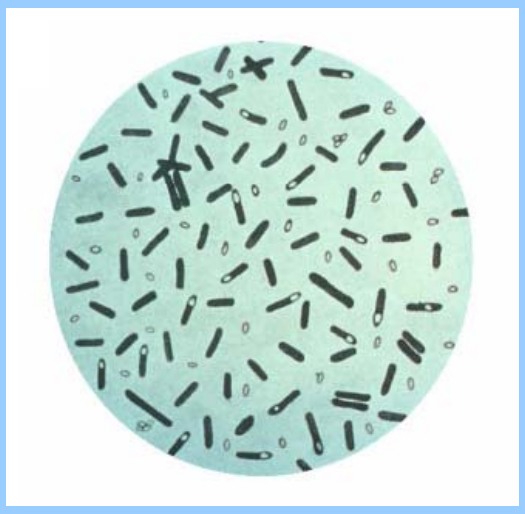

Clostridium XII

International Conference on the Genetics, Physiology and Biotechnology of Solvent- and Acid-forming Clostridia

Programme and Abstracts

Nottingham Conference Centre,
Nottingham, UK

10 - 12 September 2012

CLOSTRIDIUM XII COMMITTEES

Organizing Committee:

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Peter Duerre (Ulm, Germany)
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with the acknowledged help of Lyndy Cox.

WELCOME

There is no doubt that 2012 has been 'the' year to visit the UK. There has been the Queen's Jubilee celebrations, Andy Murray almost won Wimbledon, we have all marvelled at the London 2012 Olympic Games, and now …….. Clostridium XII.

It was my great pleasure to host the first Clostridium meeting in Salisbury over 20 years ago. Since that date (1990), meetings have continued to be held every two years, alternating between the USA and Europe, with the last two meetings in Wageningen (2008) and San Diego (2010). At all of these gatherings, research concerned with the acetone-butanol-ethanol (ABE) fermentation has figured prominently. It is, therefore, fitting that 2012 is the 100 year anniversary of the first isolation by Chaim Weizmann of *Clostridium acetobutylicum,* the microbe behind ABE process. Weizmann of course worked at the University of Manchester, just a stone's throw up the road from Nottingham.

With topics encompassing a wide spectrum of interests, and seven invited speakers from all corners of the globe, it is with great pleasure that I welcome you to Nottingham.

Nigel Minton
Host, on behalf of the Clostridium Organizing Committee.

GENERAL INFORMATION

VENUE

Nottingham Conference Centre
Burton Street
Nottingham
NG1 4BU

ORAL PRESENTATIONS

Oral presentations will be in the Adams Room, level 2, Nottingham Conference Centre. The length of oral presentations is scheduled from 15 to 35 min (check programme), within that presenters should allow 5 min for discussion. All presentations should be prepared in a form of MS Power Point slide show and stored on USB sticks or CD/DVD. The use of a personal computer or Mac is not possible. All presentations will be uploaded to the computer in the lecture hall. This can be done anytime, but at least 2-3 hours before your session or the evening before for early morning presentations.

POSTER PRESENTATIONS

Poster presentations will be in the Bowden Room, level 2, Nottingham Conference Centre. The size of each poster board is 90 cm (width) × 200 cm (height), thus the maximum recommended poster size is A0 (90 cm × 120 cm). Velcro tabs will be provided. The presenting author should stand by his/her poster for the whole length of the session.

SOCIAL EVENTS

Social events are open to all participants and accompanying persons and are included in the conference fee

Welcome Reception, Sunday, 9th September 2012, 19:30 until late, Wembley Suite, Hilton Hotel.

Dinner, Monday, 10th September 2012, 20:00- 22:00, The Old Museum, Nottingham Conference Centre.

Networking afternoon, Tuesday, 11th September 2012,14.30 - 17.30. Feel free to explore Nottingham in small groups on your own, or join one of our guided tours to Nottingham Castle or Galleries of Justice and Caves. Please sign up for the guided tours at the reception desk on registration, as places are limited.

Barbeque, Tuesday, 11th September 2012, 19:00 - 23.00, The Old Chemistry Theatre, (or Old Museum if the weather is bad), Nottingham Conference Centre.

Conference Dinner, 12th September 2012, 20:00- 22:00, The Old Museum, Nottingham Conference Centre.

EVENING ENTRANCE

Please can you use Goldsmith Street entrance for the evening dinners as Burton Street entrance will be closed.

STAYING IN TOUCH

There is complimentary phone and laptop chargers and Wi-Fi access throughout the building.

The Business Centre up the stairs on level 3 provides you with an area to work quietly, collate your presentation, or send emails.

REFRESHMENTS

The refreshment hubs just outside the Adams and Bowden rooms, offer a complimentary self-service selection of refreshments throughout the day.

YOUNG PRESENTER AWARDS

Clostridium XII has encouraged the participation of graduate and new post docs. Thanks to BBSRC funding we were able to give free registration to the following young presenters: Yi Wang (University of Illinois, USA), Torbjørn Ølshøj Jensen (Aalborg University, Denmark), Christine Voight (University of Rostock, Germany), Kousuke Yamamoto (Mie University, Japan), Kumaran Sivagnanam (McGill University, Canada), Ran Du (Tsinghua University, China), Simone Mora (University of Torino, Italy), Jonathan Lo (Dartmouth College, Germany), Swati Badgujar (Institute of Chemical Technology, Mumbai, India) and Michael Pyne (University of Waterloo, Canada).

TAXIS

We always use DG Taxis 0115 9 607607, or ask your hotel to book for you.

CLOSTRIDIUM XII PROGRAMME

Sunday, 9th September 2012

19.00-20.00 **Registration** *Wembley Suite, Hilton Hotel*

20:00-24.00 **Welcome Reception** *Wembley Suite, Hilton Hotel*

Monday, 10th September 2012

Session I: REGULATION *Adams Room, Nottingham Conference Centre*

Chairperson: Terry Papoutsakis

09.00 - 09.05 Nigel P Minton - The University of Nottingham, UK
Welcome and Introduction

09.05 - 09.40 Nigel P Minton - The University of Nottingham, UK
"A 'Road Map' for the Genetic Modification of Any *Clostridium* spp"

09.40 - 10.05 Peter Dürre - University of Ulm, Germany
"Master and Commander: Spo0A vs. SolB, a Small Regulatory RNA, in Controlling the Shift to Solventogenesis in *Clostridium acetobutylicum"*

10.05 - 10.30 Klaus Winzer - The University of Nottingham, UK
"Quorum Sensing regulates sporulation and solvent formation in *Clostridium acetobutylicum*"

10.30 - 10.45 Delyana Vasileva - University of Rostock, Germany (Young Presenter)
Kindly sponsored by the BBSRC
"Iron-dependent Response of the Strict Anaerobe *Clostridium acetobutylicum*"

10.45 - 11.15 Coffee break

Chairperson: Nigel P Minton

11.15 - 11.50 Weihong Jiang - Shanghai Institutes for Biological Sciences, China (Invited Speaker)
Kindly sponsored by the SfAM
"CcpA, a Pleiotropic Key Regulator in Butanol-producing Clostridium acetobutylicum"

11.50 - 12.15 Ralf-Jörg Fischer - University of Rostock, Germany
"Role of Granulose for Formation and SASPs for Germination of Endospores of *Clostridium acetobutylicum"*

12.15 - 12.40 Jeffrey Blanchard - The University of Massachusetts, USA
"Population Level Analysis of Mutations Underlying Improvements in Biofuel Production by *Clostridium phytofermentans"*

12.40 - 13.00 Steven Brown - Oak Ridge National Laboratory, USA
"RNA-Seq and DNA Microarray Analyses of *Clostridium thermocellum* Biomass Fermentation Transcriptomes"

13.00 - 14.30 Lunch *The Old Library, NCC*

Session II: BUTANOL ENGINEERING *Adams Room, NCC*

Chairperson: George Bennett

14.30 - 15.05 Isabelle Meynial-Salles - INSA, Toulouse, France (Invited Speaker)
Kindly sponsored by the SGM
"Metabolic Engineering of *Clostridium acetobutylicum* ATCC 824 for the High-Yield Production of Biofuels"

15.05 - 15.30 Edward Green - Green Biologics Ltd, UK
"Use of Municipal Solid Waste as a Feedstock for the Butanol Fermentation"

15.30 - 15.55 Shang-Tian Yang - The Ohio State University, USA
"Engineering *Clostridium* for High-Titer and High-Yield Biobutanol Production"

15.55 - 16.20 Ye Ni - Jiangnan University, China
"Continuous Butanol Fermentation from Low-Value Sugar-Based Feedstocks by *Clostridium saccharobutylicum* DSM 13864"

16.20 - 16.35 Yi Wang - University of Illinois, USA (Young Presenter)
Kindly sponsored by the BBSRC
"Genome-wide Transcriptomic Analysis in *Clostridium beijerinckii* 8052 using Single-Nucleotide Resolution RNA-Seq Technology"

16. 35 - 18.00 Posters I & Coffee *Bowden Room, NCC*

20.00 - 22.00 Dinner *The Old Museum, NCC*

Tuesday, 11th September 2012

Session III: CELLULOSE AND SUGAR UTILISATION *Adams Room, NCC*

Chairperson: Peter Dürre

09.00 - 09.35 Mike Himmel - NREL, USA (Invited Speaker)
Kindly sponsored by the BBSRC
"Engineering the *Clostridium thermocellum* cellulosome"

09.35 - 10.00 Lee Lynd - Dartmouth College, USA
"Clostridium thermocellum: Metabolic engineering and cellulose utilization"

10.00 - 10.25 Philippe Soucaille - INSA, France
"The Weizmann process revisited for cellulosic Butanol production"

10.25 - 10.45 David Ramey - Green Biologics Ltd, USA
"Cellulosic Butanol - A Sustainable Advanced Next Generation Biofuel"

10.45 - 11.00 Kousuke Yamamoto - Mie University, Japan (Young Presenter)
Kindly sponsored by the BBSRC
"Identification of the *Clostridium cellulovorans* cellulosomal subunits on soft biomass degradation"

11.00 - 11.25 Coffee break

Chairperson: Philippe Soucaille

11.25 - 12.00 Wilfrid Mitchell - Heriot-Watt University, UK (Invited Speaker)
Kindly sponsored by the SGM
"Glucose Sensing in the Solventogenic Clostridia"

12.00 - 12.25 Chen Yang - SIBS, China
"Pathway Reconstruction and Flux Quantification of Pentose Metabolism in Solventogenic Clostridia"

12.25 - 12.50 Katherine Germane - US Army Research Laboratory, USA
"Arabinose is Metabolized Via a Phosphoketolase Pathway in *Clostridium acetobutylicum"*

12.50 - 13.15 Ana Lopez-Contreras - Food & Biobased Research-Wageningen UR, The Netherlands
"Production of Acetone, Butanol and Ethanol from native North Sea seaweed species"

13.15 - 13.30 Kumaran Sivagnanam - McGill University, Canada (Young Presenter)
Kindly sponsored by the BBSRC
"Proteomic Analysis of *Clostridium acetobutylicum* from Butanol Fermentation"

13.30 - 14.30 Lunch *The Old Library, NCC*

14.30 - 17.30 NETWORKING AFTERNOON

19.00 - 23.00 BBQ *The Old Chemistry Theatre, NCC*

Wednesday, 12th September 2012

Session IV: PHYSIOLOGY, GENETIC TOOLS, AND MODELS *Adams Room, NCC*

Chairperson: Weihong Jiang

09.00 - 09.35 Derek Lovley - University of Massachusetts, USA (Invited Speaker)
"Feeding Acetogenic Clostridia Electricity to Produce Organic Commodities from Carbon Dioxide"

09.35 - 10.00 Michael Koepke - LanzaTech, New Zealand
"Production of Fuels and Chemicals from Industrial Waste Gases"."

10.00 - 10.25 Terry Papoutsakis - University of Delaware, USA
"Installing the Wood-Ljungdahl (WL) pathway in *Clostridium acetobutylicum"*

10.25 - 10.40 Simone Morra - University of Torino, Italy (Young Presenter)
Kindly sponsored by the BBSRC
"Clostridia from a Pilot Plant for Enhanced Hydrogen Gas Production: Isolation, Molecular Characterization and Hydrogenase Genes Cloning for Exploitation in Biotechnology"

10.40 - 11.10 Coffee break

Chairperson: Wilfred Mitchell

11.10 - 11.35 David Levin - University of Manitoba, Canada
Central metabolism of the anaerobic lignocellulolytic thermophile *Clostridium stercorarium* subsp. stercorarium DSM8532

11.35 - 12.00 Thomas Millat - University of Rostock, Germany
“Mathematical Modelling of the pH-induced Metabolic Shift Unravels a Heterogeneous Phase Transition”

12.00 - 12.25 Servé Kengen - Wageningen University, The Netherlands
“Analysis of the Transcriptional Response to n-Butanol Challenges during Acidogenic Continuous Culturing of *Clostridium acetobutylicum*”

12.25 - 12.50 Sebastian Curth - Beuth University of Applied Sciences Berlin, Germany
“Mathematical Modelling for Prediction of Strain Optimization Targets: Sensitivity Analysis and Evaluation of Dynamic Experiments”

12.50 - 13.05 Torbjørn Ølshøj Jensen - Aalborg University & Biogasol, Denmark (Young Presenter)
Kindly sponsored by the BBSRC
“Conversion of Crude Glycerol by *Clostridium pasteurianum* and by a Developed Mutant Strain”

13.05 - 14.30 Lunch *The Old Library, NCC*

14.30 - 16.00 Posters II & Coffee *Bowden Room, NCC*

Session V: PHYSIOLOGY AND HIGH THROUGHPUT TOOLS *Adams Room, NCC*

Chairperson: Klaus Winzer

16.00 - 16.35 Wolfgang Buckel - Philipps-Universität Marburg, Germany (Invited Speaker)
Kindly sponsored by the SfAM
"Radical enzymes in clostridial butyrate synthesis".

16.35 - 17.00 George Bennett - Rice University, USA
“Analysis of Redox Responses During TNT Transformation by *Clostridium acetobutylicum* ATCC824 and Mutants and Other Clostridia”

17.00 - 17.25 Tina Lütke-Eversloh - University of Rostock, Germany
“Development and Application of a High-throughput Screening System for Clostridial Biofuel Production”

17.25 - 17.50 Petra Patakova - Institute of Chemical Technology Prague, Czech Republic
“Flow Cytometry Analysis of Solventogenic Clostridia”

17.50 - 18.05 Swati Badgujar - Institute of Chemical Technology, Mumbai, India (Young Presenter)
Kindly sponsored by the BBSRC
“Carbon flux diversion in *Clostridium acetobutylicum* : Effect of Mutagenesis and Stress”

18.05 - 18-10 Closing Remarks

20.00 - 22.00 Conference Dinner *The Old Museum, NCC*

INVITED SPEAKERS

WOLFGANG BUCKEL - "Radical enzymes in clostridial butyrate synthesis"

Wolfgang Buckel is a Professor of Microbiology at the Philipps-Universität Marburg, Germany and a Fellow of the Max-Planck-Society. His research focusses on special enzymes in amino acid fermentation pathways by clostridia. The fermentation of glutamate to butyrate proceeds via three different pathways, all of which involve radical enzymes: the coenzyme B12 dependent glutamate mutase, the [4Fe-4S] cluster-containing 2-hydroxyglutaryl-CoA dehydratase that requires activation by reduced ferredoxin and ATP, as well as the [4Fe-4S] cluster and FAD-containing 4-hydroxybutyryl-CoA dehydratase. All three pathways terminate in the exergonic reduction of crotonyl-CoA to butyryl-CoA by NADH that drives the endergonic reduction of ferredoxin by NADH, a process called electron bifurcation.

MIKE HIMMEL- "Engineering the *Clostridium thermocellum cellulosome*"

Dr. Himmel has over 30 years of research experience in protein biochemistry, recombinant technology, enzyme engineering, new microorganism discovery, and the physicochemistry of macromolecules. He has also supervised research that targets the application of site-directed-mutagenesis and rational protein design to the stabilization and improvement of important industrial enzymes, especially glycosyl hydrolases. Dr. Himmel has been awarded over $100 M in research funding and contributed to over 450 journal papers, meeting abstracts, patents, and books, the latest of which “Biomass Recalcitrance” is a top selling science book and has been translated into Chinese.

WEIHONG JIANG - "CcpA, a Pleiotropic Key Regulator in Butanol-producing *Clostridium acetobutylicum*"

Weihong Jiang is a professor of microbiology in the Laboratory of Synthetic Biology and deputy director of Institut Pasteur of Shanghai, Chinese Academy of Sciences. Her research focuses on transcriptional factors related to biosynthesis of solvents and antibiotics in clostridia and streptomycetes, and genetic modification of these industry-important strains through metabolic engineering and synthetic biology approaches. Recent research on Clostridia has centered on dissection of the xylose-metabolic pathway and its regulatory mechanism in *C. acetobutylicum* and *C. beijerinckii*. The transcriptional factors, such as catabolite control protein A (CcpA) and two-component systems, were intensively investigated.

DEREK LOVLEY - "Feeding Acetogenic Clostridia Electricity to Produce Organic Commodities from Carbon Dioxide"

Derek Lovley is a Distinguished Professor in the Department of Microbiology and Director of the Environmental Biotechnology Center at the University of Massachusetts. Research in his laboratory focuses on: anaerobic microbial processes that impact the natural cycling of carbon and metals in soils and sediments; bioremediation of hydrocarbon and metal contamination; and novel bioenergy strategies that involve microbe-electrode interactions. Recent research has focused on genome-scale modelling of the metabolism of *Clostridium ljungdahlii* and its genetic manipulation to improve electron transfer from electrodes to cells and expand the diversity of products from carbon dioxide reduction.

ISABELLE MEYNIAL-SALLES - “Metabolic Engineering of Clostridium acetobutylicum ATCC 824 for the High-Yield Production of Biofuels”

Dr. Meynial-Salles is an associate professor and a senior staff member of the Biochemical Engineering Department (LISBP), INSA (Technical University), Toulouse, France. Isabelle holds a Ph.D in enzymology, awarded by INSA in 1994. However, since 1995 Isabelle has focused her research on the development of novel and original bioprocesses based on the engineering of bacterial factories. Successes include the first continuous biological process for the production of 1,3 propanediol at high yield from glycerol using recombinant *C. acetobutylicum*. Currently, Isabelle’s 15-person strong team is implementing metabolic engineering and now, synthetic biology, to construct novel microorganisms for the production of next generation biofuels and bulk chemicals.

WILF MITCHELL - "Glucose Sensing in the Solventogenic Clostridia"

Wilf Mitchell is a Reader in Microbiology in the School of Life Sciences at Heriot-Watt University in Edinburgh. He has worked on sugar uptake and its control in solventogenic clostridia for 25 years. His principal interests relate to the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which is the major mechanism for uptake and phosphorylation of monosaccharides, disaccharides and sugar derivatives and also plays a central role in regulation of carbon metabolism in response to the nutrient status of the environment. Understanding and manipulation of the control circuits can make a major contribution to construction of strains with improved fermentation performance on mixed sugar substrates.

ABSTRACTS OF ORAL PRESENTATIONS

A ‘Road Map’ for the Genetic Modification of Any *Clostridium spp*

NIGEL. P. MINTON, MUHAMMAD EHSAAN, ANNE HENSTRA, KATALIN KOVACS, WOUTER KUIT, ERIC LIEW, GARETH LITTLE, SARAH MASTRANGELO, KATRIN SCHWARZ, LILI SHENG, HENGZHENG WANG, BEN WILLSON, YING ZHANG AND KLAUS WINZER.

Clostridia Research Group, BBSRC Sustainable Bioenergy Centre, School of Life Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, UK

We have developed a ‘Road Map’ that is universally applicable to the development of gene systems for any *Clostridium spp.* amenable to DNA transfer. We have used this system to generate directed (in-frame deletions) and random (transposon) mutants in a wide range of clostridial species, including *C. acetobutylicum, C. difficile, C. beijerinckii, C. botulinum, C. sporogenes* and *C. ljungdahlii,* as well as to endow selected organisms with all manner of beneficial attributes concerned with product formation and substrate utilisation.

As a first step, ClosTron technology is used to rapidly generate a *pyrE* mutant to enable the formulation of fluoroorotic acid-based, selective media able to distinguish between a *pyrE* mutant and the wild-type. These conditions are then adopted in the selection of an in-frame deletion of *pyrE* generated using Allele-Coupled Exchange (ACE) technology. Once made, the *pyrE* mutant is used as the host for the creation of in-fame deletions by allelic exchange in any chromosomal gene, using pseudo-suicide vectors and a suitable counter selection marker, i.e., *codA* or a heterologous *pyrE* gene. The most suitable vector for pseudo-suicide is experimental chosen from the available pMTL80000 modular vectors series. Following the creation of the in-frame deletion mutant, the host is simply and rapidly (5 days) converted back to *pyrE*+ using a specially constructed ACE ‘correction’ vector. This allows the assessment of the mutant phenotype in a ‘clean’, wild type background. Crucially, using an appropriate ACE vector, it is also possible to insert a functional copy of the inactivated gene into the chromosome, concomitant with ACE-driven correction of *pyrE*. This allows complementation studies to be undertaken at an appropriate gene dosage, as opposed to the use of autonomous plasmids. As such, the use of *pyrE* mutants as the host is preferred regardless of the mutagenesis method employed (*ie*., allelic exchange, ClosTron or transposon). It may also be used, in combination with ACE, to create strains endowed with synthetic operons concerned with product formation, substrate degradation and those specialist elements needed to implement transposon mutagenesis.

