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Effect of Temperature on Structure and Function of the Methanogenic Archaeal Community in an Anoxic Rice Field Soil

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Soil temperatures in Italian rice fields typically range between about 15 and 30°C. A change in the incubation temperature of anoxic methanogenic soil slurry from 30°C to 15°C typically resulted in a decrease in the CH_4 production rate, a decrease in the steady-state H_2 partial pressure, and a transient accumulation of acetate. Previous experiments have shown that these changes were due to an alteration of the carbon and electron flow in the methanogenic degradation pathway of organic matter caused by the temperature shift (K. J. Chin and R. Conrad, FEMS Microbiol. Ecol. 18:85–102, 1995). To investigate how temperature affects the structure of the methanogenic archaeal community, total DNA was extracted from soil slurries incubated at 30 and 15°C. The archaeal small-subunit (SSU) rRNA-encoding genes (rDNA) of these environmental DNA samples were amplified by PCR with an archaeal-specific primer system and used for the generation of clone libraries. Representative rDNA clones (n = 90) were characterized by terminal restriction fragment length polymorphism (T-RFLP) and sequence analysis. T-RFLP analysis produced for the clones terminally labeled fragments with a characteristic length of mostly 185, 284, or 392 bp. Sequence analysis allowed determination of the phylogenetic affiliation of the individual clones with their characteristic T-RFLP fragment lengths and showed that the archaeal community of the anoxic rice soil slurry was dominated by members of the families *Methanosarcinaceae* (185 bp) and *Methanosaetaceae* (284 bp), the kingdom *Crenarchaeota* (185 or 284 bp), and a novel, deeply branching lineage of the (probably methanogenic) kingdom *Euryarchaeota* (392 bp) that has recently been detected on rice roots (R. Großkopf, S. Stubner, and W. Liesack, Appl. Environ. Microbiol. 64:4983–4989, 1998). The structure of the archaeal community changed when the temperature was shifted from 30°C to 15°C. Before the temperature shift, the clones (n = 30) retrieved from the community were dominated by *Crenarchaeota* (70%), "novel *Euryarchaeota*" (23%), and *Methanosarcinacaeae* (7%). Further incubation at 30°C (n = 30 clones) resulted in a relative increase in members of the *Methanosarcinaceae* (77%), whereas further incubation at 15°C (n = 30 clones) resulted in a much more diverse community consisting of 33% *Methanosarcinaceae*, 23% *Crenarchaeota*, 20% *Methanosaetaceae*, and 17% novel *Euryarchaeota*. The appearance of *Methanosaetaceae* at 15°C was conspicuous. These results demonstrate that the structure of the archaeal community in anoxic rice field soil changed with time and incubation temperature.

Methane production in anoxic freshwater environments is accomplished by a complex community consisting of hydrolytic, fermenting, syntrophic, homoacetogenic, and methanogenic microorganisms that degrade organic matter under anaerobic conditions to CH_4 and CO_2. Methane itself is produced from acetate and H_2 or CO_2, the predominant substrates of methanogenic archaea (10, 44, 52). Similar processes take place in anoxic rice field soil (8, 9, 23, 24, 47), where acetate contributes about 65 to 80% to CH_4 production (11, 32). Temperature is an important regulator of microbial activity involved in CH_4 production (8, 12, 40, 51). Soil temperatures in Italian rice fields typically range between 15 and 30°C (40).

We have previously shown that a shift of the incubation temperature of methanogenic rice soil from 30°C to 15°C not only results in a decrease in the CH_4 production rate, but also results in a change in the degradation pathway of organic matter (8). Thus, the shift to lower temperature resulted in a decrease in the steady-state H_2 partial pressure and a transient accumulation of acetate, propionate, caproate, lactate, and isopropanol (8). This observation, together with the results of inhibitor and radiotracer experiments, suggested that syntrophic conversion of fatty acids to H_2 was impeded while homoacetogenesis was enhanced at low temperatures, resulting in a relatively higher proportion of acetate-dependent compared to H_2-dependent methanogenesis (8, 12–14). A similar change in the methanogenic degradation pattern, although in the opposite direction, was also observed when the temperature of methanogenic sediments of Lake Constance was shifted from 4°C to 20°C (37, 38).

