APPLICATION OF THE DYNAMIC CULTIVATION SYSTEM FOR MICROORGANISMS – A NEW WAY TO CULTURE THE UNCULTURABLES

RENÉ KADEN[1],*, EVE MENGER-KRUG[2], KATJA EMMERICH[2], KERSTIN PETRICK[2], AND PETER KROLLA-SIDENSTEIN[2]

[1] Uppsala University, Department of Medical Sciences, Dag Hammarskjöldsväg 17, 75185 Uppsala, Sweden
[2] Karlsruhe Institute of Technology (KIT), Institute of Functional Interfaces (IFG) Herrmann-von-Helmholtz-Platz 1, 76131 Eggenstein-Leopoldshafen, Germany

Abstract—To date, ~1% of all bacteria that occur in environmental ecosystems such as soil, sedimentary rocks, and groundwater have been described. Comprehensive explanation of ecological interactions on a microscale level is thus almost impossible. The Dynamic Cultivation System (DCS) was developed in order to detect more microbial taxa than with common cultivation approaches, as well as previously undescribed bacterial species. The DCS is a quick and easy *in situ* method for the cultivation of numerous bacterial taxa in support of the description of microbial colonized ecosystems. To investigate the bacterial populations within a clay-maturation process after mining the raw material, the DCS was used to increase the microbial biomass for further molecular analysis. Two different methods were applied to extract the bacteria from the DCS and these were compared in terms of efficiency at detection of large numbers of different taxa and in terms of applicability to the detection of previously undescribed species in raw clays. A collection of different undescribed species was detected with sequencing. While direct picking of bacterial colonies leads to the detection of different genera, species mainly of the genus *Arthobacter* were proved in the phosphate-buffered saline-suspended biomass. Thus, a combination of the approaches mentioned above is recommended to increase the number of detectable species. The DCS will help to describe better the microbial content of ecosystems, especially soils that contain charged particles.

Key Words—Clay, DGGE, DCS, Ecology, Microbial Diversity, Sequencing.

INTRODUCTION

For the ceramics industry, specific properties of clay during processing and firing are necessary in order to obtain a final product of the required quality. Moistened clay is often stored in buildings with high humidity prior to industrial use. This process of clay maturation enhances the properties of the raw material for clay processing in the ceramics industry as well as for the final product quality (Telle, 2007). Thus clay maturation has a significant economic impact in the ceramics industry. On the other hand, the storage of tons of clay for the maturation process requires space which is expensive. The core questions about this procedure are: what are the main processes that cause the known improvements with respect to the plasticity of the clay during storage at high humidity and how could the processes be controlled effectively? Both clay scientists and microbiologists have attempted to answer these questions but to date have been unsuccessful. Microorganisms cause changes in the chemical (*e.g.* pH), biochemical (*e.g.* metabolites), and physical (*e.g.* distribution of water) parameters during clay aging, which lead to the known maturation effect. Knowledge

of the maturation process was used centuries ago to produce the robust Chinese egg shell porcelain, which has a wall thickness of <0.4 mm (Weiss, 1963). Microorganisms can cause these changes in material properties. An increase in bacterial and fungal biomass during clay aging was discovered by Glick (1936). Microbial interactions in clay which influenced the clay matrix were also observed by Velde (1995). Organisms interrelate directly with their habitat in ecosystems (Fiore, 2007). The question to be asked is: does the clay-maturation process drive the physical or chemical reactions that influence the composition of the bacterial population composition because of the change in environmental parameters or do the bacteria cause shifts in the ecosystem that lead to the change in the chemical and physical properties? Microorganisms have a direct impact on the properties of clays as they use ions or molecules and release other substances such as exudates, metabolites, or functional complexes, *e.g.* siderophores (Vaiberg *et al.*, 1980; Groudeva and Groudev, 1995). Those compounds are released by bacteria to capture metal ions outside of the organism and might act as lubricants to improve plasticity.

To ascertain the influence of microorganisms on clay maturation, an investigation of the composition of the microbial population is needed. While classical cultivation methods select special groups of bacteria and fungi, extraction of the complete deoxyribonucleic acid (DNA) leads to characterization of all genotypes in general.

* E-mail address of corresponding author:
Rene.Kaden@akademiska.se

Clays may be able to capture the negatively charged DNA and prevent complete extraction of nucleic acids (Takada-Hoshino and Naoyuki, 2004). Bi- or multivalent cations and humic substances enhance this effect of DNA-binding on clay minerals. Furthermore, DNA extraction from raw materials leads to co-extraction of allochthonous nucleic acids as well as of those of dead organisms. In fact, neither cultivation methods nor complete DNA-extraction methods are able to extract information about the real incidence of all active species in clayey ecosystems.