Master and Commander: Spo0A vs. SolB, a Small Regulatory RNA, in Controlling the Shift to Solventogenesis in *Clostridium acetobutylicum*

PETER DÜRRE, SONJA LINDER, TOBIAS ZIMMERMANN AND BETTINA SCHIEL-BENGELSDORF

Mikrobiologie und Biotechnologie, Universität Ulm, 89069 Ulm, Germany

Spo0A, the master regulator of sporulation in bacilli and clostridia, has long been considered as the central regulatory component for the onset of solventogenesis in *Clostridium acetobutylicum*. On the other hand, formation of butanol and acetone is still possible even after deletion of Spo0A-binding motifs (0A boxes) upstream of respective genes or in Spo0A-negative mutants, indicating the existence of additional regulatory factors. Several such components have meanwhile been identified. CodY, a pleiotropic transcription factor in Gram-positives, CcpA, the carbon catabolite regulator, AdcR and AdcS, the former being a novel transcription factor. However, of central importance is a small regulatory RNA (SolB), encoded by a gene upstream of the *sol* operon. Its overexpression leads to total loss of acetone as well as butanol formation and sporulation is severely impaired. There is also a Spo0A-independent solvent formation in *C. acetobutylicum*. This activity is completely blocked as well by overexpressing SolB. Presence of the RNA has been confirmed by Northern blot and RNA sequencing, the transcription start point has been mapped, and a model will be presented for its mode of action.

Quorum Sensing regulates sporulation and solvent formation in *Clostridium acetobutylicum*

KLAUS WINZER, ANN-KATRIN KOTTE, ELISABETH STEINER, KATRIN SCHWARZ, NIGEL MINTON.

Clostridia Research Group, Centre for Biomolecular Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, UK

Many bacteria are known to communicate via small, diffusible signal molecules to coordinate gene expression in response to cell population density (quorum sensing). Whilst quorum sensing has been extensively studied in Gram-positive species of the genera *Bacillus*, *Staphylococcus* and *Streptococcus*, very little is known about its role in *Clostridia*. However, bacteria of this class have been found to contain numerous putative quorum sensing systems in their genomes. Since quorum sensing is often involved in the regulation of stationary phase events, we investigated its role in the solventogenic *Clostridium acetobutylicum*.

Genome analysis of the ATCC 824 strain revealed the presence of at least ten putative quorum sensing systems, all of which make use of peptide-based signal molecules. These consisted of a single *agr*-type system, similar to that found in staphylococcal species, and nine RNPP-type systems, resembling those present in the *Bacillus cereus* group (by contrast, *Clostridium beijerinckii* was found to contain at least five different *agr* systems and one RNPP-type system).

Each of the identified systems was insertionally inactivated using ClosTron mutagenesis. Three independent mutants were isolated for each system and analysed for differences in metabolic profiles, sporulation, granulose accumulation, growth and (in some instances) the transcriptome. Mutants defective in the *agr* system showed normal fermentation profiles, but failed to accumulate granulose. They also produced less spores than the wild type. By contrast, seven of the putative RNPP systems appeared to be involved in the regulation of solvent production and two were involved in spore formation.

An exceptionally drastic phenotype was observed upon mutation of one particular RNPP system. The resulting strain showed very little solvent formation and no production of endospores, thus resembling a *spo0A* mutant in its phenotypic appearance. Transcriptome analysis revealed significant changes ($p<0.001$) in several hundred genes, including those required for solvent formation and sporulation, and also showed that this particular RNPP system tightly controls *agr*-based quorum sensing.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Iron-dependent Response of the Strict Anaerobe *Clostridium acetobutylicum*

DELYANA VASILEVA AND HUBERT BAHL

Institute of Biological Sciences, Division of Microbiology, University of Rostock, Rostock, A.-Einstein Str.3, Germany

Availability of transition metals for the biological systems has undergone remarkable changes during evolution from the anaerobic to the aerobic eras. One transition metal certainly common to almost all microorganisms is iron. However, upsurge of oxygen caused low availability and increased toxicity of this essential micronutrient. Due to this fact, bacteria in aerobic zones had to develop complex uptake and control mechanisms to remain viable. Pools of bioavailable iron, though, are ample in the anaerobic niches and insufficiency, as well as a fine-tuned global system for iron-dependent control in obligate anaerobes, are not expected. However, the genome of the solvent-producing, endospore-forming strict anaerobe *Clostridium acetobutylicum* revealed a gene encoding a putative ferric uptake regulator (Fur).

We inactivated the *fur* gene using the ClosTron system. Our results demonstrated that *C. acetobutylicum* senses and responds to availability of iron on multiple levels using a sophisticated system and Fur plays an important role in this process.The *fur* mutant exhibited a slow-growing phenotype and enhanced sensitivity to oxidative stress, but essentially no dramatic change in its fermentation pattern. A unique feature of its physiology was the overflowing production of riboflavin. To gain further insights into the role of the Fur protein and the mechanisms for establishment of iron balance in *C. acetobutylicum*, we characterized and compared the gene expression profile of the *fur* mutant and the iron-limitation stimulon of the parental strain.

The diverse transcriptional response to iron starvation, lack of Fur, or both highlights the need for further studies to better understand the iron-responsive networks in *C. acetobutylicum* as well as other clostridia. Starting from iron, we shall progress to the regulation and biological significance of other transition metals, since those have received little attention in clostridia and in strict anaerobes in general.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

CcpA, a Pleiotropic Key Regulator in Butanol-producing *Clostridium acetobutylicum*

CONG REN, YANG GU, YAN WU, SHENG YANG, WEIHONG JIANG

Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200032, China

Butanol, a four carbon primary alcohol, is an important solvent and transport fuel additive, which can be produced by microbe-based biological process. *Clostridium acetobutylicum* is the typical strain for this purpose with a complicated regulatory system. In this study, a catabolite control protein (CcpA) was functionally identified in *C. acetobutylicum*. It was found that deletion of *ccpA* gene could eliminate glucose repression on xylose metabolism, achieving the simultaneous fermentation of glucose/xylose mixture and will thus benefit the utilization of lignocellulosic biomass in *C. acetobutylicum*.

To dissect CcpA regulatory function globally, DNA microarray was performed to analyze the transcriptional differences between the *ccpA* mutant and its parental strain. It showed that CcpA controls not only carbon metabolism, but also other biochemical and physiological processes. Firstly, CcpA mediates carbon catabolite regulation by repressing the transcription of various genes related to metabolism of non-preferred carbon sources such as xylose and arabinose, but activating the expression of genes responsible for glucose PTS system; Secondly, CcpA is involved in positive regulation of the key genes responsible for acidogenesis and solventogenesis; Thirdly, CcpA is necessary for sporulation of *C. acetobutylicum*, an important trait adversely affecting the solvent productivity. Transcriptional alterations were observed in numerous sporulation-related genes upon *ccpA* inactivation, which should account for the lower sporulation efficiency in the mutant. Following transcriptional analysis, genetic and biochemical validation was also carried out. In summary, CcpA plays a key role in butanol-producing *C. acetobutylicum*.

Role of Granulose for Formation and SASPs for Germination of Endospores of *Clostridium acetobutylicum*

RALF-JÖRG FISCHER, HENRIQUE MACHADO, KATJA ZIMMERMANN, DANIELA WETZEL

Institute of Biological Sciences, Division of Microbiology, University of Rostock, Albert-Einstein-Str. 3, D-18051 Rostock, DE

The transition growth phase of *Clostridium acetobutylicum* is characterized by several morphological changes. Swollen and cigar shaped cells, clostridial stages, are formed in which granulose, a polymeric glycogen like carbohydrate, is accumulated. Granulose generally is considered as energy- and carbon storage for sporulation. We proved that glycogen synthase plays a crucial role in the biosynthesis of granulose. However, ClosTron® insertion mutants did not reveal uniform phenotypes. Whereas a first glgA::int strain was unable to accumulate granulose and did not form endospores, in further strains only granulose accumulation was abolished.

Here, a comparative molecular and phenotypic study will be presented including e.g. (electron)-microscopy, colony morphology, sporulation and plasmid-based complementation.

The second part of the presentation will focus on 'small acid soluble spore proteins' (SASP) of *C. acetobutylicum*. These proteins usually are located in the core of bacterial endospores and protect DNA against damage caused by desiccation, heat, or chemical agents. Furthermore, during germination degradation of SASPs provides an important amino acid source for the development of the new vegetative cell. In the genome of *C. acetobutylicum* five open reading frames are predicted to encode SASPs. Based on the analysis of insertional knock out mutants we started to elucidate their individual functions. Sporulation assays revealed varying germination capabilities of the mutant strains. Strikingly, one of the mutant strains was no longer able to germinate, independently if remaining vegetative cells were inactivated by heat, storage at -20 °C or chloroform treatment.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Population Level Analysis of Mutations Underlying Improvements in Biofuel Production by *Clostridium phytofermentans*

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Clostridium phytofermentans is a genetically tractable, anaerobic bacterium isolated from forest soil near the Quabbin Reservoir in Massachusetts, U.S.A. The combination of its broad nutritional versatility coupled with its high levels of ethanol production distinguishes *C. phytofermentans* from all other cultured microbes characterized to date. In the genome of *C. phytofermentans* there are many natural carbohydrate degradation modules consisting of extracellular glycoside hydrolases, ABC carbohydrate transporters, intracellular hydrolases, and phosphorylases. Gene expression and proteomic experiments have shown that these genes are highly expressed and the protein products accumulate in response to specific carbohydrates. These data and physiological experiments support the hypothesis that biofuel production is constrained by hydrolysis and transport.

Using experimental evolution we have have tested growth constraints of *C. phytofermentans* and developed strains with improved biofuel-related properties. Full genome re-sequencing has allowed us to identify mutations present at varying allele frequencies in cultures with increased rates of biomass degradation. Most mutations are in carbohydrate degradation modules including the promoter regions of glycoside hydrolases and amino acid substitutions in ABC transport binding proteins involved in carbohydrate uptake, signal transduction sensors that detect specific carbohydrates, proteins that effect the export of extracellular enzymes (e.g. cellulase), and regulators of unknown specificity. Protein structural modeling of the ABC transporter complex proteins identified mutations that may be involved in the recognition of carbohydrates by substrate-binding proteins and the communication between the transmembrane domains of the permease and the ATPase. The results reveal new strategies for evolving and engineering microorganisms for the production of biofuels from plant feedstocks.

RNA-Seq and DNA Microarray Analyses of *Clostridium thermocellum* Biomass Fermentation Transcriptomes

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Fuels from cellulosic biomass are among the leading options to meet sustainability and energy security challenges associated with fossil fuels. *Clostridium thermocellum* is a thermophilic, obligate anaerobic bacterium that can solubilize biomass during fermentative growth to produce ethanol and other metabolic byproducts. The bacterium possesses productivity advantages associated with thermophilic growth and is capable of producing its own enzymes for lignocellulosic biomass breakdown. However, relatively little is known about the transcriptional machinery it uses and may alter when growing on different biomass substrates.

The *C. thermocellum* ATCC 27405 genome was sequenced and annotated in 2006. Recently, we used the Prodigal gene finding program to predict 3,173 candidate protein-encoding gene models for the *C. thermocellum* ATCC 27405 genome. The majority of genes were consistent with the previous version, but there were important differences including 89 new genes, 67 that were deleted and another 147 that were modified.

To investigate what genes are important for growth on different biomasses, we cultured *C. thermocellum* ATCC 27405 at 58°C in duplicate controlled fermentors with either 5 g/L (dry weight basis) pretreated Switchgrass or Populus as substrates. *C. thermocellum* biomass fermentation profiles were collected for 140 h and showed the cells actively produced ethanol and other metabolic byproducts until 40 h post-inoculation. Samples were collected at 12 h and 37 h for transcriptomic profiling using NimbleGen high-density DNA microarrays and Illumina-based RNA-Seq analysis.

We generated approximately 100M reads for each sample, and > 99.6 % of the reads did not map to 5, 16, 23S rRNA sequences. One of the most highly expressed genes on biomass was a newly predicted gene. Differences between RNA-Seq and microarray transcript profiles and between different conditions will be discussed.

Metabolic Engineering of *Clostridium acetobutylicum* ATCC 824 for the High-Yield Production of Biofuels

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A *Clostridium acetobutylicum* strain was metabolically engineered to produce alternative biofuels at a high yield. A synthetic, polycistronic isopropanol operon expressing the *ctfAB* and *adc* genes (encoding a CoA transferase and an acetoacetate decarboxylase, respectively) from *C. acetobutylicum* and the *sadh* gene (encoding a primary/secondary alcohol dehydrogenase) from *C. beijerinckii* NRRL B593 was constructed and introduced into the pCLF1 shuttle vector under the control of the *ptb* or *thl* promoters, yielding the plasmids pCLF942 and pCLF952, respectively. Both plasmids were introduced into the *C. acetobutylicum* ATCC 824 *Δcac15* strain and *C. acetobutylicum* ATCC 824 *Δbuk* strain in which the butyrate kinase- and the phosphotransbutyrylase-encoding genes have been completely and partially deleted, respectively, consequently producing a low amount of butyrate. All recombinant strains were first evaluated in flask cultures without pH regulation. The two best strains, *C. acetobutylicum* 824 *Δbuk* (pCLF942) and *C. acetobutylicum* 824 *Δbuk (pCLF952),* were further evaluated in a controlled batch fermentation at pH 5. Our results demonstrated that the expression of the synthetic isopropanol operon under the *thl* promoter led to higher titers of butanol, isopropanol and ethanol and, subsequently, to a higher total solvent yield (0.33 g/g). Finally, the impact of a culture pH on the controlled batch fermentation of *C. acetobutylicum* ATCC 824 *Δbuk (pCLF952)* was determined. The best performance was obtained at pH 4.8, resulting in a final biofuel titer of 21 g/l at a yield of 0.35 g/g glucose and a productivity of 0.6 g/l h. Such high performances for IBE biofuel production in batch cultures open the opportunity to develop a new way to produce alternative biofuels.

Use of Municipal Solid Waste as a Feedstock for the Butanol Fermentation

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The UK government's renewable transport fuel obligation for 2013 is a 5% biofuel blend (by volume) while the EU Commission's 2020 target is 10% (by energy), the later translates into a demand for 4M t/a of biofuel in the UK alone. Biobutanol is an advanced biofuel with superior fuel properties and has the potential to substitute for both ethanol and biodiesel in the biofuel market. Biobutanol has a higher energy density (similar to petrol/gasoline) and better performance characteristics than bioethanol. Biobutanol (n-butanol) is also a high value chemical for a variety of ploymers, plastics and resins in a market worth over $5B. Today, the fermenation route to butanol is economic for the chemical market but biobutanol cannot compete in the biofuels market. The key to reducing cost and unlocking the biofuel market is to use lower cost feedstocks (approximately 70% of production cost is attributed to the feedstock).

One tremendous opportunity, relevant to the UK, is food and municipal solid waste (MSW). MSW has a negative value due to a tax imposed by Government for disposal via landfill (currently £64/T and rising to £80/T by 2014). Autoclave process technology, involving steam injection and mechanical agitation, can be used to process MSW in a clean, safe and more cost effective than conventional disposal methods. Glass, metals and textiles can easily be removed post treatment leaving a relatively clean cellulosic fibre that can be used as a fermentation feedstock.

Green Biologics Ltd., in collaboration with Aerothermal, Biocatalysts and North Energy Associates has received grant funded from the UK Technology Strategy Board to develop and test, at pilot scale, an integrated process for converting MSW to butanol using a combination of autoclave treatment and simultaneous saccharification and fermentation. We report on progress with enzymatic hydrolysis and butanol fermentation at the mid-point in this two year project.

Engineering *Clostridium* for High-Titer and High-Yield Biobutanol Production

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Butanol is an important industrial solvent and potentially a better transportation fuel than ethanol. Conventional acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* or *C. beijerinkii* is limited by its poor butanol yield (<0.25 g/g glucose), low productivity (<0.5 g/L·h), low final butanol concentration (<15 g/L), and poor process stability. To overcome these limitations, we have developed several metabolically engineered clostridia mutant strains for butanol production. A novel solventogenic Clostridium was developed by introducing butanol biosynthesis pathway and blocking acid producing pathways in non-solventogenic acid-forming *C. tyrobutyricum* which has relatively simple metabolic pathways with high flux toward butyryl-CoA, the precursor for butanol biosynthesis, and high butanol tolerance. A high butanol titer of >16 g/L with a high butanol yield of >0.30 g/g substrate were obtained in batch fermentation. Butanol was the only major fermentation product and no acetone was produced by this mutant, allowing simplified downstream processing for product purification.

We have also developed a mutant strain of *C. acetobutylicum* with high butanol tolerance and productivity through evolutionary engineering in a fibrous bed bioreactor (FBB). The high butanol tolerant strain JB200 can produce up to 21 g/L butanol in free-cell fermentation and 28.2 g/L butanol in the FBB fermentation. The fermentation with continuous gas stripping for *in situ* butanol recovery resulted in a final product containing >150 g/L of butanol in the condensate that can be purified with a low energy input. This process also allows the use of highly concentrated substrate, greatly reducing the water usage in the fermentation process. Overall, the integrated process with the metabolically engineered clostridia significantly reduces biobutanol production cost to a level that can compete favorably with bioethanol and other biofuels.

Continuous Butanol Fermentation from Low-Value Sugar-Based Feedstocks by *Clostridium saccharobutylicum* DSM 13864

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Butanol is an important industrial chemical, chemical feedstock, and also a potential biofuel. The cost of substrate is one key problem associated with the biobutanol production. To exploit the utilization of low-value sugar-based feedstock, continuous butanol fermentation by *Clostridium saccharobutylicum* DSM 13864 from cane molasses and lignocellulosic hydrolysates of agricultural residues was investigated.

Using cane molasses as substrate, total solvent of 17.88 g/L (butanol 11.86 g/L) was attained after 36 h of batch fermentation in a 5-L bioreactor, the productivity and yield were 0.50 g/L/h and 0.33 g ABE/g sugar consumption, respectively. To further enhance the productivity, a two-stage semi-continuous fermentation process was steadily operated for over 8 days (205 h) with average productivity (stage II) of 1.05 g/L/h. In a 5-stage continuous fermentation, the solvent production could be steadily operated for over 200 h at dilution rate (D) of 0.1 h^{-1}, the average total solvent and productivity were 12.41 g/L and 0.25 g/L/h, respectively.

Using an alkaline pretreatment and enzyme hydrolysis procedure, corn stover hydrolysate was prepared in this study. In the batch fermentation, total solvent of 16.1 g/L (butanol 10.59 g/L) was reached after 40 h, the productivity and yield were 0.40 g/L/h and 0.33 g ABE/g sugar consumption, respectively. A temperature-shifting strategy (37℃ to 30℃) was adapted in a 5-stage continuous fermentation for enhanced solvent accumulation. The continuous process was operated for 269 h at 0.1 h^{-1} D. The average solvent during the stable solvent-production stage (after around 80 h) was 12.28 g/L (butanol 8.50 g/L), and the average solvent productivity was 0.25 g/L/h, and the maximum total solvent was up to 15.29 g/L (including 10.82 g/L butanol).

This work provides valuable knowledge for the development of industrial continuous butanol fermentation process using renewable sugar-based feedstocks.

Genome-wide Transcriptomic Analysis in *Clostridium beijerinckii* 8052 using Single-Nucleotide Resolution RNA-Seq Technology

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Clostridium beijerinckii is a prominent solvent-producing microbe. The transcriptome structure configuration and genome-wide transcription are still not well understood.

In this study, we conducted a genome-wide transcriptomic analysis in *C. beijerinckii* 8052 during a batch fermentation using high-throughput RNA-Seq technology. We identified and confirmed the structure of important gene operons involved in metabolic pathways for acid and solvent production, including *pta-ack*, *ptb-buk*, *hbd-etfA-etfB-crt* (*bcs*) and *ald-ctfA-ctfB*-adc (*sol*) operons; we defined important operons related to chemotaxis/motility, transcriptional regulation, stress response and fatty acids biosynthesis, etc. We discovered 20 previously non-annotated regions with significant transcriptional activities and 15 genes whose translation start codons were likely mis-annotated. In addition, the gene expression profiles indicated that the glycolysis genes were comparatively more highly expressed during acidogenesis phase. The acid formation genes were down-regulated at the onset of solvent formation. The *sol* operon genes exhibited highly-coordinated expression. Out of the > 20 alcohol dehydrogenase genes in *C. beijerinckii*, Cbei_1722 and Cbei_2181 were highly up-regulated before the onset of solventogenesis, corresponding to their key roles in primary alcohol production. Most sporulation genes in *C. beijerinckii* 8052 demonstrated similar temporal expression patterns to those observed in *B. subtilis* and *C. acetobutylicum*, while sporulation sigma factor genes *sigE* and *sigG* exhibited accelerated and stronger expression in *C. beijerinckii* 8052, which is consistent with the more rapid forespore and endspore development in this strain.

The results from this work provided important supplemental information on the accuracy of genome annotation, revealed additional gene functions and regulation in *C. beijerinckii*, and provided insights for further *C. beijerinckii* strain improvement.

Engineering the *Clostridium thermocellum* Cellulosome

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Although *Clostridium thermocellum* is well known for its ability to aggressively degrade plant biomass, we have recently shown that cellulosomes themselves are not as effective acting against plant cell walls as are fungal enzymes. We are seeking to understand this difference in fundamental performance in the context of building improved cellulosomal systems. We have taken a reductionist approach to the problem by first studying the proteome of *C. thermocellum* as a function of growth on different substrates, then characterizing the performance of the primary glycoside hydrolases known to populate scaffoldins, and finally modeling these enzymes using molecular dynamics. We will present recent findings regarding the mode of action of CbhA and CelS from *C. thermocellum*. We will also compare and contrast the digestion of cellulose by key cellulase systems using TEM. Finally, early attempts to build engineered catalysts based on cellulosomes and multi-functional enzymes will be reviewed. Specifically, we used modular cooperation to improve the activity of cellulosomal cellulases, including both multi-modular cellulases with single catalytic modules and multi-functional cellulases containing two or more catalytic modules each. Three types of large “linkers”, categorized in terms of amino-acid composition and sequence as proline/threonine-rich, proline/threonine-rich with additional glycine-enrichment, and generic (not enriched in any specific residues) respectively, have been used in construction of such cellulases. Two chimeras of *C. thermocellum* CbhA were designed and constructed by replacement of its two consecutive X1 domains by each of the other two types of linkers; one of these linker-substituted CbhA constructs showed significant enhancement in activity on crystalline cellulose relative to that of wild-type enzyme. Nine artificial multi-functional cellulases were also constructed, so that all had a truncated *C. thermocellum* CbhA at the *N*-terminal residue, connected to each of three endoglucanase domains by each of three different linker segments, generating a 3x3 matrix which tests linker-catalytic-domain interactions. Some of these multi-functional cellulases showed much higher activity than that of the simple mixture of their components. This high intramolecular synergy demonstrates that construction of multi-functional cellulases is a promising approach to improve cellulase activity. Other strategies will also be discussed.