A temperature-induced change in the methanogenic degradation pathway may well be accompanied by a change in the microbial community structure. Recently, the community of methanogenic archaea in anoxic rice field soil (17) and on the surface of rice roots (18, 25) has been characterized by comparative sequence analysis of archaeal small-subunit (SSU) rRNA-encoding genes (rDNA) retrieved from environmental DNA. These analyses demonstrated a relatively large diversity of *Archaea*, including members of the families *Methanosarcinaceae*, *Methanosaetaceae*, *Methanomicrobiaceae*, and *Methanobacteriaceae* (taxonomy according to Rouviere et al. [34]). In addition, members of the kingdom *Euryarchaeota* have been detected and grouped into novel phylogenetic clusters termed rice clusters I, II, III, and V which are defined on the basis of evolutionary distance dendrograms (18). Rice clusters I and II

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seem to be methanogenic taxa, since they fall within the phylogenetic radiation of the orders *Methanosarcinales* and *Methanomicrobiales*, and since members of methanogenic enrichment cultures on H_2-CO_2 and ethanol-CO_2 have been affiliated with either of these two clusters (25). Rice clusters III and V are euryarchaeotal, but probably not methanogenic (18). A further group (rice cluster IV) that has been detected in DNA extracts from rice roots and bulk rice soil forms a phylogenetic cluster within the *Crenarchaeota* (18).

Here, we have studied the structure of the archaeal community in three different samples of a methanogenic rice field soil. These samples were obtained by incubating anoxic soil slurries at 30°C until stable CH_4 production was observed (sample ST1), by a further 2-week incubation of these slurries at 30°C (sample S30) and by shifting the temperature to 15°C and further 2-week incubation at this temperature (sample S15). The archaeal community structure was investigated by construction of clone libraries of SSU rDNA from DNA extracts from the soil samples, followed by analysis of these clone libraries by using T-RFLP and comparative sequencing.

MATERIALS AND METHODS

Soil slurry experiments. The soil samples used for slurry experiments were collected in April 1992 after plowing of yet unflooded rice fields of the Italian Rice Research Institute in Vercelli, Italy, and were stored as dry lumps at room temperature. The characteristics of the soil were previously reported (19). The same soil was used in a previous study; the preparation and incubation of slurries were carried out as described there (8). After 7 days of preincubation at 30°C, a number of samples were shifted from 30°C to 15°C to test the effects of incubation temperature on methanogenesis and turnover of intermediary substrates. The procedures for taking gas and liquid samples and the analysis of CH_4, H_2, and acetate have been described in detail previously (8).

DNA extraction from soil slurry. The sampling for molecular analysis was carried out with parallel soil slurries. One sample was taken as a control directly before temperature shift (ST1). Two other samples were taken at the end of each experiment, i.e., at 30°C (S30) and 15°C (S15). The procedure used for extraction of DNA was a modification of previously described protocols (17, 42). The modifications, which consisted of using chloroform-isoamyl alcohol instead of chloroform-phenol for extraction and using two parallel treatments for microbial lysis, were applied to obtain a large range of DNA molecules of different sizes. Two parallel slurry samples of 0.5 ml each were mixed with 0.5 ml of sodium phosphate buffer (0.12 M [pH 8.0]). One of the samples was treated by three cycles of freezing and thawing (2 min at −70°C, 2 min at 65°C), followed by treatment with lysozyme (5 mg ml^{-1}, 1 h at 37°C) and sodium dodecyl sulfate (SDS; 2.4%, 10 min at 60°C). After addition of 0.5 g of glass beads (0.17 to 0.18 mm in diameter) and three 60-s cycles of bead-beating (Mini-Bead-Beater; Biospec Products, Bartlesville, Okla.), the slurry was centrifuged (10 min, 13,000 × *g*), and the supernatant was used for DNA extraction. The other slurry sample was treated only with SDS and then was centrifuged. The DNA was extracted from the two parallel slurry samples with chloroform-isoamyl alcohol (24:1 [vol/vol]), and the extracts were combined and then purified as described previously (17, 42).

