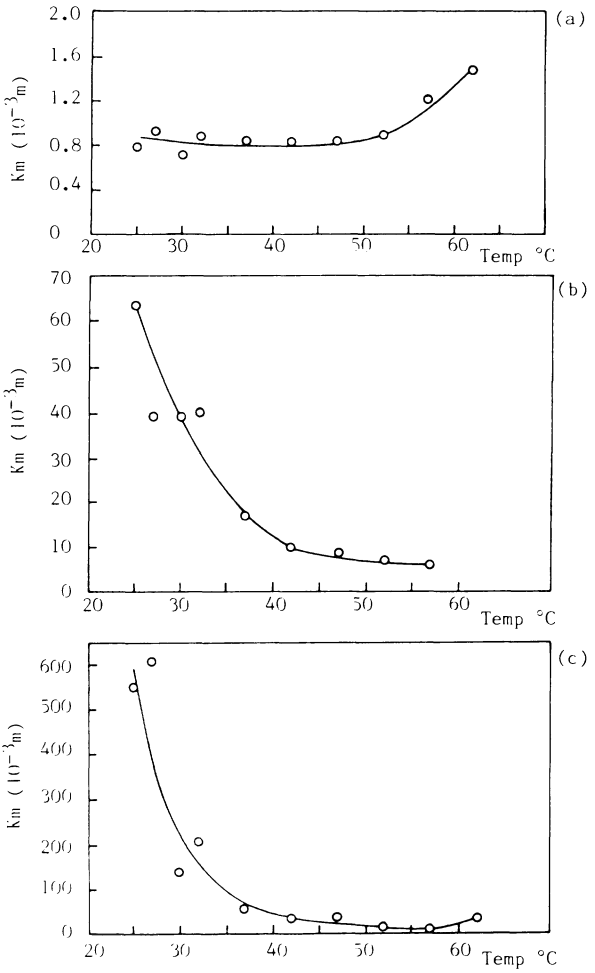


Fig. 6. Effect of temperature on the kinetics of enzyme activity. Km was determined for *Hansenula polymorpha* enzyme at various temperatures and with three substrates using an oxygen electrode. Results were corrected for changes in solubility of oxygen with temperature. Substrates were (a) methanol (b) propanol (c) butanol.

peroxisomes and the formation of a distinct lattice structure within the peroxisomes. It was also noted by those workers that when cells were allowed to grow into stationary phase they produced larger cuboid peroxisomes which contained crystalline lattice-like structures. The latter supports other evidence that alcohol oxidase production is subject to catabolite repression and that removal of glucose releases repression of alcohol oxidase synthesis. It appears that peroxisomes in these yeasts originate from the endoplasmic reticulum. However during rapid expansion and incorporation of the enzyme into these organelles, small peroxisomes separate off from the mature peroxisomes, not

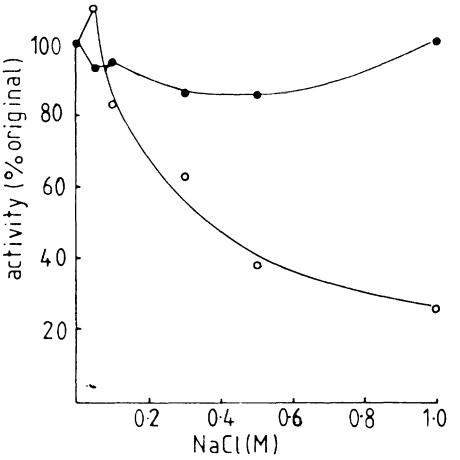


Fig. 7. Effect of sodium chloride on enzyme activity. Alcohol oxidase from *Hansenula polymorpha* (●) and *Pichia pastoris* (○) was incubated with sodium chloride at various concentrations for 10 minutes. Enzyme activity was then determined using an oxygen electrode.

from the endoplasmic reticulum (Veenhuis et al. 1978). Most interestingly the crystalloid lattice structures were always associated with peroxisomes, *Hansenula polymorpha* alcohol oxidase negative mutants never showed crystalline bodies in their peroxisomes. Thus the number and shape of peroxisomes in cells is directly related to the production of alcohol oxidase.

Very recently Veenhuis et al. (1989) have shown that mature peroxisomes which contain complete crystalline lattice structures do not import new proteins. These workers found that when they shifted cells from repressive to inductive medium containing methanol and methylamine, both alcohol oxidase and amine oxidase were incorporated into the same organelle, together with catalase. However, when methanol limited cells of *H. polymorpha* were transferred to medium containing methylamine as the nitrogen source, instead of ammonium sulphate, then the amine oxidase was only imported into the small, immature peroxisomes at the perimeter of the cells. These results are interesting in that they suggest a physical limitation to the functional size of peroxisomes which may be regulated by the presence of a mature three dimensional alcohol oxidase lattice in the organelle. It has already been reported that it is possible to osmotically shock catalase out of mature peroxisomes leaving the alcohol oxidase lattice intact (Veenhuis et al. 1979; 1983). It may be that the function of the matrix as an efficient assimilation unit is limited by diffusion of substrate through the lattice. Thus the peroxisome is size limited and new protein is excluded to ensure maximum efficiency of the substrate reaction capacity of the unit.

The mechanism by which the alcohol oxidase enters the peroxisome and is assembled into the crystalline lattice has received considerable attention in recent years. It was known that the monomer of alcohol oxidase (Mr =

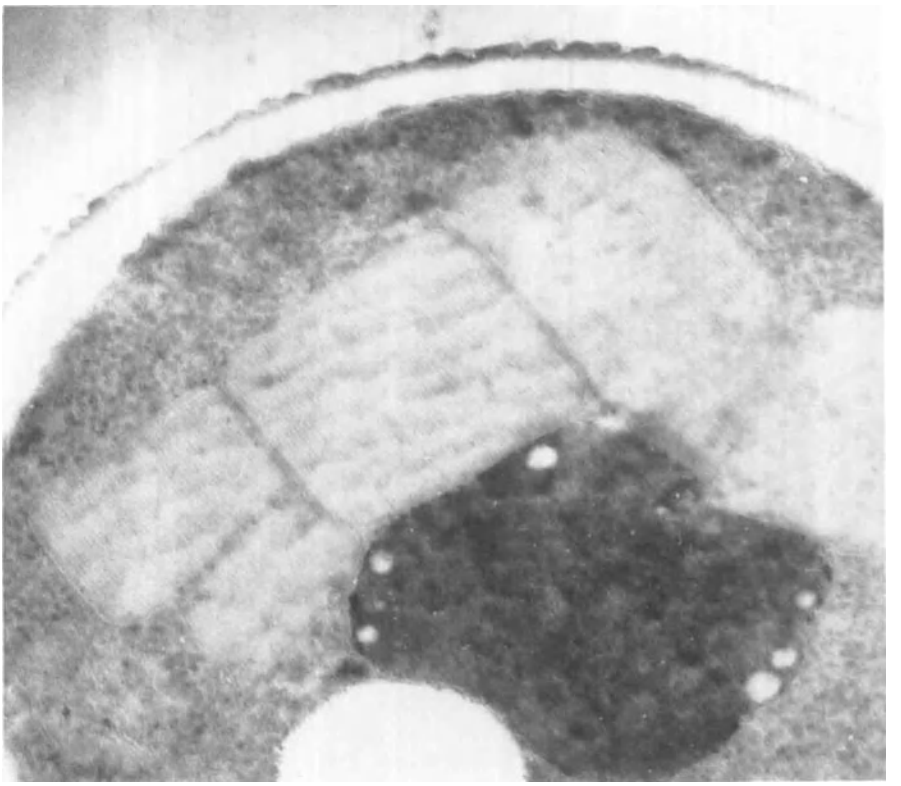


Plate 1. Electron micrograph of *Hansenula polymorpha* cells grown in continuous culture with methanol as a substrate. Note the cuboid perixosomes showing the enzyme lattice. Magnification × 66,000.