Clostridium thermocellum: Metabolic Engineering and Plant Cell Wall Solubilization

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Clostridium thermocellum exhibits among the highest rates of cellulose solubilization described and produces an elaborate complex of functionally-distinct proteins, termed the cellulosome. This organism is thus of interest for fundamental studies aimed at mechanistic understanding of the action of cellulase enzyme systems and also as a starting point for developing industrial microorganisms. A recent report [Brown et al., 2011] indicates that ethanol inhibition of wild-type strains is mediated by metabolic rather than biophysical mechanisms.

Following the development of a genetic system in collaborative work by Dartmouth and Mascoma Corp., with helpful advice along the way from attendees of the *Clostridium* meeting, several dozen targeted knockouts have been carried out, and a substantial set of genetic tools – including removable markers, inducible promoters, and temperature sensitive plasmids – has been developed. These tools will briefly be described and their application to increasing ethanol yield in *C. thermocellum* will be presented.

Knocking out genes leading to acetic and lactic acids was not sufficient to achieve near theoretical ethanol yields in *C. thermocellum* although this approach has met with success in another thermophile *Thermoanaerobacterium saccharolyticum* as well as enteric bacteria. However, manipulation of redox reactions in combination with increased understanding of distinctive features of glycolysis in *C. thermocellum* enabled development of mutant strains that produce ethanol at $\geq$ 0.45 g ethanol/g sugar equivalent fermented.

Previously-unreported comparative studies will be presented involving various cellulosic substrates (wood and grass) and conversion systems (simultaneous saccharification and fermentation using fungal cellulase, fermentation by pure cultures including but not limited to *C. thermocellum,* and fermentation by enrichments developed from environmental innocula). Among several interesting implications, our results indicate that bacterial fermentation is far more effective than fungal cellulase at mediating plant cell wall solubilization for some but not all feedstocks.

The Weizmann process revisited for cellulosic Butanol production

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Clostridium acetobutylicum ATCC 824 produces a multicomponent protein complex (with an apparent molecular weight of 665 kDa), or cellulosome, unable to hydrolyze crystalline cellulose. To try to understand why this cellulosome is inactive, the three major cellulases of the cellulosome, Cel9C, Cel9X and cel48A, were characterized from a biochemical point of view. The recombinant form of the Cel9C, Cel9X and cel48A cellulases, tagged by a C-terminal histidine tail, were over-expressed in *Escherichia coli* and purified by affinity chromatography on a Ni-nitrilotriacetic acid column. The recombinant Cel9C and Cel9X were active on carboxymethylcellulose, amorphous cellulose but also on crystalline cellulose while Cel48A was inactive on all substrate. Furthermore, the ability of Cel9C, Cel9X and Cel48A to interact with the full size CipA scaffolding protein was demonstrated by binding assays using surface plasmon resonance (SPR). These results demonstrate that the lack of activity of the *C. acetobutylicum* cellulosome on crystalline cellulose is due to the deficiency in Cel48A, the most abundant cellulase of the cellulosome. To fix this problem, we first constructed an artificial gene coding for an active hybrid protein SAFA-48, composed of the catalytic domain of Cel48F from *C. cellulolyticum* and both signal sequence and dockerin domain of Cel48A from *C. acetobutylicum*. Using our marker-less with positive selection method for successive gene deletion/integration in *C. acetobutylicum*, we first deleted the cel48A gene. Further rounds of chromosome manipulation using the same technique allowed us to replace, on the chromosome of the Δ*cel48A* mutant, the original gene by the SAFA-48 encoding gene, without any modification of the genetic context from the transcriptional or translational point of view.

The new *C. acetobutylicum* strain obtained (Δ*cel48A::SAFA*) was evaluated for its ability to grow on different cellulosic substrates.

Cellulosic Butanol - A Sustainable Advanced Next Generation Biofuel

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Global demand for liquid biofuels is rising rapidly due to the growing scarcity and security issues of crude oil. Growing regulations on greenhouse gas emissions in transportation and the underlying positive impact biofuels can have on rural and agricultural employment gives reason to produce more. The market is forecast to be worth $200 billion by 2020.

Biobutanol, produced via a fermentation route, is a superior biofuel that fits the existing fuel infrastructure; yet has a better energy density and performance than ethanol. It can also be produced from lower cost and more sustainable cellulosic feedstock. Biobutanol can substitute for both ethanol and gasoline and be blended up to 40% in diesel and biodiesel.

We report on the world's first practical demonstration of biobutanol as an advanced transportation biofuel and demonstrate the efficacy of butanol versus gasoline. We also address some of the technical and economic challenges that need to be overcome in order to make the Clostridia fermentation route commercially viable.

Identification of the *Clostridium cellulovorans* cellulosomal subunits on soft biomass degradation

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Clostridium cellulovorans can completely degrade soft biomass such as a rice straw by producing a large extracellular enzyme complex called the cellulosomes. *C. cellulovorans* changes the cellulosomal subunits for efficient degradation of carbon source. To analyze the cellulosomes, we are able to identify enzymes that contribute to degrade biomass. In this study, we identify cellulosomal subunits carrying out proteome analysis of culture *of C. cellulovorans*.

C. cellulovorans 743B was grown anaerobically except for the carbon source, which was rice straw or bagasse. The culture was centrifuged, filtered and precipitated with (NH4)2SO4 (supernatant fraction). The pellet was dissolved in Tris-HCl Buffer (50mM, pH 7.5), dialyzed against the same buffer, and incubated for an hour after mixed with Avicel. The slurry was centrifuged and separated supernatant (non-binding fraction) and pellet. The pellet was dissolved in SDS sample buffer (binding fraction). Each fraction was applied to SDS-PAGE gel. After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB) and immunoblotted with anti-CbpA antibody. The separated proteins was cut out from the gel and carried out MS/MS analyses. Sequenced amino acid sequence was compared genomics information.

CbpA was detected in supernatant fraction and binding fraction by immunoblotted analysis. The patterns of proteins band stained with CBB were different, suggesting that the different cellulosomal subunits were induced expression. The cellulosomal subunit ExgS, EngE and EngK were determined by MS/MS analysis and in comparison with databases in each culture (ExgS) and only bagasse (EngE and EngK) culture, therefore, it is able to collect the cellulosomal subunits from crude culture.

Glucose Sensing in the Solventogenic Clostridia

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For many bacteria, including the solventogenic clostridia, glucose is a dominant carbon source that represses metabolic systems for alternative carbon sources. Elimination of this carbon catabolite repression (CCR) would be beneficial for the ABE fermentation since it would allow for the simultaneous utilization of carbon sources present in the substrate. According to the mechanism proposed for firmicutes, CCR is controlled by the PEP-dependent phosphotransferase system (PTS), a multi-component system that catalyses uptake and phosphorylation of its sugar substrates and acts as a sensor of the nutrient status of the environment. A strain of *Clostridium acetobutylicum* in which a glucose PTS (*glcG* gene) was inactivated could still ferment glucose and extracts exhibited glucose PTS activity, indicating that glucose could be taken up and phosphorylated by at least one of the 12 other phosphotransferases encoded within the genome. Nevertheless, compared to the wild-type the mutant strain showed weaker repression of metabolism of xylose and arabinose in the presence of glucose, suggesting that GlcG plays a role in the repression mechanism. The *Clostridium beijerinckii* genome encodes 42 phosphotransferase systems, three of which belong to a phylogenetic cluster related to *C. acetobutylicum* GlcG. One of these systems has been shown to be a functional glucose PTS that also recognises mannose as a substrate. Progress in characterizing these three systems, which may be involved in CCR in *C. beijerinckii*, will be presented.

Pathway Reconstruction and Flux Quantification of Pentose Metabolism in Solventogenic Clostridia

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Substrate cost is a major factor impacting economics of fermentative solvent production by clostridia. To reduce the substrate cost, abundant and inexpensive lignocellulosic materials could be used, and one of their major components is pentose-rich hemicellulose. Solventogenic clostridia including *Clostridium acetobutylicum* and *Clostridium beijerinckii* are capable of utilizing the hemicellulosic pentoses, xylose and arabinose. However, the knowledge about pentose utilization pathways and their regulation in clostridia is rather limited. In this study we combined comparative genomics with experimental techniques to reconstruct pentose utilization pathways in *Clostridium* species directly from their genome sequences. The subsystems-based comparative genomic approach was used, which integrated the analysis of conserved operons and regulons with pathway reconstruction across a large variety of genomes. This approach allowed us to efficiently predict the functions of novel genes, which were then experimentally verified through a combination of genetic and biochemical techniques. We also used the ^{13}C-based metabolic flux analysis technique to identify the in vivo operation of pentose metabolism in solventogenic clostridia. Comparison of the reconstructed clostridial catabolic pathways with *Bacillus subtilis* identified multiple differences that are manifested at various levels, from the presence or absence of centain sugar catabolic pathways, non-orthologous gene replacements and alternative biochemical routes to a different organization of transcription regulatory networks.

Arabinose is Metabolized Via a Phosphoketolase Pathway in *Clostridium acetobutylicum*

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Recent studies of *C. acetobutylicum* during growth on different sugars revealed an increase in CAC1343 mRNA expression during growth on arabinose but not xylose, indicating CAC1343 contributes to arabinose metabolism. Based on sequence analysis of CAC1343, we hypothesized CAC1343 encodes a putative phosphoketolase. A novel zymogram assay revealed that recombinant CAC1343 in *E. coli* lysates exhibited bi-functional xylulose-5-p/fructose-6-p phosphoketolase (XFP) activity. Further examination showed purified XFP to be thiamine diphosphate dependant with specific activities of 21 U/mg on xylulose-5-P and 6.9 U/mg on fructose-6-P. Analysis of metabolites produced during *C. acetobutylicum* growth on xylose and glucose revealed similar final acetate: butyrate ratios of 0.72 and 0.83, respectively. In comparison, growth on arabinose induced a metabolic shift towards oxidized products, with a final acetate:butyrate ratio of 1.95. This shift towards oxidized products is expected with increased XFP expression, indicating arabinose is metabolized by a phosphoketolase pathway while xylose is likely metabolized through the pentose phosphate pathway.

Production of Acetone, Butanol and Ethanol from native North Sea seaweed species

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Seaweeds (macroalgae) have been used in East Asia since ancient times as vegetables. Currently, seaweeds are worldwide used as food and as source of chemicals (i.e. thickening agents, gelling agents and phycolloids). Annually, 7-8 million tonnes seaweeds are harvested, with an estimated total value of the products of US$ 5-6 billion[1]. Because of the special chemical composition (wide range in sugars, polymers, etc) of seaweeds and the possibility of cultivating them at large scale in the ocean with high yields, they are potential feedstocks for production of renewable chemicals and fuels [2].

Seaweed biomass is highly suited as raw material for the co-production of chemicals, biofuels and energy via the biorefinery approach. In the Seaweed Biorefinery project, adjusted and efficient biorefinery strategies and concepts are developed for seaweed biomass. In the proposed process, the seaweed is first dewatered and subsequently fractionated into its main components. In the second stage of the biorefinery, the main components, sugars, proteins and minerals are converted into bulk chemicals and energy carriers. The project aims to develop catalytic, enzymatic and fermentative conversion routes.

The conversion of sugars from brown (*Saccharina latissima*) and green (*Ulva lactuca*) seaweed species into acetone, butanol and ethanol (ABE) by Clostridial strains has been studied. Hydrolysates from seaweeds have been prepared, which contain a mix of different sugars depending on the species. Fermentations using *Clostridium acetobutylicum* and *C. beijerinckii* strains show that these strains are able to ferment (most of) the sugars in seaweeds, and that the fermentation parameters and product range vary significantly between the two strains.

Acknowledgement
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References:
[1] McHugh D. J. (2003) “A guide to the seaweed industry” . FAO Fisheries Technical Paper (FAO). 0429-9345, no. 441, ISBN 92-5-104958-0.
[2] Kraan, S. (2011) Mitigation and Adaptation Strategies for Global Change, p.1-20, online

Proteomic Analysis of *Clostridium acetobutylicum* from Butanol Fermentation

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Clostridium acetobutylicum is an obligately anaerobic bacterium capable of converting a wide variety of sugars present in the plant biomass into acetone, butanol and ethanol (ABE) through fermentation process. The traditional ABE fermentation suffers from several limitations such as poor substrate utilization rate, butanol toxicity and low productivity that make the production of butanol which is considered as a second generation biofuel an incompetent industrial process. The concurrent use of hexose and pentose sugars in the biomass by *C. acetobutylicum* for butanol production is a desirable fermentation characteristic from an economic point of view.

The potential for improving the butanol production lies in the ability to gain an in-depth understanding of *C. acetobutylicum* which constituted the aim of our research. Shotgun proteomics provides a direct approach to study the whole proteome of an organism and using this technique we have identified 894 different proteins in *C. acetobutylicum* from ABE fermentation process. This includes 717 and 826 *C. acetobutylicum* proteins using glucose and xylose substrates, respectively. A total of 649 proteins were found to be common and 22 significantly differentially expressed proteins were identified between the two substrates.

This is the first study to report the large scale investigation of *C. acetobutylicum* ATCC 824 during ABE fermentation between glucose and xylose substrate from a single-time data point at the proteomic level. Our results revealed that the chemotactic activity is lost and proteins involved in flagellar mechanism and butanol production pathway were significantly less in expression with *C. acetobutylicum* from xylose utilized ABE fermentation compared to glucose. These findings provide a detailed insight into the fermentative mechanism of *C*. *acetobutylicum* which potentially aids in the genetic and metabolic engineering of this industrially important bacterium in order to achieve an economically viable butanol production system.

Feeding Acetogenic Clostridia Electricity to Produce Organic Commodities From Carbon Dioxide

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Microbial electrosynthesis is a sustainable process for producing transportation fuels and other organic commodities from carbon dioxide by directly feeding microorganisms electrons at electrode surfaces. When solar power is the source of electricity, microbial electrosynthesis represents a form of artificial photosynthesis that offers many potential advantages over biomass-based strategies for commodity production.

Clostridium ljungdahlii is now serving as an important chassis for the development of microbial electrosynthesis because it is capable of accepting electrons from negatively poised electrodes for the reduction of carbon dioxide through the Wood-Ljungdahl pathway. Acetate is the primary product of microbial electrosynthesis with wild-type *C. ljungdahlii.* However, optimization of tools for genetic manipulation of *C. ljungdahlii* has made it feasible to produce strains with appropriate gene deletions and knock-ins to divert carbon and electron flow from the production of acetate to the synthesis of more desirable products.

Genome-scale transcriptomic and proteomic studies have provided insights into mechanisms for energy conservation during autotrophic growth and have identified bottlenecks in the production of desired commodities in engineered strains. Transcriptomic and proteomic studies under different growth conditions have revealed regulatory switches expected to be useful tools in metabolic pathway design. Adaptive evolution approaches have led to strains that can grow more effectively via carbon dioxide reduction and with simpler medium requirements.

C. ljungdahlii strains engineered for microbial electrosynthesis of organic commodities are likely to have application in other processes. These strains are capable of growth with hydrogen or carbon monoxide as the electron donor as well as growing in syngas mixtures. Conversion of lignocellulosic biomass to syngas followed by syngas conversion to fuels or other organic compounds with *C. ljungdahlii* is a promising strategy for overcoming well-documented obstacles to effect utilization of lignocellulosic materials for biofuel production. Other relatively inexpensive sources of electrons in the form of carbon monoxide or hydrogen may be available.

Thus, these studies with *C. ljungdahlii*, as well as advancements in reactor design, are advancing microbial electrosynthesis and other alternative biocommodity approaches, toward practical application. Furthermore, the ability for the fist time to genetically manipulate an acetogenic microorganism is aiding in elucidating the basic physiology of this environmentally important process.

Production of Fuels and Chemicals from Industrial Waste Gases

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LanzaTech has developed a gas fermentation platform for the production of alternative transport fuels and commodity chemicals from carbon monoxide, hydrogen and carbon dioxide containing gases. LanzaTech technology uses these gases in place of sugars as the carbon and energy source for fermentation thereby allowing a broad spectrum of resources to be considered as an input for product synthesis. At the core of the LanzaTech process is a proprietary *Clostridium* microbe capable of using gases as the only carbon and energy input for product synthesis. To harness this capability for the manufature of a diverse range of commercially valuable products, the company has developed a robust Synthetic Biology platform to enable a variety of novel molecules to be synthesised via gas fermentation.

LanzaTech initially focused on the fermentation of industrial waste gases for fuel ethanol production. The company has been operating pilot plant in New Zealand that uses direct feeds of steel making off gas for ethanol production since 2008. This platform technology has been further successfully demonstrated using a broad range of gas inputs including gasified biomass and reformed natural gas. LanzaTech has developed the fermentation, engineering and control systems necessary to efficiently convert gases to valuable products. A pre-commercial demonstration scale unit processing steel mill waste gases was commissioned in China during the 2nd quarter of 2012. Subsequent scale-up of this facility is projected for the 2013 and will represent the first world scale non-food based low carbon ethanol project.

More recently LanzaTech has developed proprietary microbial catalysts capable of converting carbon dioxide in the presence of hydrogen directly to value added chemicals, where-in CO_2 is the sole source of carbon for product synthesis. Integrating the LanzaTech technology into a number of industrial facilities, such as steel mills, oil refineries and other industries that emit Carbon bearing waste gases, can reduce carbon footprint while producing transportation fuels and chemicals to enhance overall profitability.

Installing the Wood-Ljungdahl (WL) Pathway in *Clostridium acetobutylicum*

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The ability to express the genes necessary for instating a functional Wood-Ljungdahl (WL) pathway into *C. acetobutylicum* constitutes a major advance in that the WL pathway has never before been instated into a heterologous organism. In the specific case of *C. acetobutylicum*, it would allow one to grow this organism to high cell densities and then switch the growth from a sugar to CO_2/H_2 or CO, or to a mixotrophic culture, thus enabling the production of carboxylic acids and solvents from these gases in a flexible and scaleable fermentation system. From the fundamental point of view, the ability to install this complex, primordial pathway in a heterologous host would constitute a major advance in cell/metabolic engineering that would open new horizons for pathway engineering and synthetic approaches in the genus/class *Clostridium*. Comparative genetic analysis of three sequenced acetogens, *C. ljungdahlii, C. carboxidivorans,* and *C. difficile,* showed that the WL pathway genes of these three organisms are concentrated in a highly conserved, 18 kilobase region on their respective genomes. By examining also the *C. acetobutylicum* genome, we focused on expressing 8 core genes coding for enzymes and accessory proteins. To achieve this goal, we developed a system of two co-existing plasmids combined with a method to integrate some genes (starting with a formate dehydrogenase gene) into the chromosome. We will present the new chromosomal integration method and the step-by-step verification of the expression and function of the cloned genes/proteins as well as culture experiments to test the functional installation of the WL pathway in *C. acetobutylicum*.

Clostridia from a Pilot Plant for Enhanced Hydrogen Gas Production: Isolation, Molecular Characterization and Hydrogenase Genes Cloning for Exploitation in Biotechnology

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Hydrogen is considered a promising fuel that can replace fossil fuels in the future. To this purpose, the availability of clean and efficient industrial processes is necessary and the ability of microorganisms to produce hydrogen from low cost waste materials is of high interest.

In this study a pilot plant was engineered for the fermentative production of hydrogen gas using waste materials (vegetable scraps). The microbial consortium responsible for hydrogen production at the highest efficiency was characterized. Bacteria were isolated and characterized by means of 16S rDNA amplification, RFLP and sequencing, allowing the identification; the ability of each species to produce hydrogen was also tested. The consortium was composed by several non-hydrogen producing bacteria (*Enterococcus sp., Lactobacillus plantarum* and *Staphylococcus hominis*) and four species belonging to the genus *Clostridium (C. butyricum, C. beijerinckii, C. perfringens* and *C. bifermentans)*, that were directly responsible for H_2 production. To study the role of the different enzymes responsible for H_2 production in each microorganism, *C. beijerinckii* and *C. perfringens* were selected for more detailed studies of [FeFe]-hydrogenases gene expression.

Subsequently, the [FeFe]-hydrogenase gene *hydA* from *C. perfringens* was cloned and the active enzyme (CpHydA) was recombinantly expressed and affinity purified, allowing structural and functional characterization. CpHydA has the spectral features typical of [FeFe]-hydrogenases and it is able to evolve hydrogen at high turnover frequencies (k_{cat} up to 1000 sec^{-1}) and to perform catalysis in immobilized systems on TiO_2 electrodes as electron suppliers (efficiency from to 80 to 100%).

In conclusion, the isolation and characterisation of the bacterial consortium from an efficient pilot plant fed with waste materials allowed the identification and purification of a novel [FeFe]-hydrogenase that is a valuable catalyst with extended applicative potential.