Amplification of archaeal SSU rRNA genes. The SSU rDNA fraction of each environmental DNA sample was amplified by PCR with the archaeal-specific primers described by Großkopf et al. (17), which amplifies from positions 109 to 934 (*Escherichia coli* 16S rRNA numbering [6]). Amplification of the SSU rDNA from 1 μl of DNA extracted from the slurry was performed by using Gene Amp System 2400 (PE Applied Biosystems, Weiterstadt, Germany). The reaction mixture contained 10 μl of reaction buffer supplied by the manufacturer (PCR buffer II; PE Applied Biosystems), 20 nmol of each deoxynucleoside triphosphate, 0.15 nmol of $MgCl_2$, 30 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (PE Applied Biosystems). The thermal profile used for amplification included 30 to 35 cycles of primer annealing at 52°C for 1 min, primer extension at 72°C for 1 min, and denaturation at 94°C for 45 s. For the T-RFLP analysis, the backward primer was labeled 5′ terminal with FAM (5-carboxyfluorescein).

Cloning and sequencing. The archaeal 16S rRNA gene PCR products were cloned by using the TA cloning kit with pCR 2.1 vector and *E. coli* INVαF′ (Invitrogen, Leek, The Netherlands). Further analysis of randomly selected clones was carried out as described by Rotthauwe et al. (33).

T-RFLP analysis. The principle of the T-RFLP analysis has been described by Liu et al. (27). The SSU rDNA amplicons were purified by use of the Prep-A-Gene kit (Bio-Rad, Munich, Germany) according to the instructions of the manufacturer. Aliquots of the purified SSU rDNA were digested by *Taq*I (Promega, Mannheim, Germany). Each reaction tube contained 8 μl of the SSU rDNA amplicons, 1 μl of the appropriate incubation buffer supplied by the manufacturer (Promega), and 1 μl of restriction enzyme (10 U), made up to a total volume of 10 μl with deionized H_2O. Incubations were carried out in 0.5-ml reaction tubes for 3 h at 65°C.

The digested SSU rDNA (2.5 μl) was mixed with 2.0 μl of formamide and 0.5 μl of an internal lane standard consisting of 17 different 6-carboxy-X-rhodamine (ROX)-labeled fragments ranging in length from 29 to 928 nucleotides (GeneScan-1000 ROX; PE Applied Biosystems). The samples were denatured at 94°C for 2 min and then immediately stored on ice until being loaded onto the gel. Electrophoresis was performed for 6 h through a 6% (wt/vol) polyacrylamide gel (length, 12 cm) containing 8.3 M urea and 1× Tris-borate-EDTA buffer. The restricted SSU rDNA fragments were size separated on an automated DNA sequencer (model 373; PE Applied Biosystems) under the following conditions: 2,500 V, 40 mA, and 27 W. The laser scanning system of this DNA sequencer detected only the fluorescently labeled 5′-terminal fragments.

The RFLP pattern of the 5′-terminal SSU rDNA fragments of each sample was determined in comparison to that of the internal lane standard (GeneScan-1000 ROX) by using GeneScan analysis software (version 2.1).

Phylogenetic placement. The phylogenetic analysis of sequence data (i.e., data processing and construction of trees) was carried out by using the ARB software package with its database (45). The SSU rDNA sequences, which were between 716 and 750 bp in length, were added to the database of 176 complete or partial archaeal SSU rDNA sequences (28, 31, 48). Phylogenetic placement was done in comparison to reference sequences for the main lines of descent within the archaeal kingdoms *Euryarchaeota* and *Crenarchaeota* (50), as well as *Korarchaeota* (3). The tree topology was evaluated by performing neighbor-joining analyses (35). The base frequencies of the alignment positions were determined by using the complete data set consisting of 176 archaeal sequences or using subsets of these sequences and the appropriate tool of the ARB package. The statistical significance of interior nodes was tested by bootstrap analysis by the neighbor-joining method (ARB; 1,000 data resamplings). To exclude chimeric rDNA primary structures prior to phylogenetic analysis, the terminal 300 sequence positions of the 5′ and 3′ ends of the archaeal SSU rDNA sequences were used in separate treeing analyses. Such chimerae may be produced during the mixed PCR amplification of SSU rDNA sequences (22, 26, 49).