74 000) is produced in the cytoplasm of the yeast. It was thought therefore that it might be transported into the peroxisome via a signal sequence, a polypeptide extension of the N terminus of the molecule which enables it to be translocated across a cell membrane (Walter et al. 1984). Roa and Blobel (1983) and Roggenkamp et al. (1984) observed that in vitro translation of *Hansenula polymorpha* mRNA by a rabbit reticulyte lysate, produced a protein which, when run on a sodium dodecyl sulphate dissociating gel, had the same mobility as a dissociated subunit produced *in vivo*. They concluded from these results that the *in vitro* translated product was the mature protein and that no N terminal sequence, typical of most transported proteins, exists in the case of alcohol oxidase.

The question therefore remained as to how the monomer of alcohol oxidase was translocated into the peroxisome, assembled into an octamer and then incorporated into the crystal lattice. Several facts were known; the *in vitro* produced monomer was very susceptible to trypsin digestion, unlike the

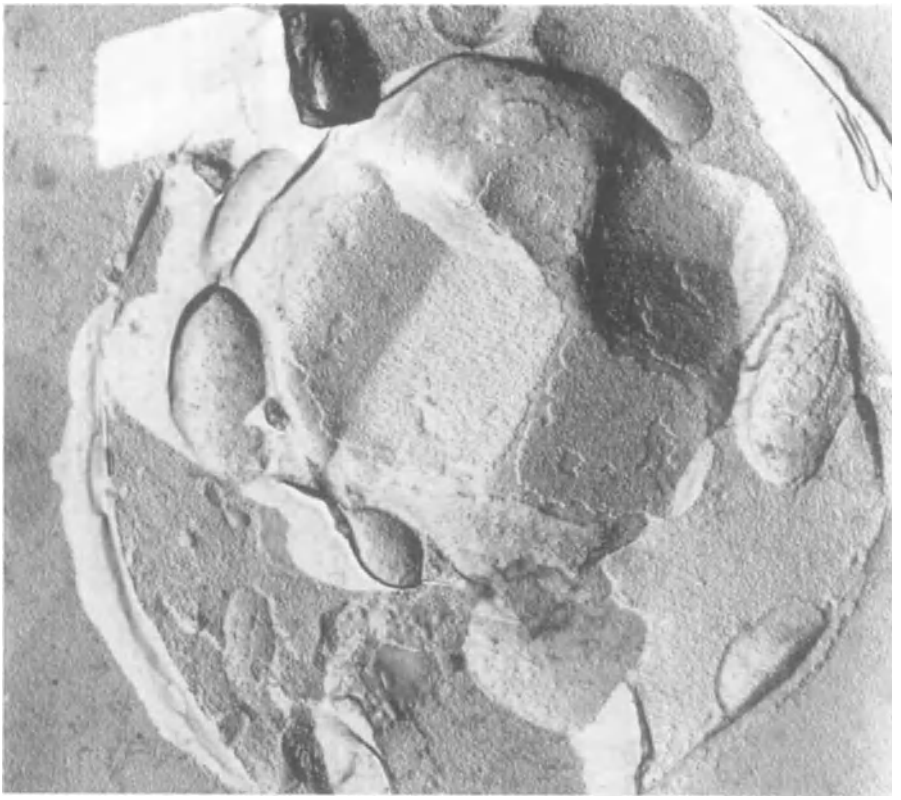


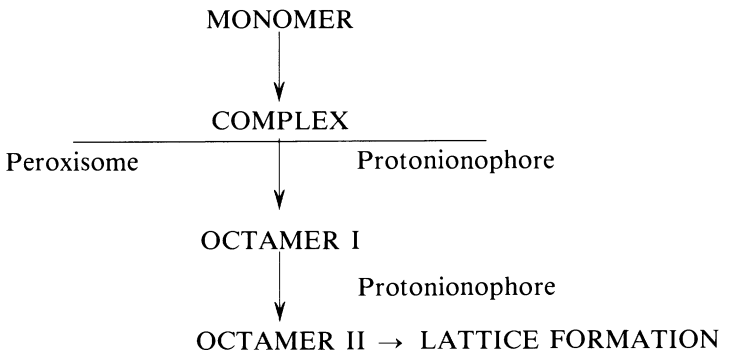
Plate 2. Freeze facture electron micrograph of cells of *Hansenula polymorpha* after shadowing with a platinum carbon. Note the cuboid shape of peroxisomes in the cells. Magnification × 36,800.

octamer (Roa & Blobel 1983). Thus the trypsin sensitive sites within the molecule were protected by assembly into octamers. Octamer protein could only be detected within the peroxisomes and never in the cytoplasm. Goodman et al. (1984) demonstrated that alcohol oxidase from *Candida boidinii* is produced as a mature monomer, using pulse chase experiments with ^{35}S labelling of proteins. They found that the mature subunit had a half-life of 20 minutes in intact cells and 73 minutes in spheroplasts before being incorporated into the peroxisomal fraction of the cell. There were no apparent intermediate organelles involved in this process, nor were any intermediate forms of oxidase observed. It was thus evident that the eight subunits assembled into the octamer without forming intermediate products. Goodman et al. (1986) observed that peroxisomal membrane proteins as well as core matrix proteins (such as dihydroxy acetone synthase and methanol oxidase) were all induced by methanol. Thus there is a possibility that it is necessary for the cell to synthesise a membrane protein which recognises the oxidase monomer and aids its translocation across the peroxisomal membrane.

Post-translational import of protein into the peroxisomal matrix is clearly indicated by the above experiments. The mechanism for this import process is, however, still unclear. Bellion and Goodman (1987) reported that proton ionophores could disrupt the translocation of monomers and their assembly into octamers. They reported that carbonyl cyanide m-chlorophenyl hydrazone (CCCP), when added to spheroplasts of *C. boidinii*, caused the appearance of proteins which could be immunoprecipitated from a supernatant fraction of the centrifuged lysate of the spheroplasts by the addition of an antiserum against alcohol oxidase. It was noted that this complex only formed several minutes after the addition of CCCP. It was concluded that this might be an interaction with the monomer as it was forming the octamer. Further studies revealed that CCCP did not interfere with the removal of monomers from the cytoplasm but did prevent octamer formation. Monomer was found to be associated with peroxisomal membranes which could be pelleted by centrifugation. Immunoprecipitation of the peroxisomal membrane-monomer complex confirmed these observations.

Goodman's work suggested that CCCP interfered with translocation and assembly of the monomer into the octamer. Removal of CCCP by addition of dithioreitol led to assembly of the accumulated monomeric complex into octamer even in the presence of protein synthesis inhibitors. Finally Goodman et al. (1984) were able to show that the CCCP complex accumulated on the outside the peroxisome whereas octamers were found on the inside of the peroxisomal membrane. This elegant work has led Goodman to put forward the scheme shown below to explain the assembly pathway for alcohol oxidase. Assembly pathway proposed by Goodman et al. (1984) for alcohol oxidase. FAD may be added at the Octamer I or Octamer II stages.

If this pathway is correct then it also suggests that some process requiring energy occurs in order to translocate and assemble the monomers into octamers. Assuming that some peroxisomal protein acts as a recognition site



for the oxidase monomer it is possible that ATP is required to assist translocation across the peroxisomal membrane. The latter appears to be the case for secretory protein translocation in *Saccharomyces cerevisiae* (Waters & Blobel 1986).