Central metabolism of the anaerobic lignocellulolytic thermophile *Clostridium stercorarium* subsp. *stercorarium* DSM8532

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Clostridium stercorarium DSM8532 (8532) is an anaerobic thermophile with high reported ethanol production on lignocellulose or its component saccharides and a unique two-gene cellulase system that contrasts with other cellulolytic clostridia. Central metabolic pathways for this organism are poorly described, therefore the goal of this study was to characterize 8532 end products in the context of whole genome sequencing (454/Roche) with automated annotation using the Integrated Microbial Genomes platform, and proteomic characterization by 2D fractionation and mass spectrometry (MS) on peptides extracted from mid-exponential cells grown on cellobiose and xylan. 8532 reached maximum density on cellobiose, xylose and xylan within 24 h, however dense growth and comparable ethanol concentrations were not observed until Day 6 on cellulose. Cellobiose and xylose were consumed simultaneouslly in cultures containing both. Ethanol concentration was highest on xylan (19mM) and cellulose (25mM) at final timepoints. Previously described cellulases/hemicellulases as well as potentially novel putative glycosyl hydrolases were annotated. Most genes of central metabolism for lactate/acetate/ethanol production from hexoses/pentoses were observed and expression confirmed by proteomic analysis. Acetaldehyde dehydrogenase and putative alcohol dehydrogenases were identified, however peptides from the bifunctional acetaldehyde/alcohol dehydrogenase (*adhE*) ortholog were highly abundant, suggesting a central role in ethanol formation. Acetate kinase was not observed by MS, despite observed acetate production. Although pyruvate kinase is present in the genome, it was not observed by MS, while peptides for pyruvate dikinase were moderately abundant, suggesting an alternative pathway for pyruvate formation as observed for *C. thermocellum*. Proteomic and transcriptomic analyses are ongoing in order to tie gene expression levels to variability in cellulose degradation and solvent:acid ratios.

Mathematical Modelling of the pH-induced Metabolic Shift Unravels a Heterogeneous Phase Transition

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The acetone-butanol-ethanol (ABE) fermentation of *Clostridium acetobutylicum* attracts new attention because it provides a potential alternative for the synthesis of value added chemicals to petroleum and other fossil reserves. This fermentative metabolic process comprises two distinct metabolic states that differ in their product spectrum. Growing on starch or sugars the predominant fermentation products are acetate and butyrate during acidogenesis (high pH). In contrast, *C. acetobutylicum* produces the solvents acetone and butanol during solventogenesis (low pH). In a continuous culture under phosphate limitation, the shift between both metabolic states can be induced by changes of the external pH level. Existing models were unable to reproduce the dynamics of this phase transition.

Here, we present a kinetic model of ABE fermentation in continuous culture that incorporates metabolic reactions, changes of the proteomic composition, pH-dependent kinetics, and population growth. Our analyses of three independent shift experiments led us to the hypothesis that the pH-induced metabolic shift is a heterogeneous process governed by two sub-populations. The extension of our kinetic model with respect to this hypothesis better reproduces the experimental data and led us to the conclusion that an acidogenic and a solventogenic phenotype coexist during the transition from acidogenesis and solventogenesis and vice versa. Furthermore, we show that the measured evolution of the optical density (OD_{600}) represents a transition between these two phenotypes.

Further analysis revealed that the metabolic shift is governed by three different time scales under the experimental conditions. The rapid decline of the external pH level affects the kinetic properties of the ABE fermentation. Interestingly, the acidogenic population seems to be able to respond to these environmental changes until the pH drops below a critical value. Then, the fermentation pathway is seriously disturbed, so that the acidogenic cells are incapable to grow further. Simultaneously, a low level solventogenic population is increasing. The rise of the solventogenic phenotype starts around 20 hours after the pH-shift and is completed after approximate 50 hours.

The origin of the solventogenic cells is still unsettled. We suggest two potential explanations: First, the clostridial population is heterogeneous during both metabolic states. Second, few acidogenic cells, selected by unknown criteria, are able to shift their phenotype. Intracellular variability inherent to microbial populations might cause this phenomenon.

Analysis of the Transcriptional Response to n-Butanol Challenges during Acidogenic Continuous Culturing of *Clostridium acetobutylicum*

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A major obstacle in the commercial production of butanol is the toxicity of the solvent itself. For *Clostridium acetobutylicum* solvent concentrations exceeding more than 1.5-2% inevitably lead to growth inhibition and cell death. To gain more insight into the butanol stress response of *C. acetobutylicum* the transcriptional response of a steady state acidogenic culture to different levels of *n*-butanol (0.25-1%) was investigated.

No effect was observed on the fermentation pattern and the expression of genes involved in glycolytic- and solventogenic pathways. Elevated levels of butanol mainly affected class I heat-shock genes (*hrcA, grpE, dnaK, dnaJ, groES, groEL, hsp90*), which were upregulated in a dose- and time-dependent manner, and genes encoding proteins involved in the membrane composition (*fab* and *fad* or glycerophospholipid related genes) and various ABC-transporters of unknown specificity. Interestingly, *fab* and *fad* genes were embedded in a large, entirely repressed cluster (CAC1988-CAC2019), which *inter alia* encoded an iron-specific ABC-transporter and molybdenum-cofactor synthesis proteins. Of the glycerol-phospholipid metabolism, the glycerol-3-phosphate dehydrogenase (*glpA*) gene was highly upregulated, whereas a glycerophosphodiester ABC-transporter (*ugpAEBC*) and a phosphodiesterase (*ugpC*) were repressed. On the megaplasmid, only a few genes showed differential expression, e.g. a rare lipoprotein (CAP0058, repressed) and a membrane protein (CAP0102, upregulated) gene. Overall, we observed that the response was most profound at 0.5% butanol or higher. In addition, we observed a two-phase response to butanol with an instant general stress response within 0.25h, and a more long-term adaptation response in the later stage.

The observed transcriptional responses suggest that *C. acetobutylicum* reacts to butanol stress by induction of the general stress response and changing its cell envelope and transporter composition, but leaving the central catabolism unaffected.

Mathematical Modeling for Prediction of Strain Optimization Targets: Sensitivity Analysis and Evaluation of Dynamic Experiments

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The biotechnological process of acetone-butanol-ethanol production by *Clostridium acetobutylicum* lately regained interest as a renewable resource for fuel and base chemicals. A key research topic is the diversion of metabolic fluxes to butanol, which is the most valuable product. Systems biology is used to tackle this challenge, providing model based tools for elucidating the regulatory interactions in the metabolic network.

It was already shown by flux analysis (Junne, 2010) that addition of acetic acid to a batch cultivation of *C. acetobutylicum* profoundly affects the metabolome. Similar dynamic experiments (Alsaker, 2010) showed a huge diversity of effects on the transcriptome level. On the other hand, the effects of addition of butanol on the transcriptome lead to contradictory results in chemostat and batch cultivation (Janssen, 2012; Alsaker, 2010). In order to cope with these results from different "-omics" levels, we use a descriptive modeling approach for data consistency checking and reconciliation (Götz, 2009).

We are presenting the application of this approach to pH shift experiments in chemostat cultivation together with implementation of new tools to extend this method. In order to predict targets for the construction of recombinant strains, a global parameter sensitivity analysis of the descriptive model is performed. Selecting sensitivities of relevant metabolites towards changes in the maximum reaction rate parameter (proportional to enzyme concentration), a hierarchy of potential targets for genetic modifications is established.

For model verification and validation stimulus-response experiments with transcriptome and metabolome data acquisition are carried out. The example stimulus acetic acid was supplied as dirac pulse or as step function after establishing steady state in continuous cultivation. Beyond model validation, such experiments also provide information on a possible role of acetic acid in regulation.

Götz, P., Reuss, M., Junne, S., 2008, Acetone-Butanol-Ethanol fermentation: Metabolic network, fluxes and reaction kinetics, Lecture at Clostridium X, Wageningen

Alsaker, K.V., Paredes, C., Papoutsakis, E.T., 2010. Metabolite stress and tolerance in the production of biofuels and chemicals: gene-expression-based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe *Clostridium acetobutylicum*. Biotechnol. and Bioeng. 105, 1131–1147

Junne, S., 2010. Stimulus response experiments for modeling product formation in *Clostridium acetobutylicum* fermentations. PhD thesis, TU Berlin, Germany

Janssen, H., Grimmler, C., Ehrenreich, A., Bahl, B., Fischer, R.-J., 2012. A transcriptional study of acidogenic chemostat cells of *Clostridium acetobutylicum* – Solvent stress caused by a transient n-butanol pulse. J. Biotechnol., *in press*

Conversion of Crude Glycerol by *Clostridium pasteurianum* and by a Developed Mutant Strain

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The production of biodiesel results in a concomitant production of crude glycerol (10% w/w). *Clostridium pasteurianum* can utilize glycerol as sole carbon source for the production of acetate, lactate, butyrate, 1,3-propanediol, ethanol, butanol, and CO_2. Crude glycerol derived from biodiesel production has, however, been shown to be toxic to the organism even at low concentrations.

To cope with the toxicity of the substrate two strategies have been applied; pretreatment of the crude glycerol to reduce the toxicity, and development of a mutant strain with increased tolerance towards the crude glycerol. Among the different pretreatments tested, it was found that storage combined with activated stone carbon facilitated utilization of the crude glycerol.

A pH controlled reactor with *in situ* removal of butanol by gas-stripping was used to evaluate the performance. The fermentation pattern of the wild type of *C. pasteurianum* on pretreated crude glycerol was almost similar to the pattern on technical grade glycerol. The organism was able to utilize 111 g/l crude glycerol. The average consumption rate was 2.49 g/l/h and the maximum consumption rate was 4.08 g/l/h. At the highest glycerol consumption rate butanol was produced at 1.3 g/l/h. These rates were higher than previously published rates by the same strain. The developed mutant *C pasteurianum* strain was less inhibited by the crude glycerol. It achieved rates significantly higher than previously published for the wild type of *C. pasteurianum* even on technical grade glycerol in fed batch reactors. In addition, high yield of the two main products butanol and 1,3-PDO were detected and these two products were efficiently separated in two steams using gas-stripping. The strain is evaluated as better suited for industrial purposes compared to the wild type strain.

Radical Enzymes in Clostridial Butyrate Synthesis

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Several clostridia and fusobacteria are able to ferment glutamate to ammonia, CO_2, acetate, butyrate and H_2. Depending on the organism the fermentation proceeds via three different pathways, all of which involve radical enzymes: the coenzyme B_{12} dependent glutamate mutase, the [4Fe-4S] cluster-containing 2-hydroxyglutaryl-CoA dehydratase that requires activation by reduced ferredoxin and ATP, as well as the [4Fe-4S] cluster and FAD-containing 4-hydroxybutyryl-CoA dehydratase. All three pathways terminate in the exergonic reduction of crotonyl-CoA to butyryl-CoA by NADH that drives the endergonic reduction of ferredoxin (Fd_{ox}) by NADH, a process called electron bifurcation.

$$\text{Crotonyl-CoA} + 2\ \text{NADH} + Fd_{ox} \rightarrow \text{Butyryl-CoA} + 2\ NAD^+ + Fd_{red}^{2-}$$

In this process the exergonic reduction of crotonyl-CoA to butyryl-CoA ($E°'$ = -10 mV) by NADH (E' = -280 mV) drives the endergonic reduction of ferredoxin (E' = -500 mV) also by NADH. In many clostridia a complex $(BcdA)_2$-EtfBC composed of the heterodimeric electron transferring flavoprotein EtfBC and the homodimeric butyryl-CoA dehydrogenase $(BcdA)_2$ catalyzes this reaction. The crystal structure of EtfBC revealed the presence of two molecules of FAD, the β-FAD bound in a flavodoxin-like domain and the loosely bound γ-FAD located with its adenine moiety at the same domain, in which the human Etf contains AMP. Both FADs are separated by 19 Å, but due to a flexible region they can move together by about 6 Å allowing electron transfer. We propose that NADH reduces γ-FAD to γ-$FADH^-$; the β-FAD takes one electron to give a stabilized radical, β-$FAD^{\bullet -}$ semiquinone, and transfers the electron to Bcd and further to crotonyl-CoA. The remaining radical, a highly reactive γ-$FADH^{\bullet}$ semiquinone, immediately reduces ferredoxin. Repetition of this process finally affords butyryl-CoA and Fd_{red}^{2-}.

A recent review [1] describes in more detail this and other flavin-based electron bifurcations (FBEB), especially in aceto- and methanogenesis.

1. Buckel W & Thauer RK (2012) *Biochim Biophys Acta*, online

Analysis of Redox Responses During TNT Transformation by Clostridium acetobutylicum ATCC824 and Mutants and Other Clostridia

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Trinitrotoluene (TNT) and other nitro-explosives are recalcitrant hazardous pollutants contaminating many military facilities. They are most actively degraded in anaerobic conditions by initial reduction of the nitro group. The transformation of TNT by several mutant strains of *Clostridium acetobutylicum* and other clostridia has been examined to analyze the maximal rate of initial transformation, determine the effects of metabolic mutations of the host on transformation rate and to access the cell metabolic changes brought about during TNT transformation. Little difference in the maximal rate of TNT degradation in acid phase cultures of strains altered in the acid forming pathways (phosphotransacetylase, or butyrate kinase) or in a high solvent producing strain (mutant B) or a degenerate strain that had lost the ability to produce solvent (M5) was found compared to the parental *C. acetobutylicum* ATCC824. A series of antisense constructs were made that reduced the expression of *hydA*, encoding the Fe hydrogenase, or *hydE* and *hydF*, genes encoding hydrogenase maturating proteins. While the antisense *hydA* strain had only ~30% of the activity of wild type, the antisense *hydE* strain exhibited a TNT degradation rate around 70% that of the parent. The redox potential, hydrogen evolution and organic acid metabolites produced during rapid TNT transformation in log phase cultures were measured. The redox potential of the acid producing culture decreased from -370 to -200 mV immediately after addition of TNT and the hydrogen evolution rate decreased, lowering the hydrogen to carbon dioxide ratio from 1.4 to around 1.1 for a period. During the time of TNT transformation, the TNT treated acidogenic cells produced less acetate and more butyrate. The results show that during TNT transformation the cells shift metabolism away from hydrogen formation to reduction of TNT and the resulting effects on cell redox cofactors generate a higher proportion of butyrate.

Development and Application of a High-throughput Screening System for Clostridial Biofuel Production

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Recent efforts on rational metabolic engineering approaches to increase butanol production in *Clostridium acetobutylicum* exhibited only limited success, demonstrating the physiological complexity of solventogenic clostridia. Since multiple largely unknown parameters determine a particular phenotype, an inverse strategy to select a phenotype of interest can be useful. However, the major constraint for explorative or combinatorial metabolic engineering approaches is the availability of a feasible screening method to select the desired phenotype from a large population in a high-throughput manner. Therefore, we developed a semi-quantitative assay to measure butanol and ethanol in microtiter cultures of *C. acetobutylicum*. The applicability of the screening system was evaluated by two examples: First, *C. acetobutylicum* ATCC 824 was chemically mutagenized and subjected to high butanol concentrations as a pre-selection step. Screening of the butanol-tolerant population resulted in the identification of four mutants with > 20 % increased butanol production as compared to the wildtype. The second application example was based on a pre-engineered *C. acetobutylicum* strain with low acetone biosynthetic activity, but concomitantly reduced butanol titer. After chemical mutagenesis, a total of 4390 clones was analyzed and three mutants revealed significantly increased butanol concentrations and similarly low acetone levels as the parental strain. Thus, the suitability of the semi-quantitative screening system was validated, opening up new perspectives for explorative metabolic engineering of solventogenic clostridia.

Flow Cytometry Analysis of Solventogenic Clostridia

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Along with light microscopy, flow cytometry (FC) is convenient for observation of *Clostridium* cell cycle due to distinct shape, optical properties and chemical composition of the cells. The analysis is based on different light scatters (forward and side scatters) of the cells in different developmental stages, i.e. vegetative, cigar-shaped or sporulating cells and released spores.

Clostridium bacteria are usually considered Gram labile; young cells after spore germination are labeled as G^+ but with fermentation progress, the response on the staining is changing and bacteria start to look like G^- ones. This phenomenon is probably elicited by an alteration in cell membranes compositions, particularly thinning of peptidoglycan layers, caused by metabolic switch from acids to solvents formation. To map ABE process, fluorescent alternatives of Gram staining together with FC were used. Different types of labeling using different *Clostridium* species (*C. beijerinckii*, *C. pasteurianum* and *C. tetanomorphum*) induce different responses. Wheat germ agglutinin labeled with alexa-fluor 488, which binds specifically to N-acetylglucosamine in the outer peptidoglycan layer was possible to use only for *C. tetanomorphum* germinating cells to get unambiguous results. For *C.pasteurianum*, double staining with hexidium iodide and Syto 13 proved to be good choice for reflection of changes in ABE batch fermention.

FC with fluorescent staining can be used for estimation of metabolically active cells in bacterial populations. Based on testing of fluorescent viability probes for membrane integrity, membrane potential and esterase activity measurements, specific probes were matched with particular *Clostridium* species. Metabolically active cells of *C.beijerinckii* could be tracked by FC, after propidium iodide (PI) staining. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol or PI were appropriate for distinguishing *C.pasteurianum* metabolically active cells.

Carbon Flux Diversion in *Clostridium acetobutylicum*: Effect Of Mutagenesis and Stress

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Biosynthesis of butanol, regarded as a superior biofuel to ethanol, is commonly considered possible through anaerobic spore forming *Clostridium* species like *C. acetobutylicum*. Attempts have been in progress for over a decade in manipulating metabolic pathways in the strain towards one or the other performance attributes like higher butanol yields, higher butanol tolerance etc. While tools and methodologies for directed genetic manipulations are being developed and applied to bring about desired changes one way or the other, simpler manipulations like subjecting the strain to physico-chemical stresses can also lead to useful results. The effect of different stress conditions on solvent production of *C. acetobutylicum* was systematically studied in present work.

Magnesium sulphate was observed to change the physiology of the organism, resulting in an increase in glucose uptake and butanol production while stress through other essential elements like Na, K and Mn was found to affect growth but not solvent production. The organism when exposed to different chemical as well as physical mutagens, ethyl methyl sulphonate generated some mutants that showed increased ethanol production. High frequency UV irradiation resulted in increased butanol yield from 0.23 to 0.33. Interestingly, the parental strain when mutagenized simultaneously by UV exposure and oxygen stress, brought forth mutants producing propionic acid (~8g/l) in addition to butanol (2g/l), which is a hitherto unreported behavior in this organism. The production of propionic acid by *C. acetobutylicum* supports the presence of complete albeit bifurcated TCA cycle in this organism where oxaloacetate flows to succinate both though citrate/ketogluatate and malate/fumarate and leads to propionic acid production. Potential mutants were found to grow more rapidly than parent strain under the similar conditions.

ABSTRACTS OF POSTER PRESENTATIONS

Function and Evolution of Bacterial Microcompartments in Clostridia

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Clostridia are a genetically diverse class of bacteria that are generally fermentative, endospore-forming, anaerobes with gram-positive cells walls. They are present as commensals in the guts of many insects and animals, as soil saprophytes, and in agricultural and industrial effluent streams. The Clostridia include potential bioremediation agents, important producers of biofuels, immune system stimulators and toxic producing pathogens. Bacterial microcompartment (BMC) genes, which express organelles composed entirely of proteins and that carry out a specific metabolic reaction, have recently been reported in several Clostridia. Using domain representations of the BMC genes we identified BMCs in genomes of over 50 Clostridia species, including human pathogens. BMC genes frequently clustered at a single locus coding for a BMC with a distinctive metabolic role. The Clostridia BMCs include functional types known to encode proteins for the metabolism of ethanolamine and propanediol, but novel classes of BMCs are present that contain genes suggesting putative functional roles. In some species there are two or three BMC loci coding for different evolutionary and/or functional classes of BMCs. Using *Clostridium phytofermentans* as a model, we demonstrate that the three BMC loci are differently expressed. The distribution of BMC-containing species was mapped onto a phylogenetic tree constructed from 16S rRNA. The presence of BMCs is sporadically distributed across the phylogenetic tree. All families that contained species with BMCs also had species without BMCs. Even within a species, BMC number varied indicative of frequent horizontal transfer and gene loss. Similarly, phylogenetic trees constructed from individual BMC genes indicates that gene transfer into/out of the Clostridia is a common occurrence. Analysis of BMC distribution in the context of a Clostridia environmental attribute database allowed us to determine physiological and genomic characters that correlate with the presence of BMCs.

Fermentation of Pretreated Biomass by Metabolic Engineered *Clostridium* and *Propionibacterium.*

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The project aims are to develop the biotechnological options and assess industrial potentials of Gram+ anaerobic fermentation of farmland wastes in a sustainable perspective.

Lignocellulosic biomass such as pretreated wheat straw is a challenging substrate for bacteria, not only because of inhibitory compounds such as furfural and HMF but also, as in this case, due to a high sugar content (135g/L total sugar). Anaerobic species of *Clostridium* and *Propionibacterium* are known to convert glucose into volatile fatty acids and gases such as acetate, butyrate, propionate and CO_2 in batch fermentations on synthetic media. But what are the fermentation products from these bacteria, if the substrate is C5 and C6 sugars extracted from pretreated wheat straw?

A selection of both mesophilic and thermophilic *Clostridium* strains, and strains of mesophilic *Propionibacterium* have been cultivated and adapted to tolerate the substrate by sequential transfer of the bacterial cells to medium with increasing amounts of pretreated biomass. The most promising strains are selected for continuous fermentations at pilot scale level. Furthermore, target genes for knockout have been identified and by applying the ClosTron plasmid vector technology, metabolic engineering will be enabled on strains which have never been *in vitro* transformed with foreign DNA before.

Regulation of the Synthesis of Hemicellulases in *Clostridium cellulolyticum*

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C. cellulolyticum is a model anaerobic mesophilic bacterium, which secretes cellulosomes to hydrolyse plant cell walls into simple sugars for its growth. Its cellulosomes are composed of a scaffolding protein bearing 8 cohesin modules able to interact with dockerin modules of 62 putative dockerin-containing enzymes. Proteomic analyses of the composition of the cellulosomes produced by *C. cellulolyticum* upon growth on different substrates indicate that modulation of cellulosomes composition occurs according to the growth substrate. In particular, the expression of a cluster of 14 hemicellulase-encoding genes (called *xyl-doc*) seems to be induced by the presence of straw and not of cellulose. Interestingly, genes encoding a two-component regulation system are present upstream of *xyl-doc*. Our aim was to demonstrate the involvement of the two-component system in the expression of *xyl-doc* genes.

First evidence for the involvement of the regulator in the expression of *xyl-doc* genes was illustrated by the analysis of cellulosome composition produced by the regulator overproducing strain when grown on cellulose. Ion-trap LC MS/MS, allowed the detection of the products of all *xyl-doc* genes and of *Ccel_1656* encoding a protein of unknown function but containing a carbohydrate binding domain targeting hemicellulose. RT-PCR experiments further demonstrated that the regulation occurs at a transcriptional level and that the transcription of all *xyl-doc* genes is linked.