The purpose of the present study was to provide a new method for the detection of some of the missing 99% of undescribed microbial species that influence clay properties during clay maturation. The method described is used for further analyses of the clay-maturation process. Once the effect of single bacterial species on clay minerals is known, those specific taxa can be used in future for manipulation of nanoparticles.

MATERIALS

For all experiments a silty clay W2, from Westerwald, Germany was used. The W2 clay is applied widely in the ceramics industry. The grain-size distribution was determined with and without Na-pyrophosphate as a dispersant (Table 1).

The mineralogy was determined by X-ray diffraction (XRD) followed by Rietveld analysis, using SEIFERT *AutoQuan* software (GE Inspection Technologies, Ahrensburg, Germany), of patterns from powdered samples and one-dimensional pattern fitting (ODPF) using *SYBILLA* (Zeelmaekers *et al.*, 2007) of patterns from textured samples to determine the mixed-layer I-S (quartz ($46\pm2\%$); kaolinite ($20\pm2\%$); illite ($19\pm2\%$); R1 I(0.8)-S($11\pm3\%$); feldspar ($3\pm1\%$); rutile ($1\pm1\%$)).

The kaolinite was distinguished from halloysite by

Table 1. Grain-size distribution with and without dispersant (data from Petrick *et al.*, 2011).

Fraction (µm)	Grain size with dispersant (%)	Grain size without dispersant (%)
>63	3	5
20−63	12	21
2−20	31	31
0.6−2	13	39
<0.6	41	4

several treatments of the textured samples. A detailed characterization of the material together with detailed description of methods used was given by Petrick *et al.* (2011).

METHODS

Five tons of raw clay (as mined) was homogenized, wetted to a total water content of 15% and divided into buckets containing 20 kg each (Figure 1). The samples were stored in a chamber with a humidity of 60% and a temperature of 21ºC. Beginning at the first day after clay preparation (=t1), samples were analyzed after 3, 6, 9, 13, 16, 20, 27, 41, and 83 days (=t3...t83).

Culturing bacteria and fungi in their original matrix (biol. *in situ*) is a possible means of overcoming the disadvantages of common culture-dependent methods and those of complete DNA extraction. The majority of bacterial species are not culturable with common methods and the analysis of whole genomes of bacterial populations to determine their potential biochemical pathways is difficult and expensive. The charges associated with clay minerals have a negative impact on all molecular analysis (Takada-Hoshino and Naoyuki, 2004). The DCS method (Figure 2), which is clay dependent, was therefore devised
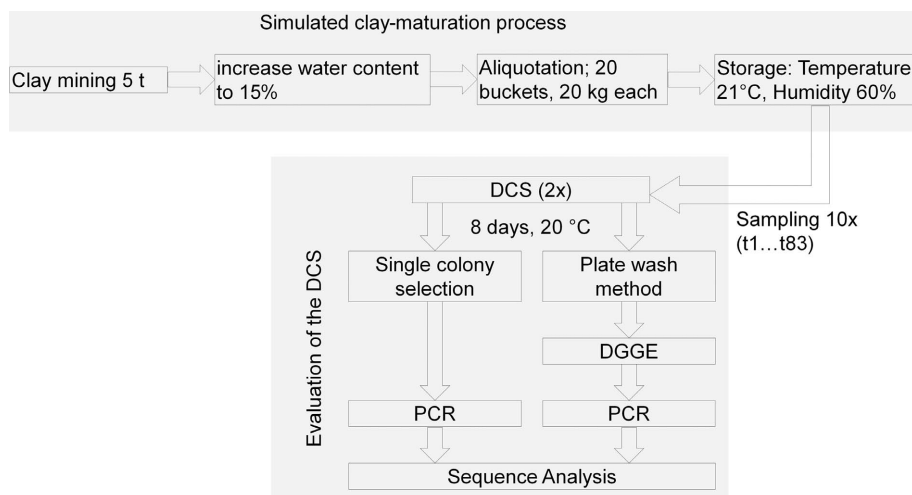

Figure 1. Experiment workflow with a simulated clay-maturation process and DCS evaluation.

(Kaden *et al.*, 2012) as a further development of the diffusion chamber of Bollmann *et al.* (2007).

The main principle of this method is to enrich the bacterial biomass (inoculum) separated from the clay above the incubation system to obtain an almost clay-mineral-free sample for molecular analysis. To separate the clay with a total water content of 30% and the inoculum in the incubation chamber, filters with a pore size of 0.2 µm were applied. Nutrients and water are available for the bacteria from the surrounding clay. A throughput of substances from the clay to the inoculum through the filters occurs due to the gravitational head of the clay-agar below the filter in addition to natural diffusion. The dilution of the sample combined with the enrichment of bacterial biomass during incubation causes a shift in the ratio between bacteria and clay minerals that allows molecular analyses with a reduced impact of inhibiting substances such as charged layer silicates. The main advantages of DCS are the cheap and easy construction and the fact that no external metal ions interact with this artificial but pseudo-natural ecosystem.

