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Abstract

■ Full Text

Figures and Tables

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Contents

Archive

Journal Homepage

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PubMed related arts

GO

**PubMed articles by:**

Sandaa, R.

Enger, Ø.

Torsvik, V.

TOP

■ ABSTRACT

MATERIALS AND

METHODS

RESULTS AND

DISCUSSION

REFERENCES

TOP

ABSTRACT

MATERIALS AND

METHODS

RESULTS AND

DISCUSSION

REFERENCES

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## Abundance and Diversity of *Archaea* in Heavy-Metal-Contaminated Soils

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### ABSTRACT

The impact of heavy-metal contamination on archaean communities was studied with sewage sludge contaminated with heavy metals to varying extents. Fluorescence hybridization showed a decrease in the percentage of *Archaea* from  $1.3\% \pm 0.3$  diamidino-2-phenylindole-stained cells in untreated soil to below the detection limit with heavy metals. A comparison of the archaean communities of the different gradient gel electrophoresis revealed differences in the structure of the archaea with increasing heavy-metal contamination. Analysis of cloned 16S ribosomal DNA revealed similarities to a unique and globally distributed lineage of the kingdom *Crenarchaeota* phylogenetically distinct from currently characterized crenarchaeotal species.

The presence of heavy metals in sewage sludge is often the main determinant in the application of sewage sludge to agricultural soil (28). After detailed assessment of the transfer of heavy metals into the food chain via crops, the Commission of the European Communities has set limits on the amount of selected heavy metals that can be added to agricultural sewage sludge (8). However, even heavy-metal contamination that is below the European Commission can have an effect on microbial community structure (1).

Many studies have focused on the effects of heavy metal on bacterial communities (30, 32, 34). However, no investigations have studied the effect of heavy-metal contamination on the archaean community. Until a few years ago, the domain *Archaea* was considered to consist of methanogens that live under strict anoxic conditions and extremophiles that inh

environments (36, 39). Recent studies have shown that *Archaea* are also present in marine (10, 11, 14, 27, 38a), freshwater (18), and terrestrial ecosystems. This suggests that the *Archaea* also have ecological significance in these environments. Phylogenetic analysis of *Archaea* from nonextreme environments shows they form a new cluster within the kingdom *Crenarchaeota* and are only distantly related to the previously described crenarchaea, as determined by 16S rRNA gene sequences. This group of nonthermophilic *Crenarchaeota* and consists of at least four distinct phylogenetic clusters. Some of the gene sequences isolated from soil are found in one of these clusters.

In this study, we analyzed the long-term effect of heavy metals on the archaean community in heavy-metal-contaminated samples from five experimental field plots (Braunschweig, Germany) between 1980 and 1991, with different amounts of sludge and heavy metals (6). We used methods such as fluorescent in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) to analyze differences in the abundance and diversity of *Archaea* in both heavy-metal-contaminated soil and uncontaminated soil. A comparative sequence analysis of 16S rDNA libraries showed that *Archaea* from heavy-metal-contaminated soils clustered with those of terrestrial nonthermophilic *Crenarchaeota*.

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[TOP](#)[ABSTRACT](#)[MATERIALS AND METHODS](#)[RESULTS AND DISCUSSION](#)[REFERENCES](#)

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## MATERIALS AND METHODS

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**Soil characteristics and samples.** Soil samples, down to a 10-cm depth, were collected in November 1994 from an experimental field site in Braunschweig, Germany. The soil was characterized by the following amendments: (i) N fertilization, (ii) low sludge and low metal, (iii) low sludge and high metal, (iv) high sludge and low metal, and (v) high sludge and high metal (1). All plots had been planted with spring rape. The soil, with a matrix consisting of 55% silt, and 45% sand, received either 100 or 300 m<sup>3</sup> of unamended or amended sludge between 1980 and 1991 (Table 1). The amended sludge was spiked with heavy metals (Cd and Zn) to increase the heavy-metal load to the upper limit set by the European Commission. During sampling, no pronounced differences in organic C (Table 1) were noticed between uncontaminated soil to the soil with the highest metal amendment was accompanied by a decrease in pH from 7.1 to 5.3 (Table 1).