Transcriptional analysis of the regulator-knock out and complemented strains confirmed the involvement of the regulator in the expression of *Ccel_1656* and *xyl-doc* genes in response to straw. These studies also indicated that the transcription of *xyl-doc* genes was linked to the transcription of the two-component system-encoding genes. EMSA experiments further demonstrated that the regulator binds to DNA regions located upstream of the sensor-encoding gene of the two-component system and *Ccel_1656*.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Detection and Toxigenic Type Characterization of *C.difficile* by Multiplex PCR From Hospitalized Patients with Diarrhea in a Tertiary Care Hospital in Manipal, India

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C.difficile is an important cause of nosocomial diarrhea worldwide. Many documented reports on *C.difficile* Associated Diarrhea (CDAD)are available in foreign medical literature in comparison to few from India.This study aimed at highlighting the increase in the number of antibiotic associated diarrheal cases due to *C.difficile* among hospitalized patients in Manipal by rapid detection of *C.difficile* toxins A&B from faeces followed by culture.

Tissue culture assays,though considered to be the gold standard, are rarely used in clinical practice owing to the high cost and time(48-72 hours)required to perform such assays.Detection of toxins A and B from feces,forms the mainstay in the rapid diagnosis of Antibiotic Associated Diarrhea (AAD) due to *C.difficile*.

Stool samples were collected from 25 patients (suspected of AAD) who had received antibiotics for various clinical problems as part of their course of treatment regimen. 25 samples collected from hospitalized patients who had received antibiotic therapy for other clinical conditions but did not develop diarrhea, served as controls for our study. A rapid membrane enzyme immunoassay for the simultaneous detection of *Clostridium difficile* glutamate dehydrogenase antigen and toxins A and B in a single reaction well was performed on all the samples (cases and controls) along with culture on Cycloserine Cefoxitin Fructose Agar. Nine out of twenty-five (18%) cases of AAD were positive for *C.difficile* by the enzyme immunoassay and were confirmed by culture. A multiplex PCR run,designed for toxigenic type characterization involving the detection of *tpi,tcd A* and *tcd B* genes showed the presence of toxin B in all the 9 toxin positive samples.

Severe diarrhea, fever with abdominal pain, loose stools with mucus and blood were important indicators of (CDAD) in our study. Pseudomembranous colitis was the cause of death in two of the positive cases, three patients had followed up and tested negative after two weeks of oral therapy with metronidazole and vancomycin.

Hydrogen Production From Cellulose in *Clostridium thermocellum*

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Clostridium thermocellum is a thermophilic anaerobe with the highest known rate of cellulose degradation. It produces an extracellular cellulosome system containing cellulase enzymes and cellulose-binding domain that functions to adhere the bacterium to solid substrate while performing saccharification and fermentation. During cellulose fermentation, the bacterium produces hydrogen (H_2), ethanol, acetate, formate, lactate, and CO_2 as byproducts.

To produce cost-competitive H_2 from renewable biomass, our goal is to increase H_2 molar yield in *C. thermocellum*. We aim to redirect the carbon flux in favor of H_2 production by knocking out genes encoding competing pathways. One such candidate pathway is the pyruvate-to-formate reaction catalyzed by pyruvate formate lyase (PFL) as our results showed that the addition of a PFL inhibitor increased H_2 yield by up to 58%. However, the lack of a tractable genetic system in *C. thermocellum* limits the ability to engineer the bacterium. Currently, we have successfully delivered a plasmid into *C. thermocellum* via a conjugative protocol co-developed with University of Manitoba. We demonstrated that vectors matching *C. thermocellum* restriction system (Dam^+Dcm^-) gain stability as recently reported in literature.

To optimize parameters to achieve high rates of cellulose conversion to H_2 in a fermenter, we operated a fully automated two-liter sequencing-batch fermentation in a cyclic mode of settle, draw, and fill. Our results demonstrated that this mode of operation (1) allows the retention of acclimated bacteria to hydrolyze cellulose at high rate and minimal lag phase, an advantage over the conventional continuous fermentation (chemostat); and (2) avoids clogging of feed and discharge lines, and pump failures, often encountered with a solid substrate in continuous fermentation. Work is ongoing to develop genetic system and optimize cellulose fermentation to improve H_2 production in *C. thermocellum*.

The resuscitation of a cellulolytic *Clostridium acetobutylicum* ATCC824

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Although the genome sequencing of the solvent producing strain *C. acetobutylicum* ATCC 824 has revealed the presence of a large cellulosomal gene cluster, the multicomponent protein complex -or cellulosome- produced (with an apparent MW of 665 kDa) is unable to hydrolyze cellulose. Previous results have shown that two of the three major cellulases of the cellulosome, Cel9C and Cel9X, were both active on crystalline cellulose and that they can form a complex with the CipA scaffolding protein.

In the first part of this study we will demonstrate that Cel48A, the most abundant cellulase of the cellulosome is inactive on all the cellulosic substrates tested.

Up to now, all the attempts to express from a plasmid a cellulase belonging to the GH48 family have been negative. In the second part of our work we try to replace on the *C. acetobutylicum* ATCC824 chromosome the inactive *cel48A* gene by a functional one, coding for an active GH48 cellulase. For this purpose, we first constructed an artificial gene coding for an active hybrid protein SAFA-48, composed by the catalytic domain of Cel48F from *C. celluloyticum* and both signal sequence and dockerin domain of Cel48A from *C. acetobutylicum*, this latter assuming the species-specific cohesion-dockerin domains interactions.

Using our marker-less with positive selection method for successive gene deletion/integration in *C. acetobutylicum* previously described (1), we first deleted the *cel48A* gene. Further rounds of chromosome manipulation using the same technique allowed us to replace on the *⊿cel48A* mutant chromosome the original gene by the SAFA-48 gene, without any modification of the genetic context from the transcriptional or translational point of view.

The new *C. acetobutylicum* strain obtained *⊿cel48A::SAFA* is currently under investigation, from the characterization of the new cellulosome produced to its ability to grow on different cellulosic substrates.

-1. SOUCAILLE P., FIGGE R and C. CROUX (2008) “Process for chromosomal integration and DNA sequence replacement in Clostridia”.Internationanl patent WO 2008/040387

Restarting the Chinese Biobutanol Industry

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Over the past decade, six large commercial biobutanol plants have been built in China designed to run with corn as a feedstock. Increasing corn prices made this process economically unfeasible so that many of the plants that were built have lain dormant for several years and other planned builds were shelved. Today, there is approximately 450,000 tonnes per annum of installed solvent production capacity in China with little to no active production.

Green Biologics Ltd. has been working closely with Laihe Rockley Biochemical Ltd. to reinvigorate the Chinese biobutanol industry through better use of lower cost and more sustainable feedstocks based on corn residues, better use of existing plant, and the utilisation of superior microbes. The process improvements have led to more attractive economics leading to the restarting of commercial biobutanol production in China. In this paper we will report on the history of the collaboration, recent developments and future plans.

Easier Genetic Manipulation of *Clostridium acetobutylicum*

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Clostridium acetobutylicum is a tough species for genetic manipulation. Typically, the DNA needs to be methylated by the Φ3TI methyltransferase before introducing into *C. acetobutylicum*, because of the presence of RM system. To skip the methylation step, a gene SMB_G1527 encoding the endonuclease Cac824I in *C. acetobutylicum* DSM1731, was disrupted using the ClosTron system. The resulted strain SMB009 lost the type II restriction endonuclease activity, and can be transformed with unmethylated DNA as efficient as with methylated DNA. Another problem for the electrotransformation process of anaerobic *C. acetobutylicum* is the dependence of anaerobic chamber, thus laborious and time-consuming. We managed the disruption of SMB_G2669 gene encoding the peroxide regulon repressor PerR in strain SMB009 to improve its aerotolerance. The resulted mutant SMB012 was proved to be electrotransformable in air with an efficiency of 1.2 - 3.1 × 10^3 transformants μg^{-1} DNA. Besides, for controlling the gene expression of a gene efficiently in *C. acetobutylicum*, we developed a new inducible expression system. This system consists of a functional chloramphenicol acetyltransferase gene promoter containing *tet* operators (tetO), PthI promoter (thiolase gene promoter from *C. acetobutylicum*) controlling TetR repressor expression cassette, and the chemical inducer anhydrotetracycline (aTc). The optimized system, designated as pGusA2-2tetO1, allows gene regulation in an inducer aTc concentration-dependent way, with an inducibility of over two orders of magnitude. The stringency of TetR repression supports the introduction of the genes encoding counterselective marker *mazF* into *C. acetobutylicum*, which will be useful for developing new gene knockout methods.

Using Assembled Consortium for Hydrogen and Ethanol Production Directly From Cellulose.

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As a series publications have reported that it was quite difficult to use just one super strain to realize one-step cellulosic ethanol production, our group brought out the idea of using assembled consortium to solve this bottleneck. A consortium named H, which can use cellulose to produce ethanol and hydrogen under anaerobic fermentation, was selected and assembled based on construction information got through DGGE and T-RFLP, then the new consortium had shown great increase on ethanol conversion rate and yield of hydrogen production. The ethanol conversion rate can reach around 81%, while the highest hydrogen production speed is around 80mL/g cellulose. T-RFLP (Terminal Restriction Fragment Length Polymorphism) was used to monitor the construction changes of assembled consortium compared to the original one. Then the key factors in metabolism flux was detected by Q-PCR, and showed certain degree of differences compared to original consortium. Therefore, it is considered it was quite possible to regulate a consortium to produce metabolin interested in by artificial assembly. As consortium have advantages such as stability, synergy, and stress assistance, compared to single engineered strains, the idea of assembled consortium may give a revolution in biological refining industry.

Expanding the Repertoire of Gene Tools for Precise Manipulation of *Clostridium* Genomes: Allelic Exchange Using PyrE Alleles and CodA.

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Sophisticated genetic tools to modify essential biological processes at the molecular level are pivotal in exploiting the beneficial properties of *Clostridium spp*. Here we have developed efficient procedures for making precise alterations to clostridial genomes by using either *codA*- or *pyrE*-based allelic exchange. The robustness and reliability of the methods have been demonstrated through the creation of in-frame deletions in a number of different genes (*spo0A*, *cwp84*, *Cac1502*, *motB, tdcC* and *mtlD*) in various different clostridial species, including *Clostridium acetobutylicum, Clostridium sporogenes* and *Clostridium difficile*. CodA may be used as a counterselection marker in wild-type backgrounds to select allelic exchange events from single crossover plasmid insertions by isolating cells resistant to 5-Fluorocytosine (5FC). The use of PyrE as a counterslection marker is reliant on the initial creation of a *pyrE* deletion mutant, using Allele Coupled Exchange (ACE), that is auxotrophic for uracil and resistant to fluoroorotic acid (FOA). This enables the subsequent modification of target genes by allelic exchange using a heterologous clostridial *pyrE* allele as a counter-/ negative-selection marker in the presence of FOA. Following modification of the target gene, the strain created is rapidly returned to uracil prototrophy using ACE, allowing mutant phenotypes to be characterised in a PyrE proficient background. Crucially, wild-type copies of the inactivated gene may be introduced into the genome using ACE concomitant with correction of the *pyrE* allele. This allows complementation studies to be undertaken at an appropriate gene dosage, as opposed to the use of multicopy autonomous plasmids. The rapidity of the 'correction' method (5-7 days) makes *pyrE*$^-$ strains attractive hosts for mutagenesis studies whatever counterselection marker is employed (CodA or PyrE).

Analysis of a Glucose Phosphotransferase System in *Clostridium beijerinckii*

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Revival of the ABE fermentation will be enhanced by the ability of bacterial strains to utilise cheap, renewable substrates containing a range of fermentable carbohydrates. The predominant mechanism of sugar uptake in the clostridia is the PEP-dependent phosphotransferase system (PTS), which not only catalyses the concurrent uptake and phosphorylation of its substrates but also plays a central role in regulation of metabolism. In general glucose is the dominant sugar which represses the metabolism of alternative carbon sources. Understanding of the mechanism of repression will be aided by a detailed characterization of glucose uptake system(s).

The *Clostridium beijerinckii* genome encodes 42 complete phosphotransferase systems, including fifteen belonging to the glucose-glucoside family. Of these transporters, the product of the *cbei0751* gene is xx% identical to the glucose PTS of *Clostridium acetobutylicum* and is thus suggested to encode a glucose PTS. The *cbei0751* gene was amplified by PCR, cloned into pCR2.1-TOPO, and transformed into *Escherichia coli* ZSC113, a mutant that is unable to grow on or ferment glucose or mannose because it lacks the ability to phosphorylate the sugars. Transformants showed a positive fermentation phenotype for glucose and mannose, and were shown to deplete the sugars from the medium during growth on LB broth. In addition, isolated cells accumulated ^{14}C-glucose, and cell free extracts exhibited PTS activity for glucose which was inhibited by mannose. Cbei0751 was therefore shown to be a functional glucose PTS, which also recognises mannose as a substrate.

Work is now in progress with the aim of inactivating the *cbe0751* gene, in order to examine the role of this PTS in regulation of metabolism of other sugars by glucose.

The Prominence of Acetogens Based on Analysis of Non-Photosynthetic Carbon Fixation Pathways for the Production of Biofuels and Chemicals

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Growing concerns about environmental sustainability and global climate change have prompted worldwide efforts to develop platform organisms capable of CO_2 fixation using electrons derived from H_2, CO, or directly from an electrode, with the intention of engineering synthetic or semi-synthetic pathways to produce biofuels and chemicals. Despite the interest in autotrophic fuel production, the maximum theoretical capacity and potential yields from these systems have yet to be explored. Therefore, we seek to evaluate the natural carbon fixation pathways on the basis of energetic efficiency and overall stoichiometry, with a primary focus on non-photosynthetic engineered strains that could be used for chemical production. Our analysis compares the pathways based on ATP and electron requirements, the number of enzymes needed to produce acetyl-CoA and, subsequently, the production of three model chemicals: ethanol, acetate and butanol.

Based on the most expensive substrate (i.e., H_2 or electron equivalent), our analysis shows that the Wood-Ljungdahl (WL) pathway is the most efficient for acetate and ethanol production. However, due to ATP-limitations, chemoautotrophic butanol production from the WL pathway is insignificant, while pathways that can co-exist alongside aerobic respiration have greater metabolic flexibility. Mixotrophic production – using a combination of sugars, CO_2 and an electron source – shows the potential for increased product yields for all of the fixation pathways. We also calculate maximal yields, both chemoautotrophic and mixotrophic, based on the WL pathway for two other important molecules: 2,3-butanediol and butyrate.

Characterization of the Ferredoxin NADP Oxidoreductase of *C. acetobutylicum* a Key Enzyme for Butanol Formation under Solventogenic Conditions

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In the strict anaerobe spore-forming bacterium *Clostridium acetobutylicum*, ferredoxin oxidoreductases enzymes play a key role in the regulation of the electron flow. In the exponential acidogenic phase of growth, in parallel of pyruvate ferredoxin oxidoreductase, the NAD(P)H ferredoxin reductase enzyme mediates the reduction of ferredoxin by consuming the excess of NADH produced during glycolysis. In the solventogenic or the alcohologenic phases, the ferredoxin NAD(P)+ reductase enzymes act in redirecting the electron flow from reduced ferredoxin toward the production of the NAD(P)H needed for ethanol and butanol production.

Although the presence of ferredoxin oxidoreductase activities in cell-free extracts from *Clostridium acetobutylicum* were reported (1,2), the enzymes responsible for these activities have never been purified or identified.

First, an overall production, extraction and purification process was designed to purify the ferredoxin NAD(P)+ reductases enzymes from solventogenic cultures of *Clostridium acetobutylicum*. At each step of extraction or purification, ferredoxin NAD+ reductase and ferredoxin NADP+ reductase activities were evaluated to select positive fractions. Using this method, a ferredoxin NAD+ reductase and a ferredoxin NADP+ reductase were identified

Second, to further characterize the putative candidates, the genes encoding these enzymes were cloned and the corresponding proteins purified and characterized for the ferredoxin NAD+ reductase and ferredoxin NADP+ reductase activities.

Finally, to evaluate the *in vivo* role of these candidates, the genes encoding each enzyme were separately disrupted in *Clostridium acetobutylicum* ATTC824. The gene encoding the ferredoxin NADP+ reductase activity was shown to play a major role in Butanol formation during solventogenic conditions.

The identification of this key enzyme and encoding gene is of great interest as it opens new perspectives for heterologous n-Butanol production.

(1) Girbal, L., C. Croux, I. Vasconcelos, and P. Soucaille. 1995. Regulation of metabolic shifts in *Clostridium acetobutylicum* ATCC824. FEMS Microbiol. Rev. 17:287-297.

(2) Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J Bacteriol 176:1443-50.

Butanol Production From King Grass (*Pennisetum hybridum*), a Lignocellulosic Substrate.

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Butanol is an alcohol that can be used as intermediate in chemical synthesis. As fuel, it has a very promissory future because of its good characteristics. The production of this compound via fermentation was developed in the early decades of the last century, but gradually lost its market against the produced by petrochemical synthesis. Due to concerns about nonrenewable resources and environmental problems associated with petroleum, it has revived the interest for this industry, furthermore the use of lignocellulosic biomass has turned very attractive due its high availability and low cost.

In our research, we found that the most suitable grass species for butanol production in Colombia, based on availability, plant conditions, theoretical yield to solvents, crop timing and composition is king grass (*Pennisetum Hybridum*). Samples of this specie were characterized according to procedures and protocols established by National Renewable energy Laboratory (NREL). Then, they were pretreated by several methods (alkaline delignification, ammonia wet soaking, ozonolysis, dilute acid hydrolysis and organosolv), to condition the raw materials for the enzymatic hydrolysis step. This step was carried out with the commercial enzymatic pull Accellerase 1500 from Genecor. Sugars content in the hydrolysate, was used as a variable to choose the best pretreatment. Content of reducing sugars (g/l) obtained through the investigated pretreatments were: alkaline 14,6, ozonolysis 4,8, amonnia 13,9 ,diluted acid 7,4, peroxide 7,2 , steam explosion 6,0. Afterwards a optimization of the alkaline pretreatment was performed, obtaining a maximum sugar content of 98 g/l.

The hydrolysate obtained with the best conditions was fermented with the bacteria *Clostridium acetobutylicum* ATCC 824. A blank run using a culture medium with glucose as carbon source was also carried out. The best solvent yield was found using the pretreated material without pH control (18 g/L solvents and 10 g/L of butanol).

Clostridium acetobutylicum Fermentation on Lignocellulose-based Hexoses and Pentoses for Butanol Production

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The Acetone-Butanol-Ethanol (ABE) fermentation is receiving renewed interest as a way to upgrade renewable resources into valuable base chemicals and liquid fuels. Abundance and un-competitiveness with food sources are desired features of a potential substrate, and they are met by lignocellulosic biomass.

Lignocellulosic biomass may be fermented provided that the hydrolysis in simple sugars – hexoses and pentoses - is carried out (Qureshi, Biopr Biosyst Eng, 2007, 30, 419). Although some reports on ability of *Clostridium* strains are available in scientific literature, no systematic investigation has been carried out.

This contribution is about the characterization of the ABE fermentation by *C. acetobutylicum* DSM 792 using sugars representative for hydrolysis products of lignocellulosic biomass: hexoses (glucose and mannose) and pentoses (arabinose and xylose).

Batch fermentations of single sugars were carried out. The conversion process was characterized as a function of time in terms of biomass, acids and solvents concentrations, pH, and total organic compounds. Effects of $CaCO_3$ supplement to the fermentation broth were investigated.

The fermentation performance of the investigated sugars decreases in the order glucose, mannose, arabinose, and xylose. The poor performance when using xylose can be explained on a metabolic level by various hypotheses:

- additional energy demand from H+ dependent symport of xylose into cells;
- inhibition or operon repression by other substrates or products (sugars/acids/solvents) (Ounine et al., Appl Environ Microbiol 1985, 49, 874);
- metabolic bottleneck in regeneration within the sugar conversion pathway by glyceraldehyde-3-P, which is a substrate for many other, competing reactions;
- metabolic bottleneck in availability of transketolase, this enzyme catalyzes two reactions.

The $CaCO_3$ supplementation improves the fermentation performance in terms of both the conversion degree of the substrate and the final solvent concentration.

Dissection and Modification of Xylose Metabolism Pathway in *Clostridium acetobutylicum* and *Clostridium beijerinckii*

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Lignocellulose is the most abundant renewable resource in nature and rich in fermentative sugars, which is available to microbes. *C. acetobutylicum* and *C. beijerinckii*, known as two important solventogenic clostridia, are able to use xylose, one of the main pentose sugars contained in lignocellulosic materials. However, their inefficient xylose metabolism is still a bottleneck in butanol production from mixed sugars of lignocellulosic hydrolysate.

For *C. beijerinckii*, its advantage in fermenting mixed hexose and pentose sugars is no “glucose repression” effect. A big gene cluster, embracing genes responsible for xylose uptake, metabolism, pentose phosphate pathway (PPP) and a predicted regulator XylR, was found in the genome of *C. beijerinckii*. These xylose utilizing genes were repressed by XylR and induced by xylose. XylR inactivation resulted in significantly enhanced xylose utilization by *C. beijerinckii*. Overexpression of a xylose transporter XylT in the *xylR*-inactivated strain further improved xylose consumption.

The xylose utilizing genes are dispersed in different genomic regions in *C. acetobutylicum*. It was experimentally confirmed that gene cac2610 and cac2612 encodes xylose isomerase (XylA) and xylulokinase (XylB), respectively. Gene cac1347 and cac1348 were proven to encode transaldolase and transketolse, two key enzymes located in PPP, respectively. Besides, gene cac1345 was experimentally assessed as a xylose proton-symporter (XylT). Through disruption of a predicted *glcG* gene, encoding enzyme II of glucose phosphoenolpyruvate-dependent phosphotransferase system, and overexpression of *xylA-xylB-xylT*, the engineered strain could efficiently co-ferment glucose, xylose and arabinose.