Diversity. Clones that had similar (<1% dissimilarity) SSU rDNA sequences and were phylogenetically placed in the same cluster were assumed to belong to the same operational taxonomic unit (OTU). Each clone was assigned a random number. The cumulative number (y) of the different OTU was determined by plotting them against the clone number (x). The plots were fitted by a hyperbolic equation [$y = ax/(b + x)$] by using ORIGIN software (version 5.0; Microcal Software, Inc., Northampton, Mass.). The Shannon-Weaver diversity index (H) was calculated from the number (n) of clones analyzed and the number of clones with identical OTU (n_i), and corrected for the maximum theoretically possible H (H_{max}) to give the equitability (J) (2): $H = \ln n - 1/n\ \Sigma(n_i \ln n_i)$; $J = H/H_{max}$.

Nucleotide sequence accession number. The sequences of the environmental slurry SSU rDNA clones obtained in this study have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the following accession numbers: clones ST1-1 to ST1-30, AJ236452 to AJ236481; clones S15-1 to S15-30, AJ236482 to AJ236511; and clones S30-1 to S30-30, AJ236512 to AJ236541, respectively.

RESULTS

Anoxic slurries of Italian rice field soil were incubated at 30°C until CH_4 was produced at a constant rate of 28.1 ± 4.6 nmol h^{-1} g of dry $soil^{-1}$ (Fig. 1). After 160 days, the temperature in one set of samples was shifted from 30°C to 15°C, whereas the other set was further incubated at 30°C. The shift to a lower temperature resulted in decrease in the CH_4 production rate (6.6 ± 0.7 nmol h^{-1} g of dry $soil^{-1}$), a decrease in the steady-state H_2 partial pressure, and transient accumulation of acetate (Fig. 1). Virtually the same results were obtained before in similar experiments and have been interpreted and discussed in detail by Chin and Conrad (8), who addressed the different functions of the microbial communities at 15 and 30°C. Soil samples from the present temperature shift experiment were used for the extraction of DNA in order to analyze the archaeal community structure (Fig. 1). One sample (ST1) was taken just before the temperature shift, i.e., from soil that had been incubated for 150 h at 30°C. Two other soil samples were taken after the temperature shift, one (S30) in the control soil that had then been incubated at 30°C for another 350 h and the other (S15) in the soil which had been shifted to 15°C and then further incubated at this lower temperature for 350 h.

The DNA extracts obtained from the three soil samples were used to construct three clone libraries of archaeal SSU rDNA. From each clone library, 30 clones were randomly selected and

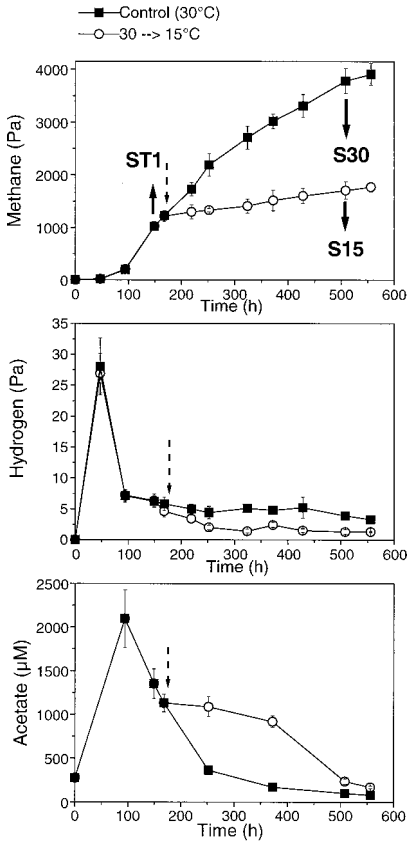

FIG. 1. Effect of temperature change on the production of CH_4, H_2 and acetate in slurries of anoxic rice field soil. Values represent the mean ± standard deviation of three experiments. Dotted arrows indicate temperature shift; solid arrows indicate sampling for molecular analysis.

characterized by T-RFLP and sequence analysis. T-RFLP analysis resulted for each clone in a characteristic fragment length (Table 1). Out of 90 clones, 53 gave a terminal restriction fragment with a length of 185 bp, 12 gave one with a length of 284 bp, and 14 gave one with a length of 392 bp. Further restriction fragments with lengths of 75, 83, 91, 169, and 490 bp were less frequent and were only represented by one clone each. Restriction fragments with a length of >700 bp were not clearly separated from each other and were represented by five clones.