The inoculum used in this study was 50 µL of diluted clay slurry, with a solid clay content of 2%, and 98% (w/w) sterile physiological NaCl. The clay at the top of the DCS and the inoculum were taken from the same samples t1 to t83.

Microorganisms grow very slowly in clay due to the oligotrophic characteristic of this matrix. Therefore, the DCS prepared from each matured sample (t1 to t83) in duplicate (Figure 1) was also incubated for 8 days at a temperature of 20ºC. After incubation, the upper filter and the clay above this filter were discarded to collect the cells on the lower filter.

The method for removing the bacteria from the lower filter might have an impact on the detectable species so two procedures within sample analyses were performed to show the differences between them.

Figure 2. Construction of a DCS for microorganisms (after Kaden *et al.*, 2012).

Method 1. All visible colonies were removed from the lower filter using a sterile toothpick. In many cases, each colony consisted of a single species. Therefore, no further steps for dividing strains for the species determination were required when applying this method. The approach described is straightforward but may lead to an incomplete population analysis. Many bacteria grow in micro-colonies or as translucent biofilm. They are not visible without a microscope and thus it is impossible to remove them with the selection method described.

Method 2. The lower filter was washed with 1 mL of sterile PBS. Furthermore, a cell scraper was used to remove the colonies completely. This method should allow an almost complete molecular screening of all grown species.

The extraction of nucleic acids from the single colonies as well as from the bacterial suspension was performed according the QIAamp DNA Mini Kit manual (QIAGEN, 2003). To increase the amount of DNA for sequencing or for subsequent Denaturing Gradient Gel Electrophoresis (DGGE), a polymerase chain reaction (PCR) was performed using the forward primer 27f (AGAGTTTGATCMTGGCTCAG) (Schuppler *et al.*, 1995) with a GC-clamp that allows an enhanced discrimination of species-specific bands in DGGE (CGCCCGCCGCGCCCCGCGCCCGCTCCGCCGCCG-CCCCCGCCCC) and the reverse primer 517r (ATTACCGCGGCTGCTG) (Gotz *et al.*, 2002). The PCR was performed by enzyme activation for 10 min at 94ºC and 27 cycles of denaturation of DNA for 30 s at 94ºC followed by a primer annealing for 30 s at 58ºC and an elongation for 45 s at 72ºC. The final elongation was for 7 min at 72ºC. The 16S rRNA gene sequences from single picked colonies were determined after the PCR at GATC Biotech in Konstanz, Germany. With 10 µL of the DNA-PBS solution-based PCR products, a DGGE (Fischer and Lerman, 1980) was implemented with a run time ratio of 1000 Vh to divide the DNA at a species-specific level. The vertical urea gradient was 40−60%. The single bands, which represent 16S rRNA gene fragments of a single species, were cut with a sterile scalpel. Same-distanced bands were accepted as the same taxon. In the present case, only one band was chosen for further analysis. The DNA was removed from the gel by diffusion while single slices of the gel were placed into reaction tubes containing PCR water at 8ºC for 12 h. A further PCR was implemented with the same primer set 27f and 517r but without the GC-clamp. The products were sequenced using the primer 27f at GATC Biotech, Konstanz, Germany. The species were deter-mined using the NCBI database (Altschul *et al.*, 1990) and the *blastn* algorithm. For phylogenetic analyses, the *Megalign* 8.0.2 *DNASTAR* software based on Clustal W (Larkin *et al.*, 2007) with the neighbor-joining algorithm was applied.

RESULTS AND DISCUSSION

As most of the single picked colonies were pure cultures, a 16S rRNA gene fragment of these organisms was amplified and sequenced directly without further steps. Within the mixed cultures in the PBS-suspension, a DGGE (Figure 3) was needed to separate the 16S rDNA molecules of the different species. No uniform bar pattern existed through multiple steps in the time series. A few species were present in multiple samples but each sample had a different population composition. This confirms the observation of Connell and Slayter (1977) that changes in ecosystems lead to changes in population composition. A microbial diversity of 27 species based on 16S rRNA genes was detected 1 day after processing of the samples (t1). This diversity persisted up to 16 days (t16) after the start

of the experiment and decreased after 20 days (t20). In their experiment, Varnam and Evans (2000) placed bacteria in a soil sample that resulted in an increased number of added allochthonous species while the amount of autochthonous species decreased within the first 20 days. Species that are brought into the system with process water at the beginning of the clay-alteration process seemed to interact with the allochthonous microorganisms directly or with secreted compounds such as signal molecules for positive growth mediation (Kaeberlein *et al.*, 2002; Peterson *et al.*, 2006) or inhibitory substances.