Novel Clostridial Isolates from an Anaerobic Switchgrass-Degrading Microcosm

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Clostridial roles in both solvent production and in biomass degradation are well known. These qualities can be harnessed for next generation biofuel manufacture. To select for microbes involved in plant biomass degradation, an anaerobic forest soil sample was inoculated into switchgrass media and serially transferred. After 43 transfers, isolations were begun to discover novel bacteria involved in plant biomass degradation.

Four strains of the cellulolytic and ethanologenic bacterium *Clostridium phytofermentans* were isolated from the microcosm. The strains vary in their cellulolytic capabilities and in their optimum temperature and pH ranges, as well as in product ratios.

KNHs209 represents a novel genus within the Clostridia. It is able to use a wide variety of sugars, is highly motile, and grows optimally between 25°C and 30°C. Its 16s sequence is 87.5% similar to its closest relative, *C. hathewayi*, and branches on its own near the XIVa group.

KNHs214 also marks the discovery of a novel genus of Clostridia. It is only 87.1% similar in 16s sequence to its closest relative, *C. tepidiprofundi*. KNHs214 is a nonmotile spore-former that can utilize sugars as well as protein hydrolysates for growth. It reduces nitrates/nitrites, iron, and sulfates/sulfites.

Another novel genus is found in KNHs216 and KNHs217, which appear to be the same species, although their 16s sequences vary by around 3%. Their 16s sequences are about 86% similar to the closest relative, *C. leptum*. They can grow on many different sugars and have an optimum temperature of 37°C and an optimum pH of 7. Butyrate is one of the main products.

The isolates are to be sequenced. The *C. phytofermentans* genomes will provide insight into the mechanism behind *C. phytofermentans*' high level of ethanol production and quick rate of cellulose degradation. Since none of the other isolates are cellulolytic, their roles in the community, specifically in relation to *C. phytofermentans,* are being investigated further.

Exemplification of Gene 'Knock-out' and 'Knock-in' in *Clostridium ljungdahlii*

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Clostridium ljungdahlii is a model organism for gas fermentation, a rapidly maturing technology for production of fuels and chemicals, such as acetate, ethanol and butanol, from industrial waste gases. In order to both optimise productivity and broaden product streams, it is desirable to be able to implement specific, as well as random, modifications to the process organism at the genetic level. Here we have implemented our clostridial 'Road Map' to gene modification of *C. ljungdahlii*, and exemplified its utility through modification of the genome by both insertional inactivation and allelic exchange. Pivotal has been the initial establishment of an improved electroporation protocol using a commercially available electroporator that allows the reproducible and efficient isolation of transformants as discrete colonies on selective agar media. Accordingly, ClosTron mutagenesis has been employed to insertionally inactivate a range of genes in *C. ljungdahlii,* including a uracil auxotroph through inactivation of *pyrE*. The latter mutant was used to identify the most appropriate concentration of 5-fluoroorotic acid (FOA) needed to distinguish a *pyrE* phenotype from wild-type. These conditions were subsequently employed to successfully generate a *pyrE* deletion mutant by Allele-Coupled Exchange (ACE) and an appropriate pyrE-ACE 'Knock-out' vector. The *pyrE* mutation can be rapidly converted back to wild-type, and uracil prototrophy, using the corresponding pyrE-ACE 'Correction' vector. Moreover, derivatives of the 'Correction' vector were also constructed that additionally carrying Multiple Cloning Sites (MCS), into which cargo DNA may be inserted. This cargo DNA is, therefore, inserted into the genome concomitant with ACE-mediated correction of the *pyrE* locus. The utility of the system has been demonstrated by the delivery of ca. 3000 bp region of cargo DNA encoding three heterologous genes into the chromosome of *C. ljungdahlii*.

The Physiologic Role of Pyruvate-Formate-Lyase in *Clostridium acetobutylicum*

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The Gram-positive *Clostridium acetobutylicum* has become a model organism for the acetone–butanol–ethanol (ABE) fermentation among solventogenic Clostridia.
To gain more insight in the biphasic fermentative metabolism, we used the ClosTron technology for targeted gene inactivation and generated knock-out mutants of the pyruvate-formate-lyase (*pflB*) and its activating enzyme (*pflA*). Pfl is catalyzing the reversible, coenzyme-A dependent and nonoxidative cleavage of pyruvate to acetyl-coenzyme-A. It has been demonstrated in a number of anaerobic bacteria, including many species of the genus *Clostridium*. The *pflB*-encoding gene CAC0980 is expressed in *C. acetobutylicum* during growth, as shown in a previous DNA time series microarray study. It was also detected in the proteome reference map of *C. acetobutylicum* ATCC 824. Since *C. acetobutylicum* contains neither a formate-hydrogen lyase as Enterobacteria, nor a formate dehydrogenase, the physiological role of Pfl in *C. acetobutylicum* remains obscure, due to the fact that so far no accumulation of formic acid in the growth medium has been observed. Therefore, it has been proposed that the clostridial enzyme may have a biosynthetic role. The formate might be used as a one-carbon-unit donor.
During batch cultivation both mutants, *pflA*::int(60) and *pflB*::int(1335), accumulate riboflavin, resulting in yellow growth medium and showed an affected growth when reaching the transition to stationary growth phase. This was accompanied by a marked decrease in glucose consumption, the absence of solvent formation and the simultaneous accumulation of the acids acetate and butyrate. A biosynthetic role of Pfl is further supported by our observation that both mutants, in contrast to the WT, are not able to grow in minimal medium.

Improvement of butnaol production from xylose mother liquor by engineering xylose metabolic pathway in *Clostridium acetobutylicum* EA 2018

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First generation biobutanol producing strain use corn as substrate, while the raw materials cost constitutes 75% of the total cost, which indicates replacement of cheaper alternative feedstocks will increase economic efficacy of biobutanol process. Xylose mother liquor (XML) is a cheap by-product of xylose production through acid hydrolysis from sugarcane bagasse or corncob, which is widely available in China or other countries. XML can be potentially used as a fermentation feedstock for the second generation biobutanol production since it generally contains 35%-40% xylose, 10%-15% arabinose, 8%-10% glucose, and a small amount of galactose. Because XML contains two major inhibitors, furfural and 5-hydroxymethylfurfural, 18 wild type butanol producing Clostridia were screened for butanol production in the synthetic P2 medium containing xylose mother liquor as carbon source with 60 g/L initial sugar (P2X60), among which *Clostridium acetobutylicum* EA2018 showed highest butanol titer (8.25 g/L) and yield (0.14 g/g sugar), while only 40% xylose (25.98 g/L) was consumed. The *glcG* gene encoding enzyme II of the D-glucose phosphoenolpyruvate-dependent phosphotransferase system (PTS) was disrupted in EA2018 (2018glcG), resulting in 15% enhancement of xylose consumption in P2X65 (10g/L glucose, 45g/L xylose and 10g/L arabinose) compared to that of EA2018. Further co-expression of the D-xylose proton-symporter, D-xylose isomerase and xylulokinase in 2018glcG (2018glcG-TBA) resulted in 68% xylose consumption (30.5 g/L) in P2X65, which was 24% or 40% higher compared to that of 2018glcG or EA2018. 2018glcG-TBA was able to ferment butanol and total solvents (butanol, ethanol and acetone) up to 12 g/L and 20 g/L with 32% and 50% enhancement of butanol yield (0.19 g/g sugar) and total solvents yield (0.31 g/g sugar) compared to that of EA2018 (0.14 g/g of butanol yield and 0.21 g/g of total solvents yield) in P2X65. P2X medium was further simplified to ZL2 medium consisted of XML, 2 g/L ammonium sulfate, 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.H_2O$, 0.01 g/L NaCl, 0.01 g/L $FeSO_4.7H_2O$, and 5 g/L calcium carbonate, which resulted in ~90% decrease of medium cost exclude carbon source XML compared to P2X without effect of butanol titer and yield.

Clostridium acetobutylicum Producing Biofuels from Lignocellulose Biomass

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Biobutanol produced from lignocellulose biomass is a promising biofuel for combustion engines due to its high heating value, low miscibility to water, low vapour pressure and low corrosion properties. The objective of our project is to create an economic process for production of biofuel mainly from hemicellulose part of wood biomass using *Clostridium acetobutylicum* which have an advantage to use all available from the biomass hexose and pentose sugars.

The wood biomass pretreatment is based on the SO_2-ethanol-water cooking, which efficiently separates cellulose, lignin and hemicellulose into different fractions. After separation hemicellulose fraction was conditioned in order to remove possible inhibitors from final fermentable liquor.

Clostridium acetobutylicum has been transformed by isopropanol dehydrogenase gene in order to produce isopropanol instead of acetone. Product of liquor fermentation by this modified strain is the mixture containing isopropanol, butanol and ethanol which can be used to replace gasoline in internal combustion engines or sold as separate chemicals.

Complete degradation of waste paper by *Clostridium cellulovorans* and its continuous enzyme recycling

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It is expected that energy problems coursed by depletion of various resources and global climate changed by carbon dioxide emissions should be figured out by change for renewable energy. On the other hand, 390 million tons of paper is produced year by year and recovery system has been established in the world. *Clostridium cellulovorans*, an anaerobic, mesophilic bacterium, produces the cellulosome that consists of cellulosomal enzymatic subunits and the scaffolding proteins CbpA, CbpB, CbpC, and HbpA. Since cellulosomal enzymatic subunits are bound to these scaffolding proteins, the cellulosome shows high cellulolytic activity. Furthermore, since CbpA, CbpB and CbpC have a CBM (cellulose-binding module), the cellulosome is capable of binding to crystalline cellulose.

In this study, we focused on waste paper as cellulosic biomass. *C. cellulovorans* was cultivated on 400 ml of 0.3% cellobiose medium. 4 g of waste paper was added and standing for 1 hour after stirring. After waste paper was precipitated, 200 ml of supernatant was replaced with fresh medium that did not contain any carbon sources. 1 ml of culture medium was sampled every 24 hours, and amount of reducing sugar was measured by DNS method. Whenever reducing sugar in the culture supernatant was decreased, 4 g waste paper was added and standing for 1 hour after stirring. After waste papers were precipitated, 200 ml of supernatant was replaced with fresh medium. The procedure was carried out continuously for a month.

As a result, waste paper was eventually added four times for a month, and total amount of waste papers were 16 g. Amount of reducing sugars were increased rapidly just after waste paper was added. In addition, 0.953 g of reducing sugars (6% (w/w) of waste paper) was recovered. Thus, it was suggested that several proteins with CBM and the cellulosome secreted by *C. cellulovorans* were precipitated with waste paper. Furthermore, after their binding to waste paper, cellulolytic enzymes could be strongly accumulated and concentrated. Therefore, it seemed that waste paper was rapidly degraded and reducing sugars were increased by *C. cellulovorans* cellulosome and other CBM-containing enzymes.

Intracellular Metabolite Analysis during the Acid/Solvent Shift in *Clostridium acetobutylicum*

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Despite the long history in using *C. acetobutylicum* for the production of butanol, still little is known about the regulation of the metabolic shift from acids to solvents. It is the goal of the collaborative systems microbiology project ´COSMIC2` to increase the knowledge on the key regulatory and metabolic events that occur during the shift. Especially key metabolites, like butyryl-phosphate and acetyl-phosphate, but also other glycolytic intermediates may play a crucial role. A set of knockout strains are being developed and their fermentative metabolism is analysed by transcriptomics, proteomics and metabolomics. While the analysis of transcript levels and proteins is rather straightforward, analysis of intracellular metabolites is still problematic. Especially the massive leakage of metabolites during the cold quenching of cells is a common problem in many metabolomics protocols. We compared various sampling techniques, quenching solutions and extraction methods. Cell leakage and metabolite recovery was determined by ATP measurements. The developed metabolomics protocol was used to analyse the metabolites from steady state acidogenic and solventogenic cells, as well as cells from different points of the intermediate shift. A targeted set of metabolites was analyzed and quantified by validated LC-MC methods (ion-pair or triple quadrupole MS). The results of the development of the metabolomics protocol and the first metabolite data will be presented.
The COSMIC2 project is carried out in close collaboration with research groups in Berlin, Munich, Rostock, Ulm and Nottingham.

Development of cellulose-degrading enzyme recycle system using cellulosome

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Lignocellulosic plant biomass is difficult to hydrolyze because cellulose is surrounded by a lignin that has covalent associations with hemicellulose, and cellulose has a tightly packed crystalline structure. Among cellulolytic microorganisms, *Clostridium thermocellum*, an anaerobic thermophilic bacterium, is the most potent cellulose degrading bacterium known to produce the cellulosome.

Recently, to isolate microorganisms that possess effective cellulose-degrading ability, new thermophilic cellulolytic strains were screened from agriculture residues in Thailand using microcrystalline cellulose as a carbon source. We isolated a new strain, *C. thermocellum* S14, which has higher cellulose-degrading ability than several type strains (1). When rice straw treated by soaking in aqueous ammonia was hydrolyzed by the combination of ß-glucosidase from *Thermoanaerobacter brockii* with the cellulosome from *C. thermocellum* S14, approximately 91% of glucan existing in the rice straw was hydrolyzed (2).

On the other hand, enzyme recycling is desired to reduce costs of saccharification process. In order to recycle the combination, CBM from CipA was fused to the ß-glucosidase. When recycling tests were carried out against crystalline cellulose and ammonia-treated rice straw, combination of cellulosome and ß-glucosidase-fused CBM could recycle at least 5 and 3 rounds, respectively, consistent with high saccharification rates. These results indicated that the enzyme recycle using combination of cellulosomes and ß-glucosidase-fused CBM has great potential as an effective lignocellulose degradation system.

(1) Tachaapaikoon C, et al., (2012) Biodegradation. 23:57-68.
(2) Waeonukul R, et al., (2012) Bioresour Technol. 107:352-357.

A Quorum Sensing System that Regulates Solvent Formation and Sporulation in *Clostridium acetobutylicum*

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The Gram-positive, endospore-forming bacterium *Clostridium acetobutylicum* is strictly fermentative and gains energy by converting sugars and starch to organic acids and solvents. In the past, it has been exploited for the large scale production of acetone and butanol, but the classical industrial fermentation process is currently considered uneconomical in most countries, despite increasing oil prices. Essential for the engineering of more efficient strains and processes is a thorough understanding of the organism's physiology and metabolism. Here we investigate, whether cell-cell communication (quorum sensing) is involved in the regulation of key features such as solvent formation and sporulation.

Eight putative quorum sensing systems were identified in the genome of *C. acetobutylicum* and inactivated by insertional mutagenesis. Each of these systems consists of a putative PlcR-type transcriptional regulator and a short linear signalling peptide. The signalling peptide is thought to accumulate during bacterial growth and to specifically interact with its cognate PlcR-type regulator.

Analysis of the obtained mutants revealed that one of the systems influenced the timing and level of solvent production and endospore formation. Overexpression of the regulator lead to a significantly decreased production of solvents and spores, inactivation resulted in earlier and increased production of solvents and spores. A similar increase in sporulation and solventogenesis was observed when the signalling peptide was overexpressed. We hypothesise that the regulator acts as a repressor of solventogenesis and that its activity is inhibited upon binding the cognate signalling peptide.

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Codon Optimisation in *Clostridium spp*

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Members of the genus *Clostridium* have a broad spectrum of biotechnological applications, ranging from the delivery of therapeutic molecules useful in the treatment of solid tumours to the fermentative generation of chemical commodities and biofuels from renewable and/or waste carbon streams. Increasingly, synthetic biology approaches are being implemented to endow the selected process strain with the enzymes required for a particular metabolic pathway or function. Pivotal to these undertakings is the optimisation of the expression of the participating proteins. A common strategy is to 'optimise' codon usage to reflect either that generally displayed by the host organism, the bias found in a subset of highly-expressed native host genes or to exclusively use the commonest codons found in highly expressed genes. Previous work in *Escherichia coli* has shown that these strategies do not necessarily result in the highest levels of protein expression [Welch *et al., PLoS One*. 2009 4(9):e7002]. Here we have measured the level of protein produced by *Clostridium sporogenes* when carrying a common expression vector containing one of 60 synthetic variants of the same gene composed of different combinations of synonymous codons. These data have allowed the formulation of an algorithm that more accurately predicts optimum codon utilisation in this clostridial species. To test the applicability of this algorithm to other clostridial hosts, we have analysed the protein expression of a subset of 8 variants in a number of other species, including *Clostridium acetobutylicum, Clostridium difficile* and *Clostridium beijerinckii.*

What's a SNP Between Friends?

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With the advent of methods for the directed mutation of specific *Clostridium* genes it is now relatively straight forward to generate isogenic mutants of selected strains and thereafter compare their metabolic properties with the progenitor strain. Moreover, the differential effects of mutating distinct genes involved in the same process can be compared in studies undertaken in disparate laboratories. But can they? This assumes that the strains used by independent research laboratories are the same. The problem is that bacterial genomes are not static, and are capable of undergoing all manner of insertions, deletions, rearrangements and point mutations. The likelihood of this happening increases proportional to the number of times the chromosome is replicated, a reflection of the number of sub-cultures a strain is subjected to. The archetypical, and much researched, example is *Clostridium acetobutylicum* strain ATCC 824. Here we compare, by Illumina genome re-sequencing, the number of SNPs and Indels present in the ATCC 824 strain currently being used in a European ERANET SysMO research programme, *C. acetobutylicum* WUR (originally obtained as ATCC 824), strain EA2018 (suggested to be a relative to ATCC 824) and the currently deposited ATCC 824 strain. A number of significant variations are apparent between strains. More striking are the considerable number of SNPs common to these isolates compared to the published genome sequence. It is clear that if researchers wish to work on the same strain of *C. acetobutylicum* that more effective means of strain curation need to be implemented. Moreover, genome re-sequencing of strains should become a routine undertaking before embarking on any programme of work, and particularly after the generation of mutant strains.

Dynamics and control of the *Clostridium acetobutylicum* metabolic network

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Clostridium acetobutylicum is an anaerobic, Gram-positive, spore forming bacteria that is characterized by its unique two-step metabolic pathway. Upon growth in presence of carbon sources, the bacterium first consumes them to produce organic acids in a phase that is known as acidogenesis. Upon accumulation of acids, the organism shuts down acid production, and in fact, consumes the acids to convert them into organic solvents (acetone, butanol, and ethanol). The second phase is called solventogenesis. In this study, using both experimental and computational techniques, we explore the dynamics of the *Clostridium* metabolic network, and more specifically, the switch from acidogenesis to solventogenesis. Our results show that the two phases are expressed independently of each other, and not in a switch-like manner. We also demonstrate that within each of the two phases, there exists a hierarchy of expression of acids and solvents. We also report presence of negative feedback loops in the network leading to a more complex picture of the *Clostridium* metabolic network.

Metabolic Engineering of *Clostridium ljungdahlii* for Production of Fuels and Other Chemicals

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Clostridium ljungdahlii has significant potential for the production of fuels and chemicals from unique feedstocks such as syngas, carbon monoxide wastes, and electricity fed to cells with negatively poised electrodes in an artificial photosynthetic process known as microbial electrosynthesis. However, biotechnological applications of *C. ljungdahlii* have been limited due to a poor understanding of its physiology and the lack of effective genetic tools for metabolic engineering. We have employed omics analyses and newly developed strategies for genetic manipulation to elucidate mechanisms for electron transfer and energy conservation during carbon dioxide reduction and to redirect metabolic pathways to generate products with greater value than acetate and ethanol.

For example, six putative hydrogenases are encoded in the *C. ljungdahlii* genome, but which hydrogenase(s) are important for hydrogen uptake was unknown. Proteomic and gene expression analyses revealed one hydrogenase that was specifically upregulated during growth with hydrogen as the electron donor. Deletion of the gene for this hydrogenase prevented growth with hydrogen as the electron donor, but had no impact on metabolism on electrodes, demonstrating that *C. ljungdahlii* was able to directly accept electrons from electrodes rather than relying on production of a hydrogen intermediate. With a similar approach, the gene encoding the bifunctional aldehyde/alcohol dehydrogenase that is responsible for ethanol production was identified. Inactivation of the ethanol production pathway resulted in a strain with higher acetate production. Insertion of genes encoding pathways for the production of fuels or other organic commodities re-directed electron flow away from acetate and ethanol production and toward the synthesis of the desired products from carbon dioxide. These results suggest that *C. ljungdahlii* can be genetically manipulated to expand the range of products produced from carbon dioxide with reduced gases and via microbial electrosynthesis.

Enhanced Production of Butanol by Metabolically Engineered *Clostridium acetobutylicum*

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It has well been known that by-product formation of A-B-E fermentation with *Clostridium acetobutylicum* results in low productivity, yield and selectivity of butanol, requiring high production cost. The recombinant strains were constructed for enhanced production of butanol.

The batch fermentation profile of the strains showed a reduction in acids, acetone formation compared to the wild type strain with the increase in yield, selectivity and productivity of butanol formation. *In-situ* butanol removal(ISBR) by addition of synthetic resin with high butanol adsorption affinity to the bioreactor results in further increased performance of the strains.

In continuous fermentation with ISBR for 100.5hr, the recombinant strain showed that the overall yield, selectivity and productivity was 37%, 80% and 3.07g/L/h, respectively ,when feeding 200g/L of glucose containing 3% corn steep liquor as a nutrient with the dilution rate of $0.042h^{-1}$

Investigation of Xylose Metabolism in Solventogenic *Clostridia*.

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Solventogenic *Clostridia* are regarded as a big hope for an efficient biotechnological fuel and solvent production in times of increasing prices of crude oil and high carbon dioxide emission. Research focuses on optimized biofuel production by metabolic engineered solventogenic *Clostridia.* They have the ability to ferment sugars from various carbon sources, including components of lignocellulosic biomass, consisting mostly of pentoses, such as xylose. Plant biomass is considered a cheap and renewable feedstock for biobutanol and solvent production.

There are several pathways for D-xylose metabolism known in nature. *C. acetobutylicum* seems to use the xylose isomerase pathway to convert the aldose into D-xylulose. For other solventogenic *Clostridia*, such as *C. saccharobutylicum*, which is known to produce high yields of butanol, the mechanism still remains unknown.

