Isolation and characterization of mine-dwelling actinomycetes as potential producers of novel bioactive secondary metabolites.

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1 Introduction

1.1. Impact of the actinomycetes in natural product research

Since the isolation of actinomycin (Waksman & Woodruff, 1940) and streptomycin (Schatz *et al.*, 1944), actinomycetes have taken their permanent and prominent place in antibiotic research. During the “golden era” in the 1940s and 1950s, almost all groups of important antibiotics, mainly isolated from *Streptomyces* species, were discovered (Bérdy, 2005). At that time, actinomycete-derived antibiotics represented some 70 to 80% of the all isolated natural products. Today, due to the nearly exponential increasing stock of known metabolites from various sources such as higher plants and fungi, this number is certainly much lower. However, members of the order *Actinomycetales* still contribute with two-thirds of the total “the lion’s share of the antibiotics producers” (Haferburg *et al.*, 2009).

In a recent genomic study, Donadio *et al*. (2007) analyzed the potential of 223 sequenced bacteria to produce secondary metabolites by searching for polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) genes. Both, PKS and NRPS are (multi)modular polypeptides involved in the biosynthesis of a large number of natural products. The authors noted that PKS and NRPS genes are rare or absent in genomes <3 MB, i.e., most bacterial genomes lack any detectable gene cluster for secondary metabolism. The percent of coding capacity devoted to PKS and NRPS genes increased with genome sizes above this threshold, rising to 1.9% in *Actinobacteria*. Within this group, the mycelially growing actinomycetes with large genomes devote up to 10% of their coding capacity to secondary metabolism genes (e.g., Ikeda *et al.*, 2003; Oliynyk *et al.*, 2007; Udwary *et al.*, 2007). None of the other taxonomic groups devote such high percentages of their coding capacity to PKS and NRPS functions. The genome of *Streptomyces avermitilis* (Ikeda *et al.*, 2003) encodes at least 30 gene clusters (6.6% of the genome) devoted for the synthesis of secondary metabolites. Of them, 13 appear to be unique to *S. avermitilis*; their corresponding metabolites remain to be identified (Donadio *et al.*, 2007). Interestingly, many representatives of the less-frequently isolated genera possess genomes similar in size and organization to those of the streptomycetes and are assumed to contain similar numbers of secondary metabolism clusters. The marine actinomycete *Salinispora tropica* strain CNB 440 for example dedicates 9.9% of its 5.18 MB genome to natural product assembly, which is greater than any previous *Streptomyces* genome sequences, with the majority of its 17 biosynthetic loci being novel (Udwary *et al.*, 2007). Furthermore,

members of many yet little-investigated genera, e.g., *Amycolatopsis* (rifamycin, vancomycine), *Actinoplanes* (teicoplanine), *Micromonospora* (gentamycine), *Streptoalloteichus* (tobramycin), and *Saccharopolyspora* (erythromycine) are producers of highly important antibiotics as well.

However, being easily adaptable to laboratory cultivation, *Streptomyces* for long has been by far the most prolific genus and the pharmaceutical industry probably isolated and screened millions of *Streptomyces* strains. In the 1980s, however, natural product research centers encountered diminishing returns from screening fermentation broths for new leads (Watve *et al.*, 2001). Although a number of recent publications indicate that new antibiotics continue to be discovered (e.g. Bérdy, 2005; Herold *et al.*, 2005), the rate of discovery has declined from about 80 new hits per year in the 1960s to a current level of less than 15 hits per year (Watve *et al.*, 2001). In the 1990s, high throughput screening (HTS) against individual antibacterial targets became the norm in antibiotic discovery but, unfortunately, has not proved to be able to meet high expectations for three primary reasons: first, most enzymes that are essential for bacterial viability are not readily druggable; second, some of the best known antibacterial targets are not accessible to *in vitro* screening (e.g. ribosomes and nascent peptidoglycan); and third, this format is particularly well suited for combinatorial libraries of chemicals, but not for natural products (Baltz, 2008). Combinatorial chemistry, however, is incapable of generating the molecular complexity found in useful natural product antibiotics (e.g., vancomycin, daptomycin, cephalosporin C, erythromycin; Henkel *et al.*, 1999). Moreover, screening one target at a time *in vitro* is much less efficient than screening all targets simultaneously in a format that demands penetration of the bacterial cell wall and cell membrane and inhibition of the growth of bacteria (whole cell screening). Antibiotics produced by actinomycetes have been evolving for 1 billion years (Baltz, 2008). Thus, they reliably reach their site of action in other microbes and are able to inhibit their target enzymes, macromolecules or macromolecular structures. As such, a major obstacle with target-based approaches, converting *in vitro* hits into whole cell active leads, has been solved during the evolution of natural product antibiotics.

Today there are two main problems with screening actinomycetes for novel secondary metabolites: (i) about 10 million strains or more have already been screened and several thousands of described microbial metabolites have been identified so far; and (ii) useful metabolites yet to be discovered will be produced by strains less abundant than those that have already been discovered.

As a consequence, known substances have become background noise in chemical screenings, interfering with the discovery of less abundant antibiotics (Donadio *et al.*, 2005). Moreover, it has been estimated that with conventional isolation methods, the chance of getting a producer of a novel antibiotic today is less than 10^{-7} (Baltz, 2005). Streptomycin, by comparison, is produced by about 1 % (10^{-2}) of randomly picked soil actinomycetes (Baltz, 2006), which means that among randomly selected actinomycetes, a streptomycin producer can be found by a chance that is five orders of magnitude as high as isolating a producer of an unknown antibiotic. The overrepresentation of *Streptomyces* isolates in most strain collections is a further problem. A random sampling of actinomycete spores usually yields about 90 % *Streptomyces* species, a fact that, in many cases, does not at all reflect their abundance in nature but rather emphasizes their remarkable adaptive abilities. It is likely, therefore, that again and again the same easy to cultivate strains have been isolated from different samples, while more fastidious "rare" genera frequently have been overlooked. "Rare" in this context does not necessarily mean less abundant but rather seldom isolated. However, the argument that less-culturable strains cannot be cultured to a high cell density has for long hindered their application to drug discovery programs. But although they initially might grow at very slow rates and/or require oligotrophic culture conditions, there is evidence that once cultured, many fastidious organisms can be grown using nutrient-rich media to satisfactory densities (e.g., Zengler *et al.*, 2002). A second reason that argues for the less-isolated actinomyctetes is their phylogenetic distance to well-studied streptomycete strains. Since secondary metabolites are produced by the concerted action of several genes, it is reasonable to expect an increased number of novel genes and gene combinations from strains phylogenetically unrelated to strains highly screened for antibiotics (Busti *et al.*, 2006).

Therefore, it has been repeatedly suggested (Baltz, 2008; Busti *et al.*, 2006; Donadio *et al.*, 2007; Williams *et al.*, 1993) to focus upon those actinomycetes that have not been in the center of previous screening progammes, to concentrate on unstudied environments or substrates, to apply novel selective isolation strategies or to miniaturize fermentations to enhance parallelism.

Although there is evidence that only a minority of the soil-dwelling actinomycetes have been explored so far, the isolation of novel soil-born strains has become increasingly challenging. More and more, therefore, unusual and/or extreme habitats get into the focus of taxonomists, ecologists, and natural products scientists. Even at the worlds most extreme sites bacterial growth has been proved; actinomycetes seem to play important

roles in many of these types of habitats. There is a continuing trend in systematically exploring unusual habitats such as strongly acidic or heavy metal contaminated sites (e.g., Haferburg & Kothe, 2007), marine and/or freshwater sediments (e.g., Bull *et al.*, 2005), the Arctic/Antarctic (e.g., Babalola *et al.*, 2009), as well as caves and mines (e.g., Groth & Saiz-Jimenez, 1999).

1.2. *Actinobacteria* in hypogean environments as potential producer strains

Caves and mines, also referred to as hypogean environments, represent one of few remaining less investigated environments in terms of microbial diversity. Such ecosystems are exposed to extreme environmental stresses and, according Peck (1986), may be based on inorganic energy sources rather than on organic sources or light. In contrast, Laiz *et al.* (1999) state that microbial communities of caves usually rely on allochthonous (extrinsic) input of organic matter, which is transported from the surface by seepage water. The limiting environmental characteristics of caves and mines, little or no light, low levels of organic nutrients, and high mineral concentrations, provide ecological niches for highly specialized organisms. Furthermore, various microhabitats, with vast differences in community structure can exist within caves and mines.

It was previously reasoned, that, due to their origin from as yet poorly studied biotopes, actinomycete isolates from caves could represent a valuable resource for the screening of novel bioactive metabolites (Groth *et al.*, 1999). However, little has been done so far to systematically explore the potential of this type of habitats to yield novel actinomycete producer strains. Most available studies that are dealing with actinomycete diversity in hypogean environments, were focusing on the biodeterioration/conservation of palaeolithic cave art and/or medieval frescoes. Actinomycetes in such art-affecting colonizations have been seen mainly as risk factors that might be able to unpredictable art destruction if environmental changes promote their massive proliferation (Groth *et al.*, 1999; Portillo *et al.*, 2009).

The existing cultural studies in caves and mines in China, Korea, Northern Spain, and Italy have demonstrated, anyway, that actinomycetes are not only the most abundant bacteria isolated from these habitats, but also reveal a great taxonomic diversity (e.g., Groth *et al.*, 1999; Laiz *et al.*, 1999; Laiz *et al.*, 2000; Urzì *et al.*, 2008). Hence, a number of novel actinomycete taxa have been described from these environments, including the genera *Beutenbergia*, *Fodinibacter* and *Knoellia* (Groth *et al.*, 1999; Groth *et al.*, 2002; Wang *et al.*, 2009) as well as several species of various less-isolated actinomycete genera e.g.,

Actinocorallia, *Agromyces*, *Amycolatopsis*, *Isoptericola*, *Jiangella*, *Kribbella*, *Myceligenerans*, *Nocardia*, *Pseudonocardia*, and *Saccharothrix* from different Korean, Italian, and Spanish caves, catacombs, and mines (e.g., Groth *et al.*, 2005; Groth *et al.*, 2006; Groth *et al.*, 2007; Jurado *et al.*, 2005a; Jurado *et al.*, 2005b; Jurado *et al.*, 2008; Lee *et al.*, 2000; Lee *et al.*, 2001; Lee, 2006a; Lee, 2006b; Lee, 2006c; Lee, 2008; Urzì *et al.*, 2008). Within the framework of a project aimed to map the taxonomic diversity of microorganisms affecting palaeolithic cave art, Groth and coworkers isolated approximately 350 heterotrophic bacterial strains from the ceilings, walls, and cave soils of the Spanish Altamira and Tito Bustillo caves (1999). More than 70% of all isolated strains were affiliated to the *Actinobacteria* and the majority of them was related to the genus *Streptomyces* (Groth *et al.*, 1999). Interestingly, a number of isolates of other, less-frequently isolated actinomycete genera have been detected as well. Members of many of these genera, e.g., *Amycolatopsis*, *Nocardioides*/*Kribbella* group, *Nocardiopsis*, *Rhodococcus*, and *Saccharothrix*, are also known for their production of antibiotic substances.

By means of culture-independent techniques, namely denaturing gradient gel electrophoresis (DGGE) and 16S rRNA/rDNA library analyses, a further number of less-common actinomycete genera, e.g., *Actinobispora*, *Frankia*, *Gordonia*, *Microbacterium*, *Propionibacterium*, *Pseudonocardia*, *Saccharothrix*, and *Tetrasphaera* have been detected in Altamira and Tito Bustillo (Portillo *et al.*, 2009; Schabereiter-Gurtner *et al.*, 2002a; Schabereiter-Gurtner *et al.*, 2002b). Interestingly, strains of *Streptomyces* that were reported to be of high diversity and abundance in the above cultural studies, have not at all been detected culture-independently. According these studies, rather strains of *Pseudonocardia*, which in turn have not been isolated (Groth *et al.*, 1999), emerged to be the most widespread *Actinobacteria* in all of the colonizations investigated (e.g., Portillo *et al.*, 2009). An important reason for the different frequencies of detection that occurred between the cultural and non-cultural studies has been seen in the use of selective media (Schabereiter-Gurtner *et al.*, 2002a). It is worth to note, however, that culture-independent techniques are also not free from bias. Limitations as, e.g., inefficient DNA extraction of less than 1% of total soil DNA (Taylor *et al.*, 2002), inefficient cell lysis of particularly Gram-Positives, preferential and selective amplification of 16S rDNA fragments (Jackson *et al.*, 2000), and the co-migration of different fragments in DGGE (Gafan & Spratt, 2005) may lead to a misinterpretation and underestimation of bacterial diversity.

Given their remarkable adaptive abilities, streptomycete isolates might be overrepresented in the above-mentioned cultural studies. Nevertheless, a range of low-abundant taxa has been exclusively detected by this approach. On the other hand, the same is true for the non-cultural studies that, among known taxa, also detected a range of yet unidentified bacteria (Portillo *et al.*, 2009; Schabereiter-Gurtner *et al.*, 2002a; Schabereiter-Gurtner *et al.*, 2002b). This clearly indicates, that for diversity analyses both, cultural and non-cultural methods, should be applied complementary.

From the Italian cave Grotta dei Cervi a similar, previously unexpected diversity of actinomycetes has been reported (Groth *et al.*, 2001). Strains of actinomycetes were most abundant, representing nearly 70% of all isolates. Again, besides a great diversity of *Streptomyces* strains, members of seldom-isolated actinomycete genera as, most notably, a number of *Amycolatopsis* isolates could be detected. In the course of a screening program for novel antibiotics that included selected strains obtained from Grotta dei Cervi, Herold *et al*. (2004) identified a bioactive complex, Cervimycins A to D from a strain of *Streptomyces tendae*. The Cervimycins have been proven to be highly active against multi-drug-resistant *Staphylococcus aureus* (MRSA) and, most remarkably, against efflux-resistant *S. aureus* EfS4 and vancomycin-resistant *Enterococcus faecalis* strains (Herold *et al.*, 2005). Mines and/or caves therefore represent promising habitats not merely in terms of bacterial diversity, but also in search for new actinomycetes featuring novel bioactive substances.

Therefore, the medieval slate mine Feengrotten was suggested to be a potential habitat of novel actinomycete strains constituting novel bioactive characteristics. To investigate the extreme environment of this mine for its actinomycete inhabitants, both, cultural as well as culture-independent techniques, have been applied. Particular attention was spent to isolates of seldom-isolated taxa.

1.3. Taxonomy of the *Actinobacteria*

When searching for novel bacteria of pharmacological interest, the probably most challenging part of work is to get hold of an interesting isolate, i.e., to adapt the strain to laboratory conditions. To be of any value to scientists or the pharmaceutical industry, the organism is required to readily grow to satisfactory cell densities under artificial, controlled conditions.

Equally, a proper identification and classification of the novel isolate is a prerequisite for various further studies concerning e.g., its metabolic capabilities. Classification also comes

into play with respect to the question of where (and where not) to focus resources. Because the ability to produce secondary metabolites is patchy even within the class *Actinobacteria* (Donadio *et al.*, 2007), correctly classified isolates may help, by prior exclusion of strains affiliated to less-promising taxa, to reduce complicity of bioactivity screenings.

As a great part of the presented work was culture-based and efforts were made to provide novel potential actinomycete isolates particularly of seldom-isolated taxa, the proper assignment of the isolated strains, which includes their identification, characterization, and classification, has been most important for the present work.

1.3.1. What taxonomy is about

Classification and identification are integral parts of a science called taxonomy or, less commonly, systematics, that deals with the allocation of correct labels to organisms. Taxonomy (or systematics) further provides an orderly arrangement (classification system) of taxa in which organisms can be classified. Bacterial taxonomy, once perceived as somewhat obscure or stuffy, has changed into a rapidly developing discipline that gives increasingly deeper insights of how bacteria have evolved, and how they should be classified, identified and characterized. As, surprisingly enough, many microbiologists still seem to shy away from taxonomic issues, it may be worthwhile to comprehensively introduce the topic.

Modern taxonomy splits into three related subdisciplines: (i) classification, i.e., the orderly arrangement of organisms into taxonomic groups (units) on the basis of similarity, (ii) nomenclature, i.e., the labeling of the units defined, and (iii) identification, i.e., the process of determining whether an organism belongs to one of the units already defined and labeled (Tindall *et al.*, 2010; Vandamme *et al.*, 1996). Taxonomy is supposed to reflect phylogeny and evolution (Brenner *et al.*, 2001). New findings therefore frequently result in changes in the existing classification system, in nomenclature, in criteria for identification, and in the recognition of new species. For taxa that have already been described, named, and classified, new characteristics may be added or existing characteristics may be reinterpreted to revise existing classifications. If the organism is new, i.e., cannot be identified as an existing taxon, it is named and described according the rules of nomenclature and placed in an existing classification.

1.3.2. Bacterial nomenclature

The scientific names of bacteria are regulated by successive editions of the *International Code of Nomenclature of Bacteria* (ICNB, the most recent edition is the 1990 Revision;

Sneath, 1990), which is currently published by the *International Committee on Systematics of Prokaryotes* (ICSP). With the starting date of the Bacteriological Code as of 1 January 1980, bacterial nomenclature became completely independent from the Botanical Code, which was applicable until then. For names published prior to this date, Approved Lists of Bacterial Names (Skerman *et al.*, 1980) were compiled. Only the names of bacteria which had been adequately described at the time of preparing the lists and for which type or neotype strains had been available, were placed on the Lists. Names not taken on the lists lost their standing in nomenclature (Rule 24a; Sneath, 1990).

As no further names will be added to the Approved Lists, a name has to be validly published to become "standing in nomenclature". However, valid publication does not take place until the name, the description of the taxon or a reference to a previously published description of the taxon, and the designation of the type have been published in the *International Journal of Systematic and Evolutionary Microbiology* - IJSEM (Rule 27; Sneath, 1990), i.e., the Bacteriological Code designates the IJSEM "validating authority". All bacterial names are therefore found in a single journal, and it is not necessary to search the rest of the scientific literature for them. This has proved a great advantage for bacterial taxonomy.

1.3.3. Taxonomic ranks

Bacterial classification uses several levels or taxonomic ranks. The highest rank is called a domain. All prokaryotic organisms are placed within two domains, *Archaea* and *Bacteria*. Phylum, class, order, family, genus, and species are successively smaller, non-overlapping subsets of the domain. The names of these subsets from class to subspecies are given formal recognition, i.e., they have standing in nomenclature (Brenner *et al.*, 2001). In addition to the above-mentioned formal categories, vernacular groups that are defined by common descriptive names are often used; the names of such groups have no official standing in nomenclature. Examples are the spirochetes, the methanogens or the actinomycetes. When using the term actinomycetes, this thesis intends to refer to the filamentous *Actinobacteria* only, i.e., nocardioforms and streptomycete-like sporoactinomycetes.

1.3.4. Nomenclatural Types

An important principle in bacterial nomenclature is the type concept. The Bacteriological Code rules that every named taxon must have a designated type (Rule 15; Sneath, 1990). Each taxon consists of one or more elements and the type of a taxon is that element with

which the name of the taxon is permanently associated. Above the rank of the species, the type of a taxon is simply a name. At the species or subspecies level the nomenclatural type is represented by a particular strain, the type strain. This strain is the permanent example of the species or subspecies, i.e., the reference specimen for the name. The Bacteriological Code recommends depositing of a culture of the type strain in at least one public culture collection. However, the IJSEM, journal of choice for publication of new taxa, requests certificates that document the deposition of type strains "in at least two or more public culture collections from two or more countries" (IJSEM online, Instructions for Authors, http://ijs.sgmjournals.org/misc/ifora.shtml).

1.3.5. The Bacterial Species Concept

The basic and most important taxonomic group in bacterial taxonomy is the species. The concept of a bacterial species is less definitive than for higher organisms and was subject of repeated change. By now, the term species as applied to bacteria has been defined as "a monophyletic and genomically coherent cluster of strains, including the type strain, that show a high degree of overall similarity in many independent characteristics and is diagnosable by a discriminative phenotypic property" (Rossello-Mora & Amann, 2001).

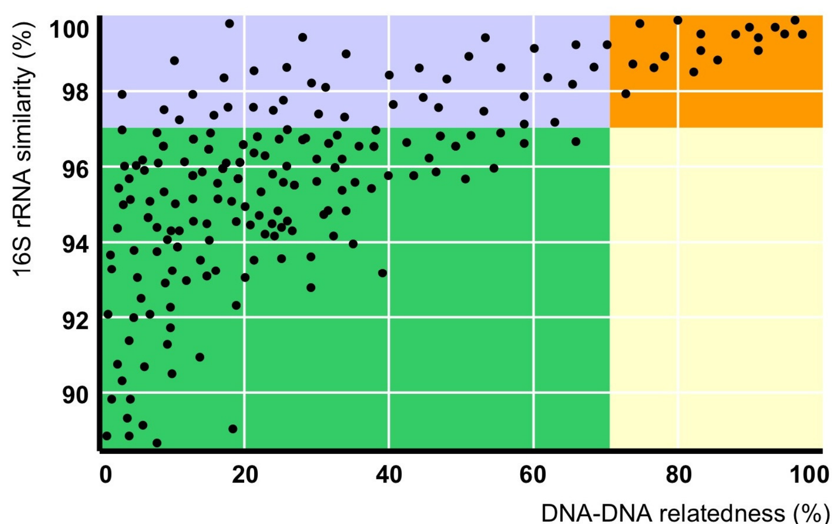

Figure 1. Comparison of DNA-DNA and 16S rDNA similarities. The orange-colored box indicates the potential "genetic space" around the type strain of one particular species as defined by the current bacterial species concept (Redrawn from Rossello-Mora & Amann, 2001).

To be classified to one particular species, strains must share an overall DNA-DNA relatedness of 70 % or greater and less than 5°C ΔT_m [1] ("genomospecies"; Wayne *et al.*, 1987). A similarity of the respective 16S rRNA genes of 97 % or greater strongly indicates that the strains under consideration are of the same species (Stackebrandt & Goebel, 1994).

[1] ΔT_m is the difference in T_m (melting temperature of a DNA-DNA hybrid) in degrees Celsius between the homologous and the heterologous hybrids formed under standard conditions (Wayne *et al.*, 1987).

Phenotypic data should further support this genotypic definition. Rosselló-Mora & Amann (2001) refer to it as a phylo-phenetic species concept. Genomospecies that cannot be separated phenotypically should not be formally named (Wayne *et al.*, 1987).

Figure 1 illustrates the interconnection between overall DNA-DNA relatedness and 16S rDNA similarity. All strains matching the nomenclatural type of a particular species within the boundaries of the orange-colored box of Figure 1 (i.e. showing a DNA-DNA relatedness of greater than 70 % and a 16S rDNA similarity of more than 97 %) by definition belong to the same species. Note the light blue box in Fig. 1: The dots therein represent strains whose 16S rRNA genes are virtually identical to their closest neighbors but which are, nevertheless, not considered as of the same species, based on their extant DNA-DNA relatedness.

The validity of the DNA relatedness based genetic definition of a species has repeatedly been questioned (e.g., Vandamme *et al.*, 1996). Interestingly, although chosen on the basis of results obtained from multiple strains of some 600 species (Brenner *et al.*, 2001), the more or less arbitrary cut-off values defined for bacterial genomospecies make the bacterial species definition much broader than of higher organisms. So if applied to higher organisms, the bacterial species definition would include lemurs in the same species as humans, as they exhibit 78 % DNA relatedness to humans (Brenner *et al.*, 2001). One consequence of the broad bacterial species definition is that very few prokaryotic species have been described, some 9.400 (Euzéby, 2009), compared with over a million animals.

1.3.6. Taxa above the rank of species

Compared to the bacterial species, the higher taxa such as genera and families are much more difficult to delineate and phylogenetic divergence is not necessarily supported by polyphasic data. Far too little attention has been paid to the way in which taxa above the rank of species should be characterized (Tindall *et al.*, 2010). The lack of a consensus approach in the delineation of genera has led to the creation of genera in which the genotypic and phenotypic divergence varies with the individual concepts of taxonomists. Except for the general statement that genera or higher taxonomic levels should reflect phylogenetic relationships, no further clear-cut recommendations exist (Gillis *et al.*, 2001). However, a 16S rRNA gene sequence similarity of well below than 97% between a particular group of novel strains and those of described strains is considered indicative for a novel genus. Although the goal remains to describe genera polyphasically, there is a rather broad consensus among taxonomists that "phylogenetic data are of superior value for the delineation of genera" (Gillis *et al.*, 2001). Hence, many recent reports on novel

taxa place emphasis particularly on phylogenetic inference. The stability of the phylogenetic position of the group in question should be demonstrable using different treeing algorithms. Confidence tests such as bootstrapping or jackknifing should support the separation of the respective subtree with a preferably high value of well above 50%. Using a preferably large dataset of related and unrelated reference organisms additionally stabilizes phylogenetic inference.

However, any new genus needs to be described (Rule 27 Bacteriological Code; Sneath, 1990). Despite lacking guidelines, however, bacterial genera should be, in a similar manner to the description of species, comprehensively described through a combination of genotypic data and suitable phenotypic and chemotaxonomic data.

1.3.7. The Polyphasic Approach in Bacterial Classification

During the last 40 years, characterization of bacteria has changed from simple procedures, in which a limited number of features of the bacterial cell (mainly morphological and physiological aspects) were studied, to a multidisciplinary "polyphasic" approach based on phenotypic, especially chemotaxonomic, and genotypic characteristics.

It has to be emphasized that there is still no officially recognized system for the classification of prokaryotes. The currently applied classification systems rely – for practical reasons – on methods and do not depend on theoretical concepts (Schleifer, 2009).

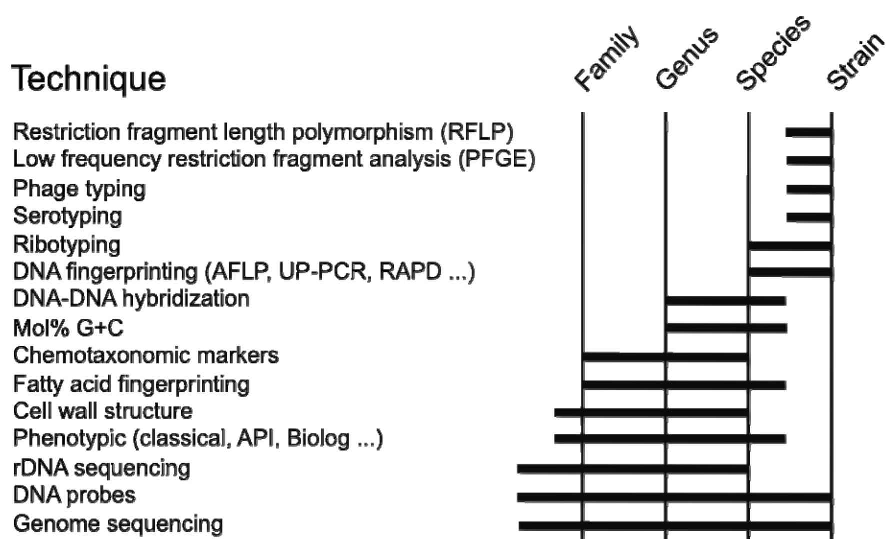

Figure 2. Taxonomic resolution of currently used techniques in bacterial taxonomy (adapted from Vandamme *et al.*, 1996).

Today, there is agreement between microbiologists that on the species level, "a reliable classification can only be achieved by the exploration of the internal diversity of taxa by

combining and measuring as much as possible phenotypic and genomic information" (Vandamme *et al.*, 1996). Numerous taxonomic techniques exhibiting quite different discriminatory taxonomic power are available and should be chosen in consideration of the taxonomic resolution needed for the particular purpose. Fig. 2 (adapted from Vandamme *et al.*, 1996) presents the resolving power of most of the methods currently used for taxonomic purposes.

Polyphasic taxonomy aims at the delineation of consensus groups using as much as possible discriminative data. A suitable strategy for obtaining useful polyphasic data encompasses: (i) an initial screening for groups of similar strains, (ii) determination of the phylogenetic position of these consensus groups, and (iii) measurement of the relationships between the groups and their closest neighbors by collecting and interpreting various descriptive data. The first step, initial screening, should be applicable to large numbers of strains and suited for the respective "taxonomic depth". Within the borders of the *Actinomycetales* consensus groups are often formed on the basis of colony morphology and/or chemotaxonomic key markers such as the isomers of diamino pimelic acid. Sequencing the 16S rRNA genes of representative strains should be the next step, which is inferring the phylogenetic position of the consensus groups. If the 16S rDNA sequence similarity between the representative strains and their nearest neighbors is 97% or above, it can be assumed that both belong to the same genus. The decision whether or not the new consensus group constitutes a novel species requires DNA-DNA hybridizations (Wayne *et al.*, 1987) that are optional at 16S rDNA similarities below 97% but become mandatory above this value (Stackebrandt & Goebel, 1994). Before a new species can be proposed and described for a consensus group that is identified as belonging to a particular genus, but not to one of its described species, the strain(s) of that group have to be thoroughly characterized by as much as possible phenotypic and genotypic data that must support the distinct position of the new taxon.