Some of the most recent work to come from the laboratories of Dr Martin Veenhuis and Professor Harder supports the idea that targeting of the monomer to the peroxisomal membrane is a function of the specific proteins in that membrane. Distel et al. (1987) showed that the expression of the methanol oxidase gene carried in a p40k - MOX51/2μ expression plasmid was possible in *Saccharomyces cerevisiae*. Ethanol grown cells of *S. cerevisiae* containing the expression plasmid exhibited synthesis of alcohol oxidase protein. However this was largely observed to be present as insoluble inclusion bodies (see also van der Klei et al. 1989). Alcohol oxidase was also imported into *S. cerevisiae* peroxisomes. When proliferation of peroxisomes was induced by growing cells on oleic acid no increased incorporation of enzyme into peroxisomes was observed. The alcohol oxidase was not functional and appeared as the monomer. Thus the targeting sequences of the oxidase monomer for peroxisomal membranes appeared to be recognised in *S. cerevisiae*. However, further work by van der Klei et al. (1989) has shown that the alcohol oxidase expressed in *S. cerevisiae* did not contain FAD despite the fact that other FAD containing proteins, normally present in *S. cerevisiae*, were produced in the usual quantities. The possibility that the gene expressed in *S. cerevisiae* was disfunctional was eliminated when protoplasts of *S. cerevisiae* and *H. polymorpha* grown on ethanol were fused and the fate of alcohol oxidase protein observed. The enzyme was shown by cytochemical staining to assemble into active octamers in the *Hansenula* peroxisomes, thus demonstrating the necessity for the correct assembly apparatus to be present within the peroxisomal membrane or lumen for octamer formation.

Once the monomer has been translocated across the peroxisomal membrane and assembled into an octamer it is incorporated into the crystalline matrix. Several workers have suggested structures for the lattice.

It is possible to examine the crystalline arrays of methanol oxidase using various methods of electron diffraction, optical diffraction and computer simulation techniques. Osumi et al. (1982) reported that two proteins are involved in the lattice structure, catalase and alcohol oxidase. Their results show that the lattice is made up of alcohol oxidase and catalase molecules alternately interspersed in the lattice and that these are positioned at 8-22nm from each other depending on the orientation of the arrays. The octamer making up the arrays is reported to have two tetramers with subunits facing each other.

Veenhuis et al. (1983), however, suggested that catalase is not required to maintain the lattice structure as it can be released from the peroxisome without loss of integrity of the lattice. These workers suggest that the lattice is formed by alternate layers of octamers. The octamer consisting of two tetramers one on top of the other but lying at a 45° rotation to each other ie. staggered. This suggests a complex inter-protein bonding system both between tetramers and

between layers of octamers. Veenhuis et al. (1983) also showed that the distance between molecules in the lattice formed *in vivo* was the same (105Å) as that between molecules in crystallised methanol oxidase suggesting that this oxidase alone is responsible for the lattice.

The work of Professor van Bruggen and Janet Vonck is very relevant to the structure and assembly of the crystal lattice. Janet Vonck has been working on the formation of two dimensional crystals of alcohol oxidase from *Hansenula polymorpha* (personal communication).

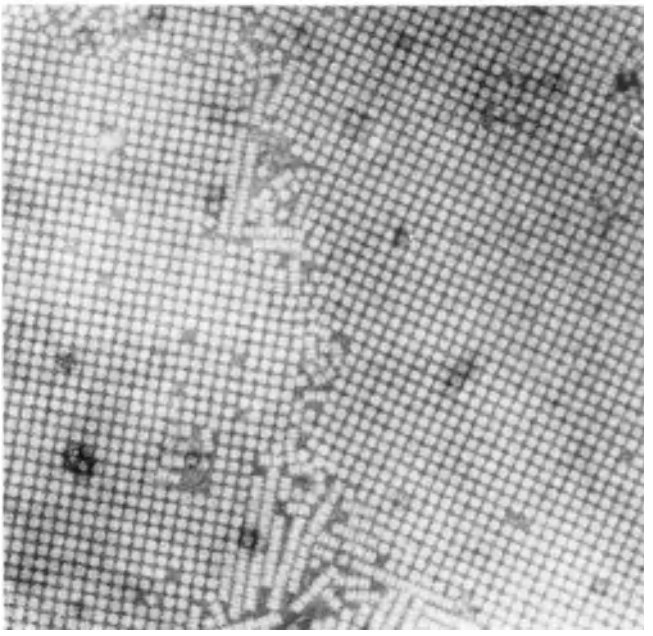


Plate 3. Electron micrograph of a two dimensional crystal of alcohol oxidase. Note the different views of molecules with some on their 'sides' (a) but most in orderly rows viewed from 'above' (b) (from Janet Vonck and Professor van Bruggen).

Although this process was not very reproducible sufficient data was collected to give the micrographs shown in Plates 3 and 4. The two dimensional crystals have a square unit cell of 125Å. Image averaging by picking the best patches of the crystal produced results as shown in Plate 5 which is a computer generated average of 100 patches from the crystal shown in Plate 3. Here it is possible to see clearly defined 'molecular bridges', two per side to each molecule. The outside shape of molecules shows clear features and some dense protein in the middle of the molecules with a less dense halo surrounding it. Reference to Plates 3 and 4 reveals that it is also possible to see rows of molecules which appear stacked on their 'sides'. These molecules never form 2D crystals but appear as random stacks of molecules.

Their results suggest that the octamers have definite sidedness. Lateral

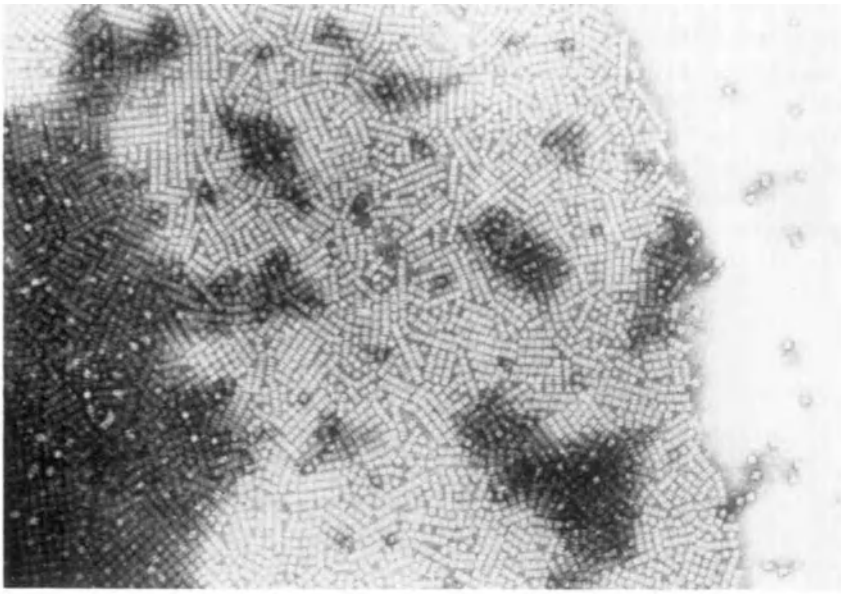


Plate 4. Electron micrograph of a two dimensional crystal of alcohol oxidase. The crystal is disordered due to molecules being on their 'sides' (from Janet Vonck and Professor van Bruggen).