In the undisturbed sample prior to mining, the lack of oxygen caused a significant diversity of anaerobic species. These were exposed to oxygen after the change in conditions while moistening. Furthermore, autochthonous species that lived in symbiosis or other dependency
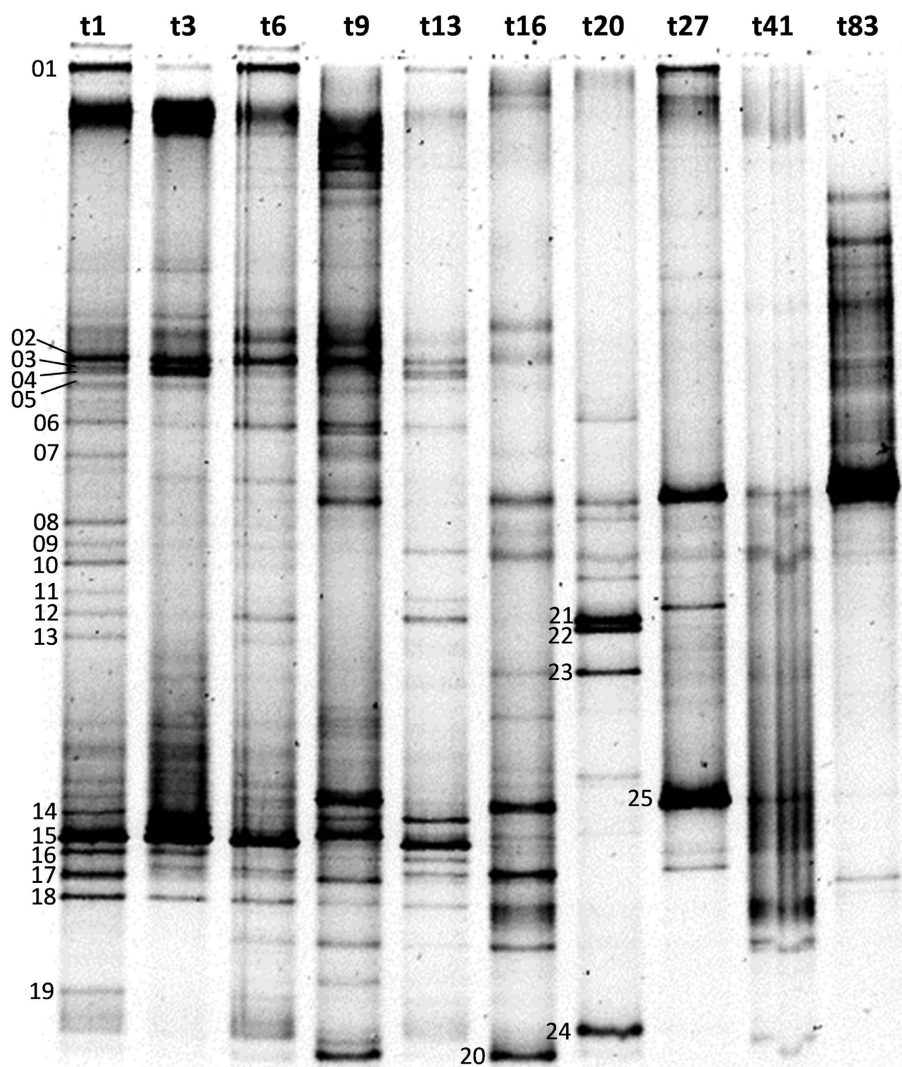

Figure 3. DGGE patterns of the time series t1 to t83 after cultivation on the DCS; bacteria removed by PBS-plate wash; DNA of numbered bands was analyzed (after Kaden *et al.*, 2012).

relationships potentially may not able to survive on a solitary basis. Within hours or days of mining and homogenization of clay, the nutrients are allocated nearly homologously. The microhabitats that are caused by irregular distribution of molecules, and that are characterized by a unique composition of bacterial species as described by Kirk *et al.* (2004), are rarely present at this manipulated state of the system. Such habitats only exist in soils with small particle sizes, such as clays, and are characterized by poor levels of water throughput and thus less physical exchange of nutrients.