1.3.8. Implications of the 16S rRNA gene approach on prokaryotic systematics

The development of a prokaryotic classification system that reflects the natural relationships between microorganisms has always been a fundamental goal of taxonomists. By the end of the 1970s, several studies had shown that ribosomal RNA held promise for phylogenetic reconstruction (e.g. Fox *et al.*, 1980). Since that time, none of the methods depicted in Figure 2 has implicated bacterial taxonomy more than the 16S rRNA gene sequencing technology. In 1987, Woese in a widely noticed paper reviewed the rRNA gene approach and launched the first eubacterial phylogeny solely based on 16S rRNA/rDNA

data (Woese, 1987). In the 1990s, comparison of 16S rRNA gene sequences finally had become a universally accepted method in bacterial taxonomy and today it is more likely that a 16S rRNA gene sequence will be the first piece of information collected for novel organisms, "rather than a Gram stain" (Lilburn & Garrity, 2004). Up to now, no other gene has been sequenced from more different bacterial sources. Release 10.9 from March 6, 2009 of the *Ribosomal Database Project II* (Cole *et al.*, 2009) contains more than 800.100 aligned 16S rRNA gene sequences of just bacterial (not archeal!) origin.

Principally, the reconstruction of phylogenetic trees is done in two steps, namely sequence alignment and treeing. However, there are a number of points that need to be considered when evaluating the data. Today, databases are full of poor-quality, chimeric sequences (Ashelford *et al.*, 2005). Therefore, it is of utmost importance to include unambiguous, at most complete, high quality sequences into the dataset. Only sequences of the respective nomenclatural types should be included; sequences of dubious quality should be resequenced (Tindall *et al.*, 2010). The multiple alignment of primary structures should be done by using either seed alignments as provided by e.g. the Ribosomal Database Project (RDP; Cole *et al.*, 2009) or the ARB project (Ludwig *et al.*, 2004), or by robust multiple alignment programs such as clustal w, clustal x or muscle, followed by manual editing (Ludwig & Klenk, 2001). Due to the loss of information, sequences should never be truncated to the region covered by partial sequences; full and partial sequences should never be mixed (Tindall *et al.*, 2010). Functional homology, if detectable or predictable, and the character of base pairing (G-C versus non-G-C, Watson-Crick versus non-Watson-Crick) should be used to improve the alignment. Furthermore, alternative treeing (distance, maximum parsimony and/or maximum likelihood) methods should be applied to weight the data in view of different models of evolution (since different treeing methods are based on different models of evolution). Confidence tests (as, e.g., bootstrap or jackknife analysis) should be used to evaluate the statistical significance of branching order (Ludwig & Klenk, 2001). Masks and filters (e.g., Swofford *et al.*, 1996) that weight down highly variable positions and that can be used to minimize branch attraction phenomena resulting from such plesiomorphic sites, should be applied repeatedly with increasing stringency; the resulting tree topologies should be carefully compared.

Furthermore, besides technical issues, there are several limitations of the method that arise from the 16SrRNA molecule itself. So, several recent studies reported a significant intragenomic heterogeneity between multiple 16S rRNA operons in a given genome (Ueda *et al.*, 1999; Ventura *et al.*, 2007), as well as horizontal gene transfer (HGT) of the 16S

rRNA gene between bacteria (Schouls *et al.*, 2003; Ventura *et al.*, 2007); facts which considerably complicate phylogenetic inference. Another limitation of the method is that considerable differences in the mutation rates between different lineages exist for the 16S rRNA gene, thus leading to artifacts in the phylogenetic tree construction (Woese, 1987). Moreover, it has been shown that the resolution power of rRNA sequences is limited when closely related organisms that diverged at almost the same time are being examined (Fox *et al.*, 1992).

The excellent agreement between phylogenies based upon 16S rRNA sequences and trees based on the sequences of other conserved macromolecules has repeatedly demonstrated that, nonetheless, 16S rRNA phylogenies rather accurately reflect organismal evolution (Höpfl *et al.*, 1989; Iwabe *et al.*, 1989; Ludwig & Schleifer, 1994). Local discrepancies in phylogenetic trees resulting from different information content or different rate or mode of change do not greatly compromise this general picture. The 16S rDNA approach tremendously expanded the knowledge of prokaryotic relationships in recent years and has taken taxonomists another step towards the ultimate goal of a systematics based on the natural relationships between organisms. The importance of the present rRNA-based concept of prokaryotic phylogeny can also be seen in that the present edition of *Bergey's Manual*, which serves as a rule for most bacteriologists, is structured according to it.

1.3.9. Towards a phylogenetic classification system of the actinomycetes

The existence of the actinomycetes has been recognized for over a hundred years. For much of this time they were regarded as an exotic group of organisms with affinities to both bacteria and fungi. However, determinations of their fine structure and chemical composition, initiated in the 1950s, confirmed their prokaryotic nature. Nevertheless, the obsolete term "ray fungi", which refers to the filamentous growth behavior typical for many representatives of this group, is still commonly used.

Traditionally, members of the class *Actinobacteria* were classified largely according to their morphology or by combining morphology by some physiological traits. Some of the earliest bacterial phylogenies that included actinomycetes have been proposed by Kluyver & Van Niel (1936). According to these schemes, morphologically complex forms, such as the actinomycetes, evolved from morphologically simple spherical forms. In contrast, Krasilnikov (1949) believed the class '*Actinomycetes*' to have evolved from a common ancestor that also led to the fungi. He also included the Gram-positive cocci into the class, as he believed they were degenerated mycobacteria. *Bifidobacteria* were seen as a bridge between mycobacteria and the lactic- and propionic-acid bacteria. In 1970, Prauser used

morphology to depict evolutionary trends within the actinomycetes (Prauser, H, 1970): the transitory-mycelial nocardioforms (Prauser, H, 1967) were believed to represent an intermediate stage between the pleomorphic rod-shaped coryneforms and the morphologically complex spore-forming sporoactinomycetes. A large problem for taxonomists were the innumerable morphological transitions which exist between nocardioform actinomycetes as e.g., *Actinomyces*, *Nocardia* or *Rhodococcus* and coryneform bacteria that have a tendency to form branched elements, such as *Corynebacterium*, *Arhrobacter* and *Cellulomonas*, (Prauser, H, 1978; Prauser, H, 1981). It became clear by that time that many of the earlier classifications of coryneform and nocardioform bacteria had been artificial. A tentative step towards remedying this situation was taken in the 1974s edition of *Bergey's Manual of Determinative Bacteriology* (Bergey, 1974) where actinomycetes and coryneform bacteria were considered together in a section entitled "*Actinomycetes and related organisms*".

Woese, in his 16S rRNA sequence-derived phylogeny (Woese, 1987), showed the phylogenetic coherent lineage of Gram positive bacteria, which was largely the same as division *Firmacutes* (Gibbons & Murray, 1978), to divide into two subdivisions, low G+C and high G+C Gram positives. The terms refer to the fact that the species covered tend to have DNA G+C ratios either well below or well above 50 %, respectively. Species in the high-G+C Gram-positive subdivision conformed to a general actinomycete phenotype with a tendency to form branched filaments. The class *'Actinomycetes'* in its traditional shape (Krasilnikov, NA, 1949) was shown to represent a collection of phylogenetically diverse families and genera. As measured by 16S rRNA analyses, most traits previously used to delineate at higher taxonomic levels, e.g. at the family level, emerged to be polyphyletic.

Thus, in 1997, Stackebrandt and coworkers proposed a novel hierarchic structure for the high-G+C Gram-positive actinomycete bacteria and relatives, which was solely delineated from 16S rDNA/rRNA sequence-based phylogenetic clustering and the presence of taxon-specific signature nucleotides in these sequences (Stackebrandt *et al.*, 1997). *Actino-mycetes* and relatives were recognized as a distinct class, *Actinobacteria*, within the Gram-positive bacteria. Except for the removal of the *Sphaerobacteridae* (Hugenholtz & Stackebrandt, 2004) and the description of an eleventh suborder, *Catulisporineae* (Cavaletti *et al.*, 2006b), the classification system of Stackebrandt *et al.* has, at least as for the higher taxonomic levels, by and large been unaltered to these days.

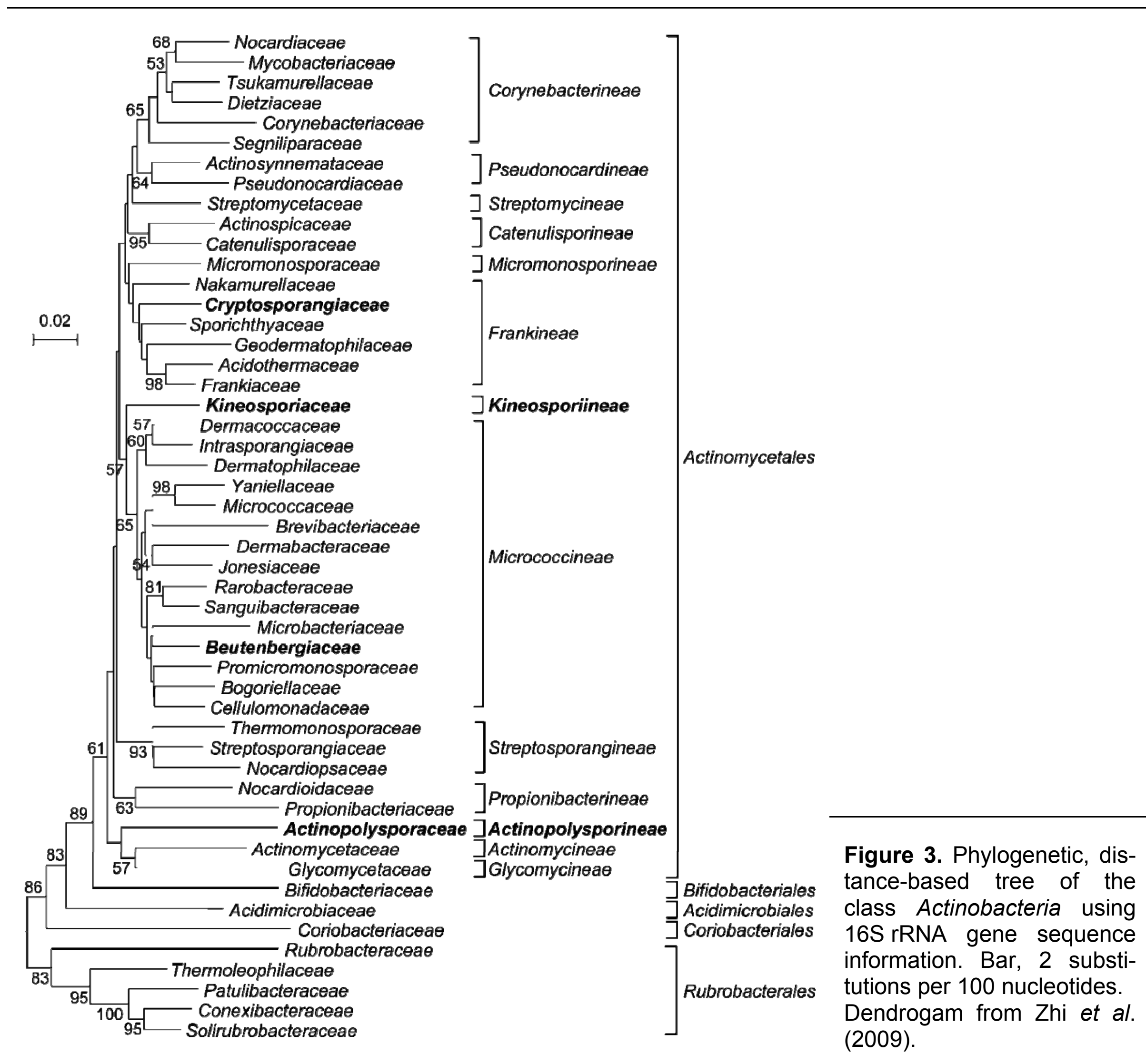

Figure 3. Phylogenetic, distance-based tree of the class *Actinobacteria* using 16S rRNA gene sequence information. Bar, 2 substitutions per 100 nucleotides. Dendrogam from Zhi *et al.* (2009).

However, within 12 years after its publication, large numbers of newly described members have been assigned to the *Actinobacteria*. At the same time, the number of 16S rRNA sequences deposited in public databases has increased tremendously. Based on these facts, Zhi *et al*. (2009) recently updated the definition of the higher ranks of the class (Figure 3). They proposed two new suborders, *Actinopolysporineae* and *Kineosporiineae*, four new families, *Actinopolysporaceae*, *Kineosporaceae*, *Beutenbergiaceae*, and *Cryptosporangiaceae* (highlighted in bold in Fig. 3), and emended descriptions of most of the existing higher-leveled taxa by providing updated sets of determinative signature nucleotides.

Today the class Actinobacteria includes four subclasses, *Acidimicrobidae* Stackebrandt *et al*. 1997, *Rubrobacteridae* Rainey *et al*. 1997[2], *Coriobacteridae* Stackebrandt *et al*. 1997, and *Actinobacteridae* Stackebrandt *et al*. 1997. A fifth subclass, *Sphaerobacteridae*

[2] In: Stackebrandt *et al*., 1997

Stackebrandt *et al*. 1997, was suggested to be retained in the class *Thermomicrobia*, phylum *Chloroflexi* (Hugenholtz & Stackebrandt, 2004).

Genotypically, membership to the *Actinobacteria* is indicated by 16S rDNA sequence similarity values above 80 % as compared with the most deeply branching members of the class, such as *Rubrobacter radiotolerans*, *Acidimicrobium ferrooxidans*, or *Coriobacterium glomerans*, and the presence of the following signature nucleotides in the 16S rRNA/rDNA: an A residue at position 906 (*E. coli* numbering; Brosius *et al.*, 1978) and either an A or a C at position 955, except for members of the subclass *Rubrobacteridae* which show U residues at these positions (Stackebrandt *et al.*, 1997). The class can be further characterized by a large insert of 79 to 100 bp in their 23S rRNA genes that is absent from all other groups of bacteria (Roller *et al.*, 1992), by the presence of some conserved insertion-deletion polymorphisms (indels) in their 23S rRNA and protein (e.g. cytochrome-*c* oxidase I, CTP synthetase and glutamyl-tRNA synthetase) sequences (Gao & Gupta, 2005; Ventura *et al.*, 2007) and by characteristic gene arrangements (Kunisawa, 2007).

Members of the *Actinobacteria* are widely distributed in both terrestrial and aquatic ecosystems, especially in soil, where they play a crucial role in the recycling of refractory biomaterials by decomposition and humus formation (Goodfellow & Williams, 1983). They contain pathogens (e.g., *Mycobacterium* ssp., *Nocardia* ssp, *Corynebacterium* ssp.), soil inhabitants (e.g., *Streptomyces* ssp., *Amycolatopsis* ssp.), inhabitants of marine sediments (e.g., *Salinispora*, *Verrucosispora* ssp.), endophytes (e.g., *Streptomyces* ssp., *Kribbella* ssp.), nitrogen-fixing symbionts (*Frankia*), and gastrointestinal tract inhabitants (*Bifidobacterium* ssp.). At the time of writing (July 2010), 286 genera (in 53 families) have been accommodated in the class *Actinobacteria*, making it one of the largest groups within the *Bacteria (Euzéby, 2010)*.

There is evidence that to date many less-cultivated actinomycete taxa are grossly underspeciated (e.g. Carlsohn *et al.*, 2008). The taxonomic position of many of these taxa therefore remains provisional. Both, the retrieval of new strains and their subsequent characterization will broaden the base for phylogenetic reconstruction and added characteristics will help to either revise or support existing classifications – at any taxonomic level. Though the cave-derived genus *Beutenbergia* (Groth *et al.*, 1999) was initially assigned to the suborder *Micrococcineae* as novel phylogenetic lineage at the genus level, it could be recently shown that their members are, in fact, part of a family-level lineage within the suborder *Micrococcineae*. The lineage had only become significant

in the dendrograms when additional sequence data of the meanwhile described genera *Georgenia* (Altenburger *et al.*, 2002) and *Salana* (von Wintzingerode *et al.*, 2001) was included into the 16S rRNA gene dataset used for phylogenetic reconstruction (Zhi *et al.*, 2009). Similarly, although separable from any previously described suborder (Kudo *et al.*, 1998), the genera *Kineococcus* and *Kineosporia* for long had been excluded from the taxonomic system of the *Actinobacteria* because of uncertainties about their exact phylogenetic position. However, both form a distinct suborder-level clade and share neighborhood with the suborder *Micrococcineae* when more sequences of newly described representatives are included into the dataset for phylogenetic reconstruction (Zhi *et al.*, 2009).

As a consequence, characterization and description of novel actinomycete taxa is reasonable not only in view of their potential pharmacological interest of the corresponding strains but also because every newly described taxon may help to gain deeper insights into phylogenetic coherencies and to expand the knowledge of bacterial diversity.

2 Summary of manuscripts

I *Amycolatopsis saalfeldensis* sp. nov., a novel actinomycete isolated from a medieval alum slate mine.

Marc René Carlsohn, Ingrid Groth, Geok Yuan Annie Tan, Barbara Schütze, Hans Peter Saluz, Thomas Munder, Jun Yang, Joachim Wink and Michael Goodfellow.

Published in: *Int J Syst Evol Microbiol* 57 (2007), 1640-1646.

Three strains HKI 457^T, HKI 473, and HKI 474 are described, which were isolated from acidic rocks of different sites of the Feengrotten. Based on a range of relevant chemotaxonomic, morphologic and molecular markers, they were shown to constitute a novel actinobacterial species, *Amycolatopsis saalfeldensis* sp. nov. Moreover, the UP-PCR fingerprinting technique was demonstrated to be a fast and simple method for the genomic differentiation of closely related bacterial strains.

Individual contributions:

Marc René Carlsohn, 40 %: isolation, propagation of strains, chemotaxonomic, morphologic, and molecular diagnostics, UP-PCR fingerprinting, sequencing of 16S rDNA, writing manuscript.

Ingrid Groth; 30 %: isolation, propagation, chemotaxonomy, and morphology of strains, writing manuscript.

Geok Yuan Annie Tan, Michael Goodfellow; 10 %: phylogenetic analyses.

Barbara Schütze; 5 %: nomenclature.

Jun Yang, Joachim Wink; 5 %: MALDI-TOF MS data (supplementary material).

Hans Peter Saluz, Thomas Munder; 10 %: professional advise, revising manuscript.

II *Kribbella aluminosa* sp. nov., isolated from a medieval alum slate mine.

Marc René Carlsohn, Ingrid Groth, Cathrin Spröer, Barbara Schütze, Hans Peter Saluz, Thomas Munder, and Erko Stackebrandt.

Published in: *Int J Syst Evol Microbiol* 57 (2007), 1943-1947.

A novel actinobacterial species, *Kribbella aluminosa sp. nov.* is proposed for a group of three related strains which share a range of morphologic, chemotaxonomic and molecular features. Given the high levels of 16S rDNA similarity between the proposed species and its closest relatives, a DNA-DNA hybridization study had to be carried out. In a second instance, the potential of UP-PCR in differentiation of closely related bacterial strains was demonstrated.

Individual contributions:

Marc René Carlsohn, 50 %: isolation, propagation of strains, chemotaxonomic, morphologic, and molecular diagnostics, UP-PCR fingerprinting, sequencing of 16S rDNA, writing manuscript.

Ingrid Groth; 20 %: isolation, propagation, chemotaxonomy, and morphology of strains, writing manuscript.

Cathrin Spröer; 5 %: DNA-DNA hybridization.

Barbara Schütze; 5 %: nomenclature.

Hans Peter Saluz, Thomas Munder; 5 % each: professional advise, revising manuscript

Erko Stackebrandt; 10 %: phylogenetic analyses.

III. *Fodinicola feengrottensis* gen. nov. sp. nov., an actinomycete isolated from a medieval mine.

Marc René Carlsohn, Ingrid Groth, Hans Peter Saluz, Peter Schumann and Erko Stackebrandt.

Published in: *Int J Syst Evol Microbiol* 58 (2008), 1529-1536.

An actinobacterial strain isolated from the "Barbara Grotto" of the Feengrotten was examined. Due to its remote relationship to known members of the suborder *Frankineae*, a novel genus, *Fodinicola gen. nov.* (derived from the latin words *fodina*, mine and *incola*, dweller), had been proposed, said strain representing the type species, *Fodinicola feengrottensis sp. nov.* of the novel genus. Both, chemotaxonomic as well as molecular data, confirmed the isolated position of said strain within the *Frankinae*. The method of Xu *et al.* (2000) of estimating genomic G+C content by monitoring fluorescence intensity was successfully applied to a real time PCR device different from that used by Xu *et al*. (2000), indicating that, independent from the system used, consistent results can be achieved.

Individual contributions:

Marc René Carlsohn, 60 %: isolation, propagation of strains, nomenclature, chemotaxonomy, morphology, scanning electron microscopy, G+C content measurements, sequencing of 16S rDNA, writing manuscript.

Ingrid Groth; 20 %: isolation, propagation, chemotaxonomy, and morphology of strains, nomenclature, writing manuscript.

Hans Peter Saluz, 5 %: professional advise, revising manuscript.

Erko Stackebrandt; 15 %: phylogenetic analyses, signature nucleotides.

3 Manuscripts

3.1. *Amycolatopsis saalfeldensis* sp. nov., a novel actinomycete isolated from a medieval alum slate mine.

International Journal of Systematic and Evolutionary Microbiology (2007), **57**, 1640–1646 DOI 10.1099/ijs.0.64903-0

Amycolatopsis saalfeldensis sp. nov., a novel actinomycete isolated from a medieval alum slate mine

Marc René Carlsohn,[1] Ingrid Groth,[1] Geok Yuan Annie Tan,[2] Barbara Schütze,[1] Hans-Peter Saluz,[1] Thomas Munder,[1]† Jun Yang,[3] Joachim Wink[4] and Michael Goodfellow[5]

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Three actinomycetes isolated from the surfaces of rocks in a medieval slate mine were examined in a polyphasic taxonomic study. Chemotaxonomic and morphological characteristics of the isolates were typical of strains of the genus *Amycolatopsis*. The isolates had identical 16S rRNA gene sequences and formed a distinct phyletic line towards the periphery of the *Amycolatopsis mediterranei* clade, being most closely related to *Amycolatopsis rifamycinica*. The organisms shared a wide range of genotypic and phenotypic markers that distinguished them from their closest phylogenetic neighbours. On the basis of these results, a novel species, *Amycolatopsis saalfeldensis* sp. nov., is proposed. The type strain is HKI 0457^{T} (=DSM 44993^{T}=NRRL B-24474^{T}).

The genus *Amycolatopsis* is classified in the family *Pseudonocardiaceae*, suborder *Pseudonocardineae* (Stackebrandt *et al.*, 1997). The genus currently contains 34 recognized species, most of which have been described in the past five years using polyphasic taxonomic approaches (Goodfellow *et al.*, 2001; Labeda *et al.*, 2003; Groth *et al.*, 2007). Members of most of these species have been isolated from geographically diverse soils (Kim *et al.*, 2002; Saintpierre-Bonaccio *et al.*, 2005; Lee *et al.*, 2006; Tan *et al.*, 2006a), and others from clinical material (Labeda *et al.*, 2003; Huang *et al.*, 2004), vegetable matter (Goodfellow *et al.*, 2001) and from the wall of a hypogean Roman catacomb (Groth *et al.*, 2007).

Amycolatopsis strains can be distinguished from members of other genera classified in the family *Pseudonocardiaceae* by using a combination of chemotaxonomic and morphological markers (Kim & Goodfellow, 1999) and genus-specific oligonucleotide primers based on 16S rRNA gene sequences (Tan *et al.*, 2006b). A range of phenotypic markers can be weighted to distinguish between species with validly published names (Saintpierre-Bonaccio *et al.*, 2005; Lee *et al.*, 2006; Groth *et al.*, 2007). The present polyphasic study was designed to determine the taxonomic position of three strains which had been isolated from a medieval alum slate mine. The strains were assigned to the genus *Amycolatopsis* on the basis of their morphological properties and ability to produce the diagnostic amplification products when probed with the genus-specific 16S rRNA oligonucleotide primers AMY2 (5′-GGTGTGGGCGACATCCACGTTGT-3′) and ATOP (5′-GTATCGCAGCCCTCTGTACCAGC-3′) as described by Tan *et al.* (2006b). The resultant data showed that the isolates represent a novel *Amycolatopsis* species for which the name *Amycolatopsis saalfeldensis* sp. nov. is proposed.

The three strains were isolated from the surfaces of acidic and heavy-metal-containing rocks of two galleries in the medieval alum slate mine Feengrotten in Saalfeld, Thuringia,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HKI 0457^{T}, HKI 0473 and HKI 0474 are DQ792500, DQ792501 and DQ792502, respectively.

A dendrogram showing relationships between the isolates and between them and the type strains of closely related *Amycolatopsis* species based on MALDI-TOF MS data is available with the online version of this paper.

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Germany. Strain HKI 0457^{T} was isolated from rock in the central grotto (second level of the mine) by touching it with a sterile cotton swab and dispersing adhering bacteria in 1 ml sterile distilled water. Aliquots of the resultant suspension were spread over casein mineral agar plates (Altenburger *et al.*, 1996), supplemented with cycloheximide (50 μg ml^{-1}), and incubated at 28 °C for 4 weeks. Strains HKI 0473 and HKI 0474 were isolated from rock surfaces towards the end of the Hess von Wichdorff Grotto (first level of the mine) by using the same procedure, but plating out onto humic acid agar (Hayakawa & Nonomura, 1987).

Working cultures of the isolates were maintained on organic medium 79 agar (Prauser & Falta, 1968). The cultures were preserved as mixtures of hyphae and fragmented spores in organic medium 79 broth and in glycerol medium (Groth *et al.*, 2007) at –80 °C. Stock cultures were also kept in liquid organic medium 79 supplemented with 5 % DMSO in the vapour phase of liquid nitrogen. Biomass for the chemotaxonomic and molecular systematic studies was prepared by growing the isolates and *Amycolatopsis kentuckyensis* DSM 44652^{T}, *Amycolatopsis lexingtonensis* DSM 44653^{T}, *A. mediterranei* DSM 43304^{T}, *Amycolatopsis pretoriensis* DSM 44654^{T}, *A. rifamycinica* DSM 46095^{T} and *Amycolatopsis tolypomycina* DSM 44544^{T} in liquid organic medium 79 and bacto-tryptic soy broth (Sigma-Aldrich) for 24 to 48 h at 28 °C. For MALDI-TOF MS analysis the strains were cultivated as described by Groth *et al.* (2007).

Chromosomal DNA was extracted from the three isolates using slight modifications of the method of Pospiech & Neumann (1995). PCR amplification of 16S rRNA genes was achieved using the conserved primers 27F (5′-AGA-GTTTGATCCTGGCTCAG-3′) and 1522R (5′-AAGGAG-GTGATCCAGCCGCA-3′) (Edwards *et al.*, 1989) and the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min; and a final extension for 10 min at 72 °C. After electrophoretic separation the 16S rRNA genes were extracted from the agarose gel using the MinElute Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Forward and reverse strands of amplified DNA fragments were directly sequenced using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit and an ABI Prism 3100 sequencer (both Applied Biosystems).

The resultant 16S rRNA gene sequences were aligned manually using the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydit/), against corresponding sequences of representatives of the family *Pseudonocardiaceae* retrieved from the GenBank/EMBL/DDBJ databases. Unrooted phylogenetic trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. Evolutionary distance matrices were generated for the least-squares and neighbour-joining algorithms, using the method of Jukes & Cantor (1969). All of the phylogenetic analyses were carried out using the PHYLIP suite of programs (Felsenstein, 1993). The robustness of the resultant trees was evaluated by bootstrap analysis (Felsenstein, 1985) of neighbour-joining data based on 1000 resamplings using the TREECON program (Van de Peer & De Wachter, 1994). The root position of the tree was estimated using *Prauserella rugosa* DSM 43194^{T} (accession no. AF051342) as the outgroup organism.

Almost complete 16S rRNA gene sequences were generated for the three novel isolates (>1447 nt), all of which had identical sequences. Comparison of the 16S rRNA gene sequences with corresponding sequences of representatives of the family *Pseudonocardiaceae* showed that the isolates belong to the genus *Amycolatopsis* (data not shown). The high 16S rRNA gene sequence similarities found between the isolates and representatives of the genus *Amycolatopsis* (94.3–98.4 %) support the addition of these strains to the genus.