Sequence analysis allowed the integration of each clone into a phylogenetic tree and its affiliation with known taxa of *Archaea* (Fig. 2 and 3). About 27% of the clones examined (i.e., 24 of 90) were affiliated with the *Crenarchaeota* and formed two distinct clusters (Fig. 2). One was the rice cluster IV described by Großkopf et al. (18), the other was a cluster (in the following discussion, termed "rice cluster VI") originally described by Bintrim et al. (5) for archaeal clones retrieved from agricultural soil. Rice cluster VI only contained clones retrieved from soil sample ST1 (i.e., soil before the temperature shift). One clone (S30-1; T-RFLP fragment length, 75 bp) was a chimera (Table 1).

About 22% of the clones examined (i.e., 20 of 90) were affiliated with the novel *Euryarchaeota* described by Großkopf et al. (18) (Fig. 3). Most (i.e., 15) clones fell into rice cluster I, which most likely represents a novel group of methanogenic microorganisms (25). One clone (S30-29) was tentatively affiliated with rice cluster II (Fig. 3). A few (five clones) fell into rice cluster V of the novel *Euryarchaeota* with an unknown phenotype (Fig. 4).

Finally, about 39 and 7% of the clones examined (i.e., 35 and 6 out of 90, respectively) belonged to the families *Methanosarcinaceae* and *Methanosaetaceae*, respectively (Fig. 4). The methanosaetaceal clones were exclusively retrieved from soil samples incubated at 15°C (S15). Only one clone was closely related to the *Plagiopyla nasuta* symbiont (family *Methanomicrobiaceae*), and another one to *Methanobacterium bryantii* (family *Methanobacteriaceae*), both retrieved from soil at 15°C (Fig. 4).

The results of the sequence analysis were combined with the T-RFLP analysis in order to characterize the major phylogenetic groups with a typical restriction fragment length (Table 1). Thus, restriction fragments with a length of 185 bp were related to either *Methanosarcina* sp. or to *Crenarchaeota*-rice cluster VI; those with a length of 284 bp were either related to *Methanosaeta* sp., to novel *Euryarchaeota*-rice cluster V, or to *Crenarchaeota*-rice cluster IV; and those with a length of 392 bp were related to the novel *Euryarchaeota*-rice cluster I.

Clones belonging to *Crenarchaeota*-rice cluster IV mostly showed restriction fragment lengths of >700 bp (Table 1). One crenarchaeotal clone, S15-28, belonging to rice cluster VI

TABLE 1. Lengths of restriction fragments of T-RFLP analysis of the different rDNA clones obtained from anoxic rice slurries (ST1, S30, and S15) and affiliation with a distinct phylogenetic lineage by rDNA sequence analysis of the clones as shown in Fig. 2 to 4

Clone type	Phylogenetic lineage	Restriction fragment length (bp)
S30-1	Chimera	75
S15-28	*Crenarchaeota*-rice cluster VI	83
S15-30	*Methanomicrobiaceae*	83
S15-14	*Methanobacteriaceae*	91
S30-29	Novel *Euryarchaeota*-rice cluster II	169
S30-3, -4, -5, -6, -7, -8, -10, -11, -12, -13, -14, -15, -16, -17, -18, -19, -20, -21, -23, -24, -25, -26, -27, -28; S15-7, -13, -15, -17, -18, -19, -21, -22, -23, -29; ST1-13, -29	*Methanosarcinaceae*	185
ST1-6, -7, -8, -9, -10, -11, -12, -14, -15, -17, -20, -21, -22, -23, -24, -26, -27, -30	*Crenarchaeota*-rice cluster VI	185
S15-8, -9, -10, -20, -24, -25	*Methanosaetaceae*	284
S30-7; S15-2, -4, -6; ST1-16	Novel *Euryarchaeota*-rice cluster V	284
S15-1	*Crenarchaeota*-rice cluster IV	284
S30-2, -9, -30; S15-3, -5, -11, -12, -16; ST1-1, -2, -5, -18, -25, -28	Novel *Euryarchaeota*-rice cluster I	392
ST1-19	Novel *Euryarchaeota*-rice cluster I	490
S30-22, S15-26, -27; ST1-3, -4	*Crenarchaeota*-cluster IV	>700