After a dwell time, the system stabilized by building new microhabitats due to local metabolic activity and interactions of specific microbial species according to the conditions present which depend on the availability of oxygen, nutrients, and many other ecological factors. The number of detectable species seemed to depend on the particle size of the matrix. Bacteria have almost the same size range (0.2−5 μm) as clay particles. In clays that contain more particles in the <0.6 μm fraction than W2, fewer bacteria might occur. Less microbial biodiversity than in clay is also expected in soils with a large proportion in the >20 μm particle size range because of the flush of nutrients with the leaching water. In contrast, clays have a large water-binding capacity and thus nutrients are much more available. A large microbial diversity was expected in the system examined.

Twenty seven different samples were analyzed and 16 species were detected within the colonies grown that were removed directly from the filter (Table 2). Six of these 16 organisms potentially represented uncultured species regarding the species-level definition with a 16S rRNA gene-homology between two organisms of >97% (Janda and Abbott, 2002). A greater homology of the 16S rRNA gene within different taxa such as *Mycobacterium marinum* and *Mycobacterium ulcerans* the 16S rRNA of which differs by only 0.2% (Stinear *et*

al., 2000) is also possible. At least 37% of all cultured and determined species within the picked colonies were undescribed bacteria.

More than 60% of DNA samples that grew on the PBS washed plates represented potentially new cultured species (Table 3). The genus detected most, *Arthrobacter*, occurs frequently in soils (Cacciari and Lippi, 1987). Aside from their very simple life cycle, some species of *Arthrobacter* are able to survive under difficult conditions such as limited nutrients or in the presence of ionizing radiation, oxygen radicals, and toxic substances (Mongodin *et al.*, 2006). The closest related species of the strains B13 and B16, according to the NCBI database, was *Arthrobacter oxydans* (Table 3), while these two 16S rRNA gene sequences differ by more than 10% (Figure 4). This demonstrates that at least one of these species is not really *Arthrobacter oxydans* and illustrates the difficulties of interpreting sequence database entries.

Within the picked colonies the diversity of genera and the genetic divergence of all taxa were greater than the microbial diversity of the PBS-suspended samples. While species of *Arthrobacter* occurred mainly in the samples obtained from the PBS-washed plates, a few species related to *Pseudomonas* and a great diversity of other taxa were detected within the picked colonies (Figure 4). Some bacteria that grow slowly under the given conditions in the DCS were unable to develop visible single colonies and were not considered in the single colony-selection approach. The differences in detection of bacterial species between these two approaches might also be influenced by bacteria with a lesser abundance but a better state of metabolism that influences directly the number of 16S rRNA copies in the bacterial cell. While starving, bacteria produce a smaller amount of rRNA which increases by the factor 30−70 after nutrients become available (Kramer and

Table 2. Species determined within the colonies analyzed; NCBI PopSet [261288875] (Kaden *et al.*, 2009a).

Colony no.	First entry of described species	Ident	NCBI Acc. No.
C01	*Pseudomonas fluorescens*	99%	
C02	*Sphingobium yanoikuyae*	96%	GU002571
C03=C06	*Enterococcus faecalis*	94%	GU002572
C04	*Nocardioides fulvus*	99%	
C05	*Arthrobacter globiformis*	98%	GU002573
C07=C08	*Pseudomonas borealis*	98%	GU002574
C09	*Pseudomonas aeruginosa*	98%	GU002575
C10=C11	*Streptomyces phaeochromogenes*	99%	
C13	*Thiobacillus plumbophilus*	92%	
C15	*Acidithiobacillus ferrooxidans*	94%	
C16	*Aquaspirillum autotrophicum*	95%	
C17=C26=C27=C28=C29	*Massilia plicata*	98%	GU002579
C18=C21	*Arthrobacter sulfonivorans*	98%	
C19=C20	*Rhizobium giardinii*	98%	GU002576
C22=C25	*Nocardioides jensenii*	95%	GU002577
C23=C24	*Streptomyces caniferus*	98%	GU002578

Table 3. Species determined using the NCBI database; samples from PBS washed plates; NCBI PopSet [261288870] (Kaden and Krolla-Sidenstein, 2009b).

Bar no.	First entry of described species	Ident	NCBI Acc. No.
B01	*Arthrobacter ramosus*	91%	
B02=B06=B08=B11=B13	*Arthrobacter oxydans*	96%	GU002566
B3=B04=B15	*Arthrobacter sulfonivorans*	98%	
B05=B09=B10=B14	*Arthrobacter oryzae*	96%	
B16=B18	*Arthrobacter oxydans*	98%	GU002567
B17	*Arthrobacter globiformis*	95%	
B20	*Terrabacter lapilli*	94%	
B22	*Duganella nigrescens*	96%	GU002568
B23	*Dyella ginsengisoli*	96%	GU002569
B24	*Arthrobacter humicola*	99%	
B25	*Aestuariimicrobium kwangyangensis*	98%	GU002570

Singleton, 1992). Within the plate-wash experiment, some species might exist that are under-represented in terms of abundance but overestimated in terms of the analysis of the 16S rRNA gene. The PCR is a method that amplifies the nucleic acid in an exponential way. The difference in the number of rRNA molecules between two species is enhanced significantly after each cycle of the PCR and may lead to the result that the species with low abundance that were detected in the colony-pick experiment were also present on the washed plate but were undetectable due to the differences in the amount of 16S rRNA molecules resulting after PCR (Figure 5).