It is apparent from Fig. 1 that the isolates are most closely associated with the *A. mediterranei* 16S rRNA subclade, though this relationship is not supported by a high bootstrap value in the neighbour-joining analysis. Strain HKI 0457^{T} was most closely related to *A. rifamycinica* DSM 46095^{T}. The two organisms shared a 16S rRNA gene sequence similarity of 98.4 %, a value that corresponded to 23 differences at 1432 locations. The isolates also shared relatively high 16S rRNA gene sequence similarities with the type strains of *A. kentuckyensis* (98.1 %), *A. lexingtonensis* (98.1 %), *A. mediterranei* (98.2 %), *A. pretoriensis* (98.2 %) and *A. tolypomycina* (98.1 %). DNA–DNA relatedness studies were not carried out between isolate HKI 0457^{T} and its closest phylogenetic neighbours, as it is known that the type strains of *Amycolatopsis* species classified in the *A. mediterranei* 16S rRNA subclade share much higher 16S rRNA gene similarities than those cited above but have lower DNA–DNA relatedness values (Labeda *et al.*, 2003; Wink *et al.*, 2003; Bala *et al.*, 2004), that is, values well below the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

The isolates were examined for a range of key chemical markers to establish whether they had a chemotaxonomic profile typical of members of the genus *Amycolatopsis*. To this end, standard HPLC and TLC procedures were used to determine the isomers of diaminopimelic acid (A_2pm) in whole-organism hydrolysates (Hasegawa *et al.*, 1983), menaquinone profiles (Collins *et al.*, 1977; Groth *et al.*, 1996), muramic acid type (Uchida & Aida, 1984), the presence of mycolic acids (Minnikin *et al.*, 1975), predominant whole-organism sugars (Becker *et al.*, 1965; Schön & Groth, 2006) and polar lipid patterns (Minnikin *et al.*, 1979; Collins & Jones, 1980), using appropriate standards. All three isolates contained *meso*-A_2pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV *sensu*; Lechevalier & Lechevalier, 1970) together with glucose, mannose and rhamnose, *N*-acetylated muramic acid, diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol,

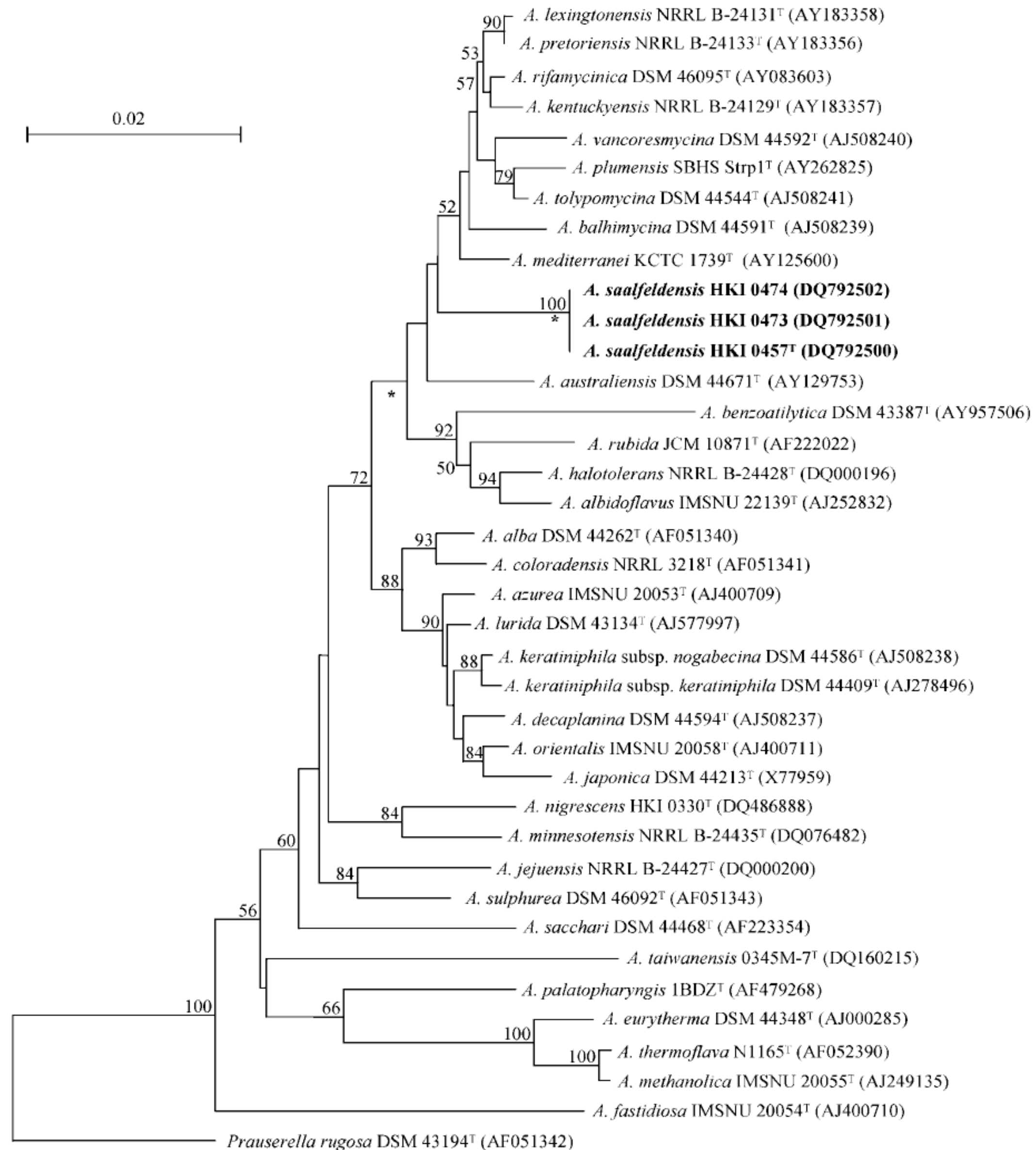

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between isolates HKI 0457^T, HKI 0473 and HKI 0474 and between them and representatives of the genus *Amycolatopsis*. Asterisks indicate branches of the tree that were also found using the least-squares and maximum-parsimony tree-making algorithms. The numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50 % are shown. Bar, 2 substitutions per 100 nt.

phosphatidylinositol, phosphatidylserine and two uncharacterized glycolipids (phospholipid pattern 2 *sensu* Lechevalier *et al.*, 1977), and tetrahydrogenated menaquinones with nine isoprene units [MK-9(H_4)] as the major isoprenologue (86–88 % of total) with minor proportions of MK-8(H_4) (4–5 %), MK-9(H_6) (1–3 %) and MK-10(H_4) (2 %), but they lacked mycolic acids. These data serve to distinguish the isolates from all other wall chemotype IV

actinomycetes, apart from those classified in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Kim & Goodfellow, 1999; Takahashi, 2001).

The fatty acid profiles of the isolates grown in bacto-tryptic soy broth for 48 h were determined using the MIDI system (www.midi-inc.com/). All of the strains had a very similar fatty acid composition in which 14-methylpentadecanoic acid (iso-$C_{16:0}$) was the major component (41–42 % of total); the fatty acids found in smaller proportions were iso-$C_{17:0}$ (8–10 %), iso-$C_{15:0}$ (8–9 %), iso-$C_{14:0}$ (7–9 %), iso-2OH $C_{16:0}$ (4–6 %), $C_{15:0}$ (6 %) and $C_{17:0}$ (5–7 %). These profiles are similar to those recorded for members of established *Amycolatopsis* species (Yassin *et al.*, 1993; Wink *et al.*, 2004; Groth *et al.*, 2007).

Morphological properties of the isolates were examined following growth on ISP media 2 and 3 agar plates (Difco; Shirling & Gottlieb, 1966) at 28 °C for up to 21 days. The pH growth range was established using shake flasks of liquid organic medium 79, adjusted to pH values between 4.5 and 10.0 with either 1 M HCl or 20 %, (w/v) Na_2CO_3 solution, and incubated for 6 days at 28 °C. The isolates were also tested for their ability to grow on solidified minimal medium (Amoroso *et al.*, 2000) supplemented with $CuSO_4$ (2 mM) and $NiCl_2$ (5 mM), respectively, following growth at 28 °C for 21 days. The remaining physiological tests, including the determination of antibiotic sensitivity and enzymic activities, were carried out as described by Groth *et al.* (2003). The isolates formed an extensively branched substrate mycelium on the ISP media tested and moderate amounts of aerial hyphae only on ISP medium 3. Substrate and aerial hyphae fragmented into rod-like elements typical of *Amycolatopsis* strains. The strains also grew in the presence of copper and nickel salts, and shared a broad range of phenotypic properties. Some of the latter can be used to distinguish the isolates from the type strains of phylogenetically close *Amycolatopsis* species classified in the *Amycolatopsis* 16S rRNA gene clade (Table 1).

UP-PCR reactions were performed on the three isolates and their phylogenetically closest neighbours using the primers AS4/AS15 of Bulat *et al.* (2000). The reactions were prepared in a final volume of 10 µl containing 0.5 U GoTaq (Promega), the GoTaq flexi buffer, 0.2 mM of each of the four dNTPs, 3 mM $MgCl_2$, 1 µM primer AS4, 2 µM primer AS15 and about 20 ng extracted DNA. For initial calculation of T_m and primer concentration, the biomath T_m-calculator provided by Promega was used (www.promega.com/biomath/calc11.htm). The different primer concentrations were empirically optimized to give the best possible size range and distribution of the resulting bands. Amplifications were performed three times using Thermo-Fast low profile PCR plates (ABgene) in an MJ research PTC 225 thermal cycler according to the following profile: an initial step at 94 °C for 2 min, 57 °C for 40 s and 72 °C for 30 s to

Table 1. Physiological properties that separate the novel isolates from the type strains of phylogenetically close *Amycolatopsis* species

Strains: 1, *A. saalfeldensis* sp. nov. HKI 0457^T, HKI 0473 and HKI 0474; 2, *A. mediterranei* DSM 43304^T; 3, *A. pretoriensis* DSM 44654^T; 4, *A. rifamycinica* DSM 46095^T. Data for the hypoxanthine and xanthine tests for the reference strains were taken from Wink *et al.* (2003), Lechevalier *et al.* (1986) and Labeda *et al.* (2003). +, Positive; −, negative; w, weakly positive; *, delayed reaction.

Characteristic	1	2	3	4
Colour of aerial mycelium	White	Absent	White to orange	White to orange
Production of soluble pigment	−	−	+(faint)	Light pale brown-yellow
Decomposition of:				
Hypoxanthine	−	+	+	+
Xanthine	+	−	−	−
Production of indole	−	+	−	−
Growth on sole carbon sources:				
Malate (0.2 %, w/v)	+	+	−	+
D-Raffinose (1 %, w/v)	−	+	+	+
API ZYM tests:				
Cystine arylamidase	−	+	+	+
α-Fucosidase	−	+	−	−
β-Galactosidase	−	+	+	+
β-Glucosidase	w	+	−	+
Valine arylamidase	+	+	−	+
Growth at:				
10 °C	w*	+	−	+
42 °C	−	+	+	+

provide templates, followed by 30 cycles at 92 °C for 20 s, 57 °C for 40 s, 72 °C for 30 s and a final extension at 72 °C for 2 min. Amplification products were separated by gel electrophoresis in native polyacrylamide gels (8 %) containing 0.5 % TBE buffer. Gels were stained with SYBR Safe (Invitrogen). Fingerprints were analysed using GelComparII software (version 4.5, Applied Maths). The banding patterns were automatically matched (optimization value 1 %, position tolerance value 0.4 %). This computed matching was refined manually afterwards. A phylogenetic clustering of the banding patterns was achieved using the built-in maximum-parsimony tree-making algorithm (Fitch, 1971). The topology of the resulting tree (Fig. 2) was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. It is clear from Fig. 2 that the isolates gave almost identical UP-PCR fingerprint patterns which serve to distinguish them from corresponding patterns of their closest phylogenetic neighbours.

For MALDI-TOF mass spectrometry, biomass of the isolates taken from cellulose acetate filters was examined as described previously (Kroppenstedt *et al.*, 2005; Groth *et al.*, 2007) using a Voyager mass spectrometer. The resultant spectra were added to an existing *Amycolatopsis* dataset, and the data were analysed using Data Explorer software (Applied Biosystems) and the peak lists compared using SARAMIS software (Anagnos Tec). It is evident from our results (supplementary Fig. S1 available with the online version of this paper) that the isolates have similar profiles that distinguish them from those of *Amycolatopsis* type strains, including those representing phylogenetically close taxa.

It is evident from the genotypic and phenotypic data that isolates HKI 0457^T, HKI 0473 and HKI 0474 form a homogeneous taxon that can be distinguished readily from representatives of phylogenetically close *Amycolatopsis* species classified in the *A. mediterranei* 16S rRNA gene clade. It is, therefore, proposed that the isolates be classified in the genus *Amycolatopsis* as *Amycolatopsis saalfeldensis* sp. nov.

Description of *Amycolatopsis saalfeldensis* sp. nov.

Amycolatopsis saalfeldensis (saal.feld.en′sis. N.L. fem. adj. *saalfeldensis* from Saalfeld, named after the place of origin, a town in Thuringia, Germany).

Aerobic, Gram-positive, non-acid/alcohol-fast, non-motile, catalase-positive actinomycete which forms an extensively branched vegetative mycelium (hyphal diameter 0.5–0.6 μm) that fragments into squarish rod-like elements. The substrate mycelium carries moderate amounts of white aerial hyphae which fragment into squarish rod-like elements. Diffusible pigments are not produced. Good growth occurs between 20 and 35 °C, but growth is not evident below 10 or at 42 °C. Grows well between pH 4.5 and 8.0 and in the presence of 2 % (w/v) NaCl, but does not grow at pH 9.0 or in the presence of 4 % (w/v) NaCl. Grows on minimal medium supplemented with $NiCl_2$ (5 mM) and $CuSO_4$ (2 mM), respectively. Oxidase is produced, aesculin and urea hydrolysed, and H_2S produced. Nitrate is not reduced to nitrite. Degrades casein, gelatin, hippurate, Tween 80 and tyrosine, but not adenine or potato starch. L-Arabinose, D-fructose, D-glucose, *meso*-inositol, D-mannitol, L-rhamnose (type strain weakly), sucrose and D-xylose are used as sole carbon sources for energy and growth, but not cellulose (all at 1 %, w/v). Similarly, acetate, aconitate, benzoate (weakly), citrate and succinate are used as sole carbon and energy sources for growth, but not DL-tartrate (all at 0.2 %, w/v). Produces α-chymotrypsin (weakly), leucine arylamidase, esterase (C4), esterase lipase (C8), *N*-acetyl-*β*-glucosamidase, α-glucosidase, naphthhol-AS-BI-phosphohydrolase, acid phosphatase and alkaline phosphatase, but not α-galactosidase, *β*-glucuronidase, lipase (C14), α-mannosidase or trypsin (API ZYM tests). Susceptible to chloramphenicol (30 μg per disc), ciprofloxacin (5 μg per disc, weakly), imipenem (10 μg per disc), kanamycin sulphate (30 μg per disc), lincomycin hydrochloride (2 μg per disc, weakly), ofloxacin (10 μg per disc, weakly), oxytetracycline hydrochloride (30 μg per disc), rifampicin (30 μg per disc), streptomycin sulfate (10 μg per disc) and

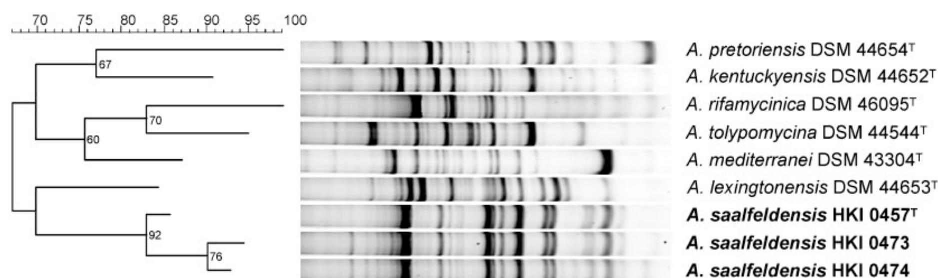

Fig. 2. Maximum-parsimony tree based on UP-PCR generated fingerprint data showing relationships between the isolates and between them and type strains of closely related *Amycolatopsis* species. Bootstrap rates (%) at the nodes are based on 1000 resamplings; only values above 50 % are shown.

vancomycin hydrochloride (30 μg per disc), but is resistant to ampicillin (10 μg per disc), methicillin (5 μg per disc), norfloxacin (10 μg per disc), novobiocin (5 μg per disc), penicillin G (10 IU per disc) and polymyxin B (300 IU per disc). Additional phenotypic properties are shown in Table 1. Chemotaxonomic characters are typical for *Amycolatopsis* species.

The type strain, HKI 0457^T (=DSM 44493^T=NRRL B-24474^T), was isolated from the surface of rocks in a medieval alum slate mine.

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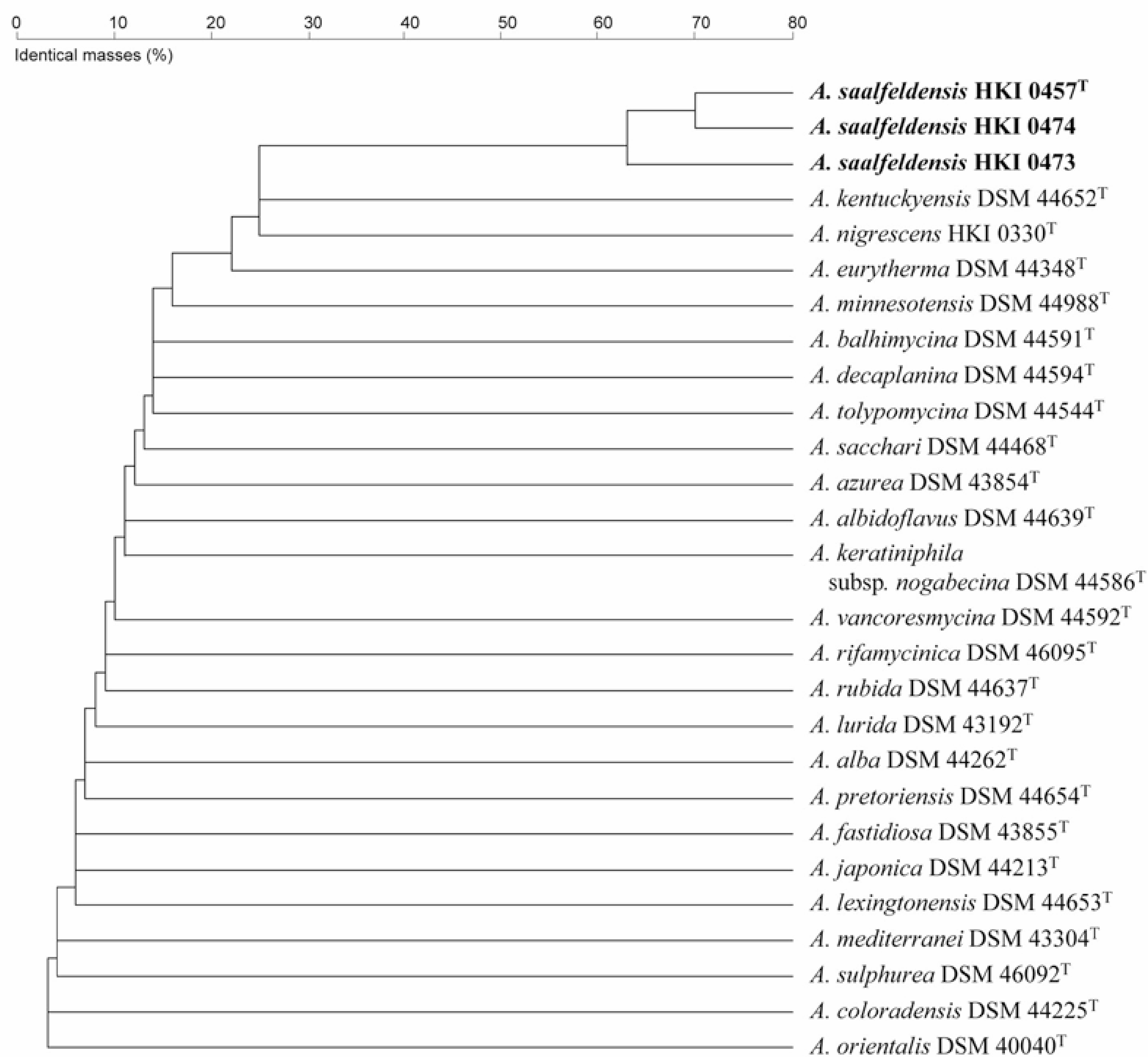
Supplementary Fig. S1. Dendrogram showing relationships between the isolates and between them and the type strains of closely related *Amycolatopsis* species based on MALDI-TOF MS data.



3.2. *Kribbella aluminosa* sp. nov., isolated from a medieval alum slate mine.

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Kribbella aluminosa sp. nov., isolated from a medieval alum slate mine

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Three actinomycetes (strains HKI 0478^T, HKI 0479 and HKI 0480) isolated from the surfaces of rocks in the Feengrotten medieval alum slate mine (Thuringia, Germany) were examined in a polyphasic taxonomic study. The following morphological and chemotaxonomic features supported their classification as members of the genus *Kribbella*: the presence of LL-diaminopimelic acid in the cell-wall peptidoglycan; glucose together with minor amounts of mannose and ribose as the whole-cell sugars; polar lipids comprising phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and unknown phospho- and glycolipids; fatty acid profiles characterized by the predominance of anteiso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{16:0}$ 9-methyl; and the presence of MK-9(H_4) as the main menaquinone. The isolates had almost identical 16S rRNA gene sequences (99.9–100 %) and were most closely related to the type strains of *Kribbella jejuensis* (98.9 % sequence similarity), *Kribbella swartbergensis* and *Kribbella solani* (both 98.8 %). A wide range of genotypic and phenotypic markers as well as the low levels of DNA–DNA relatedness between strain HKI 0478^T and the type strains of *K. jejuensis* (41.3 %), *K. swartbergensis* (18.6 %) and *K. solani* (14.2 %) distinguished the novel strains from their closest phylogenetic neighbours. On the basis of these results, strain HKI 0478^T represents a novel member of the genus *Kribbella*, for which the name *Kribbella aluminosa* sp. nov. is proposed. The type strain is HKI 0478^T (=DSM 18824^T =JCM 14599^T).

The genus *Kribbella* Park *et al.* 1999 emend. Sohn *et al.* 2003 is a member of the family *Nocardioidaceae*, which was proposed by Nesterenko *et al.* (1985, 1990) and the description of which was emended by Rainey *et al.* (in Stackebrandt *et al.*, 1997). The genus *Kribbella* is represented by Gram-positive or Gram-variable, non-motile actinomycetes that form an extensively branched vegetative mycelium and aerial hyphae that fragment into short to elongated rod-like or coccoid elements. Strains of the 11 *Kribbella* species with validly published names share the following chemotaxonomic characteristics (Park *et al.*, 1999; Sohn *et al.*, 2003): the presence of LL-diaminopimelic acid in the peptidoglycan (wall chemotype I *sensu* Lechevalier & Lechevalier, 1970), fatty acids that consist mainly of anteiso- and iso- branched components, MK-9(H_4) as the main menaquinone and polar lipids comprising phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol (phospholipid pattern III *sensu* Lechevalier *et al.*, 1977). A combination of these chemotaxonomic markers serves to separate the strains of the genus *Kribbella* from members of the other genera within the family *Nocardioidaceae*.

Three filamentously growing actinomycetes, strains HKI 0478^T, HKI 0479 and HKI 0480, were isolated from acidic and heavy-metal-containing rock surfaces in a small mining area behind the 'Märchendom', the third level of the Feengrotten medieval alum slate mine (Saalfeld, Thuringia, Germany). The rock surface was touched with a sterile cotton swab and the adhering bacteria were suspended in about 1 ml sterile distilled water. Aliquots of this suspension were spread over agar plates containing water agar supplemented with Bacto peptone (0.1 %) and yeast extract (0.02 %), mineral agar Gauze 1 (Gauze *et al.*, 1983) or starch-casein agar (Küster & Williams, 1964). All of the media were supplemented with cycloheximide (50 μg ml^{-1}). The agar plates were incubated at 28 °C for

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HKI 0478^T, HKI 0479 and HKI 0480 are EF126967, EF126968 and EF126969, respectively.

Cellular fatty acid compositions of strains HKI 0478^T, HKI 0479, HKI 0480 and type strains of closely related *Kribbella* species are presented in a supplementary table available with the online version of this paper.

about 4 weeks. Pure cultures of the isolates were maintained on organic medium 79 (Prauser & Falta, 1968) and preserved at −80 °C as a mixture of well-growing cultures in organic medium 79 broth and glycerol medium that consisted of K_2HPO_4 (1.26 %), KH_2PO_4 (0.36 %), $MgSO_4$ (0.01 %), sodium citrate (0.09 %), $(NH_4)_2SO_4$ (0.18 %) and glycerol (8.8 %). Stock cultures of the isolates in liquid organic medium 79 supplemented with 5 % DMSO were also maintained in the vapour phase of liquid nitrogen.

Bacterial growth for chemotaxonomic and molecular systematic investigations of the isolates was prepared by cultivating cells at 28 °C for 24–48 h in liquid organic medium 79 or Bacto tryptic soy broth (Sigma-Aldrich). For morphological and cultural studies, the strains were cultivated on ISP media 2, 3, 4 and 5 (Difco; Shirling & Gottlieb, 1966) and on minimal agar (Amoroso *et al.*, 2000) at 28 °C for up to 21 days. Growth parameters were determined using organic medium 79. The pH range for growth was established in shake flasks of liquid medium that was adjusted to pH values between 4.5 and 10.0 with either 1 M HCl or 20 % (w/v) Na_2CO_3 solution after sterilization. The cultures were incubated at 28 °C for up to 6 days. Physiological tests, including the determination of enzyme activities and susceptibility to antibiotics, were carried out as described by Groth *et al.* (2003). The reference strains used for comparison were *Kribbella jejuensis* JCM 12204^T, *Kribbella solani* DSM 17294^T and *Kribbella swartbergensis* DSM 17345^T.

The three isolates showed lichenous growth on all media tested. Colonies on organic medium 79 were wrinkled, pasty and cream to pale yellow in colour. The substrate mycelium was extensively branched. White aerial mycelium was produced in abundance on ISP medium 5 and less abundantly on ISP media 2, 3 and 4. No aerial mycelium was produced on organic medium 79. Substrate and aerial hyphae fragmented into irregular, elongated rod-shaped to coccoid elements. Diffusible pigments were not observed.

The morphological and physiological characteristics of the three strains under study were almost identical (see Table 1 and the species description). Although the novel strains shared numerous physiological properties with their most closely related phylogenetic neighbours *K. jejuensis* JCM 12204^T, *K. solani* DSM 17294^T and *K. swartbergensis* DSM 17345^T, they could be readily distinguished from these organisms on the basis of the characteristics listed in Table 1.

Standard HPLC and TLC procedures were used to determine the isomers of diaminopimelic acid present in whole-organism hydrolysates (Hasegawa *et al.*, 1983), the predominant whole-organism sugars (Becker *et al.*, 1965; Schön & Groth, 2006), the fatty acids present (MIDI system; http://www.midi-inc.com/), the predominant menaquinones (Collins *et al.*, 1977; Groth *et al.*, 1996) and the polar lipids present (Minnikin *et al.*, 1979; Collins & Jones, 1980).

Table 1. Physiological properties that serve to distinguish strains HKI 0478^T, HKI 0479 and HKI 0480 from their closest phylogenetic neighbours

Strains: 1, HKI 0478^T, HKI 0479 and HKI 0480; 2, *K. jejuensis* JCM 12204^T; 3, *K. solani* DSM 17294^T; 4, *K. swartbergensis* DSM 17345^T. Unless indicated, data for reference strains were taken from Song *et al.* (2004) (*K. jejuensis* and *K. solani*) and Kirby *et al.* (2006) (*K. swartbergensis*). +, Positive; w, weakly positive; −, negative; d, delayed.

Characteristic	1	2	3	4
Nitrate reduction	−	−	−	+
Decomposition of:				
Casein	+	−	−	+
Potato starch	+	−/+*	−	+*
Tween 80	+	−	+	+
Adenine	+	−	−	+
Hypoxanthine	+	−†	−†	+
Tyrosine	+	−	−	+
Xanthine	$+^d$	−	−	−*
Urea	+	+*	−	−*
Growth on sole carbon sources (1 %, w/v)				
myo-Inositol	+	−	−	w
D-Mannitol	+	−	−	w
Enzyme activity (API ZYM):				
Cystine arylamidase	−	w*	w*	+*
α-Fucosidase	+	+*	−*	−*
Lipase (C14)	−	−*	+*	+*
Trypsin	−	+*	w*	+*
Growth at/in:				
37 °C	+	+	−	+
42 °C	−	+*	−	+
45 °C	−	−	−	+
2.0 % NaCl	+	−*†	+*	+
4.0 % NaCl	−	−*	+*	w
pH 5	+	−*	−*	w^{d}*
pH 9	−	−*	−*	+*
Antibiotic susceptibility (μg per disc)				
Ampicillin (10)	+	−*	−*	−*
Imipenem (10)	+	−*	w*	−*

*Data for reference strains obtained in this study [results for growth in 2 % NaCl confirm the results of Song *et al.* (2004)].
†Conflicting results reported by Kirby *et al.* (2006).

The chemotaxonomic characteristics of the strains were consistent with an affiliation with the genus *Kribbella.* Whole-organism hydrolysates contained LL-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan and the sugars comprised glucose together with minor amounts of mannose and ribose (wall chemotype I *sensu* Lechevalier & Lechevalier, 1970). The predominant menaquinone was MK-9(H_4) (91–93 %). The phospholipids were composed of phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and unknown phospho- and glycolipids, corresponding to

phospholipid pattern III *sensu* Lechevalier *et al.* (1977). The fatty acid profiles of the three isolates were characterized by the presence of significant amounts of anteiso-$C_{15:0}$ (35.9, 44.6 and 37.2 %), iso-$C_{16:0}$ (17.6, 7.5 and 15.1 %), $C_{16:0}$ 9-methyl (8.7, 7.0 and 8.2 %) and iso-$C_{15:0}$ (6.1, 9.7 and 7.4 %), together with minor amounts of iso-$C_{14:0}$ (5.7, 2.7 and 6.1 %) and anteiso-$C_{17:0}$ (5.4, 5.1 and 4.6 %). These profiles also served to distinguish the novel strains from their phylogenetic neighbours (see Supplementary Table S1 available with the online version of this paper).