In order to understand xylose metabolism and optimize butanol production, a better understanding of the biochemistry and regulation is needed. *C. acetobutylicum* contains two operons induced by the presence of xylose in the medium. The presence of glucose in the fermentation medium inhibits consumption of large amounts of xylose by *C. acetobutylicum* until nearly all glucose is consumed. In a former study we have shown that the expression of one of the xylose degradation operons is completely repressed by glucose. To understand the role of each operon in xylose degradation, we created ClosTron and clean deletion mutants in both xylose degradation operons of *C. acetobutylicum*. We have also developed appropriate tools to genetically manipulate *C. saccharobutylicum* in order to investigate the xylose metabolism in this organism.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Screening, Characterisation and Genetic Manipulation of a New Solventogenic Clostridial Strain

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Soil and environmental samples obtained from diverse locations were screened for the presence of butanol producing *Clostridium* spp. One strain was selected from the pool of isolated mutants based on its performance across a range of assays, including its ability to grow on hexose and pentose sugars, a low ratio of acetone to butanol production and its high amenability to genetic manipulation. The strain was classified to species level based on 16S rRNA phylogeny. A draft genomic sequence was obtained by sequencing of the organism using a combination of Roche 454 and Illumina next-generation sequencing technologies. The isolated strain, in contrast to many saccharolytic clostridia, is sensitive to a wide range of antibiotics, allowing for use of antibiotic selection markers on transformed plasmids. This ability was coupled with a modified transformation protocol yielding an exceptionally high transformation efficiency, to allow for the deletion of the *pyrE* gene on the chromosome with allele coupled exchange using suicide plasmids. The advantage of a suicide plasmid (a plasmid lacking a gram-positive replicon) is the immediate loss of the delivery vector from the cell once a double crossover event has occurred. The deletion of the *pyrE* gene allows for selection of plasmid integrants based on uracil prototrophy. These factors allow for very rapid complementation of genes at the *pyrE* locus. The use of in-frame deletion as a powerful tool for gene knock-out will now be exemplified in this organism. In the future metabolic engineering will be used to improve butanol production, substrate utilisation and butanol tolerance, creating a strain that can be used as a platform for the production of butanol or relevant high value compounds

Improving ethanol production in *Clostridium thermocellum*

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Clostridium thermocellum is a candidate organism for ethanol production from cellulose via consolidated bioprocessing (CBP) and is of particular interest due to its native ability to rapidly solubilize plant cell walls. Although wild type *C. thermocellum* produces ethanol, yield and titer are too low for commercial viability.

Recent advances in techniques for genetic modification of this organism have opened the door for dramatic improvements in these areas. Systems biology analyses have allowed us to make targeted changes to the metabolism of *C. thermocellum*. Several strains have been generated with modifications in central metabolism, and many of these strains have improved ethanol yield and titer. Analyses of changes in end products reveal the importance of nicotinamide cofactor balancing for ethanol formation. These strains help us understand carbon and electron flux and lead to further targets for manipulation.

Granulose Phenotypes of *Clostridium acetobutylicum*

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The transition phase of growth of the Gram-positive, endospore forming anaerobe *Clostridium acetobutylicum* is characterized by several morphological changes. When entering the stationary phase swollen and cigar shaped cells, termed clostridial stages, are formed. In this type of cells a polymeric glycogen-like carbohydrate is accumulated in the form of granules, called granulose. This glucose-polymer is defined as an amylopectin-like structure and only slightly branched (98% α-1.4-linkages and 2% of α-1.6-linkages). Granulose is considered to store energy- and carbon, necessary as a prerequisite for sporulation.

A single glycogen synthase (GlgA) is annotated in the genome of *C. acetobutylicum* and therefore it was expected to play a crucial role in the biosynthesis of granulose. Several *glgA* insertion mutants (ClosTron® technology) were generated and the correct insertion was proved. Individual mutant strains were selected and phenotypically characterized.

From the selected *glgA* mutants, no differences were found in growth rate and fermentation pattern when compared to the wild-type strain, but all exhibited a granulose defect phenotype. Although we were able to prove the crucial role of GlgA in granulose accumulation, the influence on cell differentiation and sporulation needs further investigation. One of the strains showed a non-sporulating phenotype, while others were still able to sporulate although colonies were not iodine-stainable. Interestingly, all of the strains were able to be complemented for their defective phenotype by plasmid-based *glgA* expression.

The results indicate that there might be a cross-talk between the glycogen metabolism and the sporulation processes.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

A Phosphotransferase System for N-acetylglucosamine Uptake in *Clostridium beijerinckii*

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Environment pollution and energy supply are among the huge problems which threaten the world, especially in industrialised countries. Several studies have considered how to exploit waste materials as renewable substrates for various industries to obtain different products. Some wastes from the aquatic food industry contain a considerable amount of the N-acetylglucosamine (NAG) polymer chitin, which has potential as a substrate for the solventogenic clostridia in the acetone-butanol-ethanol fermentation. Development of an effective process will, however, depend on a detailed understanding of the mechanism and control of chitin hydrolysis and NAG metabolism.

The predominant mechanism for uptake of sugars and sugar derivatives in the clostridia is the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). Extracts of *Clostridium beijerinckii* grown on NAG exhibited a phosphotransferase activity for NAG which was also present in extracts of cells grown on glucose, consistent with the observation that glucose did not repress utilization of NAG in media containing both substrates. Genomic analysis has identified genes encoding a putative NAG-PTS that belongs to the glucose family of PTS permeases. Two divergent genes encode the IIA and IICB domains of the PTS, and are associated with a gene encoding a putative transcriptional antiterminator. These genes were found to be expressed in cells growing on NAG or glucose, but not glucitol. The role of the putative NAG *pts* genes in NAG uptake is currently being assessed by functional and mutational analysis.

Molecular Characterisation of Selected PTS systems of the Bio-butanol Organism, *Clostridium acetobutylicum* ATCC 824

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Clostridium encompass a heterogenous class of bacteria that occupy a diverse range of niches. They are able to utilise a broad range of substrates, including CO, CO_2, starch, cellulose, cellobiose, glucose, maltose, sucrose, mannitol and arabinose. To metabolise these substrates, various uptake systems are employed. Those of clostridia remain porrly charcaterised. Here we describe our efforts to genetically modify components of the phosphoenol pyruvate (PEP) dependent phosphotransferase (PTS) system of *C. acetobutylicum* ATCC 824 in a specifically created *pyr*E -ve strain using the ClosTron mutagenesis method. The use of the *pyr*E -ve strain as the host allows the subsequent rapid chromosomal complement of the mutations made through the introduction of the functional gene concomitant with correction of the *pyrE* allele using ACE (Allele-Coupled Exchange). Our aim is to further elucidate the mechanisms of carbohydrate uptake in this species, and to assess the phenotypic effects of selected PTS gene disruption. So far we have generated triplicate mutants in the genes CAC0423, CAC2956 and CAC3425, each associated with sucrose, galactose and α-glucoside uptake, respectively. Through the analysis of these strains we hope to gain a greater understanding of the physiological aspects of the cell, with a view to future biotechnological applications.

Over-expression Of Thiolase In Different Clostridial Species For Solvent Production

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The biosynthesis of value added multicarbon compounds like ethanol, butanol, acetone, acetic acid, butyric acid is restricted to species of the genus *Clostridium*, a diverse group of Gram-positive, endospore forming anaerobes. Thiolase is a key enzyme of the pathway which drags the carbon flux toward the solvents production in the most of the industrially important clostridial species. In the present study, the thiolase gene (*thl)* was cloned and expressed into different clostridial species using pSOS94 MCS *Clostridium-E.coli* shuttle vector. The recombinant thiolase enzyme is induced by constitutive ptb promoter in the acidogenic phase. The induced band of 44kDa was observed in engineered organisms attempted so far by SDS-PAGE. The over-expression of thiolase in *C. acetobutylicum* ATCC824 has resulted in increase of solvent production. Metabolite profiling of genetically engineered *C. acetobutylicum* NRRL B527, *C. beijerinckii*, and *C.butyricum* is in progress. This is the first report wherein different species of clostridia have been engineered. This study can be helpful for understanding the pathway for metabolite production in different *Clostridium* spp.

Adding a Quorum Sensing Peptide to *Clostridium acetobutylicum* Sequential Batch Cultures Increases Butanol Yield and Productivity

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The production of n-butanol and acetone by *Clostridium acetobutylicum* was one of the first large-scale industrial fermentations ever developed. The process was ultimately eclipsed as lower cost petrochemical processes for butanol (petro-butanol) manufacture were developed. More recently, increasing petro-butanol cost has renewed interest in the fermentation process, and a new round of innovation in biobutanol production has begun.

The molecular mechanisms *Clostridium* uses to control biobutanol formation are not completely understood. Many solvent-producing *Clostridium* strains have reduced ability to produce solvents after repeated subculturing, which can reduce yields in commercial production. Butrolix, LLC has discovered genetic and molecular components of an intercellular peptide signaling system (quorum sensing) in *C. acetobutylicum*. The discovery has resulted in an invention that can increase biobutanol production in sequential subcultures.

Briefly, *C. acetobutylicum* batch cultures were transferred sequentially every 24 hours for 24 days. While one set of sequential cultures remained untreated, three parallel sets were treated with different concentrations of a chemically synthesized quorum sensing peptide. Individual culture supernatants were subsequently recovered and analyzed for biobutanol concentration. The data showed that by the fourth transfer biobutanol concentration in the treated cultures was greater than in untreated, and by the tenth transfer was 46% greater. Subsequent time course studies of cultures at the tenth sequential transfer showed that biobutanol productivity of a treated culture was double that of an untreated culture during the period of peak biobutanol production.

Further development of biobutanol production processes based on manipulation of *Clostridium* quorum sensing mechanisms could result in significantly decreased production cost to a point that substantial market share could be captured from petro-butanol facilities.

Acetone from Carbon Dioxide- Screening and Engineering of Acetogens for Application with an Industrial Waste Gas Stream

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Acetone is an important raw material in the chemical industry as a solvent and for the syntheses of various products, e.g. poly(methyl methacrylate) (PMMA), also known as acrylic glass. Today, acetone still is mainly produced from fossil resources. However, future challenges require alternative strategies enabling the generation of chemicals and biofuels from renewable resources, best based on the usage of the greenhouse gas carbon dioxide (CO_2) as a substrate.

Especially CO_2 is a less expensive feedstock, available in great quantities, and is not interfering with food production, as glucose and other sugars do. Thus, the aim of the project „CO_2-based fermentation of acetone" funded by the German Federal Ministry of Education and Research (BMBF) is the development of a fermentation process in which acetogenic bacteria produce acetone by using CO_2 as starting material.

Although more than hundred acetogenic bacterial species are described so far, in part there is little knowledge about their applicability as production strains. Here, we present first results of a screening approach developing standardized heterotrophic and autotrophic growth conditions enabling the application of a synthetic industrial waste gas stream simulating a potential future biotechnological application.

As a first step to the use of recombinant anaerobic acetogens as a novel biotechnological production platform, *Clostridium aceticum* was transformed with plasmids, establishing two different pathways of acetone formation. Both, under heterotrophic conditions with fructose as a substrate as well as under autotrophic conditions with CO_2/H_2 as a substrate, acetone production could be confirmed in the recombinants.

Characterization of the cellulosomal scaffolding protein CbpB from *Clostridium cellulovorans* 743B

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Clostridium cellulovorans 743B, an anaerobic and mesophilic bacterium, produces an extracellular enzyme complex called the cellulosomes on the cell surface. Recently, we have reported the whole genome sequence of *C. cellulovorans*. As a result, a total of 4 cellulosome scaffolding proteins such as CbpA, HbpA, CbpB and CbpC were found in the genome. In particular, CbpB encoded a 429 residue polypeptide that includes a cellulose binding module (CBM), an S-layer homology module and a cohesin. CbpB was also detected in the culture supernatant of *C. cellulovorans*.

Genomic DNA coding for CbpB was subcloned into a pET-22b+ and pGEX-2T vector in order to express and produce the recombinant protein in Escherichia coli BL21(DE3), respectively. The adsorption of a CbpB to crystalline cellulose was analysed. The measured dissociation constant was 0.82 M. It is a similar to a dissociation constant of CBM from CbpA.

Also we attempted the subcloning of the region encoding the xylanase B with the dockerin from *C. cellulovorans* and analyzed the interaction between xylanase B and CbpB by GST pull-down assay. It was observed CbpB has the ability to bind xylanase B. The activity of xylanase B bound to CbpB increased by one and a half times compared to that of only xylanase B. These results showned that *C. cellulovorans* produces the minimal cellulosome containing CbpB.

Regulation of *adhE2* in *Clostridium acetobutylicum*

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In continuous culture, *C. acetobutylicum* can be maintained in three different stable metabolic states: acidogenic (production of acetic and butyric acids) when grown at neutral pH on glucose, solventogenic (production of acetone, butanol and ethanol) when grown at low pH on glucose, and alcohologenic (formation of butanol and ethanol but not acetone) when grown at neutral pH under conditions of high NAD(P)H availability (Girbal and Soucaille, 1998). In alcohologenic culture, the genes involved in the butanol formation (*bdhA, bdhB,* and *adhE*) were not transcribed and the *adhE2* gene of *C. acetobutilycum* ATCC 824 is expressed specifically. The *adhE2* gene was characterized from molecular and bio-chemical points of view by Lisa Fontaine in our laboratory (Fontaine *et al.* 2002). This is the second AADH identified in *C. acetobutylicum* ATCC 824. Both of the genes *adhE* and *adhE2* are carried by the pSOL1 megaplasmid of *C. acetobutylicum* ATCC 824. Regulatory mechanism of *adhE2* expression is unknown. A redox sensing protein encoded by *CA_C2713* was found to bind to the promoter regions of *thl, ldh, crt* and *adhE2* (Wietzke and Bahl 2012). Interestingly, we found that there are two additional palindromes upstream of *adhE2* that potentially contribute to concomitant control of *adhE2*. We are elucidating the functions of these palindromic sequences by employing pGUSA reporter system developed by Girbal (Girbal *et al.* 2003). In parallel, we are isolating the potential repressors/activators by using magnetic bead technology. ClosTron knock-out system will be used for validating their regulatory effects. Understanding the mechanism of *adhE2* regulation would be helpful for further engineering *C. acetobutylicum* to achieve a highly efficient process for the production of butanol.

References

Fontaine *et al.,* 2002. J*ournal of Bacteriology* **184:** 821.
Girbal *et al.,* 2003. *Appl Environ Microbiol* **69:** 4985-8.
Wietzke & Bahl. 2012. *Appl Microbiol Biotechnol* May 12.
Girbal & Soucaille 1998. *Trends in Biotechnol.***16:** 11-16

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A robust method based on an inducible counter-selection marker for isolating rare genomic recombination events in *Clostridium acetobutylicum*

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The limited availability of genetic tools and the inherent difficulty of genetically modifying *Clostridium* organisms have been a hindrance in both basic science research and applied research towards the development of desirable strains. Specifically, gene inactivation and chromosomal integrations for fundamental investigations and practical applications in strain development have been difficult to achieve. The lack of a counter-selection marker for identifying the extremely (or very) rare double-crossover recombination events for use in *C. acetobutylicum* and other *Clostridium* organisms has been described as a major obstacle. Development of such a counter-selectable system and identifying the appropriate conditions for its use is one of the most challenging features for such a system. We will report on the development of such a system and show its utility and application. We utilized a lactose inducible promoter and its divergent regulator that was adapted from *C. perfringens* strain 13 (HARTMAN, AH et al. 2011. *Appl. Environ. Microbiol*. **77:** 471-478) to temporally control the expression of a codon optimized, synthetically generated gene coding for the *E. coli* toxin MazF, which is a part of a family of toxin-antitoxin systems in many prokaryotes. MazF is an endoribonuclease that cleaves RNA at specific sequences leading to rapid cell growth arrest and ultimately cell death. We successfully isolated double-crossover based knock outs of all four genes (*ca_p0157, ca_p0167, sigF and sigK*) we have attempted to knock out. We also integrated a 3.65 kb heterologous “cargo” DNA (this includes a 2.1 kb formate dehydrogenase gene plus a FRT-flanked thiamphenicol resistance marker) into the *C. acetobutylicum* chromosome. Our methods allows to knock out any gene and also knock in heterologous DNA in any chromosomal location, thus enabling sophisticated synthetic-biology strategies, such as the introduction of heterologous, multi-gene-based cellular programs.

Development of an Electrotransformation System and Gene Knockout Mutants of *Clostridium pasteurianum*

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Despite continued interest within the scientific community, efforts to reestablish an ABE-based biobutanol industry have been largely unsuccessful. Economics remains the chief barrier to viability, where feedstock costs represent up to 80 % of total process costs for biobutanol production using the prototypical solventogens, *Clostridium acetobutylicum* or *C. beijerinckii*. As a result of its overabundance and exceptionally low cost, the fermentation of waste biodiesel glycerol to butanol, a metabolic capability reserved exclusively for *C. pasteurianum*, currently holds the greatest industrial potential amongst the solventogenic clostridia. However, in spite of a recent upsurge in the number of scientific publications detailing *C. pasteurianum* butanol production and expressed interest in the genetic engineering of this organism, no genetic tools have been established, nor has any genome sequencing been conducted. Accordingly, the industrial potential of *C. pasteurianum* remains untapped.

As an entry point to allow genetic manipulation of *C. pasteurianum*, we have developed the first electrotransformation system for DNA transfer to *C. pasteurianum*. Similar to *C. acetobutylicum*, efficient transformation (10^4 transformants/µg DNA) is dependent upon in vivo or in vitro methylation of plasmid DNA to protect against an endogenous Type II restriction endonuclease. Using our optimized protocol, we have also utilized the powerful TargeTron Gene Knockout technology to first disrupt canonical knockout genes, such as *spo0A*, as well as a number of targets hypothesized to improve butanol yield on crude glycerol. Finally, we have undertaken genome sequencing of *C. pasteurianum* and will share preliminary insights on the unique metabolic capabilities gleaned from the sequencing data and our ongoing gene knockout work. These results represent the first development of a comprehensive set of genetic tools for *C. pasteurianum*, which will pave the way for its viable production of biobutanol.

Exploring Nitrogen Requirements in Fermentation Using Novel South African NCP *Clostridium beijerinckii* and *Clostridium saccharobutylicum* Strains.

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The products of the ABE (Acetone Butanol Ethanol) fermentation are of commercial value as solvents used in both laboratory and industrial settings. Understanding the role of nitrogen in feedstocks is crucial for optimal cell growth which leads to improved solvent yields and economic profit. Our study focuses on the role of nitrogen metabolism in solventogenic clostridia and its effect on solvent production in different nitrogen media conditions to increase solvent yields. This study showcases a large number of the NCP strain collection isolated from the National Chemical Products (NCP) ABE fermentation plant in Germiston South Africa before the plant's shutdown in 1982.

The studied NCP strains were classified according to both species and strain grouping by a quick species-specific colony PCR method and by Random Amplification of Polymorphic DNA (RAPD) methods respectively. This approach indicates that the University of Cape Town has a collection of 19 saccharolytic *Clostridium beijerinckii* and 11 *Clostridium saccharobutylicum* NCP strains. RAPD screening separated the *C. beijerinckii* strains into four distinct groups and *C. saccharobutylicum* strains into 2 groups at the strain level.

Candidate strains from the NCP collection were tested for preferred nitrogen sources including various amino acid organic nitrogen conditions and assayed by Gas Chromatography (GC) for acetone, butanol and ethanol solvent production relative to complex casamino acid media fermentation conditions. The different strains show considerable variation in fermentation substrate specificity and solvent yields, indicating that specific strains can be selected for use in fermentation plants to suit the fermentation substrate availability of the region in question. This study shows insights into the optimum nitrogen requirements for NCP strains and will improve nitrogen conditions in existing industrial feedstocks.

Characterization of the Ferredoxin NAD Oxidoreductases of *C. acetobutylicum*

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In the strict anaerobe spore-forming bacterium *Clostridium acetobutylicum*, ferredoxin oxidoreductases enzymes play a key role in the regulation of the electron flow. In the exponential acidogenic phase of growth, in parallel of pyruvate ferredoxin oxidoreductase, the NAD(P)H ferredoxin reductase enzyme mediates the reduction of ferredoxin by consuming the excess of NADH produced during glycolysis. In the solventogenic or the alcohologenic phases, the ferredoxin NAD(P)+ reductase enzyme acts in redirecting the electron flow from reduced ferredoxin toward the production of the NAD(P)H needed for ethanol and butanol production.

Although the presence of ferredoxin oxidoreductase activities in cell-free extracts from *Clostridium acetobutylicum* were reported (1,2), the enzymes responsible of these activities have never been purified or identified.

First, an overall production, extraction and purification process was designed to purify both NADH-ferredoxin reductases and ferredoxin NAD+ reductases enzymes from acidogenic and alcohologenic cultures of *Clostridium acetobutylicum*. At each step of extraction or purification, NADH-ferredoxin reductase or ferredoxin NAD+ reductase activities were evaluated to select positive fractions. Using this method two different proteins or protein complexes were identified.

Second, to further characterize the putative candidates, the genes encoding these enzymes were cloned and the corresponding proteins purified and characterized for the NADH-ferredoxin reductases and ferredoxin NAD+ reductases activities.

Finally, to evaluate the *in vivo* role of these candidates, the genes encoding each enzyme were separately disrupted in *Clostridium acetobutylicum* ATTC824. The resulting mutant strains (if viable), were then characterized from a physiological point of view.

The identification of these key enzymes and encoding genes is of great interest as it opens new perspectives for heterologous n-Butanol production.

(1) Girbal, L., C. Croux, I. Vasconcelos, and P. Soucaille. 1995. Regulation of metabolic shifts in *Clostridium acetobutylicum* ATCC824. FEMS Microbiol. Rev. 17:287-297.