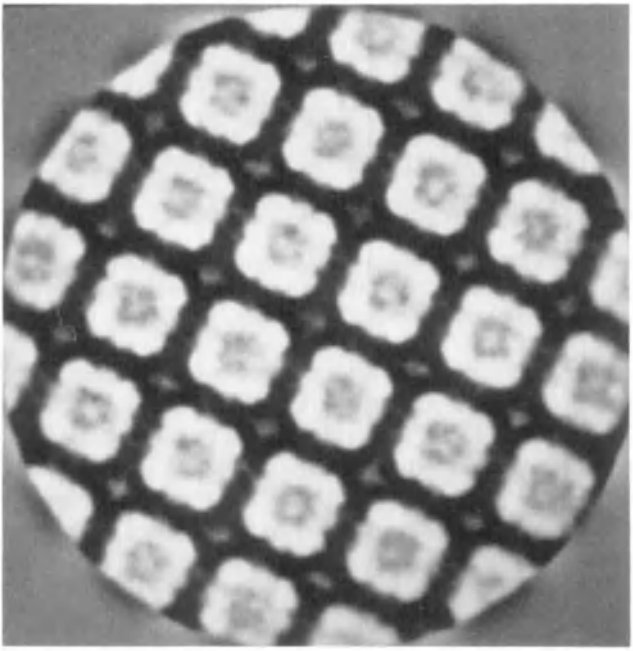


Plate 5. Image enhanced view of Plate 3. The electron micrograph was subjected to image averaging by using a computer generated average of 100 patches from an electron micrograph (from Janet Vonck and Professor van Bruggen).

bridges which allow 'horizontal' assembly into a regular pattern seen in mosperoxisomes *in vivo* are clearly different to those which allow 'vertical' stacking. Using this work it is possible to deduce the structure of the octamer which closely resembles that proposed by Veenhuis et al. (1983).

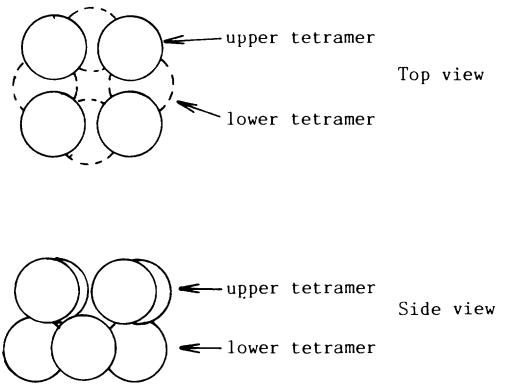


Fig. 8. Schematic representation of an alcohol oxidase octamer.

Figure 8 shows a schematic view of an alcohol oxidase molecule arrived at by observation of Janet Vonck's data.

Crystal Structure of Alcohol Oxidase

Several authors have reported the formation of microcrystals of alcohol oxidase from *Candida* and *Pichia* (Patel et al. 1981; Fuji & Tonamura 1972 and Hopkins & Muller 1987). However, it is only recently that good diffracting crystals of alcohol oxidase have been produced. Here at Leeds we have spent three years developing techniques for the production of crystals capable of diffracting X rays in an ordered fashion. Although it is possible to crystallise alcohol oxidase from *Pichia, Torulopsis* and *Hansenula*, only crystals from *Pichia* have produced good diffracting crystals which are sufficiently ordered to produce good diffraction patterns. Figure 9 shows the narrow range of sodium chloride and polyethyleneglycol concentration under which crystals will form.

We also observed that although it was possible to obtain diffracting crystals from *Pichia* these gave poor results unless the enzyme was reduced with sodium azide. Previously, we had observed (Hill, Woodward unpublished results) that the alcohol oxidases had flavin molecules which existed in different forms. It was never possible to obtain completely oxidised FAD molecules in the crystals of *Hansenula polymorpha* oxidase, nor could these be reduced by addition of methanol or sodium sulphite. Hopkins & Muller (1987) observed that *Pichia* alcohol oxidase bound one molecule of azide per subunit of the octamer. The nature of the complex is unknown. However, it is red, and other research

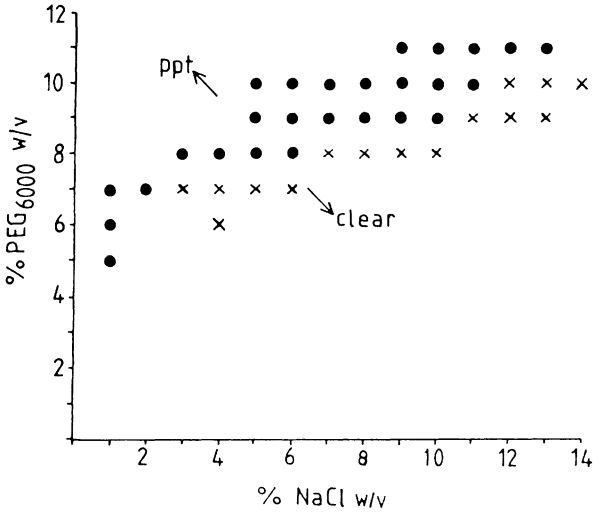


Fig. 9. Conditions for the formation of crystals of alcohol oxidase. Crystal growth took place in 50 μl dialysis buttons containing enzyme and buffer. The dialysis buffer contained various concentrations of salt and polyethylene glycol (●). Small crystals, not suitable for X-ray diffraction; (×) large crystals giving good diffraction patterns.

workers have observed red forms of the enzyme when purifying it (J.P. van Dijken, personal communication). The enzyme is still active and in this form produces large crystals which can be used for X ray crystallographic data acquisition. Plate 6 shows some of these crystals and Plate 7, shows the a and b planes of a typical precession photograph of a *Pichia* crystal.

The diffraction of X-rays by the molecules gives a three-dimensional pattern of spots. Plate 7 shows a two-dimensional slice through the centre of such a diffraction pattern (known in the case as the hKO zone). The patterns shows approximately 4-fold symmetry and would be consistent with octameric molecules whose local symmetry axes were slightly out of line with the crystal unit cell.

Precession photographs show the space group to be $P2_1$ with cell dimensions a = 157.3Å, b = 171.47Å, c = 231.6Å, β = 94°. The unit cell volume corresponds to 2 octamers per asymmetric unit with a solvent content of about 49%.

Professor van Bruggen and Janet Vonck have produced an optical transform of the 2D crystal array (Plate 8). This shows 4-fold rotational symmetry though, interestingly, not exact mm symmetry, this could be a consequence of differential penetration of stain into the upper and lower tetramers.

Thus it seems likely that the *H. polymorpha* and *Pichia* enzymes have a similar sub-unit arrangement and the crystallographic and electron microscopic methods can yield complementary information. By combining the X-ray and electron microscope results, we also hope to identify the bridging domains

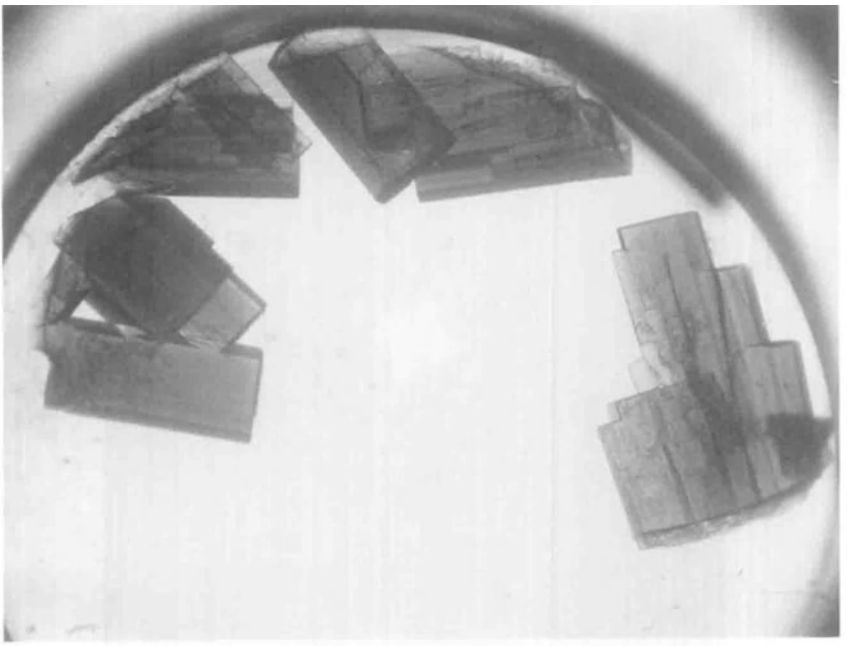


Plate 6. Crystals of *Pichia pastoris* formed in microdialysis buttons using PEG6000 and sodium chloride. The largest crystals are 3-4mm.