Chromosomal DNA was extracted from the three isolates by using a slightly modified version of the method of Pospiech & Neumann (1995). Amplification of the 16S rRNA genes and subsequent purification and direct sequencing of the respective PCR products were performed as described by Carlsohn *et al.* (2007).

The 16S rRNA gene sequences were aligned with actinobacterial sequences from the database of DSMZ by using the ae2 editor (Maidak *et al.*, 1997). Evolutionary distances were calculated by using the Jukes–Cantor method (Jukes & Cantor, 1969). Dendrograms were constructed by using the neighbour-joining and maximum-likelihood algorithms (Felsenstein, 1993) and by using the algorithm of De Soete (1983). Bootstrap analysis (500 resamplings) was used to evaluate the tree topology (Felsenstein, 1985).

Almost-complete 16S rRNA gene sequences (1429–1463 nt) were obtained from the three novel strains. These sequences were virtually identical (99.9–100 %) and showed between 98.8 and 98.9 % similarity to the corresponding genes of the closest relatives, namely the type strains of *K. solani*, *K. swartbergensis* and *K. jejuensis*. The levels of similarity with the 16S rRNA genes of the other *Kribbella* species were lower, ranging between 97.7 and 98.7 %. The topologies of both the maximum-likelihood and distance-matrix dendrograms (Fig. 1) were identical in that the three isolates and the three closest relatives in the genus *Kribbella* formed an individual clade. The topologies differed in that the positions of the type strains of *K. jejuensis* and *K. swartbergensis* were transposed. Most of the bootstrap percentages were below 70 %, indicating the low statistical significance of the branching points of the majority of the type strains.

For DNA–DNA relatedness studies, the DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977).

DNA–DNA hybridization was carried out between strain HKI 0478^T and its closest relatives as described by De Ley *et al.* (1970) taking into account the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/Vis spectrophotometer (Varian) equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe. The low levels of DNA–DNA relatedness (in 2 × SSC plus 12 % formamide at 70 °C) between strain HKI 0478^T and *K. jejuensis* JCM 12204^T (46.9 and 35.7 %), *K. solani* DSM 17294^T (13.0 and 15.3 %) and *K. swartbergensis* DSM 17345^T (20.7 and 16.4 %) clearly indicate that the novel organism cannot be affiliated with any of these species.

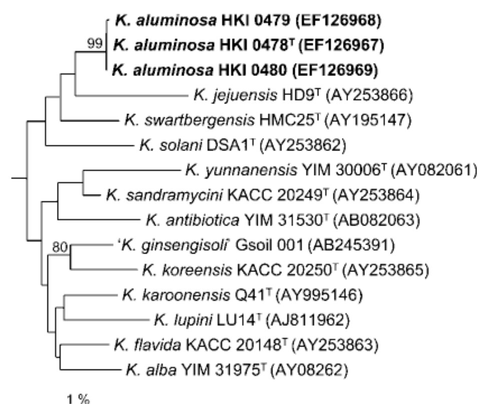

Fig. 1. Additive phylogenetic tree (De Soete, 1983), based on 16S rRNA gene sequences, showing the nearest neighbours, within the genus *Kribbella*, of the three novel strains. Numbers shown at branch points indicate bootstrap percentages (from 500 datasets); only values greater than 70 % are shown. Bar, 1 % difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting any two organisms.

The genome structure of the three isolates and their closest phylogenetic neighbours was examined using universally primed PCRs with primers AS4/AA2M2 (Bulat *et al.*, 2000). The set-up and protocol were as described previously (Carlsohn *et al.*, 2007). The resultant fingerprints were analysed using GelCompar II software (version 4.5; Applied Maths). The banding patterns were aligned using the pGEM DNA marker (Promega) as the reference system. The corresponding bands were automatically matched (optimization value, 0.3 %; position tolerance value, 0.18 %); the resultant matching was examined and then refined manually. Phylogenetic clustering was obtained using the built-in maximum-parsimony tree-making algorithm (Fitch, 1971). The resulting most parsimonious tree (Fig. 2) was artificially rooted; the topology was evaluated by using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. It is clearly shown in Fig. 2 that the isolates shared almost identical fingerprint patterns from universally primed PCRs, distinguishing them from the corresponding patterns of their phylogenetic neighbours.

The results of our genotypic and phenotypic investigations revealed that the three isolates are distinct from all *Kribbella* species with validly published names. Therefore it is proposed that strains HKI 0478^T, HKI 0479 and HKI 0480 represent a novel species of the genus *Kribbella*,

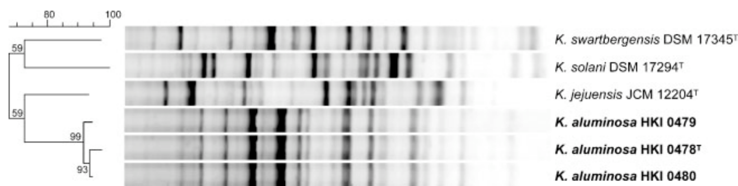

Fig. 2. Maximum-parsimony tree, based on the fingerprint data shown generated by universally primed PCRs, showing the relationships among the isolates and type strains of closely related *Kribbella* species. Bootstrap percentages shown at nodes are based on 1000 resamplings; only values above 50 % are shown.

for which the name *Kribbella aluminosa* sp. nov. is proposed.

Description of *Kribbella aluminosa* sp. nov.

Kribbella aluminosa (a.lu.mi.no′sa. L. fem. adj. *aluminosa* aluminous, full of alum, alum-containing, referring to the source of isolation of the first strains).

Gram-positive, aerobic, non-motile actinomycete that produces an extensively branched, beige to pale yellow substrate mycelium and a white aerial mycelium. Substrate and aerial hyphae fragment into irregular rod-shaped elements. Colonies on organic medium 79 are wrinkled and of a pasty consistency. Diffusible pigments are not produced. Grows between 20 and 37 °C and between pH 5 and 8. Tolerates 2 % NaCl in the culture medium. Grows on minimal medium in the presence of $NiCl_2$ (5 mM) and $CuSO_4$ (0.5 mM). No growth occurs at 6 or 42 °C, at pH 9.5 or in the presence of 4 % NaCl. Catalase-positive and oxidase-negative. Aesculin and hippurate are hydrolysed. H_2S is produced, but indole is not. Acetate, aconitate (delayed), citrate, malate, succinate, L-arabinose, D-fructose, D-glucose, D-raffinose, L-rhamnose, sucrose and D-xylose are utilized as sole carbon sources, but benzoate, DL-tartrate and cellulose are not. Produces leucine arylamidase, valine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase and alkaline phosphatase, but not α-chymotrypsin (type strain, weak) or β-glucuronidase (API ZYM tests). Susceptible to the following antibiotics (amounts per disc shown in parentheses): chloramphenicol (30 µg), ciprofloxacin (5 µg, weakly), novobiocin (5 µg, weakly), ofloxacin (10 µg, weakly), oxytetracycline hydrochloride (30 µg) and polymyxin B (300 IU, weakly). Resistant to the following antibiotics (amounts per disc shown in parentheses): lincomycin hydrochloride (2 µg), meticillin (5 µg), nalidixic acid (30 µg), norfloxacin (10 µg) and penicillin G (10 IU). Additional phenotypic properties are shown in Table 1. Chemotaxonomic characteristics are typical of those for *Kribbella* species.

The type strain, HKI 0478^T (=DSM 18824^T =JCM 14599^T), was isolated from a medieval alum slate mine in Thuringia, Germany. Strains HKI 0479 and HKI 0480 are also members of this species.

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Supplementary Table S1. Cellular fatty acid compositions (%) of strains HKI 0478^T, HKI 0479 and HKI 0480 and related type strains

Fatty acid	HKI 0478^T	HKI 0479	HKI 0480	*K. jejuensis* JCM 12204^T	*K. solani* DSM 17294^T	*K. swartbergensis* DSM 17345^T
Saturated						
14:0					0.4	
15:0	0.4	1.2	0.6		2.2	
16:0	0.4	1.2	0.4	0.8	3.4	0.4
17:0	0.4	1.7	0.6		3.1	
18:0					0.8	
Unsaturated						
15:1 B*	1.6	1.6	1.6		1.1	0.4
16:1 *cis*9	0.6	1.4	0.8		0.9	0.6
17:1 *cis*9	2.8	2.5	2.8		1.6	2.3
18:1 *cis*9	0.4	0.4	0.4			0.4
Branched						
iso-14:0	5.7	2.7	6.1	10.4	10.4	2.2
iso-15:0	6.1	9.7	7.4	11.3	10.0	5.7
iso-16:0	17.6	7.5	15.1	17.3	16.9	27.4
iso-16:0 2-OH	3.0	2.0	3.1	6.3	1.0	1.3
iso-16:1 H*	3.0	1.1	2.0	1.5	1.5	4.7
iso-16:1 2-OH				1.1		
iso-17:0	1.2	5.2	2.1	3.0	2.9	2.4
iso-18:0	0.4		0.4	0.5	0.4	0.8
iso-19:1 I*				0.6		
anteiso-15:0	36.0	44.6	37.2	27.1	31.2	16.4
anteiso-15:0 2-OH	2.1	2.2	2.7	2.8	1.2	
anteiso-15:1 A*					0.6	
anteiso-17:0	5.4	5.1	4.6	1.4	2.2	11.7
anteiso-17:0 2-OH	1.6	1.3	1.5			
anteiso-17:1 C*						1.9
Methyl fatty acids						
16:0 9-methyl	8.7	7.0	8.2	7.9	4.0	13.8
17:0 10-methyl	1.6	0.5	1.1	0.3		3.2
iso-16:0 10-methyl				4.0	3.6	4.3
Feature 4†	1.0	1.2	1.3	3.6	0.5	

*The double-bond position is unknown.

†Unknown fatty acids: these compounds have no names listed in the Peak Library file of the MIDI system and therefore could not be identified.

3.3. *Fodinicola feengrottensis* gen. nov, sp. nov., an actinomycete isolated from a medieval mine.

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Fodinicola feengrottensis gen. nov., sp. nov., an actinomycete isolated from a medieval mine

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A filamentous, Gram-positive actinobacterium was isolated from acidic rocks in a medieval alum slate mine and was investigated by means of a polyphasic taxonomic approach. A 16S rRNA gene sequence similarity study indicated that strain HKI 0501^T forms an individual line of descent and is related to certain members of the suborder *Frankineae*, order *Actinomycetales* (<95 % sequence similarity). Distance-matrix and neighbour-joining analyses set the branching point of the novel isolate between two clades, one being represented by members of the genus *Cryptosporangium* (family '*Kineosporiaceae*') and the other by members of the genera *Frankia* and *Acidothermus* (family *Frankiaceae* and family *Acidothermaceae*, respectively). The organism had *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan and xylose as the characteristic cell-wall sugar. The muramic acid in the peptidoglycan was found to be *N*-acetylated. The major menaquinones were MK-9(H_4), MK-9(H_6) and MK-9(H_8) and the fatty acid profile was characterized by the predominance of iso-$C_{16:0}$, 10-methyl $C_{17:0}$, $C_{17:1}$ *cis*9 and 10-methyl iso-$C_{18:0}$. The polar lipids comprised diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and several unknown phospholipids and glycolipids. Mycolic acids were absent. The DNA G+C content was 65 mol%. The distinct phylogenetic position and the phenotypic markers that clearly separate the novel organism from all other members of the suborder *Frankineae* indicate that strain HKI 0501^T represents a novel genus and species, for which the name *Fodinicola feengrottensis* gen. nov., sp. nov. is proposed. The type strain of *Fodinicola feengrottensis* is HKI 0501^T (=DSM 19247^T =JCM 14718^T).

The suborder *Frankineae* Stackebrandt *et al.* 1997 currently accommodates 12 genera classified within six families (Garrity *et al.*, 2007): *Frankiaceae* Becking 1970 emend. Hahn *et al.* 1989 emend. Normand *et al.* 1996 emend. Stackebrandt *et al.* 1997 (genus *Frankia* Brunchorst 1886); *Geodermatophilaceae* Normand *et al.* 1996 emend. Stackebrandt *et al.* 1997 (genera *Geodermatophilus* Luedemann 1968, *Blastococcus* Ahrens and Moll 1970 and *Modestobacter* Mevs *et al.* 2000); *Nakamurellaceae* Tao *et al.* 2004 [substitute for the illegitimate name *Microsphaeraceae* Rainey *et al.* 1997 in Stackebrandt *et al.* (1997)] [genera *Nakamurella* Tao *et al.* 2004 (substitute for the illegitimate name *Microsphaera* Yoshimi *et al.* 1996), *Quadrisphaera* Maszenan *et al.* 2005 and *Humicoccus* Yoon *et al.* 2007]; *Sporichthyaceae* Rainey *et al.* 1997 in Stackebrandt *et al.* (1997) (genus *Sporichthya* Lechevalier *et al.* 1968); *Acidothermaceae* Rainey *et al.* 1997 in Stackebrandt *et al.* (1997) (genus *Acidothermus* Mohagheghi *et al.* 1986); and '*Kineosporiaceae*' (genera *Kineosporia* Pagani and Parenti 1978, *Cryptosporangium* Tamura *et al.* 1998 and *Kineococcus* Yokota *et al.* 1993).

The members of the suborder *Frankineae* are morphologically and biochemically heterogeneous: the genera can be readily distinguished from one another by using a combination of chemotaxonomic and morphological properties (Table 1). Members of this suborder have been isolated from various specialized habitats (plants, hot springs, stone surfaces, activated sludge and geographically diverse soils). Most of the strains are characterized by low growth rates and fastidious growth requirements. At the time of writing, all of the genera affiliated to this suborder are grossly underspeciated.

The present polyphasic study was designed to determine the taxonomic position of a filamentous bacterial strain

Abbreviation: A_2pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HKI 0501^T is EF490376.

Table 1. Characteristics that serve to differentiate strain HKI 0501^T from members of genera classified in the suborder *Frankineae*

Data for reference genera were taken from Lechevalier (1994) and Mirza *et al.* (1991) (*Frankia*), Luedemann & Fonseca (1989), Kroppenstedt (1985) and Collins *et al.* (1984) (*Geodermatophilus*), Urzì *et al.* (2004) (*Blastococcus*), Mevs *et al.* (2000) (*Modestobacter*), Yoshimi *et al.* (1996) and Yoon *et al.* (2007) (*Nakamurella*), Yoon *et al.* (2007) (*Humicoccus*), Maszenan *et al.* (2005) (*Quadrisphaera*), Tamura *et al.* (1999) and Rainey *et al.* (1993) (*Sporichthya*), Mohagheghi *et al.* (1986) (*Acidothermus*), Itoh *et al.* (1989) and Kudo *et al.* (1998) (*Kineosporia*), Tamura *et al.* (1998) (*Cryptosporangium*) and Yokota *et al.* (1993) (*Kineococcus*). +, Present; −, absent; ND, no data available.

Taxon	Cellular morphology	Spore/bud formation	Motility	Cell-wall diamino acid(s)	Major menaquinone(s)	Polar lipid(s)*	Predominant fatty acid(s)	DNA G+C content (mol%)
Fodinicola gen. nov. (strain HKI 0501^T)	Substrate and aerial hyphae	− (Fragmentation of aerial hyphae)	−	*meso*-A_2pm	MK-9(H_4), MK-9(H_6), MK-9(H_8)	DPG, PE, PS, PI, PL, GL	iso-$C_{16:0}$, 10-methyl $C_{17:0}$, $C_{17:1}$ *cis*9	65
Frankiaceae								
Frankia	Substrate hyphae; no aerial mycelium; multilocular sporangia	Sporangiospores	−	*meso*-A_2pm	MK-9(H_4), MK-9(H_6), MK-9(H_8)	PI, PIM, DPG	iso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:1}$	66–71
Geodermatophilaceae								
Geodermatophilus	Thallus consisting of cuboid to oval cells; multilocular sporangia; rudimentary hyphae; no aerial mycelium	Zoospores	+/−	*meso*-A_2pm	MK-9(H_4)	PE, PIM, PI, DPG	iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$	73–75
Blastococcus	Cocci, rods, vibrios; pairs, tetrads; clusters	Buds	+/−	*meso*-A_2pm	MK-9(H_4), MK-9	DPG, PG, PI, PE	iso-$C_{16:0}$, iso-$C_{16:1}$, iso-$C_{15:0}$, $C_{18:1}\omega 9c$, $C_{17:1}\omega 8c$, $C_{17:0}$	74
Modestobacter	Rods and cocci	Buds	+/−	*meso*-A_2pm	MK-9(H_4), MK-8(H_4), MK-9(H_6)	DPG, PE, PI, PG	$C_{18:1}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$	68–70
Nakamurellaceae								
Nakamurella	Cocci; pairs; clusters	−	−	*meso*-A_2pm	MK-8(H_4)	DPG, PE, PE-dimethyl	iso-$C_{16:0}$, iso-$C_{15:0}$, $C_{18:1}$	68
Humicoccus	Cocci	−	−	*meso*-A_2pm	MK-8(H_4), MK-9(H_4)	DPG, PE, PE-dimethyl	anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{17:0}$	73
Quadrisphaera	Cocci; tetrads; clusters	−	−	*meso*-A_2pm	MK-8(H_2)	DPG, PG, PI	anteiso-$C_{15:0}$, $C_{16:0}$	75
Sporichthyaceae								
Sporichthya	Short aerial hyphae; no substrate mycelium	Coccoid to rod-shaped spores	+	LL-A_2pm	MK-9(H_8), MK-9(H_6), MK-8(H_6)	PI, PG, DPG, PL	$C_{16:0}$, iso-$C_{16:0}$, $C_{17:1}$, $C_{17:0}$	71
Acidothermaceae								
Acidothermus	Slender rods, filaments	−	−	A_2pm, Ser, Ala	ND	ND	ND	61
'*Kineosporiaceae*'								
Kineosporia	Substrate hyphae; no aerial mycelium; elongated, club-shaped sporangia	Spherical to ovoid spores	+	LL-A_2pm and *meso*-A_2pm	MK-9(H_4), MK-8(H_4), MK-9(H_6)	PC, DPG, PI, PIM	$C_{16:0}$, $C_{18:1}$, 10-methyl $C_{18:0}$	69–71
Cryptosporangium	Substrate and aerial mycelia; sporangia	Sporangiospores	+	*meso*-A_2pm	MK-9(H_6), MK-9(H_4), MK-9(H_8)	PE	iso-$C_{16:0}$, $C_{17:1}$, $C_{18:1}$	70
Kineococcus	Cocci; pairs, tetrads; clusters	−	+	*meso*-A_2pm	MK9(H_2)	DPG, PG, GL	anteiso-$C_{15:0}$	74

*DPG, Diphosphatidylglycerol; GL, unknown glycolipid(s); PC, phosphatidylcholine, PE, phosphatidylethanolamine; PE-dimethyl, phosphatidyldimethylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PL, unknown phospholipid(s).

that had been isolated from a medieval alum slate mine. The resultant phylogenetic and phenotypic data showed that the novel organism should be classified in the suborder *Frankineae* within a novel genus and species.

Strain HKI 0501^T was isolated from acidic and heavy metal-containing rocks in the 'Barbara Grotto' of the Feengrotten medieval alum slate mine in Saalfeld, Thuringia, Germany. Material from the rock surface was scraped off with a sterile cotton swab and the adhering bacteria were dispersed in about 1 ml sterile distilled water. Aliquots of the resultant suspension were spread over starch-casein agar plates (Küster & Williams, 1964) supplemented with cycloheximide (50 μg ml^{-1}). The agar plates were incubated at 28 °C for about 4 weeks. Subcultivation of the isolate was done on solidified organic medium 79 (Prauser & Falta, 1968; http://www.dsmz.de/microorganisms/html/media/medium000426.html) and ISP 2 medium (Difco; Shirling & Gottlieb, 1966). Pure cultures of strain HKI 0501^T were preserved at −80 °C as a mixture of well-growing cultures in organic medium 79 broth and glycerol medium that consisted of K_2HPO_4 (1.26 %), KH_2PO_4 (0.36 %), $MgSO_4$ (0.01 %), sodium citrate (0.09 %), $(NH_4)_2SO_4$ (0.18 %) and glycerol (8.8 %). Stock cultures of the novel isolate in liquid organic medium 79 supplemented with 5 % DMSO were also maintained in the vapour phase of liquid nitrogen.

Bacterial growth for chemotaxonomic and molecular systematic studies was prepared by cultivating strain HKI 0501^T at 28 °C for 2–7 days in liquid organic medium 79 or Bacto tryptic soy broth (Sigma-Aldrich). Chromosomal DNA was extracted from the isolate by using a slightly modified version of the method of Pospiech & Neumann (1995). Amplification of the 16S rRNA gene and subsequent purification and direct sequencing of the respective PCR products were performed as described by Carlsohn *et al.* (2007).

The 16S rRNA gene sequence (1441 bp) was aligned to actinobacterial sequences from the database of the DSMZ using the ae2 editor (Maidak *et al.*, 1997). Evolutionary distances were calculated by using the Jukes–Cantor method (Jukes & Cantor, 1969). Dendrograms were constructed by using the neighbour-joining and maximum-likelihood algorithms (Felsenstein, 1993) and by using the algorithm of De Soete (1983). Bootstrap analysis (500 resamplings) was used to evaluate the tree topology (Felsenstein, 1985).

Similarity values calculated for isolate HKI 0501^T indicated a remote relationship (<95 % similarity) with members of the suborder *Frankineae*. Although similar values were shared with respect to type strains of both *Cryptosporangium* (family '*Kineosporiaceae*') and *Sporichthya* (family *Sporichthyaceae*) (92.9–94.8 and 93.9–94.5 %, respectively), the two treeing algorithms applied to the Jukes–Cantor-corrected dissimilarity values clearly indicated an individual line of descent for strain HKI 0501^T (see Fig. 1 for the neighbour-joining dendrogram); in view of its phylogenetically deep branching point within the family, HKI 0501^T could be considered as representing a novel genus. Members of the genera *Frankia* and *Acidothermus* are slightly less closely related to isolate HKI 0501^T (92.5–93.0 %) than members of the genus *Cryptosporangium*. According to the neighbour-joining

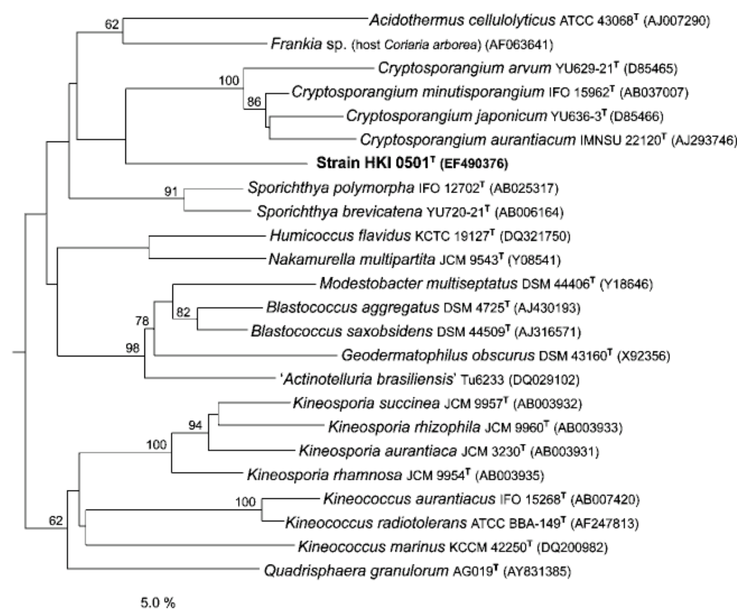

Fig. 1. Phylogenetic dendrogram, based on 16S rRNA gene sequences and constructed from evolutionary distances (De Soete, 1983), showing the position of strain HKI 0501^T within the radiation of members of the suborder *Frankineae*, order *Actinomycetales* (Stackebrandt *et al.*, 1997). Numbers at branching points refer to bootstrap percentages (based on 500 resamplings); only values above 60 % are shown. Members of the family *Microbacteriaceae* were used to root the tree. Bar, 5 inferred nucleotide substitutions per 100 nucleotides.

dendrogram, the family '*Kineosporiaceae*' does not emerge as a phylogenetically coherent family, perhaps because of novel entries into *Frankineae* that were not present when the suborder was originally defined (Garrity *et al.*, 2007; Stackebrandt *et al.*, 1997).

Strain HKI 0501^T possesses most of the 16S rRNA gene sequence signatures defined for the suborder *Frankineae* (Stackebrandt *et al.*, 1997), the exception being a U residue instead of a C residue at position 222. Several new genera have been described since the definition of the signatures for *Frankineae*, but no families have been proposed for these genera. Table 2 provides an update of the signatures for families described by Stackebrandt *et al.* (1997) and for novel genera that are not yet assigned to families. The intermediate branching position of strain HKI 0501^T (Fig. 1) is confirmed by the moderate number of signatures shared with members of the *Frankiaceae*. Strain HKI 0501^T shares more signatures with members of the *Frankiaceae* (40–60 %) than with members of the genera *Kineosporia*, *Kineococcus* and *Quadrisphaera* (35–40 %).

For morphological and cultural studies, strain HKI 0501^T was cultivated on agar plates containing ISP media 2, 3, 4 and 5 (Difco; Shirling & Gottlieb, 1966), humic acid agar (Hayakawa & Nonomura, 1987), Bennett's agar (Jones, 1949) and organic medium 79 for up to 21 days at 28 °C. Cell morphology and cell dimensions were examined using a phase-contrast microscope (Axioscope 2; Zeiss) equipped with image-analysing software (AXIO VISION 2.05; Carl Zeiss).

For scanning electron microscopy, samples were prepared by cutting agar blocks containing growing cells of strain HKI 0501^T (humic acid agar, 21 days) and fixing them in a solution of 2.5 % glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, for about 20 h at room temperature. Subsequently, samples were rinsed with 0.1 M sodium cacodylate solution and dehydrated through a graded ethanol series (30–100 %); this was followed by critical-point drying and sputter-coating with gold before observation under a LEO 1450 VP scanning electron microscope at an acceleration voltage of 15 kV and a working distance of 12 mm. Motility was checked as recommended by Tamura *et al.* (1999). Growth parameters (temperature, pH and tolerance of NaCl) were determined using organic medium 79. The pH range for growth was established in shake flasks of liquid medium adjusted to pH values between 4.5 and 10.0 with either 1 M HCl or 20 % (w/v) Na_2CO_3 solution after sterilization. The cultures were incubated at 28 °C for up to 7 days. Physiological tests, including the determination of enzyme activities and antibiotic susceptibilities, were carried out as described previously (Groth *et al.*, 2003).

Strain HKI 0501^T formed a branched substrate mycelium and sparse to abundant, short, white aerial hyphae that fragmented into irregular rod-like elements (Fig. 2). Spore chains and motility of the fragments, as reported for the coccoid to rod-shaped spores of the closely related *Sporichthya* strains (Lechevalier *et al.*, 1968; Tamura *et al.*, 1999), were not observed. Furthermore, the unique morphological properties of *Sporichthya* strains (lack of a substrate mycelium and the presence of a basal cell as a holdfast in solid medium) serve to distinguish strain HKI 0501^T from members of that genus. Representatives of the equally closely related genus *Cryptosporangium* (Tamura *et al.*, 1998) can be also readily distinguished from isolate HKI 0501^T as they produce spherical to irregularly shaped sporangia with spores that show motility when they are

Table 2. 16S rRNA signature nucleotides for families of the suborder *Frankineae* and some related genera

Taxa: 1, strain HKI 0501^T; 2, *Frankiaceae*; 3, *Geodermatophilaceae*; 4, *Nakamurella*; 5, *Humicoccus*; 6, *Sporichthyaceae*; 7, *Acidothermaceae*; 8, *Kineosporia*; 9, *Cryptosporangium*; 10, *Kineococcus* and *Quadrisphaera*. var., Variable sequence observed at these positions.