(2) Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J Bacteriol 176:1443-50

The Project Profiles of 25 kt/a ABE from Sweet Sorghum Juices

BANGXIONG RONG, LIJUN SHAO

Xingan League Yuan Xing Bio-energy CO.,LTD.,
Inner Mongolia Berun Holdings Group CO., LTD., Erdos, China

Inner Mongolia Berun Holdings Group CO., LTD. is a private joint-stock enterprise, mainly engages in the chemical industry, the energy, the real estate and financial service industry. In recent years, Berun group tightly follows the national macroeconomic policies and industrial guidance, focuses on the industrial structural adjustment and realizing its transformation and upgrading.

For Berun group, the “Twelfth Five Years” period would be the second establishment stage and the new development one. During the five years, Berun group will make Xingan League the breakthrough and grasp the chance from the preferential policy adopted by the autonomous region and Erdos to Xingan League, and then carry on the strategic layout with the advantage of the local resources. Therefore, a large number of high quality projects would gradually be implemented step by step resulting in the final total capital investment in Xingan League to 30 billion Yuan, including the non-ferrous metal, the coal chemical industry and the biochemical industry with sweet sorghum as raw material.

Xingan League Yuan Xing Bio-energy CO., LTD. is Berun group's wholly-owned subsidiary, located in Inner Mongolia Xingan League Zhalaite County industrial park, committed to biochemical technology development and industrialization of sweet sorghum stalks as raw materials. The company has built the China’s first sweet sorghum renewable energy engineering laboratory with the platforms of material handling, microbe culture, fermentation, analysis and detection. Moreover, the company has established a 300 t/a fermentation pilot plant.

The project of 25 kt/a ABE (acetone-butanol-ethanol) from the sweet sorghum juices is a cutting edge one with which Berun group step into the bio-energy field. We welcome the friends from all around the world to cooperate, to progress with hand in hand and enjoy the prospects.

Promoter Activity-Studies using a novel *in vivo* Reporter System for *Clostridium acetobutylicum* ATCC 824

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Fluorescent proteins such as the green fluorescence protein and its derivatives strictly require oxygen similar to luciferase-based reporter systems, which excludes these gentle *in vivo* reporters for applications in anaerobes. Recently, novel flavin mononucleotide (FMN)-based fluorescent proteins harboring light-oxygen-voltage domains were engineered for non-invasive reporter systems applicable for both aerobic and anaerobic conditions in *Escherichia coli* and *Rhodobacter capsulatus* (Drepper *et al.*, Nat. Biotechnol. 25:443-445). We have optimized these fluorescence-based reporters for monitoring gene expression in *Clostridium acetobutylicum*. To examine the applicability of the *in vivo* reporter system, we comparatively analyzed the promoter activities of four native *C. acetobutylicum* genes (*thlA*, *ptb*, *adc*, *hydA*). Furthermore, we studied the *adhE2* promoter activity in the wildtype and recently described knock-out mutants with an ethanologenic phenotype. The results demonstrated the effectiveness of this fluorescence reporter system to differentiate promoter strength and growth-dependent profiles.

Expression of Cellulosomal Elements in *Clostridium acetobutylicum*

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Clostridium acetobutylicum is well known for its ability to ferment sugars into the solvents acetone, butanol and ethanol and has in the past been used in the industrial ABE fermentation process. However, sustainable biofuel, especially bio-butanol, production must use renewable non-food biomass as feedstock. A challenge, which can be met by the deployment of cellulosomes, extracellular multi-enzyme complexes capable of degrading plant cell wall material, or 'free' carbohydrate-active enzymes (CAZy). Our aim, therefore, is to equip *C. actobutylicum* with the ability to degrade lignocellulosic material. Using synthetic biology approaches and well-characterised elements of the known cellulosomal systems of *Clostridium thermocellum* and *Clostridium cellulolyticum,* we have successfully expressed native (i) *C. thermocellum* and (ii) *C. cellulolyticum* cellulosomal subunits as well as (iii) chimeric hydrolases in *C. acetobutylicum.* Cellulosomal components were stably integrated into the genome and secretion was demonstrated.

Rapid, Simple and Routine Method for Identifying Proprietary Industrial Strains

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With the depletion of petroleum resources and mounting pressure to reduce carbon dioxide emissions linked to climate change, there is an increasing demand for sustainable sources of fuel and chemicals. Butanol is particularly valuable both as an intermediate in the synthesis of commercially important chemicals and as a biofuel. Green Biologics Limited (GBL) is a leading industrial biotechnology company focused on the fermentative production of biobutanol by solventogenic clostridial species in the Acetone-Butanol-Ethanol (ABE) process. GBL has a large number of proprietary strains modified by random mutagenesis or targeted genetic manipulation to improve commerically relevant characteristics. To enable quick and efficient identification of these resultant strains in a commercial environment, allele coupled exchange (ACE) tools developed at the University of Nottingham (Heap *et al*., 2012) are being used to integrate short, synthetic DNA sequences into the host genome. Universal primers will be used to rapidly and directly identify the DNA tag by means of a simple tag detection kit. This will be used on customer sites, allowing easy identification and monitoring of GBL strains from other strains or contaminants present. This project is funded by the Knowledge Transfer Partnership (KTP) scheme.

Heap *et al*., (2012) Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. *Nucleic Acids Research* 40(8):e59

Differential Regulation of Genes Involved in the Metabolism of Arabinose and Galactose

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C. acetobutylicum can ferment carbohydrates from a variety of industrial and agricultural waste products to acetone and butanol. The complex nature of these feedstocks means regulation of the multiple metabolic pathways will determine the efficiency of conversion. In this report we begin to elucidate metabolic pathway regulation during growth on mixed carbohydrates by examining mixed galactose/arabinose fermentations. When cells were fed a 0.25% galactose/ 0.25% arabinose mixture both sugars were used simultaneously. Arabinose was preferentially used while arabinose concentrations were above ~4 mM with a maximal arabinose:galactose utilization rate of ~7.8. Transcriptional analysis during early log growth on an arabinose/galactose mixture revealed the pentose phosphoketolase pathway for arabinose utilization and the tagatose-6-P pathway for galactose utilization were induced. The Leloir pathway for galactose utilization was not induced. The tagatose-6-P pathway generates pyruvate and glyceraldehydes-3-P and the Leloir pathway produces upper glycolytic intermediates. During simultaneous metabolism of arabinose and galactose the pentose phosphoketolase pathway can provide upper glycolytic intermediates via fructose-6-P. This would make the Leloir pathway redundant and could explain why it is not induced. It can also be suggested that the primary role of the Leloir pathway during galactose metabolism is to provide upper glycolytic intermediates while the tagatose-6-P pathway provides lower glycolytic intermediates.

Advanced Continuous Fermentation Process for Butyric Acid using Engineered *Clostridium tyrobutyricum*

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Butyric acid is an important C4 chemical in animal feed, and for the chemical and pharmaceutical applications. Currently butyric acid is produced chemically from the oxidation of butyraldehyde, but there is renewed interest in the production of renewable butyric acid via bacteria fermentation of sugars. Typical strains for butyric acid production suffer from low titres, yield and productivity. In this study, we report on a superior engineered strain and improved fermentation performance using continuous culture with a mutated *Clostridium tyrobutyricum strain* in an immobilized fibrous bed bioreactor (FBB).

C. tyrobutyricum PAK-Hyd-Em (*ack-KO)* was first created using integrational mutagenesis to disrupt the acetate kinase (*ack*) gene. A derivative that produced higher butyric acid yields was then selected and isolated from the continuous FBB system at the Ohio State University. Strain performance was evaluated in batch, fed-batch and 3-stage continuous systems. Overall, the 3-stage continuous FBB proved advantageous resulting in a butyric acid titre of 41.4 g/L, yield of 0.41 g/g and an overall productivity of 1.3 g/L/hr.

The continuous 3-stage FBB system with immobilized cells of *C. tyrobutyricum* PAK-Hyd-Em is a promising method for industrial production of butyric acid. The results demonstrate the potential of using *Clostridia* and continuous FBB technology for the production of high value chemicals, in addition to acetone and butanol, from lower cost renewable feedstocks.

Modelling of Glucose and Acid-Pulsed Chemostatic Continuous Culture Experiments in *Clostridium acetobutylicum*

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Clostridium acetobutylicum is being considered as a suitable bacterium for the conversion of glucose substrates into the useful solvents acetone, ethanol and butanol: with the latter providing a major component of new biofuels. In order that the yield is maximised, many mutants are being considered in order to modify the metabolism in order to preferentially produce one solvent over the others.

Experimental evidence suggests that imposing acetate stress on batch cultures increases the production of acetone and butanol relative to unchallenged cultures, whereas butyrate stress reduces acetone and butanol production relative to unchallenged cultures (Alsaker et al., *Biotechnology and Bioengineering*, 105(6), 1131-1147, 2010). This is explored for pulsed acetate and butyrate stresses on continuous cultures using a metabolic model with parameters fitted to existing pH-shift chemostat continuous culture experiments (Haus et al., *BMC Systems Biology*, 5:10, 2011) which is extended to take account of the different physiologies of acidogenic and solventogenic *C. acetobutylicum*. Finally, the equivalent experiments using glucose pulses are simulated and discussed.

Dynamics of Cecum Microbiota During Antibiotics Treatment in Clostridial Murine Model Revealed by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

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Clostridium difficile is the major leading cause of nosocomial antibiotic-associated diarrhea with increasing incidence and fatality. Since antimicrobial resistance is not clinically problematic, the risk of recurrent *C. difficile* infection (CDI) is increased in patients. Antibiotic treatment alters the composition of gut microbiota which may diminish protective immunity. Although alterations in gut microbial communities are widely expected to be associated with CDI disease states, the nature of these transitions are mostly unknown. Therefore, we established a mouse model of antibiotic-associated CDI to investigate how infection with *C. difficile* affects the spatial and temporal dynamics of the full gut microbial community. Bactria from feces and cecum contents were compared in the groups before antibiotic treatment, after antibiotic treatment and after CDI. We used the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) Biotyper system to identify microorganisms directly from cultured colonies. While the major components of the intestinal microbial flora, *Bacteroidetes*, *Firmicutes*, and *Lactobacilli*, were significantly decreased after antibiotic treatment, *Enterobacteriaceae* and *Enterococcus* were significantly increased after CDI. Our results demonstrate that antibiotics perturb the commensal microbiota that could disrupt the colonic immune homeostasis and set the stage for intestinal domination by bacteria associated with hospital-acquired infections. Understanding the complex microbial populations and determining their composition using MALDI-TOF MS approaches will increase our ability to identify patients at risk for developing clostridial infections and allow us to prevent CDI.

Host Innate Responses to *Clostridium diffcile* Colitis

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Clostridium difficile is the major leading cause of nosocomial antibiotic-associated diarrhea with increasing incidence and fatality. An inability to mount a protective immune response to *C. difficile* appears to underlie susceptibility to recurrent infection. Gaps in our understanding of the immunopathogenesis of CDI present major challenges to the development of better preventive and therapeutic strategies against this problem. An animal model is extremely important in elucidating the immunopathogenesis of CDI. Many different CDI animal models including piglet, guinea pig, hamster or gnotobiotic mice had been reported, but many limitations and weaknesses exist. Here we established a practical mouse model of antibiotic-associated CDI that has disease spectrum being more closely to that in human beings, including weight loss, symptomatic disease, cecal and colonic ulceration, a relatively minor neutrophile infiltration, a measurable cytokine response, and even death. Bacteria from fecal cultures grew the respective strain that were infected with. To directly address the inflammatory state on the spatial and temporal pattern *in vivo*, we induced CDI on a previously generated NF-κB-dependent reporter mouse model containing the luciferase transgene under the transcriptional control of NF-κB. Clostridial infected mice exhibited an increase in luminescence in the abdominal region and the precise location of luminescence in colon was confirmed by autopsy examination. Histologic examination of colonic tissues obtained from infected mice showed inflammatory colitis with superficial epithelial necrosis and inflammatory cells infiltration.

The Phosphotransferase System CAC0231-CAC0234 Controls Fructose Utilization of *Clostridium acetobutylicum*

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Clostridium acetobutylicum is well characterized by its biphasic fermentation metabolism. At higher pH values exponentially growing cells usually produce acetate and butyrate as main fermentation products whereas when the pH has dropped below 5.0 the metabolism switches to 'solventogenesis' with the dominating fermentation products butanol and acetone. As a carbon and energy source a variety of carbohydrates like glucose, fructose or xylose can be utilized by *C. acetobutylicum*.

Generally, carbohydrates were taken up via three types of transporters: symporter, ATP-binding cassette (ABC) transporter and phosphotransferase systems (PTS). For the uptake of hexoses, hexitols and disaccharides thirteen PTS have been identified in *C. acetobutylicum*. Among them, three PTS are supposed to be responsible for the uptake of fructose. The apparent primary fructose transport system is encoded by a polycistronic operon (*cac0231-cac0234*) including a putative DeoR-type transcriptional regulator (CAC0231), a 1-phosphofructokinase (CAC0232), a PTS IIA (CAC0233) and a PTS IIBC (CAC0234). To analyze the role of the PTS during growth on fructose as sole carbon source, each single gene of the operon (*cac0231-cac0234*) was specifically interrupted using the ClosTron® system. All mutant strains showed impaired growth due to reduced fructose consumption. Interestingly, a concomitant loss of solvent production was monitored indicating a threshold of sugar concentration for initiation of the metabolic switch.

Moreover, the transcriptional regulator CAC0231 was overexpressed in *E. coli* and purified for electrophoretic mobility shift assays (EMSA). Here, a putative binding motif was identified and proved by a specific binding of CAC0231 to the promoter region of *cac0231-cac0234*.

A Small Acid-Soluble Spore Protein is Essential for Germination of *Clostridium acetobutylicum* Spores

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Clostridium acetobutylicum is a potent solvent producer and it has become a model organism for the understanding of the molecular biology of non-pathogenic clostridia. We are interested in the events during the cell cycle of *C. acetobutylicum*. Here, we focus on aspects of the resistance and germination capability of its spores.

Alpha/beta-type small acid-soluble spore proteins (SASP) are usually located in the core of the endospores. They exhibit important functions like protection of spore DNA against damage caused by desiccation, heat, or chemical agents. Furthermore, during germination fast degradation of SASPs provides an important amino acid pool for the development of the new vegetative cell.

In the genome of *C. acetobutylicum* five open reading frames are predicted to encode SASP-like proteins (1). In order to unravel the function of each of these proteins, we generated insertional knockout mutants using the ClosTron technology, which is based on selective retargeting of group II intron (2).

The constructed mutants did not exhibit distinguishable difference in growth rate and ability to form spores relative to the parental strain. However, sporulation assays (3) showed that germination capabilities of these strains are affected to varying degree. Most interestingly, one SASP appeared to be essential for this process. Lack of this protein resulted in production of morphologically intact spores with an aberrant coat, which was visualised using transmission electron microscopy. The germination defect was restored by a plasmid-based complementation with the wild type copy of the gene encoding the corresponding SASP protein.

References:
(1) Nölling *et al*., 2000, J Bacteriol. 183:4823-4838
(2) Heap *et al.,* 2007, J Microbiol Methods 70:452-464
(3) Burns *et al.,* 2010, J Bacteriol. 192:657-664

The redox sensor Rex controls product formation in *Clostridium acetobutylicum*

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The anaerobic bacterium *Clostridium acetobutylicum* is well known for its biphasic fermentation metabolism. The exponential growth is characterized by acetic and butyric acid formation and during the stationary phase the solvents acetone, butanol and ethanol are the main products.

However, very little is known about regulatory and molecular mechanisms controlling the carbon and electron flow during the metabolic shift. The sensing of the redox status of the cell is expected to play an important role within this regulatory network.
The genome of *Clostridium acetobutylicum* encodes the protein Cac2713, which is annotated as “redox sensing transcriptional repressor Rex“. The deduced amino acid sequence of Rex shows a high similarity to well-known NADH/NAD^+ redox regulators. To analyze the function of Rex in *C. acetobutylicum*, a Rex negative mutant of *C. acetobutylicum* was constructed by insertional inactivation of the gene. The mutant exhibited an interesting phenotype. In batch culture this strain produced high amounts of ethanol and butanol production started earlier at higher pH-value compared to the parental strain. The production of butyric acid and acetone was significantly reduced.

In agreement with the physiological data the genes of several dehydrogenases, including the bifunctional aldehyde/alcohol dehydrogenase AdhE2 (Cap0035) were upregulated as shown by Northern blot analysis. Furthermore, the purified Rex protein was able to bind to putative Rex boxes in front of these genes.
We concluded that Rex plays an important role in product formation by sensing the redox status of the cell and adjusting the metabolic flux accordingly.

Understanding *Clostridium thermocellum* Cellulosome System By Deletion Of Scaffoldin Genes

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The *C. thermocellum* cellulosome system, consisting of numerous different cellulosomal cellulases that are organized through specialized binding domains onto non-catalytic scaffoldin peptides, is extremely effective in the digestion of cellulose. In order to evaluate the individual contributions of the different scaffoldin proteins to overall activity, we are systematically creating mutants in which we have knocked out the genes encoding individual scaffoldins or combinations thereof. Performance of these mutants in degradation and utilization of Avicel in fermentation have been tested, and some interesting results have been found: cellulosomal enzymes organized on scaffoldins are much more effective than a simple mixture of the same enzymes expressed as “free enzymes” (i.e., with scaffoldin genes knocked out), and the primary scaffoldin CipA appears to be more important for cellulosome digestion of Avicel than are the secondary scaffoldins, combined. Further insights are provided by comparisons of direct biochemical analyses of the mixtures of cellulosomal complexes present with the organism-level performance observed.

Analysis of Metabolic Mutants of *Clostridium acetobutylicum*

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Clostridium acetobutylicum is an anaerobic, gram-positive, spore-forming bacterium which is able to convert various sugars and polysaccharides into organic acid and solvents. In continuous culture, *C. acetobutylicum* can be maintained under three different metabolic states according to the pH of the culture and availability of NAD(P)H : i) an acidogenic state (production of acetic and butyric acids), ii) a solventogenic state (production of acetone, butanol, and ethanol) and iii) an alcohologenic state (formation of butanol and ethanol but not acetone). The complex metabolism of *Clostridium acetobutylicum* has been studied for a long time, but still the factors involved in the metabolic shift and the physiological transition from acidogenesis to solventogenesis are not well understood.

For a better comprehension of the regulation of solvent formation, we have constructed several metabolic mutants (*buk, adhE2, alsD*) by the "double crossing over gene deletion" method developed by Metabolic Explorer We will present a physiological characterization of these mutants in batch and chemostat cultures under different metabolic states.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Controlling the redox potential during the fermentation of *Clostridium acetobutylicum* to improve solvent production

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Fermentative production of solvents (acetone, butanol, and ethanol) by *Clostridium acetobutylisum* typically uses submerged anaerobic process. Comparing with the precise monitoring and controlling of the dissolved oxygen in aerobic fermentation processes, anaerobic fermentation processes are less well monitored and controlled, due to lack of meaningful monitoring and controlling parameters. We developed an ORP (oxidoreductive potential) auto-controlling system so as the ORP of the broth can be controlled automatically throughout the fermentation. Using such a newly developed facility, we detected and controlled the ORP during fermentation of C. acetobutylicum. Though the strain is obligate anaerobic, we found that when controlling the broth ORP at a more oxidative-status of -290 mV (under ORP-uncontrolled condition the ORP is around -320 mV) by automatically inputting air, both cell growth and solvent production were improved. At ORP of -290 mV, an earlier initiation of solventogenesis was achieved, and solvents production reached 25.6 g/L (2.8 g/L acetone, 16.8 g/L butanol and 6.0 g/L ethanol), increased by 35% compared with the ORP-uncontrolled process. By using metabolic flux analysis, we found that the central carbon flux was increased significantly when ORP was controlled at -290 mV, which might provide more ATP for solvent production. Specifically, the solvent ratio (acetone:butanol:ethanol) was changed from 25:64:11 to 11:66:23 at ORP level of −290 mV, which might be resulted from the rigidity at acetyl-CoA node and the flexibility at acetoacetyl-CoA and butyryl-CoA nodes in response to ORP regulation.

Targeted Mutagenesis of the *Clostridium acetobutylicum* Acetone-Butanol-Ethanol fermentation Pathway

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Using ClosTron technology, a large-scale targeted mutagenesis in *C. acetobutylicum* ATCC 824 was carried out, generating a set of 10 mutants, defective in alcohol/aldehyde dehydrogenases 1 and 2 (*adhE1, adhE2*), butanol dehydrogenases A and B (*bdhA*, *bdhB*), phosphotransbutyrylase (*ptb*), acetate kinase (*ack*), acetoacetate decarboxylase (*adc*), CoA transferase (*ctfA/ctfB*), and an previously uncharacterised putative alcohol dehydrogenase (CAP0059). However, inactivation of the main hydrogenase (*hydA*) and thiolase (*thl*) could not be achieved. Constructing such a series of mutants is paramount for the acquisition of information on the mechanism of solvent production in this organism, and the subsequent development of industrial solvent producing strains. Unexpectedly, *bdhA* and *bdhB* mutants were not affected in solvent production, whereas inactivation of the previously uncharacterised gene CAP0059 resulted in increased acetone, butanol, and ethanol formation. Other mutants showed predicted phenotypes, including a lack of acetone formation (*adc*, *ctfA*, and *ctfB* mutants), an inability to take up acids (*ctfA* and *ctfB* mutants), and a much reduced acetate formation (*ack* mutant). The *adhE1* mutant in particular produced very little solvents, demonstrating that this gene was indeed the main contributor to ethanol and butanol formation under the standard batch culture conditions employed in this study. All phenotypic changes observed could be reversed by genetic complementation, with exception of those seen for the *ptb* mutant. This mutant produced around 100 mM ethanol, no acetone and very little (7 mM) butanol. The genome of the *ptb* mutant was therefore re-sequenced, together with its parent strain (ATCC 824 wild type), and shown to possess a frameshift mutation in the *thl* gene, which perfectly explained the observed phenotype. This finding reinforces the need for mutant complementation and Southern Blot analysis (to confirm single ClosTron insertions), which should be obligatory in all further ClosTron applications.

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