Furthermore, all primer pairs are selective and are unable to amplify the DNA of all bacterial species, even the universal 16S rRNA specific primer used in this study. Those alleged disadvantages of the method lead to the question of why no other genetic marker was chosen for the molecular analyses. For species determination, the largest number of entries in the NCBI database exists for the 16S rRNA gene. Furthermore, no PCR primers currently exist that indicate more bacterial species than a general 16S-specific primer combination (Stackebrandt, 2009).

Aside from the methodological challenges described, natural processes in clay may have influenced the results. Many direct interactions exist between clay minerals and microorganisms. Thus, it would be almost impossible to describe and explain the whole ecosystem 'clay' with the recent methods. Culturing bacteria in a clay-free approach might lead to enormous differences in contrast to DCS-cultivation of the inoculum, which
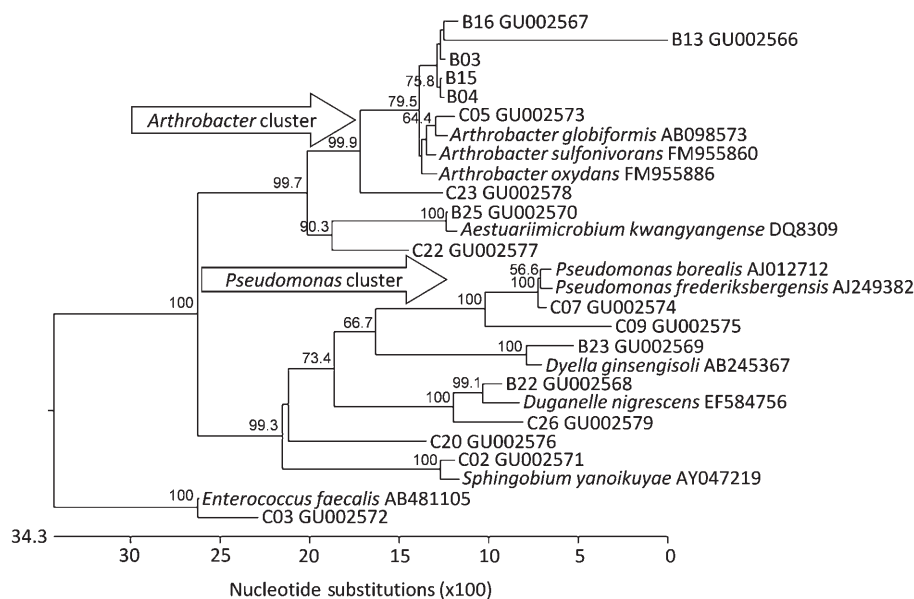

Figure 4. Taxonomic tree (Clustal W neighbor joining method and bootstrap, 1000 trails, seed of 111) of all analyzed sequence samples obtained by cultivation on the DCS, PBS-plate wash technique (BXX) and picked colonies (CXX), PCR, DGGE and sequencing; next related taxa were included (full name and NCBI accession number).
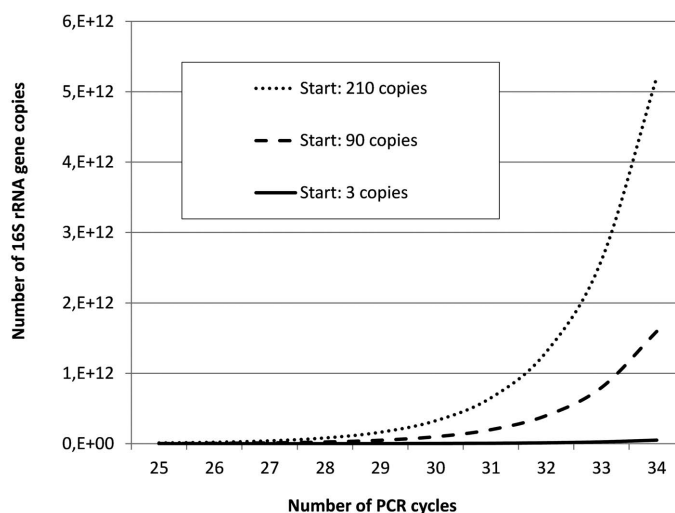
Figure 5. Model of the dependency of the number amplified gene fragments on the number of cycles in PCR assuming that all species occur in the same abundance and species 1 contains three copies of 16S rRNA (poor metabolic state), species 2 and 3 (good metabolic state), 30 and 70 times more 16S rRNA copies, respectively.