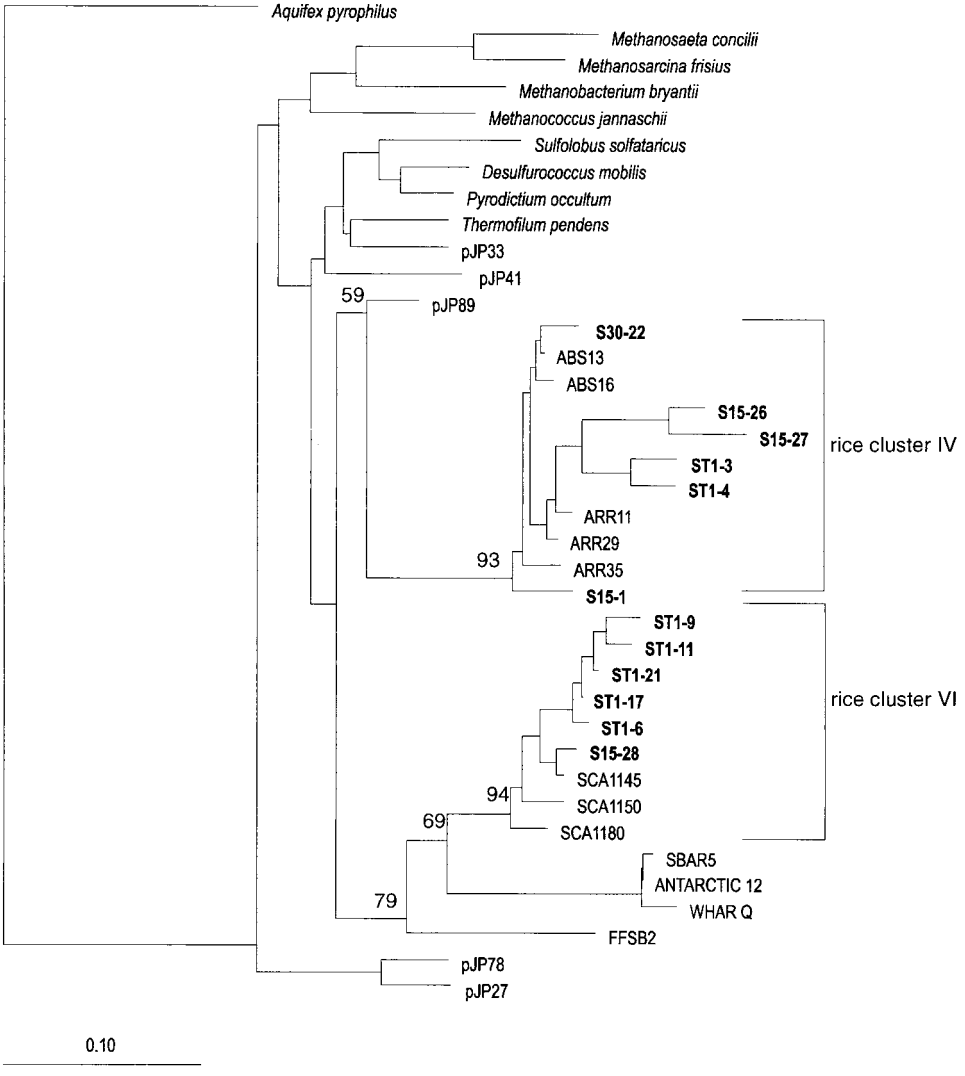

FIG. 2. Evolutionary distance dendrogram showing SSU rDNA sequences of representative soil clones (complete list in Table 1) in relation to known sequences of *Crenarchaeota*, including environmental sequences retrieved from a hot spring in the Yellowstone Park (pJP27, pJP78, pJP33, pJP41, and pJP89 [4]), from coastal marine environments (SBAR5, ANTARCTIC 12, and WHAR Q [15]), from forest soil (FFSB2 [21]), from agricultural soil (SCA1145, SCA1150, and SCA1180 [5]), from rice roots (ARR11 and ARR29 [18]), and bulk rice soil (ABS13 and ABS16 [17]). The scale bar indicates the estimated number of base changes per nucleotide sequence position.