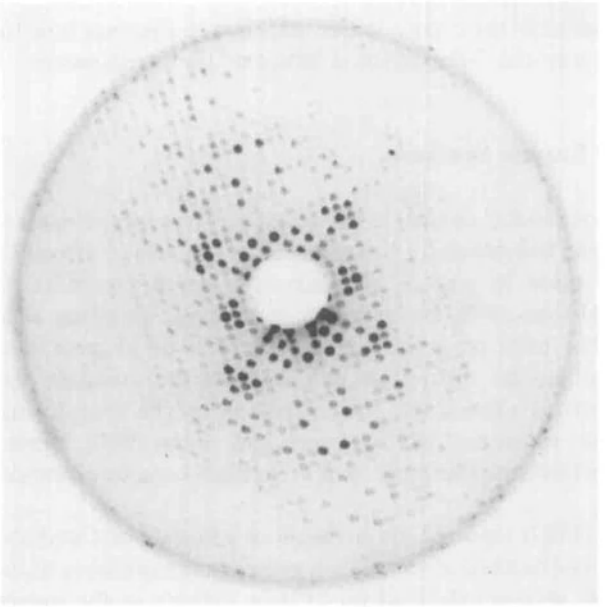


Plate 7. 6°precession photograph of the X-ray diffraction pattern produced by a 3 dimensional crystal of *Pichia pastoris.*

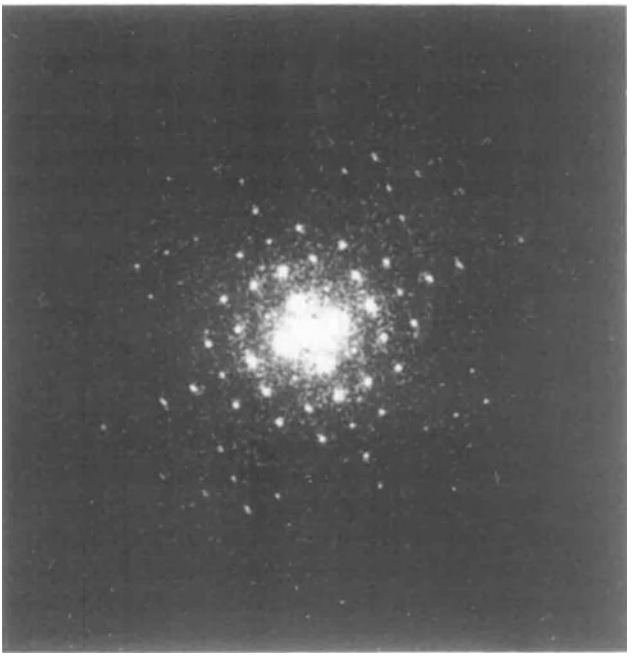


Plate 8. Optical transform of a 2-dimensional crystal of *Hansenula polymorpha*.

between molecules in the crystal lattice and then to discover how the molecules are assembled into the 3-dimensional lattice of the peroxisomes.

Regulation of Enzyme Synthesis

The synthesis of alcohol oxidase is regulated by three mechanisms. Repression, derepression and induction. In the presence of glucose or ethanol synthesis of alcohol is repressed by what is generally considered catabolite repression or inactivation (Holzer 1976; Bruinenberg et al. 1982; Eggeling & Sahm 1980). However, it has been observed that if the level of glucose is substantially reduced or exhausted during growth and alcohol oxidase synthesis will commence albeit at a low level. Similar effects can be seen during growth on glycerol, xylose, ribose and xylitol (Eggeling & Sahm 1980). Derepression to a level of 70% of that seen during growth in methanol can be observed using these substrates.

Sakai et al. (1987) reported the isolation of a mutant of *Candida boidinii* A5 exhibiting alcohol oxidase activity when grown in the presence of methanol and glucose. Parent strains exhibited no oxidase activity in the presence of both substrates. Their work also showed co-regulation of catalase production, both catalase and alcohol oxidase were derepressed in the mutants and both

remained repressed in the parent strain. However, the catabolite inactivation of alcohol oxidase by glucose was not affected by the mutation to strain ADU15 as transfer of cells from glucose/methanol to glucose medium brought about the inactivation of the alcohol oxidase in the normal manner. Interestingly the ADU15 mutant was also completely repressed by ethanol. The mechanism of catabolite repression in methylotrophic yeasts is still not understood and although the ADU15 mutation in *Candida boidinni* A5 if characterised, might give some insight into glucose repression, catabolite inactivation and ethanol repression appear to be associated with some other genetic mechanism.

As well as derepression, synthesis of alcohol oxidase can be induced (Eggeling & Sahm 1980). The exact mechanism of induction is not understood, nor is the compound which acts as an inducer understood as yet. It is known however that in batch cultures methanol is most likely to act as an inducer whereas in continuous cultures it is possible to use both formaldehyde and methanol as inducers (Eggeling & Sahm 1980; Giuseppin et al. 1988a). Giuseppin et al. (1988a) have produced work which sheds some light on the molecular regulation of enzyme production in continuous culture. These workers used cultures in which mixed substrates were supplied to the fermentation. Glucose and methanol at a ratio of 4:1 (w/w) produced cultures in which the glucose level was usually limiting growth and the methanol acted as an inducer as well as a partial carbon source. Giuseppin et al. (1988a) followed alcohol oxidase production, FAD content and mRNA levels of alcohol oxidase and dihydroxyacetone synthase during growth of cells at different dilution rates. Figures 10a, 10b and 10c show the results they obtained. Total methanol oxidase as protein reached its highest level at a dilution rate of 0.05 and decreased rapidly at dilution rates above 0.2-0.25. mRNA poroduction levels did not fall until a dilution rate of 0.3 was reached, thus demonstrating that maximum methanol oxidase production is not limited by synthesis of mRNA. The ratio of FAD to alcohol oxidase dropped simultaneously with the fall in the total production of the enzyme. Thus the results indicate that the efficiency of alcohol oxidase production is regulated by a translational event or monomer degradation, as glucose accumulates at high dilution rates, leading to catabolite inactivation.

Brooke et al. (1986) also showed a direct correlation between alcohol oxidase synthesis and the synthesis of FAD. Their results showed that addition of glucose to cultures producing alcohol oxidase led to inactivation of alcohol oxidase and release of FAD and also the inactivation or repression of some of the enzymes synthesising FAD. It appeared that the regulation of FAD synthesis was not governed directly by glucose concentration as reported above for alcohol oxidase. Instead the authors observed that free FAD accumulating intracellularly led to the inactivation of FAD synthesising enzymes. The two synthetic pathways are thus independently but positively regulated.