Positions	1	2	3	4	5	6	7	8	9	10
66 : 104	G–C	G–C	A–U	A–U	A–U	A–U	G–C	A–U	G–C	A–U
139 : 224	C–G	G–C	C–G	C–G	U–A	U–A	C–G	U–A	U–A	U–G
140 : 223	A–U	A–U	A–U	A–U	A–U	A–U	U–U	G–C	C–G	G–Y
157 : 164	G–U	G–C	A–U	G–C	G–C	G–C	G–C	G–G	G–C	G–C
158 : 163	G–C	G–C	A–U	G–C	G–C	G–C	G–C	G–C	G–C	G–C
186 : 191	G–C	G–C	C–G	C–G	C–G	G–C	G–C	var.	G–C	var.
293 : 304	G–C	G–C	G–U	G–C	G–C	G–C	G–C	G–C	G–C	G–Y
600 : 638	G–C	G–C	G–C	G–C	G–C	U–G	C–G	G–C	G–C	U–G
602 : 636	U–G	C–G	C–G	A–U	A–U	C–G	C–G	C–G	C–G	C–G
839 : 847	C–G	A–G	Y–R	U–A	U–A	U–A	A–U	a–u/u–a	Y–G	var.
986 : 1219	A–U	A–U	U–A	A–U	A–U	A–U	U–U	A–U	A–U	A–U
987 : 1218	A–U	G–C	A–U	A–U	A–U	A–U	G–C	A–U	A–U	A–U
998 : 1043	G–C	G–C	G–C	G–C	G–C	A–U	G–C	G–C	A–U	G–Y
999 : 1042	G–U	C–G	C–G	C–G	C–G	U–A	C–G	G–U	C–G	C–G
1308 : 1329	U–A	C–G	U–A	U–A	U–A	U–A	C–G	C–G	C–G	Y–R
1059 : 1198	C–G	C–G	U–A	U–A	U–A	U–A	C–G	U–A	U–A	U–A

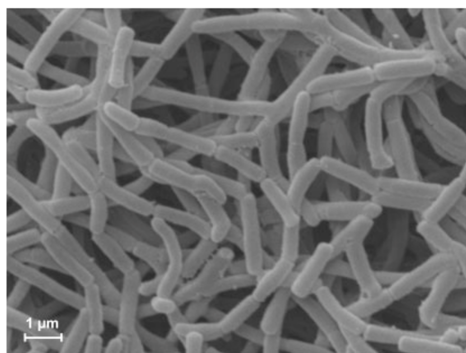

Fig. 2. Scanning electron micrograph of cells of strain HKI 0501^T from cultures grown on humic acid agar for 21 days. Fragmentation of aerial hyphae is visible. Bar, 1 µm.

suspended in water. As shown in Table 1, the less closely related members of the genus *Frankia*, which comprise nitrogen-fixing root symbionts of dicotyledonous plants, and the only strain of the monospecific thermophilic genus *Acidothermus* can also be differentiated easily from strain HKI 0501^T on the basis of their typical morphological properties. The cultural characteristics of strain HKI 0501^T on different media are listed in Table 3. For laboratory cultivation, growth was generally improved when the culture media were acidified with HCl (after sterilization) to about pH 6.0–6.4. The physiological properties of strain HKI 0501^T are given in the species description.

Standard HPLC and TLC procedures were used to determine the isomers of diaminopimelic acid (A_2pm) present in whole-organism hydrolysates (Hasegawa *et al.*, 1983), the predominant cell-wall sugars (Becker *et al.*, 1965; Schön & Groth, 2006), the muramic acid type (Uchida & Aida, 1984), the fatty acid composition (MIDI system; http://www.midi-inc.com/), the predominant menaquinones (Collins *et al.*, 1977; Groth *et al.*, 1996), the presence of mycolic acids (Minnikin *et al.*, 1975) and the polar lipid pattern (Minnikin *et al.*, 1979; Collins & Jones, 1980). For determination of the molar ratios of the cell-wall amino acids, highly purified peptidoglycan preparations were obtained according to the method of Schleifer & Seidl (1985). The amino acid composition of the peptidoglycan hydrolysate (4 M HCl, 16 h, 100 °C) was determined by one-dimensional TLC on cellulose plates (Merck) using the solvent system of Rhuland *et al.* (1955) and by GC and GC/MS of amino acids (Schumann *et al.*, 1997) after derivatization according to MacKenzie (1987).

The G+C content of the DNA was estimated by monitoring the fluorescence intensity during DNA denaturation according to Xu *et al.* (2000) but using a MiniOpticon real-time PCR system (Bio-Rad) at a ramping rate of 0.1 °C s^{-1} and genomic DNA from *Bacillus subtilis* subsp. *subtilis* strain 168 (DSM 402; G+C content 42 mol%; Kunst *et al.*, 1997) as the reference. The resulting value (65 mol%) was in good agreement with the 65.3 mol% obtained with the HPLC method (Mesbah *et al.*, 1989).

The cell-wall peptidoglycan of strain HKI 0501^T contained *meso*-A_2pm, alanine, glycine and glutamic acid in a molar ratio of 1 : 0.9 : 2.4 : 1, respectively. The presence of *meso*-A_2pm is a feature shared by most of the genera of the *Frankineae* (Table 1), the exceptions being the strains of the phylogenetically close genus *Sporichthya* (which possess LL-A_2pm) and those of the distantly related genus *Kineosporia* (which are characterized by the common occurrence of both LL-A_2pm and *meso*-A_2pm). In *Acidothermus cellulolyticus*, the A_2pm isomer has not been defined (Mohagheghi *et al.*, 1986). The main menaquinones in the novel isolate were MK-9(H_4), MK-9(H_6) and MK-9(H_8) (similar proportions; ratio of HPLC peak areas, 31 : 29 : 29), this profile being shared with the majority of the members of the *Frankineae*. Comparable menaquinone profiles are not present in the coccoid genera *Nakamurella* and *Quadrisphaera* (family *Nakamurellaceae*). The predominant menaquinones in strains of these genera possess eight isoprene units. However, the type strain of the only species of the genus *Humicoccus*, which belongs to the same family, contains both MK-8(H_4) and MK-9(H_4) (similar amounts) as the main menaquinones. No data on

Table 3. Cultural characteristics of strain HKI 0501^T

The strain was grown at 28 °C for 21 days.

Medium	Growth	Substrate mycelium	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Good	Wrinkled, pale orange	Short, white	None
Oatmeal agar (ISP 3)	Poor	Wrinkled, orange	Sparse, short, white	None
Inorganic salts-starch agar (ISP 4)	Good, flat	Wrinkled, pale orange	Abundant, short, white	None
Glycerol-asparagine agar (ISP 5)	Poor, flat	Wrinkled, beige	Sparse, short, white	None*
Bennett's agar	Good	Wrinkled, pale orange	Abundant, short, white	None
Humic acid agar	Good, flat	Orange–brown	Abundant, short, white	None
Organic medium 79 agar	Good	Wrinkled, beige to pale orange	None	None

*After storage at room temperature for about a further 6 weeks, growth increased and a soluble yellow pigment was observed.

menaquinones are available for *A. cellulolyticus*. Although these two chemotaxonomic traits are shared with other organisms, strain HKI 0501^T can be readily distinguished from its closest phylogenetic neighbours and from all other representatives of the suborder *Frankineae* on the basis of its cellular fatty acid profile, its polar lipid composition, the presence of xylose as its diagnostic cell-wall sugar and its genomic DNA G+C content (65 mol%). The fatty acid profile (analysed from freeze-dried biomass grown in shake flasks of Bacto tryptic soy broth for 48 h at 28 °C) was characterized by a predominance of iso-$C_{16:0}$ (32.2 %), 10-methyl $C_{17:0}$ (10.7 %), $C_{17:1}$ *cis*9 (8.9 %) and 10-methyl iso-$C_{18:0}$ (8.5 %) and the presence of smaller proportions of $C_{17:0}$ (7.4 %), iso-$C_{17:0}$ (5.8 %), $C_{18:1}$ *cis*9 (4.8 %), 10-methyl $C_{18:0}$ (4.7 %), iso-$C_{18:0}$ (3.2 %), anteiso-$C_{17:0}$ (3.0 %), iso-$C_{16:0}$ 2-OH (2.7 %) and $C_{16:0}$ (2.6 %). The phospholipids comprise diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and several unknown phospholipids and glycolipids together with unknown ninhydrin-positive compounds.

The data obtained from the phenotypic characterization underline the separate position of strain HKI 0501^T in the 16S rRNA gene sequence-based phylogenetic tree for the suborder *Frankineae*. It is evident from both the phylogenetic and phenotypic studies that strain HKI 0501^T cannot be classified within any genus of the suborder *Frankineae* and thus merits classification within a novel genus and species, for which the name *Fodinicola feengrottensis* gen. nov., sp. nov. is proposed.

Description of *Fodinicola* gen. nov.

Fodinicola (Fo.di.ni′co.la. L. n. *fodina* a pit, mine; L. suff. *-cola* from L. n. *incola* dweller; N.L. masc. n. *Fodinicola* a mine dweller).

Gram-positive, aerobic, non-motile, catalase-positive, oxidase-negative actinomycetes that form branched substrate mycelium and sparse to abundant, white aerial mycelium. The aerial hyphae break up into irregular rod-like elements. The cell-wall peptidoglycan contains *meso*-A_2pm, alanine, glycine and glutamic acid. The muramic acid in the peptidoglycan is *N*-acetylated. The cell-wall sugars are xylose and minor amounts of an unknown compound. The predominant menaquinones are MK-9(H_4), MK-9(H_6) and MK-9(H_8). The polar lipids comprise diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and several unknown phospholipids and glycolipids together with unknown ninhydrin-positive compounds. Mycolic acids are absent. The cellular fatty acid profile is characterized by the predominance of iso-$C_{16:0}$, 10-methyl $C_{17:0}$, $C_{17:1}$ *cis*9, 10-methyl iso-$C_{18:0}$ and $C_{17:0}$. The G+C content of the genomic DNA of the type strain of the type species is 65 mol%. Phylogenetically, the genus is affiliated to the suborder *Frankineae*. The type species is *Fodinicola feengrottensis*.

Description of *Fodinicola feengrottensis* sp. nov.

Fodinicola feengrottensis (fe.en.grot.ten′sis. N.L. masc. adj. *feengrottensis* pertaining to the Thuringian cave Feengrotten, the origin of the type strain).

Displays the following properties in addition to those given in the genus description. Hyphal diameter is 0.35–0.52 μm. Diffusible pigments may be produced. Colonies are wrinkled and beige to orange in colour. Good growth occurs between 20 and 28 °C, but growth is not evident below 10 °C or above 32 °C. Grows well between pH 5.0 and 6.0, but does not grow at pH 4.0 or 8.0. Growth at pH 7.0 is delayed and reduced. 1 % NaCl in combination with organic medium 79 is tolerated, but 2 % NaCl is not. Aesculin, casein, gelatin, potato starch and urea are hydrolysed. Nitrate is not reduced to nitrite. Adenine, hypoxanthine and tyrosine are not degraded. L-Arabinose, D-fructose, D-glucose (weakly), D-mannitol, raffinose, L-rhamnose, sucrose and D-xylose are used as sole carbon sources for energy and growth, but *myo*-inositol and cellulose are not (all at 1 %, w/v). Produces α-chymotrypsin (weakly), cystine arylamidase, leucine arylamidase, valine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, α-glucosidase, lipase (C14) (weakly), α-mannosidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase and alkaline phosphatase, but not α-fucosidase, β-glucosidase or β-glucuronidase. Production of trypsin is variable (API ZYM tests). Susceptible to the following antibiotics (μg per disc): chloramphenicol (30), ciprofloxacin (5), imipenem (10), kanamycin sulphate (30), norfloxacin (10), novobiocin (5), oxytetracycline hydrochloride (30), streptomycin sulphate (10), sulfonamide (200) and vancomycin hydrochloride (30). Resistant to the following antibiotics (μg per disc, unless otherwise indicated): ampicillin (10), lincomycin hydrochloride (2), meticillin (5), nalidixic acid (30), penicillin G (10 IU), polymyxin B (300 IU) and rifampicin (30).

The type strain, HKI 0501^T (=DSM 19247^T =JCM 14718^T), was isolated from rocks from a medieval alum slate mine in Thuringia, Germany.

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4 Discussion

Natural products are among the most important sources of anticancer and antiinfective agents. More than 60 % of approved and pre-new drug application candidates are either natural products or related to them (Demain & Zhang, 2005). Members of the order *Actinomycetales* produce two-thirds of these natural products, and the genus *Streptomyces* is reported as the most prolific producer (Bérdy, 2005).

However, in the last two decades microbe-based drug discovery programs are characterized by diminishing returns of novel structures and leads. With conventional isolation methods the chance of getting a producer of a novel antibiotic today is less than 10^{-7} (Baltz, 2005). The primary reason for this phenomenon is that fast growing strains accumulate under laboratory conditions - at the expense of slow-growing microbes (Baltz, 2008; Donadio *et al.*, 2005; Sosio *et al.*, 2000). Therefore it is likely, that again and again the same abundant strains mainly of the genus *Streptomyces* have been analyzed. However, it has been repeatedly reasoned (Baltz, 2008; Donadio *et al.*, 2005) that previously uncultured actinomycetes might represent a potential source of novel bioactive metabolites. Strategies that favor access to rare or unculturable bacterial strains could possibly increase the chances of discovering novel compounds.

Being for long considered as mainly confined to soil environments (Porter, 1971), actinomycetes have been reported from nearly all habitat types, ranging from the deep sea to the arctic. From endangered and extreme environments like naturally or anthropogenically acidified and/or metal-enriched outcrops, mining sites and caves a surprising diversity of actinomycetes has repeatedly been reported as well (Amoroso *et al.*, 1998; Groth & Saiz-Jimenez, 1999; Haferburg *et al.*, 2007; Johnson & Hallberg, 2003; Van Nostrand *et al.*, 2007; Zhou *et al.*, 2007).

Unfortunately, many recent diversity studies mainly rely on total DNA/RNA, merely listing impressive numbers of yet uncultured taxa. However, in the search for novel bioactive secondary metabolites, strain isolation, cultivation and characterization remains an essential part of the analysis of microorganisms.

4.1. Habitat characterization

To succeed in the isolation of novel and potential strains, a profound knowledge of the limiting environmental conditions that characterize the habitat is essential.

From the ecological point of view, the bacterial habitat divides into a complex system of microhabitats, in which environmental parameters like e.g., pH, ionic strength or nutrient availability can greatly vary and that provides various ecological niches for specially adapted organisms over short distances. Soil-born microbes are not only affected by their environment, but they also control particular habitat parameters, directly and indirectly. Growth and metabolism can lead to changes in pH, redox potential or ionic strength. For example, the excretion of organic acids leads to a pH decline within the habitat and thereupon to a higher mobility of heavy metals. This process of metal mobilization, in turn, determines the species composition of the habitat to a great extent. The microflora, again, strongly participates in processes like decomposing soil constituents as well as particle aggregation and influences availability of nutrients (Krasilnikov, 1961).

4.1.1. The Feengrotten – a medieval mine

The actinomycete strains discussed in the presented publications (3.1 – 3.3) are isolates derived from the Feengrotten, a cave system that was built in medieval times in connection with the underground mining of alum slate.

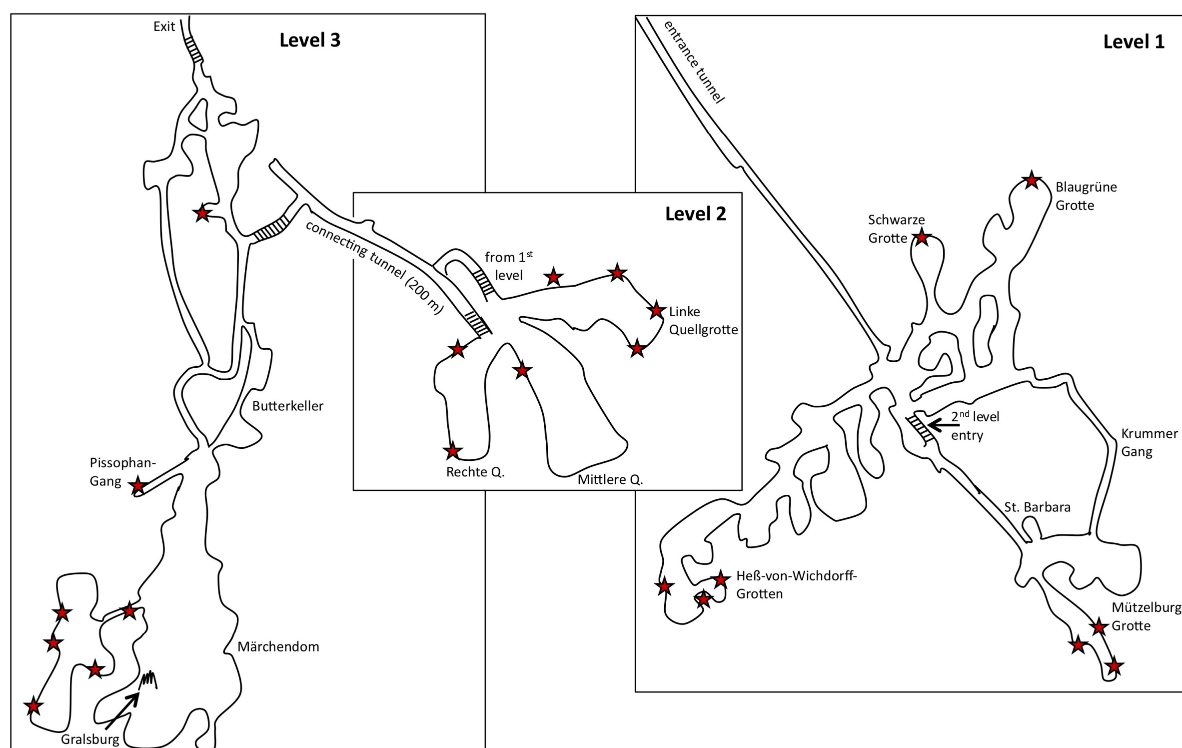


Figure 4. Map of the mine; drawing not true-to-scale. The 2nd level locates below the 1st one but was drawn separately for the sake of clarity. Red marked asterisks indicate main sampling sites.

The Feengrotten mine is situated south of the small town Saalfeld at the north rim of the Thuringian Slaty Mountains ("Thüringer Schiefergebirge"). Though the mining area

geologically belongs to the Saxonian-Thuringian Anticlinorium, the northeastern flank of the Schwarzburg-Anticlinorium directly adjoins to it. The bedrock rock arose in the Paleozoic era. It basically consists of different horizons of alum slates and lydite but layers of Silurian ochre colored limes ("Ockerkalk") and Ordovician Thuringian "Lederschiefer" crop out in minor parts, too.

The pyrite-rich blue to black colored slate was raised to extract alum or vitriol, which was used in medieval times, e.g., to tan leather, to stain cloth or to conserve timber. Over the years, three independent floors were drifted, the third floor (Märchendom) being the most ancient one. By following the local geographical conditions (Fig. 4), mining took place in two shale cavities: levels 1 and 2 were built in the southeast one and level 3 in the north one. Accordingly, the third floor is separated from levels 1 and 2 by a narrow gallery of more than 200 m length.

Over centuries of digging, miners built a complex system of stopes, working and transporting tunnels, ventilation and day drifts. Mined-out rooms partly were filled with waste rock. After closing it, the mine slowly sank into oblivion. In 1912, explorers that expanded into the mine were stunned by it's beauty. The walls and ceilings glowed in all tonalities of red and brown imaginable and bizarre formations of big dripstones (Fig. 5) arose under the dim light of the pit lamps. Due to its highly diversified secondary mineral features, the grottoes nowadays are considered to be the worlds most colorful cave system.

Figure 5. Big stalactides (left) and stalacmites (right picture) of up to two meters in length. Unlike to calcium carbonate precipitates which form speleothems in limestone caves, the Feengrotten formations mostly have been built from diadochite. Dripstones of diadochite can grow at a rate of up to 1 cm per year, which is at least ten times faster than calcite speleothem formation in limestone caves.

4.1.2. Geochemical and hydrological situation of the Feengrotten

The microclimate in the Feengrotten is relatively constant, exhibiting a temperature of about 10 degrees Celsius throughout the year and a relative humidity that is constantly near saturation. The rock surfaces therefore are always damp. Great parts of the walls and ceilings are covered with acidic dripping waters and/or encrusted with precipitates and layers of various secondary minerals.

The geochemistry of the Feengrotten is strongly influenced by the characteristic hydrological situation. During mining, the artesian alkaline groundwater that had protected the ground from leaching events was lowered to the working level. As a result, oxygenic, acidified surface waters could intrude into the pyrite-containing overburden. This initiated a process similar to that of acidic mine drainage (AMD) formation typical for open pit mines. The pyrite of the bedrock, when exposed to the oxygenic and acidified seepage waters, is oxidized to ferrous ions (Fe^{2+}) and sulfate ions (SO_4^{2-}). The emerging sulfuric acid further acidifies the environment causing more pyrite and other metal sulfides to be oxidized. Iron-oxidizing acidophilic bacteria like *Acidothiobacillus ferrooxidans* actively promote this (slow) spontaneous processes by (i) oxidizing ferrous iron to ferric iron (Fe^{3+}), which in turn functions as an oxidizing agent in the above-mentioned process, and by (ii) directly oxidizing pyrite and other metal sulfides to their soluble sulfate salts. The charged percolating waters transport the soluble metal salts to the internal rock surfaces, where they in parts precipitate, due to a significant pH increase facilitated by additional O_2 uptake from the surrounding air. Over the years, a great variety of speleothems and secondary mineral features developed at the Feengrotten walls and ceilings and within a few hundred years dripstones of up to 2 meters in length were formed.

The acidic dripping waters strongly determine the character of the microhabitats and their colonization of microorganisms. On the internal rock surfaces they mediate an acidic milieu of pH 1.5 to 4. The high acidity and the high amounts of dissolved heavy metals generally lead to an extreme toxicity to most organisms (Pentreath, 1994). Nevertheless, there are microbes thriving even in this type of environment. The phylogenetic diversity of prokaryotes dwelling in acidic, heavy metal-influenced habitats can reach unexpected dimensions as has been shown, e.g., for the extremely acidic environments of the Tinto River in Spain (González-Toril *et al.*, 2003; Mirete *et al.*, 2007). Furthermore, isolation of highly adapted organisms from anthropogenic metal rich habitats is not unusual. Publications on isolation of metal resistant bacteria from the sewage sludge of wastewater treatment plants and metal-processing industry are numerous. However, cultural studies of acidophilic,

metalliferous and/or hypogean environments that were specifically focused to actinomycetes are rare. Most of work in this field was done by Groth and coworkers, who used cultural approaches when investigating heterotrophic, Gram-positive bacteria of cave art deteriorating biofilms (e.g., Groth & Saiz-Jimenez, 1999; Groth *et al.*, 1999; Groth *et al.*, 2001). In a completely different context, Haferburg *et al.* focused on actinomycetes when investigating different AMD-influenced sites of a former uranium mine area and isolated surprising numbers of remarkably metal-resistant actinomycete strains (Haferburg *et al.*, 2007; Haferburg *et al.*, 2009). Interestingly, the geological situation of the Ronneburg district in which the investigations of Haferburg *et al*. were located, is well comparable to that found around the Feengrotten's cave system. Both sites are characterized by the presence of pyrite-containing shales, whose oxidative wheathering here and there is the causative agent for the formation of extreme acidic and heavy-metal-polluted environments.

4.1.3. Availability of organic nutrients

Being heterotrophic organisms, actinomycetes need organic nutrients for maintaining themselves and for their reproduction. For the public-accessible parts of the Feengrotten, it can be assumed that visitors, guides and scientific personal introduce organic matter sufficient to feed heterotrophic bacterial populations. However, one goal of the study was to monitor microbial communities found in regions of the mine that are not accessible to the public. The occurrence of dissolved organic matter in cave dripping waters (e.g., Saiz-Jimenez & Hermosin, 1999; Laiz *et al.*, 1999) shows that heterotrophic life is possible even in such remote regions. The organic matter thereby is most likely entrained from overlying soil. For example, the cave waters of Altamira (Spain) contain, amongst others, aliphatic organic acids, phenolic compounds and humic substances (Laiz *et al.*, 1999). However, heterotrophic growth in the caves might also be based on inorganic life. *A. ferrooxidans*, for example, is frequently associated with heterotrophic bacteria belonging to the genus *Acidiphilium*, which maintain themselves in large parts by utilizing organic material of its "prime-producing" partner, e.g. by using fragments of pili or flagella or other surface material, as well as excreted metabolites (Harrison, 1984). It can be assumed, therefore, that also the Feengrotten's remote regions possess suitable conditions for heterotrophic life. Given that actinomycetes seem to be widely distributed in similar environments (Groth *et al.*, 1999; Haferburg *et al.*, 2009; Portillo *et al.*, 2009), the Feengrotten were supposed to be a potential habitat of a variety of specially adapted actinomycetes and thus were expected to yield novel actinomycete strains of potential pharmacological interest.

4.2. Cultural studies

4.2.1. Strain isolation

In three independent sampling campaigns, a total of 32 samples was taken from different surfaces of the Feengrotten caves, including blank as well as decorated shale, active stalactites, sediments, and mine timber. Each sample was documented and assigned a 4-digit ID number which essentially is the serial number given to any sample that is deposited in the HKI soil sample collection. Isolates from a particular sample were tagged by composite IDs consisting of the number of the respective sample and a 3-digit tag starting with 001. Isolates that were sent to a recognized culture collection as e.g., DSMZ, NRRL or JCM were additionally given official HKI culture collection numbers.

Figure 6. Exemplary pictures of several sampling sites. The rock widely was decorated with ferrous secondary minerals. Thereon, on blank shale or even active stalactites, white to grayish biofilms were visible.

A map of the mine indicating the main sampling sites is given in Fig. 4. The sites generally were acidic, with a pH of 1.5 to 4. To a great extent, the surfaces were covered with white or grey patinas of presumptive microbial origin (Fig. 6).

Samples were taken by either touching the rock with a sterile cotton swab and subsequent dispersion of adherent bacteria into 1 ml sterile distilled water or scratching material with a sterile spoon from the wall concretions into 15 ml test tubes filled with 1 ml sterile distilled water; the resultant suspension was not pretreated any further. Aliquots were spread over different agar-solidified actinomycete isolation media that had been proven to be useful in earlier studies (Table 2).

4.2.2. Optimization of isolation parameters

While it is widely recognized that less than 1 % of the microorganisms in an environment can be readily cultivated in the laboratory using standard techniques (e.g. Amann *et al.*, 1995), it

has been repeatedly shown that when exhaustive plating conditions and long incubation times are employed, up to 7.5 % of the total microbial community is recovered (Davis *et al.*, 2005; e.g., Cavaletti *et al.*, 2006a; Janssen *et al.*, 2002).

To optimize isolation, critical parameters as, e.g., media composition, pH of the media, duration and temperature of incubation were evaluated based on four samples taken during the first sampling campaign. For this purpose, ten selective actinomycete isolation media known from the literature were tested, namely humic acid (HV) agar (Hayakawa & Nonomura, 1987), peptone (0.1 %) supplemented water agar, half-strength yeast extract-starch agar (Kudo *et al.*, 1993), artificial soil agar (KEHE) (Henssen & Schäfer, 1971), Gauze 1 mineral agar (Gauze *et al.*, 1983), actinomycete isolation agar (Difco), casein mineral agar (Altenburger *et al.*, 1996), starch casein mineral agar (Küster & Williams, 1964), maltextract (ME) agar (Reiss, 1972), and brain heart infusion agar (PY-BHI; Yokota *et al.*, 1993a) that (except for ME agar) were adjusted to pH 7.2 prior sterilization. Five media, namely casein mineral agar, starch casein mineral agar, half strength yeast extract-starch agar, peptone supplemented water agar and actinomycete isolation agar, additionally were set to pH 5.5 with sterile 0.1 M HCl solution after sterilization so that 15 media were tested in total. After inoculation (dilution series up to 10^{-3}), the plates were incubated for at least 6 weeks at three different incubation temperatures (15°C, 21°C and 28°C).

Although microcolonies of a number of slow-growing strains developed not until four to six weeks after inoculation, prolonged incubation times of up to ten weeks (at 21°C) did not yield any further colonies. Interestingly, acidification of the isolation media did neither significantly enhance absolute numbers nor diversity of the appearing microcolonies, as judged under the stereomicroscope. However, on pH 5.5-adjusted casein mineral agar plates inoculated with a cell suspension made of sample 2423, naked and wrinkled brownish colonies appeared that were not present on the corresponding pH 7.0-adjusted plates. In addition, ME agar, which has a pH of about 5.0, yielded interesting strains of a new phylogenetic lineage that later could be assigned to the novel and acidophilic genus *Catenulispora*. Hence, the use of pH-adjusted media may enhance the recovery of particularly acidophilic or neutrotolerant isolates. Incubation below 15°C resulted in delayed growth; an enhancing effect on colony diversity could not be observed. Therefore, for further isolations, inoculated plates were incubated at 28°C or room temperature (21°C), respectively.

Although differences in both, absolute numbers (colony forming units, cfu) of microcolonies and diversity of colonies, were easily visible (Figure 6), each of the used media supported the

growth of actinomycetes. Some taxa readily grew on a range of isolation media, e.g., strains of *Kribbella* (Carlsohn *et al.*, 2007a) which could be isolated from water agar, starch casein agar, and Gauze's mineral agar 1. Colonies of *Amycolatopsis* and *Pseudonocardia*, by contrast, developed most abundantly and in great numbers on HV agar or casein mineral agar, respectively, but only occasionally appeared on other media. Strains of *Catenulispora* have been isolated from plates containing ME agar and acidified casein mineral agar.