was a dilute clay slurry. As a result of direct interaction between clay minerals and microorganisms, the rate of sulfur oxidation is enhanced in *Thiobacillus thiooxidans* in the presence of clay minerals (Rinder, 1979). The products of this process lead to a decreased pH which also influences clay minerals as well as the microbial populations. The type of clay mineral is also important in terms of its effect on microorganisms. *Pseudomonas cepatia* and probably some other undescribed species are able to solubilize inorganic phosphorus in feldspar-rich silicate rocks only in the presence of kaolinite or montmorillonite (Bar-Yosef *et al.*, 1999). Comparable interactions with other available minerals might occur in the clay examined. Some bacteria are able to transform clay minerals. *Shewanella oneidensis* converts smectite to illite (Kim, 2004; Zhang, 2007). The changed properties in terms of the charge of the minerals have an effect on other microbial species. Other than clay, it is possible that for microorganisms no other ecosystem exists that is characterized by such extensive interactions. With the help of DCS, it was possible to culture many known and unknown species that exist in clay. The number of culturable species was enhanced from ~1% up to 60% in the present study.

SUMMARY AND CONCLUSIONS

In the present study, the dynamic cultivation system was applied to culture *in situ* a large number of currently unknown bacteria. Two methods for the removal of biomass from the system were compared. One approach was based on single-colony selection and another on suspension of the whole biomass from the filter. A large abundance of species related to the genus *Pseudomonas* and *Arthrobacter* was detected. Because of the different results from the two approaches used, a combination of

these methods is recommended to obtain the largest number possible of taxa. While 60% of the bacteria in the plate-wash approach represented new species, only 37% of the picked-colonies approach were previously undescribed species. In contrast, ~1% of all bacterial species are culturable with common culture methods. The DCS is therefore more applicable to the detection of new species in several clay-mineral-containing matrices than standard media. The method may contribute to a better understanding of several ecosystems, once the bacterial diversity is described. Only with knowledge of the species which occur in clays and how they metabolize the substances available will it be possible to describe interactions between clay minerals and whole bacterial populations. Furthermore, the processes during clay maturation can only be described in detail when all the contributing bacteria are known. This will lead to more time- and cost-efficient clay-maturation procedures in future.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403−410.

Bar-Yosef, B., Rogers, R., Wolfram, J., and Richman, E. (1999) *Pseudomonas cepacia*-mediated rock phosphate solubilization in kaolinite and montmorillonite suspensions. *Soil Science Society of America Journal*, **63**, 1703−1708.

Bollmann, A., Lewis, K., and Epstein, S.S. (2007) Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. *Applied and*

Environmental Microbiology, **73**, 6386−6390.

Cacciari, I. and Lippi, D. (1987) Arthrobacters: Successful arid soil bacteria: A review. *Arid Soil Research and Rehabilitation*, **1**, 1−30.

Connell, J.H. and Slayter, R.O. (1977) Mechanisms of succession in natural communities and their role in community stability and organization. *The American Naturalist*, **111**, 1119−1144.

Fiore, S., Dumontet, S., and Huertas, J.F. (2007) Clay minerals and bacteria. *Proceedings of Euroclay 2007*, Aveiro, Portugal, pp. 62−65.

Fischer, S.G. and Lerman, L.S. (1980) Separation of random fragments of DNA according to properties of their sequences. *Proceedings of the National Academy of Sciences of the United States of America − Biological Sciences*, **77**, 4420−4424.

Glick, D.P. (1936) The microbiology of aging clays. *Journal of the American Ceramic Society*, **19**, 169−175.

Gotz, D., Banta, A., Beveridge, T.J., Rushdi, A.I., Simoneit, B.R.T., and Reysenbach, A. (2002) *Persephonella marina* gen. Nov., sp nov and *Persephonella guaymasensis* sp nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 1349−1359.

Groudeva, V.I. and Groudev, S.N. (1995) Microorganisms improve kaolin properties. *American Ceramic Society Bulletin*, **74**, 85−89.

Janda, J.M. and Abbott, S.L. (2002) Bacterial identification for publication − when is enough enough? *Journal of Clinical Microbiology*, **40**, 1887−1891.

Kaden, R., Menger-Krug, E., and Krolla-Sidenstein, P. (2009a) NCBI Pop Set [261288875]: Population analysis of clay. NCBI database.