The understanding of the regulation of alcohol oxidase synthesis is very important from an industrial point of view. There is a necessity to obtain maximum yield of enzyme to improve efficiency of production if viable

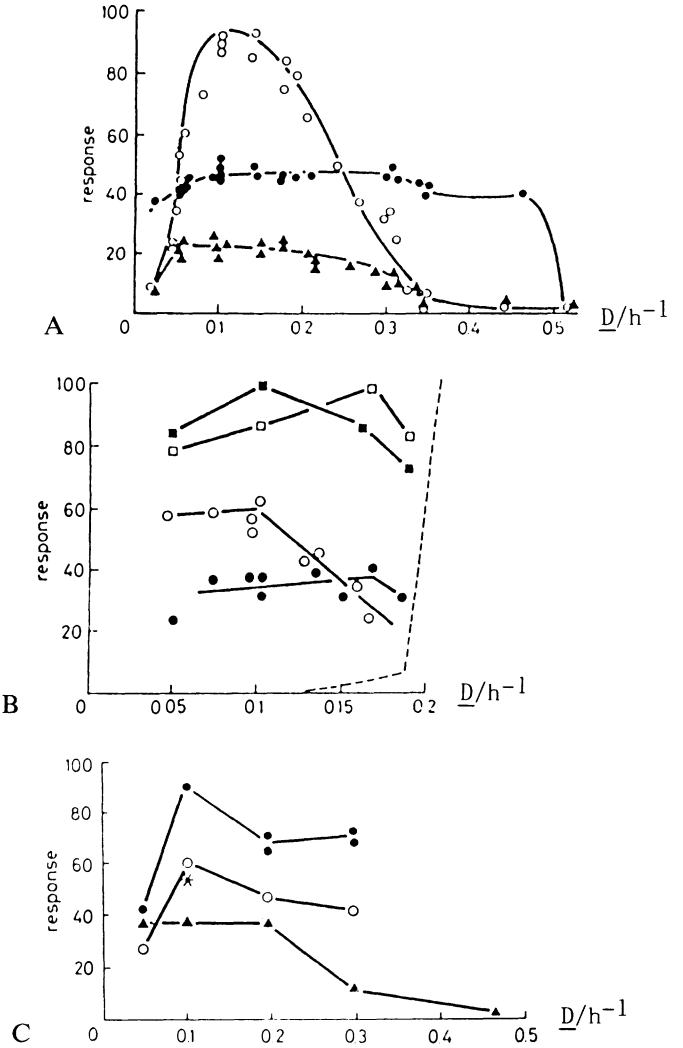


Fig. 10. (a) Steady-state values of MOX activity and cell yield in *H. polymorpha*. Substrate: glucose/methanol 4:1 (w/w). ● *Y*sk [(gX·gS⁻)·100); ○ activity [(MOX-units·gX^{-1})·0.04]; Δ MOX '*in vivo*' [umol O_2·gX^{-1})·0.04].

(b) Steady-state values of MOX activity and cell yield and MOX and DHAS-mRNA levels in *H. polymorpha*. Substrate: glucose/methanol 1:1 (w/w). □ MOX mRNA (%); ■ DHAS mRNA (%); ○ MOX activity [MOX-units·gX^{-1})·0.02]; ▲ *Y*sx [(gX·gS^{-1})·100); methanol concentration (mg/l)·0.1.

(c) FPLC analysis of MOX at several dilution rates. Units have been corrected for catalase activity. Molecular weight: 592.5kDa. Substrate: glucose/methanol 4:1 (w/w) ▲ MOX protein (%)·10; ○ MOX activity [(MOX-unit·mg protein^{-1}·10]; FAD/MOX (mol/mol)·10; *reference FAD/MOX ratio with methanol as substrate.

Reproduced with permission from Giuseppin et al. (1988c) Biotechnol Bioeng 32: 377-583.

industrial fermentation processes are to be developed for the synthesis of the enzyme. However, more important than this is the production of an enzyme free of catalase. The major cost in preparing alcohol oxidase is in the separation of the enzyme from catalase. The enzyme is usually used commercially for the production of hydrogen peroxide. Thus it is necessary to reduce catalase contamination of the enzyme to a maximum in order to improve the efficiency of hydrogen peroxide production, especially when given the poor kinetics of the enzyme. This is usually carried out by incorporating an ion exchange chromatography step within the purification protocol. Alcohol oxidase binds to DEAE sepharose but catalase is only poorly bound and easily washed off the column by 0.4% NaCl solution at pH 7.8 (JR Woodward, unpublished results). Alcohol oxidase is then eluted from the column using 3-4% NaCl or a 0.4-4% NaCl gradient.

It is also possible to destroy catalase using an anionic detergent. It can be shown that C_{12} anionic detergent such as sodium dodecyl sulphate will destroy catalase preferentially but leave the alcohol oxidase intact. The ability of the alcohol oxidase to withstand detergents is remarkable and will be discussed in the section on applications of the enzyme. Although this method does not require a purification step it is difficult to control and there are problems in recovering the protein from solution. Thus there has been interest for some years in the possibility of using catalase negative mutants of a methylotrophic yeast to obtain catalase free alcohol oxidase without a chromatography step in the downstream processing. The existence of catalase negative mutants has been known for some time. These mutants are easily prepared by simple mutagenic techniques such as the use of nitrosoguanamine. We have isolated a number of such mutants in our own laboratory and have demonstrated the absence of catalase upon their derepression by growth on substrates such as ribose and xylose. However, induction of these mutants to a high level of expression of the AOX gene is more difficult. Mutants cannot be induced using glucose/methanol mixtures as there is no catalase to destroy the hydrogen peroxide produced by the enzyme. Thus it has been necessary to devise a method of inducing the AOX gene in the absence of methanol. Giuseppin et al. (1988b) have reported the induction of a catalase negative strain of *H. polymorpha* with glucose/formaldehyde or glucose/formate mixtures. They found that the best inducer concentration mixtures lay within the ratio of the 0-1.8 range. Formaldehyde was a better inducer than formate and the optimal ratio of formaldehyde to glucose was 1.4. At higher ratios production of the enzyme was less efficient and above 2.2 poisoning of cells occurred with resultant washout of the continuous culture system. Using formate/glucose mixture at a ratio of 3.6 dilution rates of 0.05-0.15 were optional for maximum production of enzyme. In general the authors found that it was possible to obtain final yields within 60% of those obtained in wild type cells under the same conditions. These results are probably due to other mutations to the promoter or regulator sites on the AOX gene. However, it is not possible to rule out some co-regulation of expression of the catalase gene which affects the final

yield of alcohol oxidase in catalase negative mutants. The above process is now the subject of patent applications by Unilever who clearly see this process as the best method of economical production of the enzyme.

Applications of Alcohol Oxidase

Practically all the applications of alcohol oxidase are in the production of hydrogen peroxide or the detection of alcohols, although there are some reports of its use for formaldehyde production.

Several methods for the production of alcohol oxidase have already been patented or are the subject of patent application. These cover two organisms, *Hansenula polymorpha* (Eggeling et al. 1981 and Giuseppin 1988) and *Pichia pastoris* (Hopkins 1980). In the case of *Hansenula polymorpha* protection is sought for methods of inducing high levels of alcohol oxidase, in the case of Giuseppin et al. (1988b) in the absence of catalase. In the case of *Pichia* alcohol oxidase protection was sought for the method of purification, in which the enzyme crystallises from solution at low ionic strengths. There is only one major source of alcohol oxidase as a bulk enzyme and this is from Provesta, a subsidiary of Phillips Petroleum. Enzyme is also sold from a *Candida* source although it is usually only found in biochemical catalogues for research purposes.