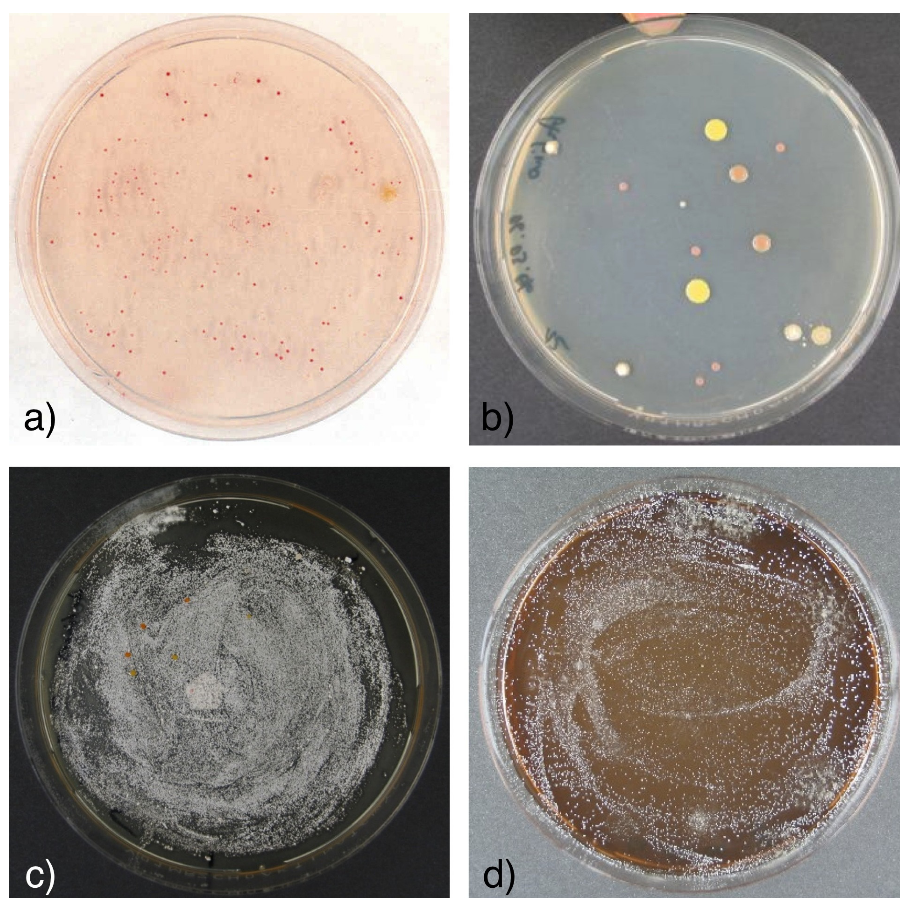


Figure 7. Representative isolation plates containing different isolation media after 4 weeks of incubation at 28°C using aliquots of 1:10 diluted sample 2445. Water agar, a); actinomycete isolation agar, b); half-strength yeast extract-starch agar, c); humic acid agar, d).

Therefore, i.e., to maximize numbers and diversity of isolated strains, a combination of 11 media was used in the following, including HV agar, peptone supplemented water agar, half-strength yeast extract-starch agar, KEHE agar, Gauze 1 agar, actinomycete isolation agar, casein mineral agar, starch casein mineral agar, ME agar, PY-BHI agar and pH 5.5-adjusted casein mineral agar.

4.2.3. Strain identification and classification

For morphological and cultural studies, isolated strains were cultivated on agar plates containing ISP media 2, 3, 4 and 5 (Difco; Shirling & Gottlieb, 1966), humic acid agar

(Hayakawa & Nonomura, 1987), Bennett's agar (Jones, 1949) and organic medium 79 for up to 28 days at 28 °C. Bacterial growth for chemotaxonomic and molecular systematic studies was prepared by cultivating the isolates at 28 °C for 1–7 days in liquid organic medium 79, ISP 2 or Bacto tryptic soy broth (Sigma-Aldrich). If needed, the pH of the solid and liquid media was lowered to values between 5.0 and 6.5.

Introduction of the 16S rRNA sequence based identification as a routine technique

The process of strain identification should advantageously rely on a few suitable key characteristics that should be easily determinable. It should be relatively inexpensive and give results rapidly (Krieg, 2001). The particular pattern of characteristics used for the identification of a bacterial group must not be found in any other bacterial taxon. With the advent of 16S rDNA sequencing, however, traditional identification schemes have been more and more replaced or modified.

At the beginning of this study, sequencing and interpreting of the 16S rRNA gene was managed by external partners and, due to the costs, had been done only for special purposes, not for routine identification of strains. Basically, actinomycete isolates were identified following a procedure established by Groth and coworkers (Fig. 8; Groth *et al.*, 1999).

Determination of the morphological characteristics of the isolate, detection and identification of the isomers of the diamino pimelic acid present in the cell wall's peptidoglycan and determination of the menaquinone pattern in many cases gave the information needed for a tentative affiliation on the genus level. However, these tests require significant amounts of biomass. Often, their production was virtually impossible, particularly from fastidious strains.

Therefore, one of the first aims of this study was to establish the 16S rRNA gene sequence based identification of actinomycetes as a routine technique. The chosen combination of methods (DNA extraction, amplification and purification of the 16S rRNA gene, sequencing of the PCR products) is detailed in the above manuscript 3.1 (Carlsohn *et al.*, 2007b). For reasons of rapidity, sequencing was done directly from the PCR product using the sequencing primers 27F, 519F, 907R and 1522R (Carlsohn *et al.* 2007b). Using the Chromas Pro software tool, which can display chromatograms and automatically assembles overlapping sequences into a consensus while showing ambiguities (http://www.technelysium.com.au/ChromasPro.html), the raw data was edited and assembled into the final, virtually complete consensus sequence. In the case of ambiguities, corresponding nucleotide positions were resequenced. The phylogenetic position of the final sequence then was estimated by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against publically available bacterial 16S rRNA gene sequences. For exhaustive phylogenetic inference, an alignment (usually using

the clustal w algorithm) was generated comprising high quality sequences of the type strains of related taxa. The alignment was then used as input file for different treeing algorithms and the resulting tree topologies were compared to each other.

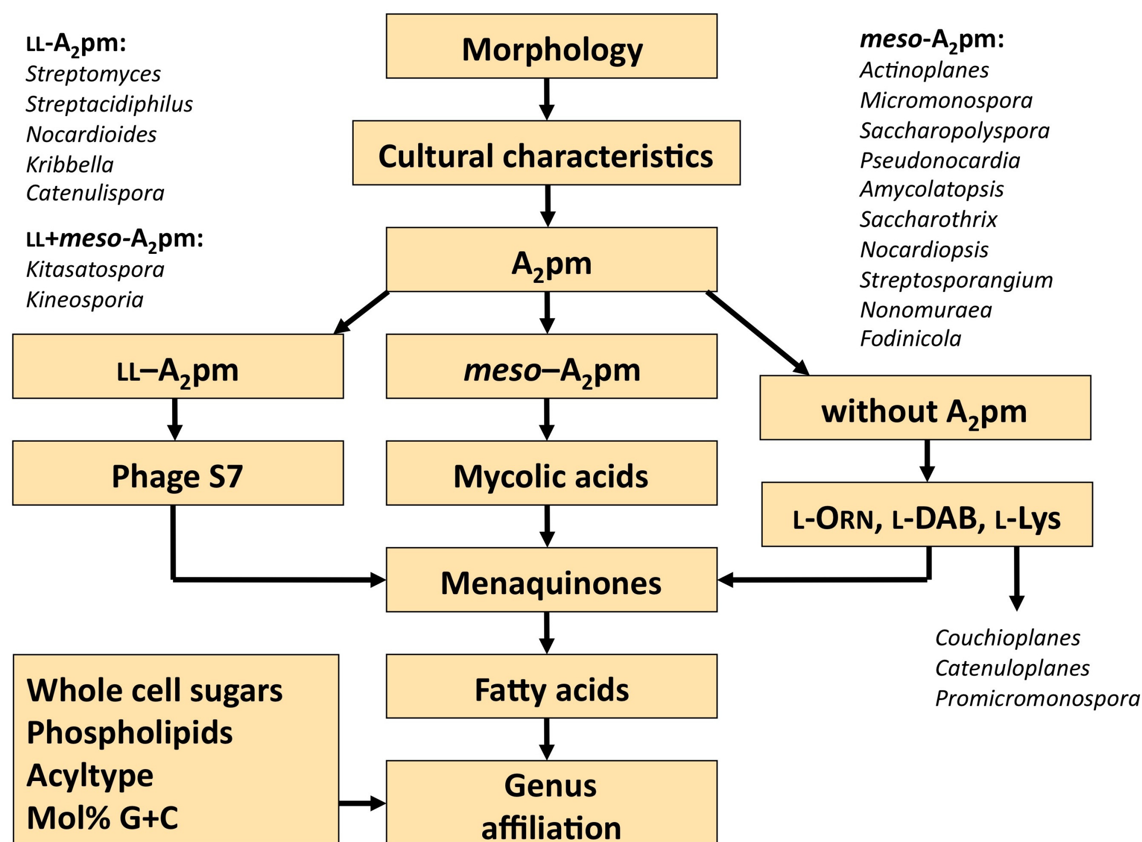


Figure 8. Actinomycete strain identification strategy as established at the group of Groth, HKI Jena (Groth *et al.*, 1999). A_2pm, 2,6-diaminopimelic acid; Orn, ornithine; DAB, 2,4-diaminobutyric acid; Lys, lysine.

As the thorough characterization of potential strains forms the basis of the successful implementation of the strains concerned into standardized screening programs, additional phenotypic information (as, e.g., relating to colony morphology, cell shape, chemical and physiological properties of the cell) was provided, if possible. Standard procedures were used for this purpose that are detailed or referenced in the above manuscripts. Distinctive features and/or discrepancies to the distribution patterns of known taxa served to distinguish the isolates from their described relatives.

Application of colony PCR for identification of fastidious colonies

To provide sequence information of particular fastidious colonies, a simple colony PCR protocol was applied. Using this approach, it was possible to analyze microscopic colonies without prior cultivation directly from the isolation plates.

Colony PCR is widely used to quickly screen for plasmid inserts directly from *E. coli* colonies. As *E. coli* cells are relatively easy to disrupt and standard cloning vectors normally are high copy number, template DNA is provided even at low disintegration efficiencies. The walls of Gram-positive cells, by contrast, are much more resistive. Chromosomal target sequences only exist in one single or a few copies in the cell. Perhaps these reasons have hindered the more widespread application of colony PCR protocols to actinomycetes yet.
However, Ishikawa and coworkers (2000) successfully amplified 16S rRNA gene sequences of several strains of the genus *Streptomyces* using a simple colony PCR approach, in which they added the cells directly to the PCR mixture. To minimize the influence of PCR-inhibiting cellular substances, the protocol of Ishikawa *et al*. was adapted in the present study in that picked cells were treated separately before adding them to the PCR mixture. Briefly, a microscopic amount of cell mass was picked with a sterile and DNA-free tip, the cells were suspended in 20 µl of sterile ultrapure water. The suspension then was heated to 98°C for 10 min. After that, cellular mass was centrifuged for 2 min at 16.000 x g. From the supernatant, 1 µl was taken for a PCR volume of 10 µl. The conditions of subsequent PCRs then were as described in manuscripts 3.1 to 3.3. The efficiency of the method was tested with material of several type strains of the genera *Amycolatopsis* and *Streptacidiphilus*, namely *A. alba*, *A. decaplanina*, *A. fastidiosa*, *A. mediterranei*, *A. orientalis*, *S. albus*, *S. carbonis*, and *S. syringae*.
Using this approach, PCR products of the 16S rRNA gene that were amenable to direct sequencing have been generated for any bacterial colony tested, including representatives of the genera *Streptomyces*, *Amycolatopsis* (see also page 70), *Pseudonocardia*, and *Catenuli-spora*.

Application of universally primed PCR for actinomycete strain identification

Universally primed (UP) PCR (Bulat *et al.*, 1991) is a genome-wide PCR-based finger-printing method similar to the well-known randomly amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990). It likewise is capable of amplifying DNA from any organism without previous knowledge of DNA sequences and generating multibanding profiles following gel electrophoresis (Bulat *et al.*, 1998).
Although alternative genotypic methods as e.g., nucleic acid fingerprinting (AFLP, ribotyping, RAPD, UP-PCR etc.) or taxon-specific PCR, are considered to be of limited value for species descriptions, they can be used to support identification and classification at the species or subspecies level (Tindall *et al.*, 2010).

Originally published in the same year as RAPD, UP-PCR has never gained its popularity. However, there are several advantages compared to RAPD. UP-PCR uses longer (16-20 nt) and semi-random primers with unique design that allow relatively high annealing temperatures and therewith ensure a high reproducibility. The resulting fingerprints consist of higher numbers of bands than most RAPDs, thus facilitating identification of specific markers while at the same time showing species-conservative bands (Bulat *et al.*, 1998). The primers are composed of a variable part of 8-10 nt at the 3' end and conserved "minisatellite-like sequences" of 6-10 nt at the 5' end that can be found in any genome (Guo *et al.*, 2003). The 5' end generates numerous PCR products with almost any kind of template DNA and at the same time stabilizes hybridization at high annealing temperatures (Bulat *et al.*, 1998). The random 3' end avoids amplification of phylogenetically conserved regions of the genome. UP-PCR markers have been used in studies on the genetic variation within fungi (Bulat *et al.*, 1998; Lübeck *et al.*, 2000), plants (Guo *et al.*, 2003) and bacteria (Bahrmand *et al.*, 1996). In fungi, UP-PCR had repeatedly been used for phylogenetic classification purposes (Lübeck *et al.*, 1999; Nielsen *et al.*, 2001; Zhao *et al.*, 2009).

Within the framework of the present thesis, the UP-PCR method was evaluated for its significance in actinomycete taxonomy, too. Although its suitability in the identification of tightly related actinomycetes was first published in connection with the description of the novel species *Amycolatopsis saalfeldensis* (see section 3.1; Carlsohn *et al.*, 2007b), the method was initially evaluated with nine similar isolates from the Feengrotten relating to the genus *Kribbella* and the type strains of *Kribbella antibiotica*, *K. jejuensis* and *K. koreensis* (Fig. 9).

Therefore, the isolates and type strains were grown at 28 °C for 48 h in liquid organic medium 79. Chromosomal DNA was extracted from the strains using a slightly modified version of the method of Pospiech & Neumann (1995). The set-up and protocol of the method was the result of empirical optimization of parameters. The protocol was described in detail in manuscript 3.1 (Carlsohn *et al.*, 2007). The primers 3-2, AS4, AS15, AA2M2, Fok 1 and L 21 described by Bulat *et al.* (2000) were compared to optimize the sensitivity and reliability of the resulting banding patterns. In absolute numbers and size distribution of bands, primers AS4, AS15 and AA2M2 worked best for the tested kribbellae strains.

Using AS4 and AS15 in combination, a resolution of up to 55 evenly distributed bands per reaction was achieved. As the combination of AS4 and AS15 likewise generated banding patterns of similar resolution for strains of the genera *Amycolatopsis* and *Catenulispora*, they both may be an adequate initial choice when investigating actinomycete strains.

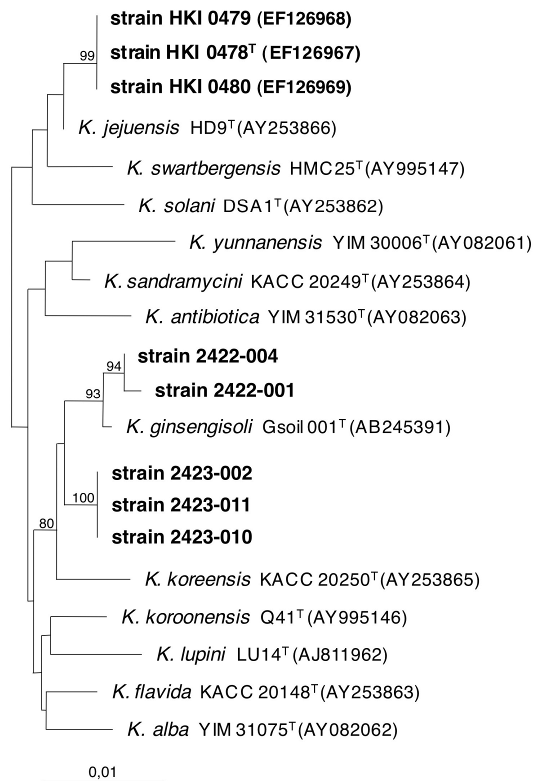


Figure 9. Additive phylogenetic tree (De Soete, 1983), based on 16S rRNA gene sequence data, showing relationships between the 9 isolates assigned to the genus *Kribbella* and between them and representatives of the genus. Numbers shown at branching points indicate bootstrap percentages; only values greater than 70 % are shown. Bar, 1 substitution per 100 nt.

However, one has to bear in mind that UP-PCR parameters generally need to be individually optimized for the particular biological system under investigation. The quality of the chemicals used, their precise mixture, the used devices and machines and the thermal PCR profile are further factors that can influence the reaction and thus the banding as well.

The fingerprints were analysed using the GelComparII software tool (Applied Maths, version 4.5) and the corrected banding patterns were phylogenetically clustered (Fig. 10).

The clustering obtained from the banding patterns was in accordance with the grouping that had been done before based on based on morphologic and chemotaxonomic data as well as by 16S rRNA gene sequence based phylogenies (Fig. 9). The UP-PCR method therefore was concluded to be of value also in the identification of tightly related actinomycete strains at and below the species level. It was used in manuscripts 3.1 and 3.2 (Carlsohn *et al.*, 2007a; Carlsohn *et al.*, 2007b) to further support the distinctiveness of the therein described isolates from their closest phylogenetic neighbors. The technology, however, is equally well suited to quickly dereplicate ambigous strains at or below the species level in order to facilitate efficient screening and thereby minimize costs and time of sorting large collections of identical isolates.

In further experiments aimed to measure the performance of different available machines, it was found that the reproducibility of independent UP-PCRs could be drastically improved

when a fast-ramping, temperature-stable PCR machine (Speedcycler, Analytik Jena) in combination with ultrathin-walled low-profile PCR plates was used (Fig. 11).

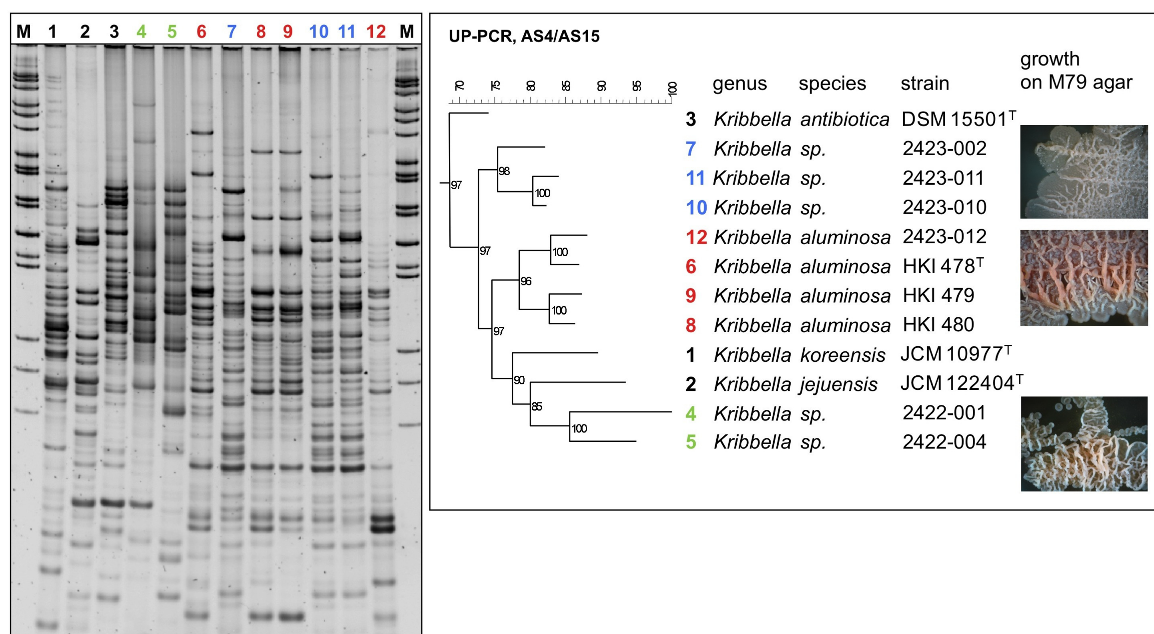


Figure 10. The left image shows UP-PCR fingerprints of nine isolates related to the genus Kribbella and three type strains of described Kribbella species. The image was analysed using GelComparII software by i) detecting, ii) correcting and iii) matching the individual bands of each fingerprint. A phylogenetic clustering of the banding patterns was achieved using the maximum parsimony algorithm (Fitch, 1971). Bootstrap rates (Felsenstein, 1985) at the nodes are based on 1.000 resamplings.

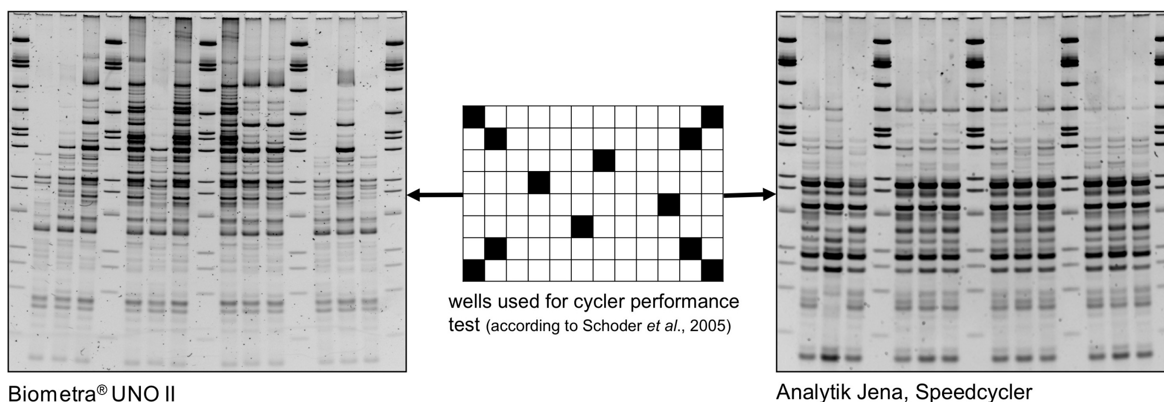


Figure 11. Performance test of two 96-well block cyclers using the 12 block positions as indicated in the central scheme according to Schoder *et al*. (2005) showing the fast-ramping, high precision Speedcycler to produce much more uniform banding patterns than the classical UNO II cycler.
One single UP-PCR reaction (400 µl) was prepared (200 ng DNA of *Kribbella aluminosa* HKI 0478^T, 25 U Go*Taq* polymerase (Promega), 0.2 mM of each of the four dNTPs, 3 mM $MgCl_2$, 1 µM primer AS4, 2 µM AS15) that was split afterwards in equal parts of 15 µl each into the respective wells of both PCR plates. The fastest available ramping rate and calculated temperature control mode was chosen for each cycler, and the following PCR profile was programmed: one initial step of 120 s at 94 °C, 20 s at 57 °C and 30 s at 72 °C, followed by 30 cycles of 92 °C for 15 s, 57 °C for 20 s and 72 °C for 30 s and a final extension of 120 s at 72 °C. Size standard: pGEM DNA marker (Promega).

The Speedcycler allowed fast heating and cooling rates of 12 °C/s and 8 °C/s, respectively and, in combination with the particular low-profile plates intended to enhance the thermal efficiency of the system, ensured a highly accurate thermal profile. This is of great practical relevance especially in genotypic techniques as RAPD and UP-PCR, since the in-well thermal accuracy of the annealing step is considered one crucial element for producing reproducible banding patterns (Penner *et al.*, 1993).

The cycler performance tests further showed that a good reproducibility could be achieved also in independent runs on the same machine. However, as different machines of the same model could not be tested it is not clear to what extent an inter-laboratory reproducibility would be achievable using the same equipment under the same conditions.

4.3. Isolates

A culture collection encompassing 86 actinomycete strains was established based on 32 samples (Table 3). The diversity of the isolates was restricted to a limited number of genera. Out of the 86 isolates, 51 strains (61 %) affiliated to non-streptomycetes. This value is far above average to that obtained in conventional isolation campaigns where usually less than 10 % are non-streptomycetes (Baltz, 2006).

Table 3. Feengrotten strain collection.
ND, not determined; ?, uncertain

Genus	Suborder	Geno-types	Isolates	Novel taxa	Occurrence (on isolation plates)
Streptomyces ssp.	*Streptomycineae*	ND	33	ND	rather rare
Catenulispora ssp.	*Catenulisporineae*	2	22	?	frequent
Pseudonocardia ssp.	*Pseudonocardineae*	3	15	3	frequent
Kribbella ssp.	*Propionibacterineae*	3	9	2	rare
Amycolatopsis sp.	*Pseudonocardineae*	1	3	1	frequent
Fodinicola sp.	*Frankineae*	2	2	2	rare
Related to AB062380 'Clavisporangium sp.'	*Streptosporangineae*	1	1	1	rare

As also such classical media recommended for the isolation of streptomycetes like starch casein mineral agar, yeast extract starch agar (table 2) yielded only low numbers of streptomycete strains, the lack of streptomycete isolates most likely is a result of the extreme conditions found in the caves that might rather favor rare and slow-growing actinomycetes. In addition, most *Streptomyces* strains were isolated from a single sample taken from a big,

sintered, into the mine expanding, tap root of an old oak tree. The walls, however, seemed to be almost completely naked from *Streptomyces* strains.

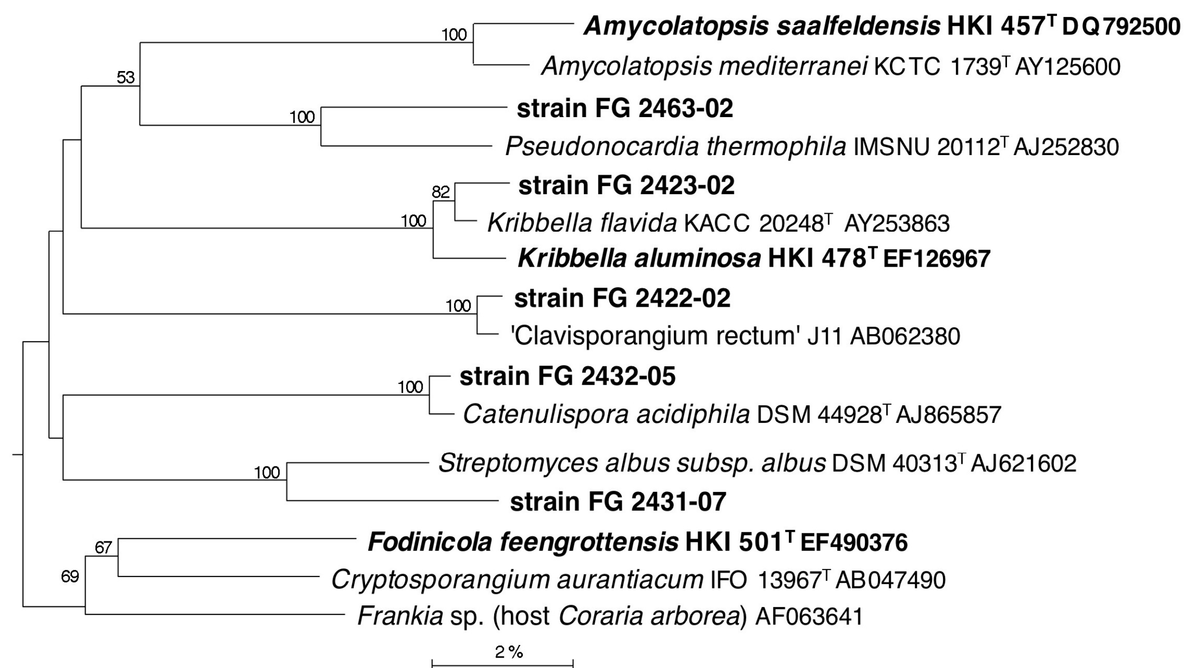


Figure 12. Phylogenetic dendrogram, based on 16S rRNA gene sequences and constructed from evolutionary distances (De Soete, 1983) showing the positions of representative strains within the radiation of the order *Actinomycetales*. Numbers at the nodes refer to bootstrap percentages (based on 1.000 resamplings); only values above 50% are shown. Members of the *Firmicutes* were used to root the tree. Bar, 2 inferred nucleotide substitutions per 100 nucleotides.

Despite the reduced diversity, most suborders from which mycelial growing representatives are known were covered (Fig. 12), ranging from the *Pseudonocardineae* (genera *Amycolatopsis*, *Pseudonocardia*), *Streptomycineae* (*Streptomyces*), *Catenulisporineae* (*Catenulispora*), *Frankineae* (*Fodinicola*), *Streptosporangineae* (‘*Clavisporangium’*) to *Propionibacterineae* (*Kribbella*). Interestingly, from all identified genera potent producer strains are known, with exception of the herein newly described genus *Fodinicola* Carlsohn *et al*. 2008.