Kaden, R. and Krolla-Sidenstein, P. (2009b) NCBI PopSet [261288870]: The Dynamic Cultivation System, a new method to show temporal shifts in microbial community structure. NCBI database.

Kaden, R., Menger-Krug, E., Emmerich, K., Petrick, K., Mühling, M., and Krolla-Sidenstein, P. (2012) The dynamic cultivation system: A new method for the detection of temporal shifts in microbial community structure in clay. *Applied Clay Science*, **65−66**, 53−56.

Kaeberlein, T., Lewis, K., and Epstein, S.S. (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science*, **296**, 1127−1129.

Kim, J., Dong, H.L., Seabaugh, J., Newell, S.W. and Eberl, D.D. (2004) Role of microbes in the smectite-to-illite reaction. *Science*, **303**, 830−832.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Khironomos, J.N., Lee, H., and Trevors, J.T. (2004) Methods of studying soil microbial diversity. *Journal of Microbiological Methods*, **58**, 169−188.

Kramer, J.G. and Singleton, F.L. (1992) Variations in rRNA content of marine vibrio spp. During starvation-survival and recovery. *Applied and Environmental Microbiology*, **58**, 201−207.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947−2948.

Mongodin, E.F., Shapir, N., Daugherty, S.C., DeBoy, R.T., Emerson, J.B., Shvartzbeyn, A., Radune, D., Vamathevan, J., Riggs, F., Grinberg, V., Khouri, H., Wackett, L.P., Nelson, K.E., and Sadowsky, M.J. (2006) Secrets of soil survival revealed by the genome sequence of arthrobacter aurescens tc1. *PLoS Genet*, **2**, e214. (doi: 10.1371/journal.pgen.0020214)

Peterson, S.B., Dunn, A.K., Klimowicz, A.K., and Handelsman, J. (2006) Peptidoglycan from bacillus cereus mediates commensalism with rhizosphere bacteria from the cytophaga-flavobacterium group. *Applied and Environmental Microbiology*, **72**, 5421−5427.

Petrick, K., Diedel, R., Peuker, M., Dieterle, M., Kuch, P., Kaden, R., Krolla-Sidenstein, P., Schuhmann, R., and Emmerich, K. (2011) Character and amount of I-S mixed-layer minerals and physical-chemical parameters of two ceramic clays from Westerwald, Germany: Implications for processing properties. *Clays and Clay Minerals*, **59**, 58−74.

QIAGEN (2003) QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit 02/2003. User manual, pp. 33−36.

Rinder, G. (1979) Einfluß von tonmineralen auf der verhalten von acidophilen thiobacillus-arten in suspensionen. *Zeitschrift für Allgemeine Mikrobiologie*, **19**, 643−651.

Schuppler, M., Mertens, F., Schon, G., and Gobel, U.B. (1995) Molecular characterization of nocardioform actinomycetes in activated-sludge by 16S rRNA analysis. *Microbiology*, **141**, 513−521.

Stackebrandt, E. (2009) *Phylogeny Based on 16S rRNA/DNA.* eLS. John Wiley & Sons Ltd, Chichester, UK. doi: 10.1002/9780470015902.a0000462.pub2.

Stinear, T., Jentkin, G.A., Johnson, P.D.R., and Davies, J.K. (2000) Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. *Journal of Bacteriology*, **182**, 6322−6330.

Takada-Hoshino, Y. and Naoyuki, M. (2004) An improved DNA extraction method using skim milk from soils that strongly adsorb DNA. *Microbes and Environments*, **19**, 13−19.

Telle, R. (2007) *Salmang scholze keramik*, 7th edition. Springer, Berlin, Heidelberg.

Vaiberg, S.N., Vlasov, A.S., and Skripnik, V.P. (1980) Treating clays with silicate bacteria. *Glass and Ceramics*, **37**, 387−389.

Varnam, A.H. and Evans, G.G. (2000) *Environmental Microbiology*. Manson Publishing, London.

Velde, B. (1995) *Origin and Mineralogy of Clays. Clays and the Environment*. Springer-Verlag, New York, 358 pp.

Weiss, A. (1963) A secret of Chinese porcelain manufacture. *Angewandte Chemie International Edition in English*, **2**, 697−703.

Zeelmaekers, E., McCarty, D., and Mystkowski, K. (2007) *SYBILLA* user manual. Chevron proprietary software, Houston, Texas, USA.

Zhang, G.X., Kim, J.W., Dong, H.L., and Sommer, A.J. (2007) Microbial effects in promoting the smectite to illite reaction: Role of organic matter intercalated in the interlayer. *American Mineralogist*, **92**, 1401−1410.