Four samples from each of the five plots were sieved, pooled, and mixed before being analyzed. The plots were investigated by DGGE analysis and FISH. For *Archaea* 16S rDNA analysis, in two plots were studied, the low-sludge–low-metal- and high-sludge–high-metal

**FISH.** Soil samples of 5 g each were fixed in 4% paraformaldehyde–phosphate buffer (0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, and 3 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.2] in water) on ice. The soil suspensions were centrifuged at 4,000 × g for 5 min, and the pellets were subsuspended in phosphate-buffered saline, resuspended in 20 ml of 96% ethanol, and stored at -20°C. A concentration of 25 mg of soil (wet weight) ml<sup>-1</sup>. Before application to slides, the soil suspension was dispersed in 960 µl of 0.1% sodium pyrophosphate in distilled water. After sonication for 30 s at a setting of 8 (B-12 sonifier; Branson, Danbury, Conn.) (400 µl), 200 µl was subsequently spotted onto gelatin-coated slides [0.1% gelatin, CMC, dried at 45°C for 30 min, and finally dehydrated sequentially in 50, 80, and 96% ethanol (each)].



loaded onto 8% acrylamide gels (bisacrylamide gel stock solution, 37:5:1; Bio-Inc.) and run with 0.5× TAE buffer (1× TAE is 0.04 M Tris base, 0.02 M sodium EDTA [pH adjusted to 7.4]). DGGE gels contained a 20 to 60% gradient of solution increasing in the direction of electrophoresis. A 100% urea-formamide 40% (vol/vol) formamide plus 7.0 M urea. DGGE was conducted at 60°C at a v min and thereafter at 200 V for 3 h. The gels were stained for 1 h with a 1:10,00 Green II (Molecular Probes, Eugene, Oreg.) in 0.5× TAE buffer before photogr

**Cloning and restriction fragment length polymorphism.** PCR products PCR products were purified by preparative gel electrophoresis (1% low-melting-point T BioProducts, Rockland, Maine), followed by purification with the PCR cleanup The products were concentrated by precipitation by a standard procedure with s and 70% ethanol (31). Cloning into the pMOSBlue T vector was performed as a manufacturer (Amersham International Plc., Little Chalfont, Buckinghamshire, were screened for  $\alpha$  complementation by using X-Gal (5-bromo-4-chloro-3-ind galactopyranoside) and IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) (31). One of ampicillin per milliliter was added to Luria agar plates (10 g of tryptone, 5 g of NaCl, 15 g of agar, 1,000 ml of distilled water) for the selection of positive c 150 blue colonies were picked from each cloning reaction from the two soil tre; 300 blue colonies were screened for positive inserts by PCR with T7 (TAATACGACTCACTATAGGG) and U-19mer (GTTTTCCCAGTCACGAC (Amersham). PCR was performed as described above but in a total volume of 5 *Taq* DNA polymerase (Perkin-Elmer). Template was added as whole cells. The verified by gel electrophoresis.

Amplified cloned inserts were cut with the restriction enzymes *Cfo*I and *Hpa*II Restriction was carried out for 2 h at 37°C in a total volume of 10  $\mu$ l containing enzymes and 9  $\mu$ l of PCR product and restriction buffer. The resulting restrictic polymorphism products were separated by gel electrophoresis at 7 V/cm for 2 h containing 0.1  $\mu$ g of ethidium bromide  $\text{ml}^{-1}$ . The restriction products were visu excitation (Electronic Dual Light transilluminator; Ultra Lum, Carson, Calif.) a device camera (Ultra Lum).

**Sequence analysis.** A total of 11 of the cloned amplicons with different restri containing 16S rDNA from the low-sludge–low-metal and high-sludge–high-m sequenced. Plasmid DNA was prepared from the clones by using the Qiaprep P (Qiagen, Inc., Chatsworth, Calif.). Sequencing was performed by the Advance Centre (Charing Cross and Westminster Centre, London, England). The whole 450 bp, was sequenced by using the T7 primer as a sequencing primer. These s checked for chimeric artifacts by the CHECK-CHIMERA service provided by 1 Database Project (23). Sequences were aligned with the PILEUP program of th Group Software package. Distance matrices and phylogenetic trees were made CLUSTAL W program. The sequences were taxonomically assigned by using t database at GenBank and BLAST (National Center for Biotechnology Informat

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TOP

ABSTRACT

MATERIALS AND

METHODS

■ RESULTS AND

---

## RESULTS AND DISCUSSION

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Application of DGGE revealed differences in the archaean community structur heavy-metal contamination (Fig. 1). While the control soil resulted in a banding