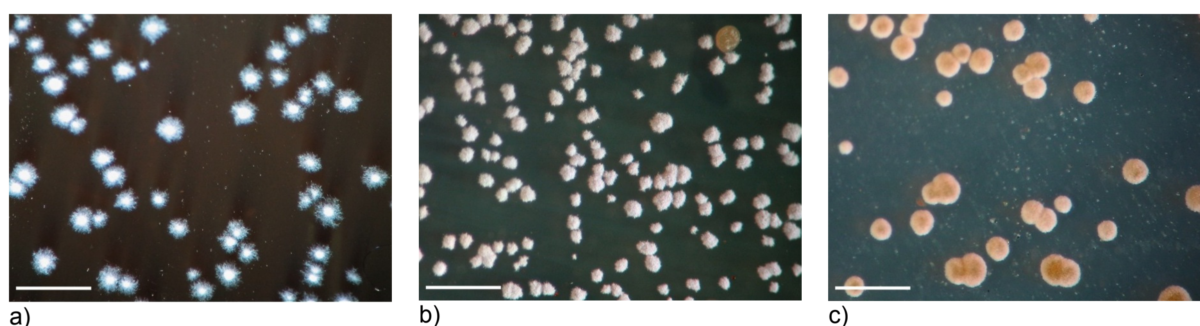


Figure 13. Sections of different isolation plates. Plates often were dominated by one or few colony-types. Microcolonies of a) *Amycolatopsis* sp. on HV agar, b) *Catenulispora* sp. on casein mineral agar (pH 5.5), and c) *Pseudonocardia* sp. on casein mineral agar. Bar, 2 mm.

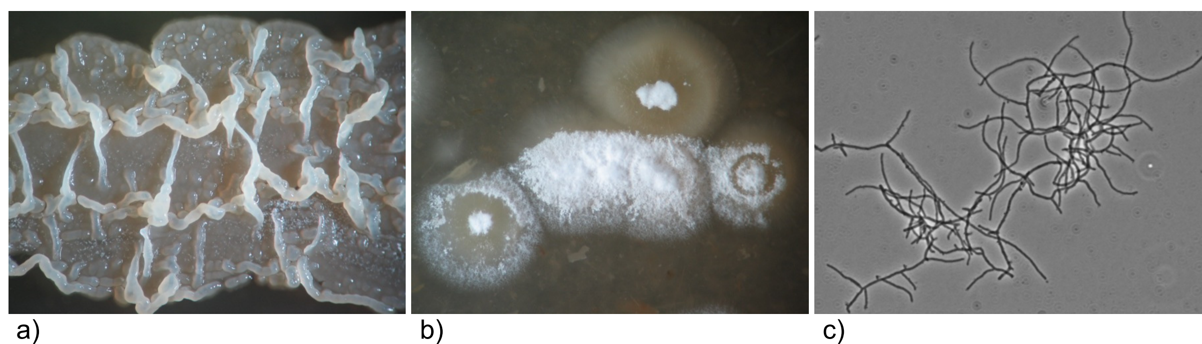
The isolation plates were often dominated by only one or few (2 to 4) colony types (Figs. 7 and 13). This confirms previous reports that stated reduced actinomycete diversity for similar habitats (Portillo *et al.*, 2009; Schabereiter-Gurtner *et al.*, 2002a; Zhou *et al.*, 2007).

4.3.1. Genus *Amycolatopsis*

HV agar plates frequently were covered with large numbers of tiny white colonies that somewhat resembled snowflakes or cotton balls, as they almost exclusively seemed to consist of aerial hyphae (Figure 12a). Material of 24 of these colonies from different samples representing all three mine-levels was picked and tested by 16S rDNA-specific colony PCR using slightly advanced primers of that described by Tan *et al*. (2006), showing the following sequences: Amyc 874f CGT TGT CCG TGC CGT AGC and Amyc 1344r AGC GAC TCC GAC TTC ACG CA (numbers indicating hybridization sites according the *E. coli* numbering system of Brosius *et al*., 1978). The amplicons were eluted from the agarose gel, and directly sequenced. The resulting sequences in all cases could be verified as being identical to those sequences belonging to strains of *A. saalfeldensis* Carlsohn *et al*. 2007. This suggests the taxon to be widespread in the Feengrotten and to represent an important constituent of the Feengrotten's microbial life. However, besides their massive occurrence on different isolation plates, only three isolates (HKI 0457^{T}, HKI 0473, HKI 0474; Carlsohn *et al*. 2007) have been maintained in culture; a fact that underpins the fastidious growth requirements of strains of *Amycolatopsis*. This has repeatedly led to the assumption that the genus yet is grossly underspeciated (Groth *et al.*, 2007; Tan *et al.*, 2006b). Once adapted, the three isolates grew well on a range of media, including organic medium 79, ISP media 2 and 3, HV agar and Bennett's saccharose agar. They preferred a slightly acidic milieu, with the pH optimum laying between pH 5 and 7 (determined on Bennett's saccharose agar). Interestingly, growth at 10°C, which is the mine's mean temperature, was weak and delayed but growth was abundant between 20 and 35°C.

Amycolatopsis saalfeldensis sp. nov.

Within the framework of this thesis, a polyphasic study was designed to determine the taxonomic position of the three isolates. This study, as discussed in manuscript 3.1 (Carlsohn *et al.*, 2007b), led to the assignment of the isolates to the genus *Amycolatopsis*. It was concluded that the strains represented a novel *Amycolatopsis* species for which the name *Amycolatopsis saalfeldensis* sp. nov. was proposed (Carlsohn *et al.*, 2007b).



a) b) c)

Figure 14. Macroscopic (a,b) and microscopic (c) appearance of strain HKI 0457^T when grown on organic medium 79 agar, a); on ISP 3 agar, b), and in liquid organic medium 79, c).

Within the scope of this study, the isolates were examined for a range of key chemical markers that clearly revealed a chemotaxonomic profile typical of members of the genus *Amycolatopsis*. All three isolates contained *meso*-A_2pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV sensu; Lechevalier & Lechevalier, 1970) together with glucose, mannose and rhamnose. The muramic acid was N-acetylated, mycolic acids were lacking. With 86–88% of the total, tetrahydrogenated menaquinones with nine isoprene units [MK-9(H_4)] was the major isoprenologue, followed by minor proportions of MK-8(H_4) (4–5%), MK-9(H_6) (1–3%) and MK-10(H_4) (2%). Distribution of polar lipids was according phospholipid pattern 2 *sensu* (Lechevalier *et al.*, 1977). Almost-complete 16S rRNA gene sequences, which were generated from the three strains, showed high sequence similarities (98.4-94.3%) to representatives of the genus *Amycolatopsis* that further supported their addition to the genus. Their sequences were most closely related to that of *A. rifamycinica* DSM 46095^T; with the type strain HKI 0457^T (Fig. 14) sharing a 16S rRNA gene sequence similarity of 98.4% to it. However, as it is known that type strains of *Amycolatopsis* species classified in the *A. mediterranei* subclade share much higher 16S rRNA gene sequence similarities than that cited above but have DNA-DNA relatedness values well below the 70% cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987), DNA-DNA relatedness studies were not carried out. Instead, the newly introduced UP-PCR technique (manuscripts 3.1 and 3.2) and the MALDI TOF MS approach (e.g., Lay, 2001; Russell, 2009) was applied. Both, UP-PCR (see Fig. 2 in Carlsohn *et al.*, 2007b) and MALDI TOF MS analysis (supplementary Fig. S1; Carlsohn *et al.*, 2007b), showed similar profiles for the isolates that distinguished them from those of phylogenetically close taxa. A range of further differentiating physiologic and chemotaxonomic features likewise supported the distinctness of the isolates to representatives of their phylogenetically close neighbors (table 1; Carlsohn *et al.*, 2007b). Thus, the genotypic and phenotypic data clearly showed the isolates to form a homogenous taxon that can be distinguished readily from representatives of

phylogenetically close *Amycolatopsis* species. It was, therefore, proposed that the isolates be classified in the genus *Amycolatopsis* as *Amycolatopsis saalfeldensis* sp. nov.

PFGE analysis of the isolates

Using pulsed-field gel electrophoresis (PFGE), the isolates were screened for the presence of bioactive extrachromosomal plasmids. Gel plugs for PFGE were prepared by modifying the method described by Schenk *et al*. (1998). Briefly, the cells were grown for 24 h at 28°C in 20 ml ISP 2 medium supplemented with 0.5 % glycine. Cells were harvested (5000 x g, 20 min., 4°C) and washed twice in 4 ml SucTE buffer (25 mM Tris, 25 mM EDTA, 0,3 M saccharose, pH 8.0). The resuspended cells were diluted to an optical density (OD_{600}) of about 2.0. 400 µl of the cell suspension was mixed with 800 µl of 1 % low-melting agarose. Then the mixture was poured into ice-cooled molds and allowed to solidify. The plugs were pushed out of the molds and incubated for 1 h at 37°C in SucTE supplemented with 2 mg lysozyme / ml. After removing the lysozyme solution, plugs were incubated for 16 to 24 h at 37°C with gentle agitation in ESP solution (0,5 M EDTA, 1 % laurolylsarcosinate, 0,1 M Tris, pH 9.5) supplemented with 1 mg proteinase K / ml. After repeated washing in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), plugs were dialyzed overnight in 0.5 % TBE buffer and then stuffed into the slots of a 0.8 % agarose gel made from SEAKEM gold agarose, with the slots sealed with low-melting agarose afterwards. Separation of plasmids from genomic DNA was achieved using a *Biorad*® PFGE apparatus filled with 0.5 % TBE buffer using appropriate current and pulse times.

In strain HKI 0457^T, a giant extrachromosomal plasmid of about 300 kb was found (Fig. 15a); strains HKI 0473 and 0474 were lacking this plasmid (data not shown). Additional incubation with S1 nuclease, which can be used to linearize circular supercoiled plasmids (Barton *et al.*, 1995), failed to change the mobility of the DNA molecule in the PFGE gel, even when run at different pulse times (Fig. 15b). This behavior is indicative of linear plasmids.

Similar plasmids occurring in actinomycetes are reported to encode antibiotic biosynthetic genes (e.g., Kinashi *et al.*, 1987; Suwa *et al.*, 2000), heavy metal resistance (e.g., Ravel *et al.*, 1998), or catabolic traits (e.g., Overhage *et al.*, 2005). In *Amycolatopsis* ssp., large linear plasmids have not yet been reported.

To show, whether the plasmid contained secondary metabolite genes, the plasmid DNA was probed with primers encoding the KS domain (ca. 700 bp) from modular polyketide synthases (PKS-I; Courtois *et al.*, 2003), the KSα domain (600 bp) from PKS-II genes (Metsä-Ketelä *et al.*, 1999) and the adenylation (A) domain (450 bp) from non-ribosomal peptide

synthetases (NRPSs; Busti *et al.*, 2006).

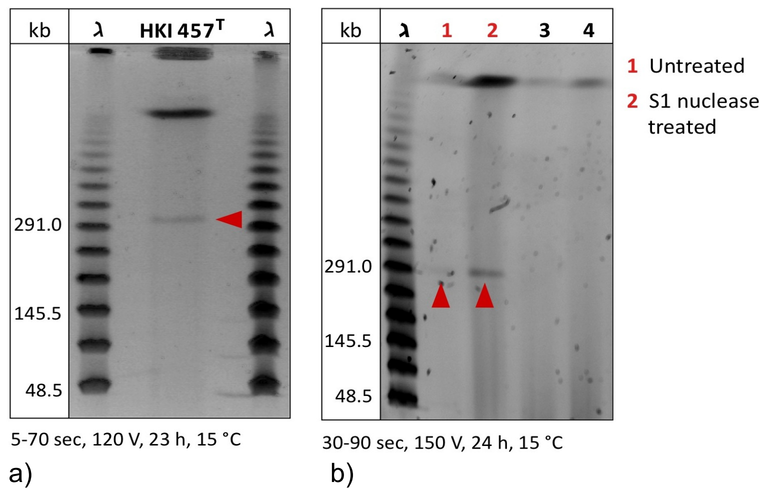


Figure 15. PFGEs of strain HKI 457^{T} native DNA following different conditions that found an extrachromosomal plasmid of about 300 kb, a). Incubation with S1 nuclease did not change the mobility of the palsmid band in relation to the untreated sample, b).

Therefore, the plasmid DNA was electro-eluted from the gel as follows: The PFGE gel was stained with SYBR green I, and the plasmid DNA was exited from the gel using the DarkReader transilluminator to avoid DNA damage typically observed from UV light. Then, the gel slice was stuffed into a cathode-oriented well of a gel made from low-melting agarose (0.5 %) that exhibited two rows of wells, a first near the cathodic end and a second directly below. The well was sealed with low-melting agarose and the corresponding well directly below was filled with $Aqua_{\text{bidest.}}$. The gel was placed into a non-fluorescent electrophoresis chamber; the chamber was placed onto the DarkReader. Without flooding the gel, the chamber was filled with TBE buffer. Subsequently, the gel was run in the dark, but using the DarkReader to control progress, until the plasmid DNA was tapped in the second capturing well. After the current was reversed for several seconds, the DNA solution was pipetted from the well, quantified and, without further purification, used for PCR. The plasmid DNA yielded a distinct band of the expected size (450 bp) with the NRPS-specific primer set (data not shown). The fragment was exited, and cloned. The inserts of seven clones were sequenced and aligned. The alignment showed the fragments to be split into two distinct sequence types (Fig. 16); both, however, being related to adenylation domains.

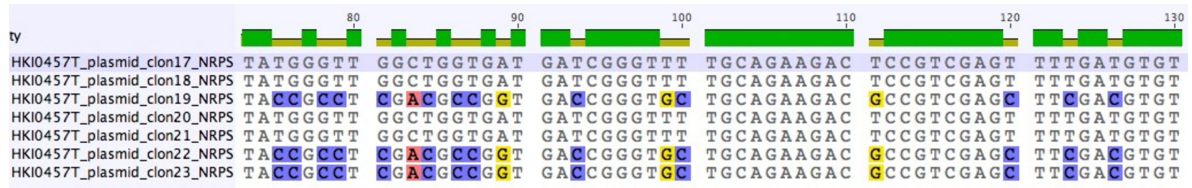
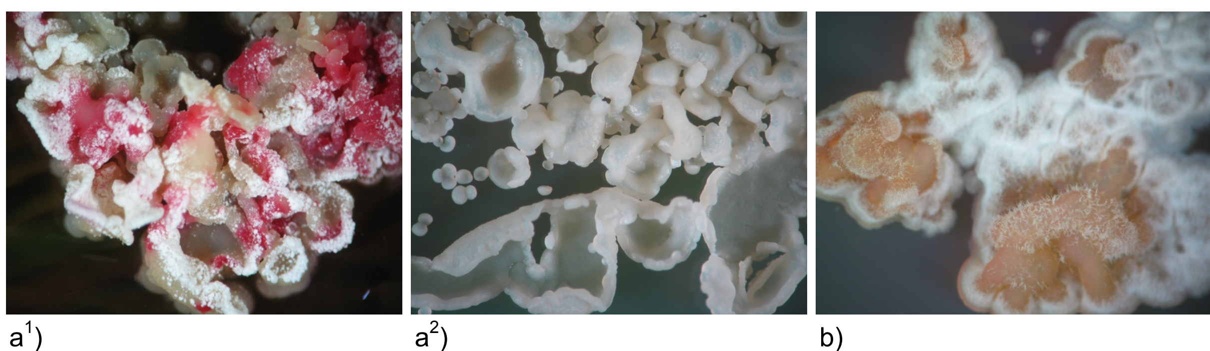


Figure 16. The seven A domain sequence fragments obtained from HKI0457^T plasmid DNA split into two sequence types that showed a pairwise identity of 81.7 %. Alignment, clustal W.

As NRPS genes present a modular organization, with a repetition of similar gene segments within a single gene cluster, it is not clear whether the sequences belong to the same NRPS cluster. The preliminary findings, however, clearly indicate that at least one NRPS gene cluster may be encoded by the large linear plasmid of strain HKI0457^T.

4.3.2. Genus *Catenulispora*

Twenty-two strains were isolated from acidified starch casein mineral agar plates (Fig. 13b) and malt agar plates. The strains grew well on ISP agar 2 (Shirling & Gottlieb, 1966) adjusted to pH 5.0 – 5.5 and contained LL-A2pm in whole organism hydrolysates. No growth was observed at pH 6.5 and above. The isolates diverged into two clearly distinguishable consensus groups that showed differences in colony morphology, pigmentation and in the production of aerial hyphae (Figure 17). On acidified (pH 5.5) ISP 2, strains of group 1 (cat 1) formed white colonies, that, when matured, produced a strong red pigment and only sparsely developed aerial mycelia whereas strains of group 2 (cat 2) formed compact orange-colored colonies which lacked the red pigment and instead developed well-visible amounts of aerial mycelia.



a^1) a^2) b)

Figure 17. Macroscopic appearance of strains 2432-005 (cat 1) and 2444-006 (cat 2). Strain 2432-005 on malt-extract agar, a^1) and ISP 2 agar (pH 5.5), a^2). Strain 2444-006 on ISP 2 agar (pH 5.5), b). Cultures grown for 28 days at 28°C.

Almost-complete 16S rRNA gene sequences (>1432 bp; from strains 2432-005 (cat 1), and 2444-006 and 2444-008 (cat 2)) allowed their classification into the genus *Catenulispora* Busti *et al.* 2006. Interestingly, representatives of the genus generally seem to favor acidic

environments, since strains of all for species known yet where isolated from acidic soil samples. Another point that makes *Catenulispora* strains attractive to natural products research is that they simultaneously encode type I and II PKS genes as well as NRPS (non-ribosomal peptide synthetase) genes (Busti *et al.*, 2006). Thus, they could possess several gene clusters for secondary metabolite production, a feature that, based on the present state of knowledge, is limited to the antibiotic producing *Actinomycetales*. Since secondary metabolite production is the result of the concerted interaction of several genes or gene combinations, it is reasonable to expect *Catenulispora* strains, which are phylogenetically unrelated to strains highly screened for antibiotics, to contain an increased number of novel genes and gene combinations dedicated to the production of novel antibiotics and other biologically active substances (Busti *et al.*, 2006).

However, the present 22 isolates neither have been subjected to an initial screening nor are fully characterized yet. Whereas the cat 2 strains, 2444-006 and 2444-008, had identical sequences, the sequences between cat 1 and cat 2 differed in six nucleotide positions (99,6% similarity), i.e. their morphologic distinctiveness (Fig. 17) is not reflected on the level of their 16S rRNA gene sequences.

While the 16S rRNA gene sequence of strain 2432-005 (cat 1) was most closely related to that of *C. acidophila* DSM 44928^T (AJ865857; 99,7% similarity; Busti *et al.*, 2006), the cat 2 sequences were most closely related to *C. rubra* DSM 44948^T (AB180773; 99.5% similarity; Tamura *et al.*, 2007)). As the genus *Catenulispora* is reported to exhibit remarkably low 16S rRNA gene sequence variability (Busti *et al.*, 2006), the above high similarity values, although they are far above the recommended cut-off point of 97 % (Stackebrandt & Goebel, 1994), cannot identify the isolates to be strains of already described species, when taken alone. Instead, further taxonomic studies will be necessary for a proper species affiliation of the 22 isolates.

4.3.3. Genus *Fodinicola*

Fodinicola feengrottensis gen. nov., spec. nov.

A filamentous, Gram-positive actinobacterium was isolated from acidic rocks in a medieval alum slate mine and was investigated by means of a polyphasic taxonomic approach. A 16S rRNA gene sequence similarity study indicated that strain HKI 0501^T forms an individual line of descent and is related to certain members of the suborder *Frankineae*, order *Actinomycetales* (<95 % sequence similarity).

Since publication of manuscript 3.3 (Carlsohn *et al.*, 2008), the classification of the higher taxa within the suborder *Frankineae* has changed significantly. So, the genera *Kineococcus*,

Kineosporia and *Quadrisphaera* have been removed from the suborder, a new family, *Cryptosporangiaceae*, has been proposed and all taxa above the rank of genus have received emended descriptions (Zhi *et al.*, 2009). Currently, the suborder *Frankineae* Stackebrandt *et al*. 1997 emend. Zhi *et al*. 2009 accommodates 11 genera (including *Fodinicola* Carlsohn *et al*. 2008) classified within six families: *Acidothermaceae* Rainey *et al*. 1997[3] emend. Zhi *et al*. 2009 (genus *Acidothermus* Mohagheghi *et al*. 1986); *Cryptosporangiaceae* Zhi *et al*. 2009 (genus *Cryptosporangium* Tamura *et al*. 1998); *Frankiaceae* Becking 1970 emend. Hahn *et al*. 1989 emend. Normand *et al*. 1996 emend. Stackebrandt *et al*. 1997 emend. Zhi *et al*. 2009 (genus *Frankia* Brunchorst 1886); *Geodermatophilaceae* Normand *et al*. 1996 emend. Stackebrandt *et al*. 1997 emend. Zhi *et al*. 2009 (genera *Geodermatophilus* Luedemann 1968, *Blastococcus* Ahrens & Moll 1970 and *Modestobacter* Mevs *et al*. 2000); *Nakamurellaceae* Tao *et al*. 2004 [substitute for the illegitimate name *Microsphaeraceae* Rainey *et al*. 1997 [3]] [genera *Nakamurella* Tao *et al*. 2004 (substitute for the illegitimate name *Microsphaera* Yoshimi *et al*. 1996), *Humicoccus* Yoon *et al*. 2007 and Saxeibacter Lee *et al*. 2008] and *Sporichthyaceae* Rainey *et al*. 1997 [3] emend. Zhi *et al*. 2009 (genus *Sporichthya* Lechevalier *et al*. 1968). The genera *Kineococcus* Yokota *et al*. 1993 and *Kineosporia* Pagani & Parenti 1978, which had been excluded from the taxonomic system of *Actinobacteria*, together with *Quadrisphaera* Maszenan *et al*. 2005, were assigned within a novel suborder and family *Kineosporiinae* Zhi *et al*. 2009 and *Kineosporiaceae* Zhi *et al*. 2009, apart from the suborder *Frankineae*.

The members of the suborder *Frankineae* are morphologically and biochemically hetero-geneous: the genera can be readily distinguished from one another by using a combination of chemotaxonomic and morphological properties (Carlsohn *et al.*, 2008). Most of the strains are characterized by low growth rates and fastidious growth requirements.

Strain HKI 501^T (Carlsohn *et al.*, 2008) was isolated from the Barbara Grotto using a starch-casein agar plate that had been incubated for about 4 weeks. Fig. 18 shows the macroscopic appearance of strain HKI 0501^T on several growth media. The cultural characteristics of strain HKI 0501^T as well as its physiological and chemotaxonomical properties have been detailed in manuscript 3.3 (Carlsohn *et al.*, 2008). As measured from 16S rRNA gene sequence similarity studies, strain HKI 0501^T showed a remote relationship (<95% similarity) to members of the suborder *Frankineae*. Although similar values were shared with respect to type strains of both genera, *Cryptosporangium* (family *Cryptosporangiaceae*) and *Sporichthya* (family *Sporichthyaceae*) (92.9–94.8 and 93.9–94.5 %, respectively), the applied

[3] in Stackebrandt *et al*. (1997)

treeing algorithms (neighbor-joining and maximum likelihood) clearly indicated an individual line of descent for strain HKI 0501^T. Members of the genera *Frankia* (family *Frankiaceae*) and *Acidothermus* (family *Acidothermaceae*) have been slightly less closely related to isolate HKI 0501^T (92.5–93.0 %). It is worth to note here that the phylogenetic incoherencies that prompted Zhi *et al.* (2009) to remove the genera *Kineococcus*, *Kineosporia* and *Quadrisphaera* from the suborder *Frankineae* had already been seen and discussed in manuscript 3.3 (Carlsohn *et al.*, 2008).

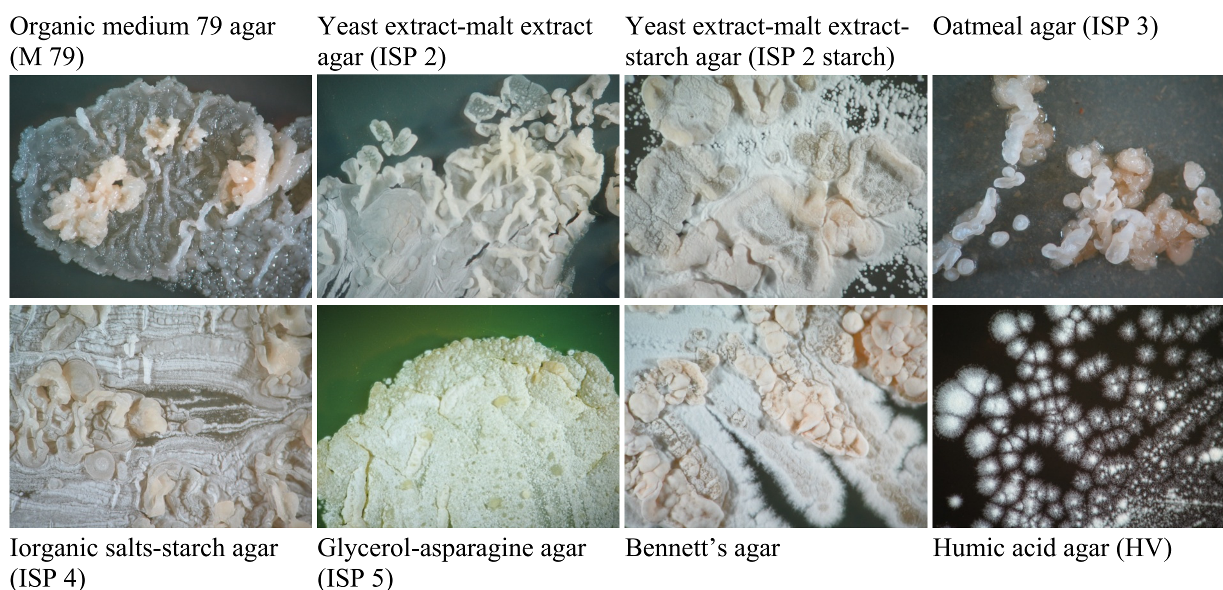


Figure 18. Macroscopic appearance of strain HKI 0501^T on different solid media. Cultures grown for 21 days at 28°C.

Strain HKI 0501^T possessed most of the 16S rRNA gene sequence signatures defined for the suborder *Frankineae* (Stackebrandt *et al*., 1997), the exception being a U residue instead of a C residue at position 222. Strain HKI 0501^T shared more signatures with members of the genera *Frankia* (40–60 %) and *Cryptosporangium* (50-60 %) than with members of the genera *Kineosporia*, *Kineococcus* and *Quadrisphaera* (35–40 %). In view of its phylogenetically deep branching point within the family *Cryptosporangiaceae* (Fig 1; Carlsohn *et al*., 2008), HKI 0501^T could be considered as representing a novel genus. Moreover, the isolate was readily distinguished from its closest phylogenetic neighbors and from all other representatives of the suborder *Frankineae* on the basis of a range of chemotaxonomic and morphologic traits (Table 1 and genus description; Carlsohn *et al.*, 2008). Therefore, a novel genus and species, *Fodinicola feengrottensis* gen. nov., spec. nov. was proposed to accommodate strain HKI 501^T.

4.3.4. Genus *Kribbella*

Eight filamentous growing strains have been isolated from a gallery within in the third level of the mine near the Märchendom grotto not accessible to the public. As similar organisms were not detected in other regions of the mine, neither by cultural work nor culture-independent investigations based on genus-specific amplification of the 16S rRNA gene, representatives of the genus seemed to be restricted to this insulated area.

The chemotaxonomic characteristics of the isolates were consistent with an affiliation with the genus *Kribbella* Park *et al*. 1999 emend. Sohn *et al*. 2003, since the novel strains shared chemotaxonomic key characteristics with type strains of 11 *Kribbella* species with validly published names.

Whole-organism hydrolysates of the isolates contained LL-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan. The fatty acids consisted mainly of anteiso- (anteiso–$C_{15:0}$, 30–45 %) and iso- branched (iso–$C_{16:0}$, 5–18 %) components. The predominant menaquinone was MK-9(H4) (84–93 %). The phospholipids were composed of phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and unknown phospho- and glycolipids, corresponding to phospholipid pattern III *sensu*, Lechevalier *et al*. (1977). The combination of these markers served to separate the novel strains from members of the genera other than *Kribbella* within the family *Nocardioidaceae* (Park *et al*., 1999; Sohn *et al*., 2003; Carlsohn *et al*., 2007a).

Based on differentiating physiological data (Table 4) that has been obtained using standard procedures (Carlsohn *et al.*, 2007a), the isolates were divided into three consensus groups, K1 (HKI 478^T, HKI 479, HKI 480; Carlsohn et al., 2007a), K2 (2422-001, 2422-002) and K3 (2423-002, 2423-010, 2423-011). The grouping was also in accordance with morphologic, genotypic and phylogenetic analyses. On all tested media (ISP media 2, 3, 4, 5 and organic medium 79 agar), the novel isolates showed lichenous growth. On ISP 2 and organic medium 79 agar, colonies of K1 and K2 were clearly more wrinkled than that of K3 (Fig. 19). Whereas colonies of the K3 strains were cream to pale yellow in color on organic medium 79, strains of K1 and K2 developed light salmon-colored colonies. Comparative UP-PCR fingerprinting analyses (Fig. 10) likewise supported the provisional classification of the novel strains. Phylogenetic analyses based on 16S rRNA gene sequences of the novel isolates and the type strains of 12 *Kribbella* species with validly published names finally revealed the same classification (Fig. 9). As a result, the physiological and molecular systematic data

indicated that the consensus groups K1, K2 and K3 created from the novel strains might constitute three novel species.