showed a restriction fragment length of 83 bp. The same restriction fragment length of 83 bp was also found for the *Methanomicrobiaceae* clone, whereas that of the *Methanobacteriaceae* clone was 91 bp. The novel *Euryarchaeota* clone, S30-29, which was tentatively affiliated with rice cluster II, exhibited a characteristic restriction fragment length of 169 bp. This clone was tentatively assigned to rice cluster II, since a similar *Taq*I restriction site causing the T-RFLP fragment length of 169 bp was detected in the rDNA sequences of the clones ARR11 and ARR29 that form rice cluster II (18). However, these latter clones would have given a shorter T-RFLP fragment length because of a second *Taq*I restriction site further upstream.

When DNA extracts obtained from the three different soil samples (ST1, S30, and S15) were directly analyzed by T-RFLP prior to the construction of clone libraries, typical fragment length patterns were obtained (Fig. 5). These patterns showed that all of the restriction fragments detected in the clone libraries (Table 1) were also found when the environmental DNA was directly analyzed by T-RFLP (Fig. 5). However, the percentage of representation of each restriction fragment type in the clone libraries and the T-RFLP analysis was not always the same. For example, the representation of the restriction fragments >700 bp in length were generally higher in the T-RFLP analysis (20 to 44% of the total peak area) than in the clone libraries (3 to 7% of the clones). In sample ST1, the restriction fragment with 91 bp was only detected in the T-RFLP analysis, representing 20% of the total peak area (Fig. 5), but was not detected in the clone library, where only one clone from sample S15 was represented (Table 1).

Nevertheless, both the T-RFLP patterns and the sequence information of the clone libraries indicated a change in the archaeal community structure from the initial sampling (ST1) to the later sampling at either 30°C (S30) or 15°C (S15). The pattern of sample ST1 showed peaks at lengths of 185, 284, and 392 bp of similar intensity and a large peak at a length of 91 bp