## DISCUSSION

## REFERENCES

the low-metal soils, A and B, gave patterns of 6 bands each. However, soils with high amounts of heavy metals (C and D) gave banding patterns of 11 bands each. T<sub>1</sub> (positions 5.2 and 9.6) were detected in all five plots (Fig. 1). The A and B soil banding patterns, except for one band that was unique to soil A (position 10.0) was unique to soil B (position 2.2) (Fig. 1). Likewise, the C and D soils also showed similarities in DGGE banding patterns (Fig. 1). Three distinct bands (positions 2.5, 3.1, 4.1, 8.4, and 8.9) were detected only in these two plots. In addition, soils C and D each showed six distinct bands (positions 2.5, 3.1, 4.1, 8.4, and 8.9). Only one distinct band was detected in the high-sludge-contaminated soil (position 7.4). The banding patterns from the low-sludge–low-metal plot (A) and the low-sludge–high-metal plot (B) were different from the control soil. The control soil was somewhat similar with respect to pH (Table 1). Thus, the differences in archaeal community structure between the control soil and soils A and B could in part be due to an increase in the concentration of the heavy metals or the addition of sludge. This was confirmed by another study showing a substantial reduction in total bacterial diversity in the control soil and soil A (low sludge–low metal) (32).

The pHs of the A and B soils were in the same range as that of the control soil, while the C and D soils were substantially lower (Table 1). The overall differences in the archaeal community structure between the control and A and B soils on one hand and the C and D soils on the other therefore may not be the effect of heavy metals alone but also that of an increase in pH or a lowering in the pH (Table 1). pH has a great effect on the solubility of heavy metals; a twofold increase in heavy metals (Cd, Ni, and Zn) in solution, for example, has a one-unit decrease in pH (7, 33). However, differences in the banding patterns between the low-sludge soils (A and B) and between the two high-sludge soils (C and D) must be the effect of heavy metals alone (Fig. 1).

FISH with probe ARCH915 showed that the number of *Archaea* belonging to the *Halorubrum* group decreased from 1.3% ± 0.3% of the DAPI-stained cells in the control soil to numbers below the detection limit (<1% of the DAPI-stained cells) in contaminated soils (5, 40). This indicates that heavy metals were somewhat toxic to the growth or survival of some of the *Archaea*. The *Archaea* observed in this study are in accordance with numbers found in other ecosystems (5, 40).

Phylogenetic analysis of the *Archaea* 16S rDNA clone libraries revealed from 95% similarity to a novel group within the kingdom *Crenarchaeota* (2), also known as the nonthermophilic *Crenarchaeota* (4). Members within this group of *Archaea* have been detected from different environmental sources (4, 18, 19, 24, 26, 27), and they are phylogenetically distinct from currently characterized crenarchaeotal species. Phylogenetic analysis of the clones from these studies has shown that the clones belong to at least four distinct groups that appear to have a common ancestry (4). The sequences in this study fell into the terrestrial cluster of *Crenarchaeota*. Two groups (clones a-195a, a-161d, a-177) were similar to clones from an agricultural soil in Wisconsin (U62811 and U62814) and a third group (a-9a, a-10a, a-18a, and a-3a) showed sequence similarities to clones from freshwater sediments (U59973) (18) (Fig. 2). In another study, sequences from freshwater sediments were shown to group within the terrestrial cluster of nonthermophilic *Crenarchaeota*.

Compared to noncontaminated soil, a significant reduction in the percentage of archaeal community structure, was observed even at concentrations below the upper limit set by the European Commission. Molecu

demonstrated that nonthermophilic *Crenarchaeota* are found in diverse environments globally distributed (2–4, 10, 18, 21, 24). Nonthermophilic *Crenarchaeota* have been found to occur in heavy-metal-contaminated soil. The findings in this study, that heavy metals are toxic to the growth or survival of some of the *Archaea*, may contribute to our knowledge of the ecological role of this novel group of organisms. Little is still known about their characteristics and ecological significance, and further investigations are needed to determine their ecological importance.

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TOP

ABSTRACT

MATERIALS AND  
METHODS

RESULTS AND  
DISCUSSION

■ REFERENCES

## REFERENCES

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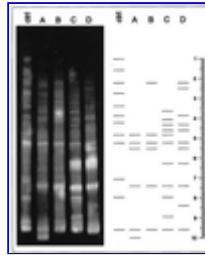
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## FIGURES AND TABLES

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**FIG. 1**

DGGE profile and schematic representations of the main bands of the C amplified 16S rDNA from *Archaea* in soil with different amendments. ctrl A, low sludge-low metal; B, low sludge-high metal; C, high sludge-low (r



**FIG. 2**

Neighbor-joining analysis showing the relationship of eight partial sequence libraries from low-sludge-low-metal soil (with suffix "a") and high-sludge-low-metal soil (with suffix "d") to (more ...)

**TABLE 1**

Soil characteristics

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