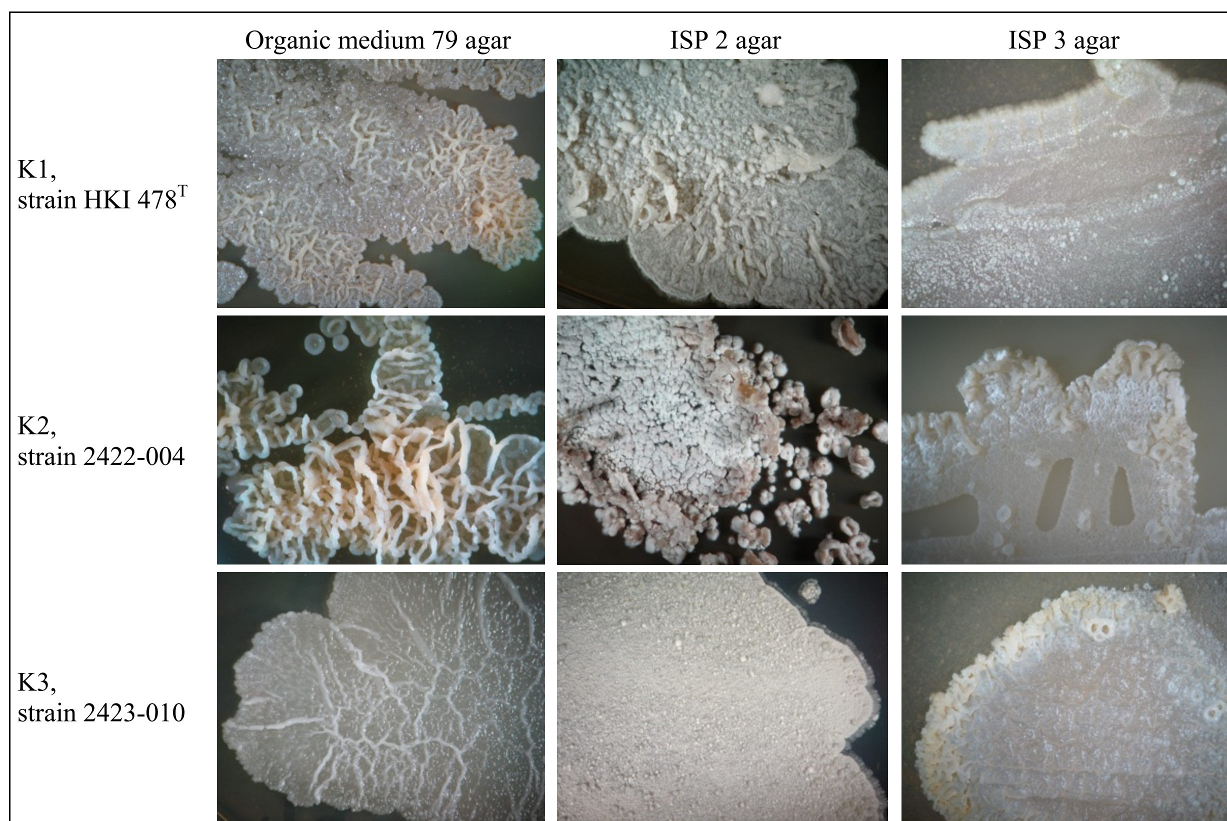


Figure 19. Macroscopic appearance of strains HKI 0478^{T} (K1), 2422-004 (K2) and 2423-010 (K3) on different solid media (organic medium 79, ISP 2 and ISP 3). Cultures grown for 21 days at 28°C.

However, the 16S rRNA gene sequences of the novel strains showed between 98.8 % and 99.9 % similarity to the corresponding genes of their closest relatives. These values are far above the recommended cut-off point of 97 % similarity that is held appropriate for the designation of a novel species based on 16S rRNA gene phylogenies (Stackebrandt & Goebel, 1994). Above this value, the currently applied genomo-species concept (Wayne *et al.*, 1987) at any rate requires DNA-DNA relatedness studies (Stackebrandt & Goebel, 1994). For the K1 strains, such studies were externally done by the DSMZ. The resultant data provided the basis for the description of the strains of this group as a novel species within the genus *Kribbella*, *Kribbella aluminosa* (Carlsohn *et al.*, 2007a).

Kribbella aluminosa spec. nov.

Strains of the consensus group K1, HKI 0478^{T}, HKI 0479 and HKI 0480, were shown to form a tight group next to the type strains of *K. jejuensis*, *K. swartbergensis* and *K. solani* in 16S rRNA gene sequence based phylogenies (Fig. 9). They could be separated from their nearest phylogenetic neighbors by a range of chemotaxonomic, physiologic, morphologic and

Table 4. Physiological characteristics that served to distinguish between the isolates and between them and type strains of their closest phylogenetic neighbors.

Strains: K1, *K. aluminosa* HKI 0478[T], HKI 0479, HKI 0480 (Carlsohn *et al.*, 2007a); K2, strains 2422-001 and 2422-004; K3, strains 2423-002, 2423-010 and 2423-011; 4, *K. jejuensis* JCM 12204[T] (Song *et al.*, 2004); 5, *K. solani* DSM 17294[T] (Song *et al.*, 2004); 6, *K. swartbergensis* DSM 17345[T] (Kirby *et al.*, 2006); 7, *K. koreensis* JCM 10977[T] (Lee *et al.*, 2000); 8, *K. ginsengisoli* DSM 17941[T] (Cui *et al.*, 2010). +, Positive; w, weakly positive; –, negative; d, delayed; ND, no data available. K1-3, data from this study, see also section 3.2: Carlsohn *et al.* (2007a).

Characteristic	K1	K2	K3	4	5	6	7	8
Oxidase	–	–	–	+	+	–	+	+
Nitrate reduction	–	–	–	–	–	+	–	–
Decomposition of:								
Casein	+	+	–	–	–	+	+	+
Potato starch	+	+	+	–/+*	–	+*	+	w
Tween 80	+	+	+	–	+	+	–	ND
Adenine	+	+	–	–	–	+	–	ND
Hypoxanthine	+	+	–	–†	–†	+	+	ND
Tyrosine	+	+	–	–	–	+	+	–
Xanthine	+ d	+	–	–	–	–	+	ND
Urea	+	+	–	+*	–	–*	+	–
Growth on sole carbon sources (1 %, w/v)								
myo-Inositol	+	+	+	–	–	w	+	+
D-Mannitol	+	+	+	–	–	w	+	+
Enzyme activity (API ZYM):								
Cystine arylamidase	–	–	–	w*	w*	+*	+*	ND
a-Fucosidase	+	–	–	+*	–*	–*	w*	ND
Lipase (C14)	–	–	–	–*	+*	+*	w*	ND
Trypsin	–	+	–	+*	w*	+*	w*	ND
Growth at/in:								
6 °C	–	w d	w d	–	–	–	w d	–
10 °C	w d	+	+	–	–	w d	+	+
32 °C	+	+	–	+	+	+	+	+
37 °C	+	+	–	+	–	+	+	+
42 °C	–	–	–	+*	–	+	–	–
45 °C	–	–	–	–	–	+	–	–
2.0 % NaCl	+	+	–	–*†	+*	+	+	+
4.0 % NaCl	–	–	–	–*	+*	w	–	+
pH 4.5	+ d	+ d	–	–	–	–	–	–
pH 5	+	+	–	–*	–*	w d*	–	–
pH 8	+ d	+	–	+*	+*	+*	+*	+
pH 9	–	–	–	–*	–*	+*	+*	–
Antibiotic suceptibility (µg per disc)								
Ampicillin (10)	+	–	+	–*	–*	–*	–*	ND
Ciprofloxacin (5)	w	w	+	–	–	w	+	ND
Novobiocin (5)	w	+	+	+	+	+	+	ND
Imipenem (10)	+	+	+	–*	w*	–*	+*	ND
Polar lipids#	DPG, PC, PG, PI	DPG, PC, PG, PI	DPG, PC, PG, PI	DPG, PC, PI	DPG, PC, PI	ND	DPG, PC, PG, PI	DPG, PC, PG, PI

* Data for reference strains obtained in this study. † Conflicting results reported by Kirby *et al*. (2006).

DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

Grey-shaded colums, characteristics that differentiate the novel isolates.

genotypic characteristics as well as DNA-DNA relatedness data (see section 3.2; Carlsohn *et al.*, 2007a). The three isolates therefore were suggested to constitute a novel species, for which the name *Kribbella aluminosa* spec. nov. was proposed.

4.4. Initial biological and chemical screenings of selected strains

Selected strains, including strains of the genera *Amycolatopsis*, *Fodinicola*, *Kribbella*, *Pseudonocardia* and *Streptomyces*, have been preliminary tested for their antimicrobial activity and, in a chemical screening, for their production of secondary metabolites (Table 5). After 7 days of shaking flask cultures at 28°C, supernatant and biomass were separated by centrifugation and subsequently extracted. The extracts were used in a chemical and a biological screening.

When strains are cultured solely in the usual fermentation media, compounds derived from sleeping gene clusters might be overlooked. It has been shown that strains, when grown in minimal medium, produce metabolites that do not appear in the extracts of rich-media grown cells (Haferburg *et al.*, 2009). Similarly, tracing media with heavy metals has been found to induce secondary metabolite production, too (Haferburg *et al.*, 2009). Therefore, a minimal medium (MM; Amoroso *et al.*, 2000) was used for growing the strains in addition to the standard media, namely soy-mannite medium (M01, 20 g l^{-1} soy meal and 20 g l^{-1} mannite, pH 7.2), oatmeal medium (M02, 20 g l^{-1} oat meal and 2,5 ml l^{-1} trace element solution (3.0 g l^{-1} $CaCl_2$ $2H_2O$, 1.0 g l^{-1} Fe(III)-Citrat, 0.2 g l^{-1} $MnSO_4$, 0.1 g l^{-1} $ZnCl_2$, 0.025 g l^{-1} $CuSO_4$ $5H_2O$, 0.02 g l^{-1} $Na_2B_4O_2$ $10H_2O$, 0.004 g l^{-1} $CoCl_2$, 0.01 g l^{-1} $NaMoO_4$ $2H_2O$), pH 7.2), glucose medium (M10, 4 g l^{-1} glucose, 10 g l^{-1} malt extract, 4 g l^{-1} yeast extract, pH 7.0) and soja E2 medium (M27, 20 g l^{-1} glucose, 20 g l^{-1} soy meal, 5 g l^{-1} NaCl, 3 g l^{-1} $CaCO_3$, pH about 5.5, without adjustment). Furthermore, for induction of stress response, heavy metals were added to both, minimal medium and soy mannite medium to give a final concentration of 0.3 mM $NiCl_2$ $6H_2O$.

Bioassay

Extracts of supernatant and mycelia were tested for antibacterial and antifungal effects in an agar diffusion assay. *Staphylococcus aureus*/MRSA + chinolon-r 134/93, *Escherichia coli* SG 458, *Mycobacterium smegmatis* SG 987 and *Candida albicans* BMSY 212 were used as test organisms. Fifty microlitre of the single extracts were filled in 9 mm agar wells. The antibiotic effect was evaluated by measuring the zones of inhibition.

Table 5. Biological and chemical screening of extracts of supernatant (S) and mycelial fraction (M). Only strains that have been striking in biological and/or chemical screenings are listed.

S. aur. 134/93, *Staphylococcus aureus*/MRSA + chinolon-r 134/93; *E. coli* 458, *Escherichia coli* SG 458; *M. smeg*. 987, *Mycobacterium smegmatis* SG 987; *C. alb*. 212, *Candida albicans* BMSY 212; M01, soy mannite medium; M02, oatmeal medium; M10, glucose medium; M27, soy 2E medium; MM, minimal medium, M01 (MM) + Ni, M01 (MM) spiked with 0.3 mM $NiCl_2$ $6H_2O$.

		Biological screening								Chemical screening [TLC]	
Strain	Medium	*S. aur.* 134/93		*E. coli* 458		*M. smeg.* 987		*C. alb.* 212			
		S	M	S	M	S	M	S	M	S	M
2431-002 *Streptomyces*	M01	3	3			3	3			2	1
	M01 + Ni	3	3			3	3	1	1	2	2
	MM + Ni		1					2	2		3
2431-006 *Streptomyces*	M01	3	3			3	3	2	1		1
	M02	3	3			2	2	3			1
	MM	2	3					3	3	3	
2431-007 *Streptomyces*	M01	3	2			2	3			3	2
	M01 + Ni	3	3			3	3				2
	MM	3	3					3	3		
	M27	3				1		3		1	
2431-009 *Streptomyces*	M01	3	3	2		2	3			1	3
	M01 + Ni	3	3			3	3			2	3
	M27	3	3	2		2	3			1	
2422-002 rel. to AB062380	M01	3	3			3	2	3		3	3
	M10	3			3		2			3	2
	MM	3	3			3				2	
2422-001 *Kribbella*	M01	1				1	3			1	
	M01 + Ni	1				2	3				1
	MM			3		2	2	2	3	1	
	MM + Ni					3	3	3	3		
2422-004 *Kribbella*	M01		1			2	3			2	2
	M01 + Ni	1	3			2	3	1	2	2	2
2423-002 *Kribbella*	M01	1				2	3			3	2
	M02					3	3			2	1
HKI 0473 *Amyc. saalf.*	M01	3	3				2			3	3
	M02	3	3				2			1	
	M10	3	3							3	1
	M27	3	3					3		3	3
	MM		3								1

Ranks assigned for the biological screening: blank, no inhibition; 1, moderate inhibition; 2, inhibition; 3, strong inhibition; ranks assigned for the chemical screening: blank, non-relevant; 1, less relevant; 2, relevant; 3 particular relevant.

Some of the crude extracts showed a high biological activity towards several medically relevant test organisms. Both, mycelial fraction and supernatant of the strains of *A. saalfeldensis* displayed a potent antibiosis towards *S. aureus* over a broad range of media. The mycelial fraction of *A. saalfeldensis* strain HKI 0473 furthermore showed a strong inhibition of *C. albicans* after growth in soy E2 medium M27. Several strains of *Kribbella* were highly active against *M. smegmatis*. Strain 2422-004, when grown in minimal medium, was additionally effective against *C. albicans*, the mycelial fraction of it displayed a potent antibiosis against Gram-negative *E. coli*. Most interestingly, the filamentous-growing strain 2422-002, which is phylogenetically unrelated to any described taxa, showed strong activities in either supernatant or mycelial fraction or both of cultures grown in soy-mannite medium against all 4 test organisms, *S. aureus*, *E. coli*, *M. smegmatis*, and *C. albicans*. In addition, several strains of the genus *Streptomyces*, namely strains 2431-002, 2431-006, 2431-007 and 2431-009, emerged to be particularly effective against *S. aureus* and *M. smegmatis*. Moreover, strains 2431-006 and 2431-007 were highly active against *C. albicans.*

Chemical screening by thin-layer chromatography (TLC)

Extracts of supernatant and mycelia were analyzed by thin-layer chromatography. Two different running systems were applied; butanol:glacial acetic acid:water = 4:1:5 and chloroform:methanol = 9:1. For detection of chemical compounds of different substance groups, thin layer chromatograms were developed using UV light at wavelengths 254 and 366 nm as well as staining with anis aldehyde, Ehrlichs reagent and orcinol.

Depending on the number of extractable bands and their appearance (color, shape, distinctiveness), the chromatograms were ranked into 4 categories (table 5), ranging from non-relevant to particular relevant.

Strains of *A. saalfeldensis* displayed large numbers of metabolite bands, among them a prominent blue-colered one. The metabolite pattern of strain 2422-002 was similarly diversified and ranged far above average. Most kribbellae strains, by comparison, showed significantly fewer bands. However, individual bands worth to analyze have been detected. So, the supernatant of strain 2423-002, when grown in soy mannite medium, displayed an interesting greenish-colored band visible in UV light.

Strains that displayed an exceptional antibiotic activity and additionally showed unique banding patterns in the TLC screening should be further processed. In particular, this applies for strains HKI 457^T, HKI 473, HKI 474, 2422-001, 2422-004, 2423-002, 2431-002, 2431-006, 2431-007, 2431-009 and 2422-002 (Table 5). Detected bands should be analyzed by

HPLC-MS to confirm its assumed novelty. The strains of interest should be fermented to high densities to isolate pure compounds for structural elucidation.

5 Conclusions

Leaving the beaten track increases the chances in the hunt for novel bioactive compounds and leads. This is true not only for the screening part, but is valid also in the search for novel actinomycete strains.
It is widely acknowledged that the most promising source of new drugs remain natural products (Bull *et al.*, 2000). Actinomycetes, which still account for more than 60 % of the known antibiotics (Haferburg *et al.*, 2009), are amongst the most prolific organisms and therefore remain in the focus in the search for novel antibiotics. Currently, the traditional perception of actinomycetes as merely bound to soil substrates is being radically changed as actinomycetes are among the most successful colonizers of all kinds of extreme environments (Goodfellow & Fiedler, 2010). There is much evidence today that secondary metabolites, amongst them antibiotics, have important ecological functions (Jensen, 2010). Highly adapted organisms coming from extreme environments frequently show unusual metabolic activities that likely are connected with their adaptation capabilities. Therefore, several authors (Baltz, 2008; Donadio *et al.*, 2007; Goodfellow & Fiedler, 2010; Williams *et al.*, 1993) have repeatedly recommended focusing search efforts to extreme or neclected environments.

It can be concluded from the present study that extreme acidic and heavy metal burdened environments generally are of value in providing novel actinomycete strains of potential pharmacological relevance.
Although the actinomycete strain diversity isolated from the Feengrotten was restricted to a limited number of genera, more than 60 % belonged to non-streptomycetes, a value far above average to that obtained in conventional isolation campaigns (Baltz, 2006). Surprising numbers of the isolates could be affiliated to rare taxa as for example to the genera *Amycolatopsis*, *Catenulispora*, *Kribbella*, or *Pseudonocardia*. Some strains lacked close phylogenetic relatives and thus can be seen to represent novel phylognetic lineages. Of these, strain HKI 501^T, based on molecular systematic and phenotypic data, was shown to represent a novel genus and species, *Fodinicola feengrottensis* gen. nov., sp. nov. within the suborder *Frankineae* (manuscript 3.3; Carlsohn *et al.*, 2008).
However, the application of additional selective isolation methods can be expected to yield additional taxonomic diversity from the Feengrotten and/or similar environments. So, cultural procedures that have been specifically adapted to individual taxa such as for the genera

Amycolatopsis (Tan *et al.*, 2006) or *Catenulispora* (Busti *et al.*, 2006) might be applied. This is true all the more considering the fact that members of both genera have been frequently detected in the Feengrotten by culture-independent methods, but were isolated only in few cases. In addition, novel strains of *Amycolatopsis* and *Catenulispora* are desirable also because members of both genera either have been proven (e.g., McCormick *et al.*, 1956; Hashizume *et al.*, 2010; Sensi *et al.*, 1959; Wink *et al.*, 2003) or are expected (Busti *et al.*, 2006) to represent a promising source of clinically relevant antibiotics. Other approaches that might be used to further gain the diversity of cultured *Actinobacteria* may include in situ procedures (e.g., Ben-Dov *et al.*, 2009; Gavrish *et al.*, 2008) or the use of the different cave dripping waters in media formulations to mimic environmental conditions to a higher extent.

The present study provides further indication that actinomycetes derived from acidic heavy metal polluted hypogean environments might be an important source of new secondary metabolites. Although both, bioassay and chemical screening, do no more than scratch the surface of the isolated strain's secondary metabolite production abilities, they nevertheless indicated several potent strains worth to be further analyzed, in particular strains HKI 457^{T}, HKI 473, HKI 474 which were shown herein to represent a novel species of the genus *Amycolatopsis*, *Amycolatopsis saalfeldensis*. Strains 2422-001, 2422-004, 2423-002 relating to the genus *Kribbella* and strains 2431-002, 2431-006, 2431-007, 2431-009 affiliating to *Streptomyces* were of equal interest. Strain 2422-002 that, based on 16S rRNA gene sequence data, lacks any close described relatives, has been shown of particular interest not only concerning its strong metabolic activity but also regarding its proper classification. However, additional phenotypic data has to be collected for this purpose.

Confirming the presumptive novelty of hits found in the screenings is one of the next logical steps that have to be taken. This can be done for example by HPLC-MS analysis of detected bands, followed by scale-up fermentation of strains, and isolation and structural elucidation of pure compounds. The use of modern screening methods as e.g., reversed-phase HPLC coupled with diode array monitoring (Huber & Fiedler, 1991) might significantly enhance the sensitivity and reliability of the chemical screening part as the application of this technique to freshly isolated strains has resulted in the detection of a high number of identified novel compounds (Goodfellow & Fiedler, 2010) compared to a relatively low throughput of strains. The use of additional stress-inducing media and media that mimic conditions of the natural habitat, as recently exemplified by Hertweck and coworkers (Lincke *et al.*, 2010), may lead to the production of further metabolites whose biosynthesis genes remain dormant under

standard laboratory conditions. Current sequencing technologies will drastically gain availability of whole genome sequences, enabling extensive bioinformatics analyses of genomes for the presence of biosynthetic genes (genome mining). Promising candidates for sequencing and genome mining would be strain 2422-002, *A. saalfeldenis* strain HKI 457^{T} and strains 2432-005 and 2444-006, designated representatives of the two detected consensus groups affiliated to the genus *Catenulispora*.

6 Summary

New antibiotics are urgently needed, since antibiotic-resistant pathogens have evolved to a serious risk to human health. Their high replication rates, short generation times and ability to transferring genetic information foster the rapid spreading of resistance mechanisms. Unfortunately, recent strategies in the discovery of antibiotics, such as combinatorial chemistry and fragment-based drug design have been proven to be relatively unproductive. Thankfully it is widely acknowledged yet again that in the hunt for novel antibiotics, natural products remain the leading edge. Amongst prokaryotes, actinomycetes, notably strepto-mycetes, are the most prolific source of new natural products. Diminishing returns from conventional screening methods are likely caused by the excessive re-screen of common actinomycetes which leads to the costly rediscovery of known compounds. It is, therefore, essential in drug discovery programmes to leave beaten tracks. As it has become increasingly clear, that actinomycetes are among the most successful colonizers of all kinds of extreme environments and that strains coming from these habitats frequently show unusual metabolic activities, it has repeatedly been recommended focusing search efforts to extreme environments. Therefore, within the framework of this study, the medieval alum slate mine and today's cave system Feengrotten was investigated for its actinobacterial life. In terms of acidity and heavy metal pollution, the Feengrotten caves were characterized by particular extreme environmental conditions. Mechanisms similar to that observed during the formation of acidic mine drainage caused pH values as low as 1.5 at some sites, which led to high concentrations of various soluble heavy metals. As bioactive actinobacteria have been previously isolated from similar environments, the Feengrotten were expected to be a potential source of novel actinomycete isolates. During the study, a total of 86 dereplicated strains have been isolated, with 51 strains (61 %) affiliating to non-streptomycetes. This value is far above average to that obtained in conventional isolation campaigns and most likely resulted from the acidic and metal polluted conditions found in the caves, which favored rare actinomycete taxa above streptomycetes. Apart from *Streptomyces*, the isolates have been classified into the genera *Amycolatopsis*, *Catenulispora*, *Fodinicola*, *Kitasatospora, Kribbella* and *Pseudonocardia*. Classification further led to the description of two novel species, *Amycolatopsis saalfeldensis* and *Kribbella aluminosa* and to one novel genus and species, *Fodinicola feengrottensis*. Formal descriptions have been published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) and are essential parts of the present work. It was further shown that *Amycolatopsis saalfeldensis* strain HKI

0457^T contains a large linear plasmid of about 300 kB which likely dedicates some of its coding capacity to secondary metabolite genes. In a biological and chemical screening, a number of strains have been active against various test organisms and/or produce compounds worth to be further analyzed.

7 Zusammenfassung

Multiresistente Keime haben sich in den letzten Jahren zu einer ernsthaften Bedrohung für den Menschen entwickelt. Kurze Generationszeiten und die Möglichkeit des Austauschs genetischer Informationen auch zwischen entfernt verwandten Stämmen begünstigen die Ausbreitung von Resistenzmechanismen. Neue und gegen moderne Problemkeime hochwirksame Antibiotika werden deshalb dringend benötigt. Die heute bei der Suche nach neuartigen Strukturen angewandten Technologien wie die Kombinatorische Chemie oder das sogenannte Fragment-basierte Wirkstoffdesign (fragment-based drug design) haben sich leider als eher unproduktiv erwiesen. Erfreulicherweise sind in der jüngsten Zeit aus der Natur isolierte Stoffe wieder verstärkt in den Mittelpunkt der Bemühungen um neue Antibiotika gerückt. Actinomyceten, und hier vor allem die Vertreter der Gattung *Streptomyces*, rangieren nach wie vor über allen anderen prokaryotischen Organismen als lukrativste Quelle neuartiger bioaktiver Naturstoffe. Allerdings sind auch hier die Erfolgsmeldungen selten geworden. Der wichtigste Grund hierfür ist, dass in konventionellen Screening-Programmen immer wieder dieselben häufigen und leicht zu isolierenden Organismen untersucht werden mit der Folge, dass mit hohem Kostenaufwand bereits bekannte Strukturen wiederentdeckt werden. Es ist deshalb wichtig, in der Suche nach neuen Wirkstoffen die vielfach begangenen Pfade zu verlassen. Eine der oft vorgeschlagenen Möglichkeiten ist die gezielte Suche nach neuen Produzentenstämmen an bisher wenig oder nicht untersuchten Habitaten, was beispielsweise auf die meisten Extremhabitate zutrifft. Im Rahmen der vorliegenden Arbeit wurde ein früheres Alaunschieferbergwerk, die Feengrotten nahe Saalfeld in Thüringen, hinsichtlich der dort heimischen Actinomyceten untersucht. Das heutige Höhlensystem besticht durch die äußerst vielfältige Mineralisierung der Höhlenwände. Diese wird durch Prozesse hervorgerufen, die dem Phänomen des acidic mine drainage (AMD) offener Erztagebaue ähneln und die für vorzufindenden extreme sauren Bedingungen und hohe Konzentrationen an verschiedenen löslichen Schwermetallen sorgen.

Im Rahmen der vorliegenden Studie wurden 86 zur Ordnung *Actinomycetales* gehörende Stämme isoliert, von denen 35 zur Gattung *Streptomyces* gehörten. Mehr als 60 % der Isolate sind somit anderen Gattungen zuzurechnen. Dieser Wert ist viel höher als bei konventionellen Isolierungskampagnen, wo in der Regel weniger als 10 % der isolierten Stämme in andere Gattungen als *Streptomyces* klassifiziert werden. Die gewonnenen Isolate wurden den Gattungen *Amycolatopsis*, *Catenulispora*, *Fodinicola*, *Kitasatospora, Kribbella* und *Pseudonocardia* zugeordnet. Vertreter der Gattungen *Amycolatopsis*, *Catenulispora* und

Pseudonocardia gelten als weltweit verbreitet und divers. Außerdem sind Vertreter dieser Gattungen Lieferanten hochwirksamer Antibiotika. Die Charakterisierung und Identifizierung der Feengrotten-Isolate führte vorliegend zur formalen Beschreibung zweier neuer Arten, *Amycolatopsis saalfeldensis* sp. nov. und *Kribbella aluminosa* sp. nov.. Außerdem konnte eine neue Gattung und Art, *Fodinicola feengrottensis* gen. nov., sp. nov. beschrieben werden. Die entsprechenden Gattungs- und Artbeschreibungen sind zentrale Bestandteile der vorliegenden Arbeit und wurden in der wisssenschaftlichen Zeitschrift "International Journal of Systematic and Evolutionary Microbiology (IJSEM)" veröffentlicht. Im Rahmen der vorliegenden Arbeit konnte außerdem gezeigt werden, dass HKI 0457^T, Typstamm der hierin beschriebenen neuen Art *Amycolatopsis saalfeldensis*, ein etwa 300 kB großes lineares Plasmid trägt, das wahrscheinlich unter anderem auch Biosynthesegene kodiert. Entsprechende Plasmide sind bisher aus der Gattung *Amycolatopsis* nicht bekannt. Zahlreiche der Feengrotten-Isolate wiesen zudem hochwirksame biologische Aktivitäten gegen verbreitete Testkeime auf. Es bleibt zu testen, ob die zugrundeliegenden Wirkmechanismen neuartig sind oder nicht. Vertiefende Untersuchungen sollten jedenfalls an denjenigen Stämmen erfolgen, die in einem parallel durchgeführten DC-basierten chemischen Screening ebenfalls Auffälligkeiten zeigten.

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10 Eigenständigkeitserklärung

Ich erkläre, dass ich die Dissertation "ISOLATION AND CHARACTERIZATION OF MINE-DWELLING ACTINOMYCETES AS POTENTIAL PRODUCERS OF NOVEL BIOACTIVE SECONDARY METABOLITES" selbständig und nur mit der darin angegebenen Hilfe verfasst habe. Die Dissertation wurde in keiner anderen Fakultät oder Universität eingereicht.

Jena, den	Marc René Carlsohn
24.01.2011	gez.