Hydrogen Peroxide Production

Two major detergent manufacturers have filed patents on the use of alcohol oxidase in detergent systems. The reason for interest in the ability of alcohol oxidase to produce hydrogen peroxide (H_2O_2) is found in the fact that chemically produced H_2O_2 cannot be incorporated into a liquid detergent. All detergent systems use H_2O_2 to bleach clothes during washing. This is either done by heating the wash water to temperatures above 60°C when the H_2O_2 breaks down to form bleaching radicals or by using a bleach activator such as N.N.N.N. tetra acetyl ethylene diamine, which allows bleaching to occur at temperatures below 40°C and allows 'cold' wash powders to be produced. Powders contain perborate which, on contact with water produces H_2O_2. Clearly this cannot be added to liquids and H_2O_2 in liquids rapidly breaks down. Thus it was necessary to find a way in which bleach could be generated on dilution of a liquid detergent. Schreiber et al. (1975) filed a patent application in which alcohol oxidase was incorporated into a tablet which could be added to a liquid detergent containing the substrate. This method was effective but not commercially acceptable. The manufacturers wished to market a single product incorporating the enzyme.

In order to attain the goal of a single product containing both enzyme and substrate it was necessary to reduce the water activity of the liquid detergent to

a state in which the enzyme became inactive but was not inactivated. A formulation was thus arrived at in which tne enzyme exhibited no activity even in the presence of 8% ethanol. Cox et al. (1982) gave a number of examples of liquid detergent compositions containing aionic and non-ionic detergent at very high concentrations. No damage was apparent to the enzyme even at these high concentrations. Upon dilution 1:100 in water the enzyme became hydrated and activated almost immediately, producing up to 25mM hydrogen peroxide *in situ* over a period of 30 minutes at 40°C. This was sufficient H_2O_2 to give bleaching of tea stained cloth equivalent to that obtained with similar quantities of chemically derived H_2O_2. It is interesting to note that the enzyme from *Hansenula* is remarkably resistant to the effects of anionic and non-ionic but not cationic detergents (Fig. 11).

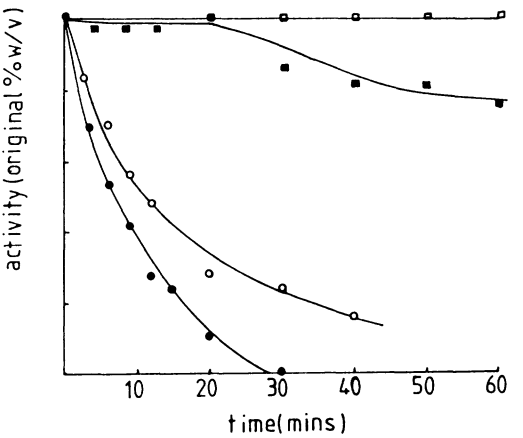


Fig. 11. Effect of detergent on enzyme activity. Enzyme was incubated at 45°C and activity was measured on an oxygen electrode. (□) No additions; (■) 1% synperonic (non-ionic detergent); (○) 1% sodium dodecyl sulphate; (●) 1% Arquad (cationic detergent).

There is thus the ability to produce a liquid detergent capable of *in situ* H_2O_2 production upon dilution which can achieve results as good as those with concentrated powders. Why does it remain unused? The answer at present is simple. The cost of enzyme is still too high to allow its use in a bulk product. Even at the price of $ 8000 per million units or at a considerable discount for bulk supplies of *Pichia* enzyme the product could not be manufactured at an economic price. This is therefore an elegant solution to an impossible problem, which fails on economic grounds.

Perhaps a more hopeful area for the use of *in situ* production of H_2O_2 is as an antimicrobial system. Many environmentally aware people are beginning to question the use of harsh chemicals such as hypochlorite and formaldehyde as sterilising agents. Both are irritants and they should not contaminate foods or come into contact with wounds etc. However, most other antiseptics are also chemicals, usually phenolic in origin. It is therefore interesting to note that in experiments carried out in our laboratory we have shown that *in situ* generation

of H_2O_2 using alcohol oxidases and ethanol produces a very effective antimicrobial system (See Fig. 12). Furthermore the *in situ* production of H_2O_2 is more effective than the addition of much higher quantities of chemically produced hydrogen peroxide. Table 5 shows a comparison of the width of zones of inhibition produced in *E. coli* and *Candida* lawns on agar plates by *in situ* enzyme produced H_2O_2 and chemically produced H_2O_2. Separate experiments on which the cells were added to solutions containing enzyme and alcohol showed that in some cases the in situ production of H_2O_2 was up to 1000 times more effective than chemically produced H_2O_2. In the case of the inhibition of yeast growth there was little effect of chemical H_2O_2 until levels of 40-60 mM were reached.

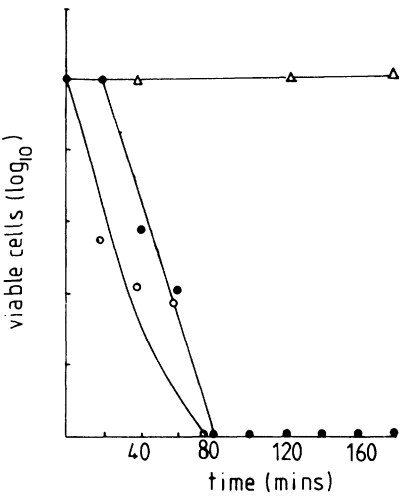


Fig. 12. Antimicrobial activity of de novo hydrogen peroxide production by alcohol oxidase. Cells were incubated with alcohol oxidase, with and without ethanol. At various times samples were removed subject to serial dilution and plated onto suitable media. Viable counts were then determined. (○) *E. coli* incubated at 37°C; (●) *Candida boidinii* incubated at 30°C: (Δ) control numbers for both microorganisms.

E. coli and *C. boidinii* were spread as lawns on agar plates. 8mm holes were cut in the agar and the wells filled with enzyme plus substrate or suitable dilutions of commercial H_2O_2. H_2O_2 levels were determined in separate assays.

It has also been observed in agreement with previous work (Berglin et al. 1982) that addition of cysteine to H_2O_2 produced *in situ* potentiates its antimicrobial effects. Addition of 10mM cysteine increased the zone of inhibition for *E. coli* from 4.5 to 10.0mm and *C. boidinii* from 3.0mm to 7.0mm. These antimicrobial systems have not yet been investigated commercially for use in kitchens and bathrooms but certainly warrant further investigations, especially as legislation may soon reduce the levels of formaldehyde allowed in toilet cleaners and other hygiene products.

Table 5. Effect of enzyme generated and chemically produced hydrogen peroxide on the inhibition of the growth of *E. coli* and *C. boidinii*.

	Molarity of H_2O_2	Enzyme generated H_2O_2. Zone width (mm)	Chemically generated H_2O_2. Zone width (mm)
E. coli	0.008	2.5	2.5
	0.016	4.5	3.5
	0.022	8.0	6.0
	0.031	-	6.5
C. boidinni	0.008	1.0	0
	0.016	4.5	0
	0.020	7.0	0
	0.031	-	1.5

Alcohol Detection

The most active area for the application of alcohol oxidase has been in the area of alcohol detection. No less that five dry test strip assays for blood or saliva alcohol are on the market or close to the market place at the present moment. Four of those tests use *Pichia pastoris* enzyme. A number of patents have been filed in this area but it is not clear as to which has priority or indeed whether any can be defended, as much of this technology is already in the public domain (Bauer 1985; Phillips 1984; Majkic-Singh & Berkes 1980).

All the tests using alcohol oxidase involve a dual enzyme system.

$$CH_3CH_2OH + O_2 \xrightarrow{\text{Alcohol oxidase}} CH_3CHO + H_2O_2$$

$$H_2O_2 + \text{acceptor} \xrightarrow{\text{Peroxidase}} H_2O + \text{coloured acceptor}$$

This reaction is generally the Trinder reaction in which a phenol reacts with 4-aminoantipyrine and peroxidase to produce a red coloured product (Trinder 1969).

All the reactive dry test strips use peroxidase coupled reaction which produces a colour. Dried preparations of the enzyme however undergo a form of autooxidation producing H_2O_2 in the absence of substrates. This leads the mixtures of enzymes and colour coupling reagents to spontaneously form colour during the drying process. This can generally be prevented by incorporation a non-volatile H_2O_2 scavenger to the mixture during drying. The

oxidisable substrate on the alcohol oxidase is proposed to be formaldehyde by Hopkins and Muller (1987) in the reaction

$$\begin{array}{l} \text{H} \\ \text{E-N-CH}_2\text{OH} \end{array} \xrightarrow{\text{spontaneous}} \text{E} + \text{HCHO} \xrightarrow[\text{O}_2]{\text{Alcohol oxidase}} \text{E. HCOOH} + \text{H}_2\text{O}_2$$

All the dry reagent strips require stabilisers for the enzyme. Mannitol and similar saccharides have been used to help preserve the stability of the enzyme. Addition of protein such as horseradish peroxidase, haemoglobin, cytocrome C and myoglobin have also been proposed as stabilisers (Hopkins 1988). However, to date none of these tests has proved particularly successful commercially. The major drawback is in the fact that visual assessment of colour density against a simple chart of three or four colours is very difficult. The more a person has had to drink the less able they are in making such a judgement. Also the stability of some tests has been erratic and in some cases, very poor.

A new test recently developed in our laboratory in collaboration with Dr J. P. van Dijken of the Technical University of Delft overcomes these problems by incorporating a patented stabilisation system into the drying process which assures a shelf life of at least one to two years for the test cards. The test itself does not rely on the judgement of the individual in matching colours but on the development of colour above certain threshold levels of alcohol in saliva or blood. Two reagent pads are placed on the test card and each develops colour only when the alcohol level exceeds a pre-set value. Thus in a typical card, if the user has over 50mg/dl alcohol in their blood or saliva, but under 80mg/dl, then only one disc will develop colour. If the user has over 80mg/dl both discs will develop colour when exposed to saliva. The system is simple and foolproof. It is to be hoped that this type of test will enable drivers to assess the level of alcohol in their blood before setting off in a car and thus reduce the level of accidents due to alcohol impaired driving.

Alcohol oxidase has also been used in the measurement of methanol when measuring the methyl ester content of pectins after hydrolysis with sodium hydroxide (Klavans & Bennett 1986; Herzenberg & Rogerson 1985). Alcohol determination systems based on alcohol oxidase and very sensitive colour coupling reagents can be used to detect yeast contamination of food products including yoghurt and starch products (Woodward & Gibson, unpublished data).

Immobilisation of the enzyme has received considerable attention. Its most helpful application is in the automation of alcohol determination. We have immobilised alcohol oxidase onto the walls of nylon tubing by derivatising the nylon with triethyloxonium tetrafluoroborate, reacting this with diamino-

alkane and glutaraldehyde and then coupling enzyme to the free aldehyde groups (Gibson & Woodward 1986). This technique allows the immobilised enzyme to be incorporated into an automated system and used on line to measure alcohol during fermentation (Gibson & Woodward 1988). In the latter case it was possible to incorporate a dialysis probe into the fermentation vessel and directly measure the alcohol diffusing across the membrane. The results obtained gave exactly the same answers as those obtained by direct sampling of the culture. These immobilised enzyme coils have a life of up to one month in regular use. Interestingly the coils show activation with repeated injections of alcohol, there is no obvious explanation for this phenomenon although we have noticed it occurring in biosensors in previous work.

Biosensors

The invention of the oxygen of 'Clarke' electrode in the early 1950's by Leland Clarke was a milestone in biosensor technology. Clarke & Lyons showed in 1962 that it was possible to incorporate an enzyme behind the gas permeable membrane of an oxygen electrode such that if the enzyme reacted to produce hydrogen peroxide, by setting the electrode at the correct voltage the peroxide could be destroyed with the concomitant passage of electrons into the electrode. The early alcohol electrodes were thus made by sandwiching the enzyme between a cellulose acetate and a gas permeable polycarbonate membrane using glutaraldehyde crosslinking to attain enzyme immobilisation (Clarke 1987). These membranes were effective and could be used to measure solutions containing up to 5 g/l ethanol; they have been incorporated into the Yellow Springs Instrument Inc. (Ohio, USA) analyser range. A variation on this type of electrode has been produced by Verduyn et al. (1983) who immobilised their enzyme onto nylon net and placed it over the oxygen permeable membrane. The electrode performed well and had a lifetime of at least 15 days. In both these electrodes the source of alcohol oxidase was *Hansenula polymorpha*. Observations by J.P. van Dijken and also in our own laboratory suggest that the *Hansenula* enzyme is more robust than the *Pichia* or *Candida* enzymes in these applications. Belghith et al. (1987) also reported the production of a biosensor in which the enzyme was immobilised in gelatine by glutaraldehyde treatment of a thin gelatine/enzyme layer. Their probe was stable for at least 500 assays of 10mM ethanol.

As an alternative to the use of oxygen electrodes it is possible to link the reaction between alcohol and alcohol oxidase to a glassy carbon probe via peroxidase and a mediator. In this case (Parker, Higgins & Woodward, unpublished results) we have found that the salt can mediate electron transfer from peroxidase to the glassy carbon probe. The carbon is first covered with the mediator salt (NMP-TCNQ) and then treated with carbodiimide. Enzymes are immobilised onto the probe and the whole is then encapsulated in a gas permeable membrane.

The result is a biosensor which, when covered with a polymer membrane, acts as a diffusion limited enzyme reactor and has a linear response to ethanol up to 16mM, the upper limit of the blood alcohol content permitted in a vehicle driver.

It is hoped that this sensor can be developed into a disposable system which will be of use in measurement of blood and saliva alcohol determination.

Conclusion

Alcohol oxidase remains an enzyme of considerable industrial potential especially within the field of diagnostics, provided it can be presented to the public in a user friendly form. We are likely to see a considerable expansion in its use for the measurement of alcohol both clinically and in the food and beverage industry. However, the bulk use of the enzyme for industrial H_2O_2 production and in household cleaners and disinfectants is unlikely to become reality until the cost of production has been drastically reduced.

Biochemically the enzyme remains of great interest. The solving of the X-ray cyrstallographic structure and hence the mechanism of assembly of the octamer and the crystalline lattice of the peroxisome remain challenging prospects which are nevertheless within our grasp. This enzyme will continue to provide a fascinating if sometimes frustrating living for many of us for some years to come!

Acknowledgements

I wish to acknowledge the kind help and advice of Dr J.P. van Dijken during the preparation of this manuscript. I am also indebted to Dr M. Giuseppin for permission to use figures from his thesis and to Janet Vonck and Professor van Bruggen of the University of Groningen for permission to use their electron micrographs of two dimensional crystals of *Hansenula polymorpha*.

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