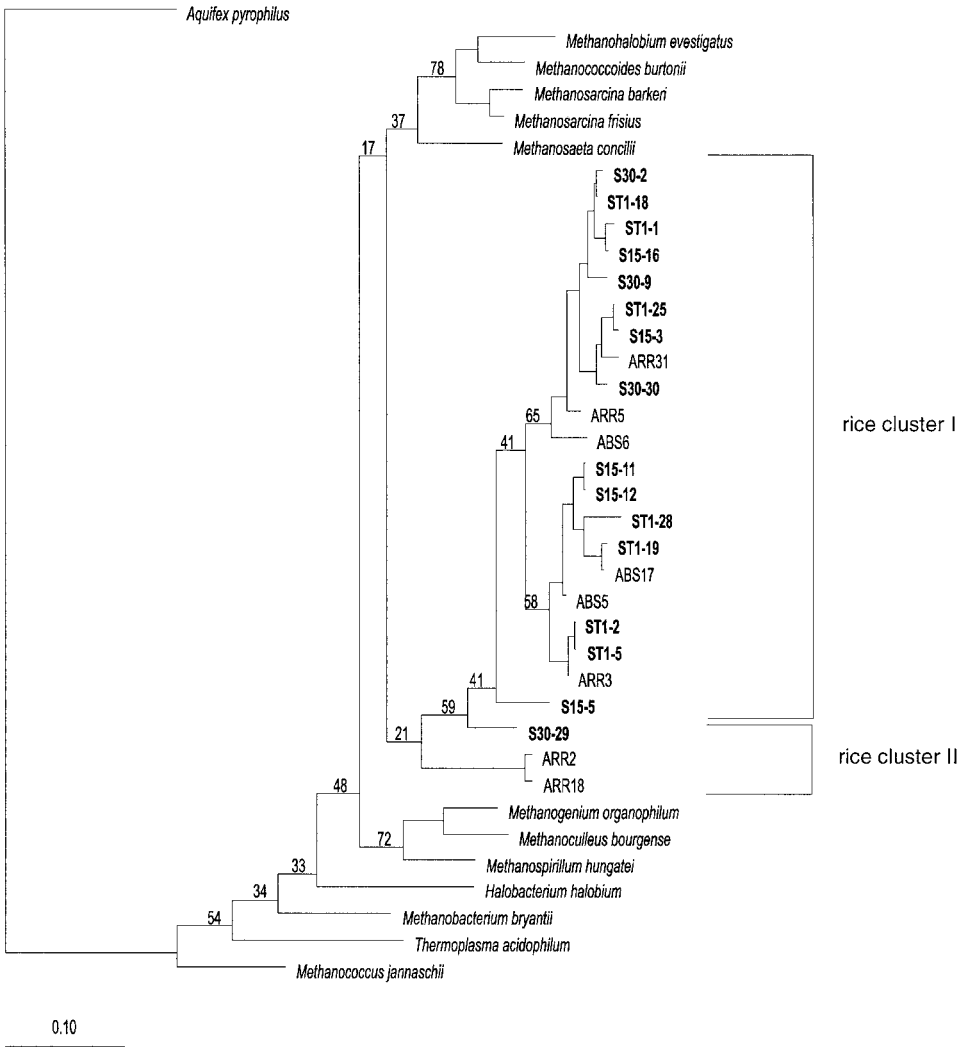

FIG. 3. Evolutionary distance dendrogram showing SSU rDNA sequences of all the soil clones exhibiting a relation to known sequences of *Euryarchaeota*, including the environmental sequences to novel *Euryarchaeota* retrieved from rice roots (termed ARR) and bulk rice soil (termed ABS) (17, 18). The scale bar indicates the estimated number of base changes per nucleotide sequence position.

(Fig. 5). According to the classification of the characteristic fragment length (Table 1), this pattern indicates the presence of *Methanobacteriaceae* (91 bp) *Methanosarcinaceae* (185 bp), *Methanosaetaceae* (284 bp), novel *Euryarchaeota*-rice cluster I (392 bp), and *Crenarchaeota*-rice cluster IV (>700 bp). Unfortunately, the peaks "characteristic" for *Methanosarcinaceae* did not differentiate against *Crenarchaeota*-rice cluster VI, many members of which may have the same restriction fragment length (Table 1), and the peaks "characteristic" for *Methanosaetaceae* did not differentiate against *Crenarchaeota*-rice cluster IV and novel *Euryarchaeota*-rice cluster V. A pattern similar to that in sample ST1 was seen in sample S15, but the relative intensities of the peaks were such that fragments with 185 and 392 bp were relatively more abundant than fragments with 91 and 284 bp (Fig. 5). However, the pattern in sample S30 was different, in that only fragments of 185 and 392 bp (i.e., *Methanosarcinaceae* or *Crenarchaeota*-rice cluster VI and novel *Euryarchaeota*-rice cluster I) were dominant (Fig. 5).

A change in the archaeal community structure was also observed when the results of the sequence analysis of the clone libraries (Fig. 2 to 4) were evaluated with respect to the different soil samples (Fig. 6). In contrast to the T-RFLP analysis, the evaluation of the sequence analysis did clearly differentiate between *Methanosarcinales*, novel *Euryarchaeota*, and *Crenarchaeota*. Before the temperature shift (ST1), the clones (n = 30) retrieved from the community were dominated by *Crenarchaeota*-rice cluster VI (60%), novel *Euryarchaeota*-rice cluster I (23%), and *Methanosarcinacaeae* (7%). Further incubation at 30°C (S30) resulted in a relative increase of *Methanosarcinaceae* to 77% of the clones, whereas further incubation at 15°C (S15) resulted in a much more diverse community consisting of 33% *Methanosarcinaceae*, 20% *Methanosaetaceae*, 17% novel *Euryarchaeota*-rice cluster I, and 13% *Crenarchaeota* (mostly rice cluster IV). In addition, *Methanomicrobiaceae* and *Methanobacteriaceae* were detected by one clone each. Novel *Euryarchaeota*-rice cluster V clones were found at all temperatures. The presence of *Methanosaetaceae* at only 15°C was conspicuous.

The diversity of the archaeal communities in the different soil samples (ST1, S15, and S30) was determined by comparison of the SSU rDNA sequences of the individual clones. Some of the clones had similar sequences which differed by less than 1% (i.e., <8 bp) of the rDNA segment (825 bp) analyzed. We assume that

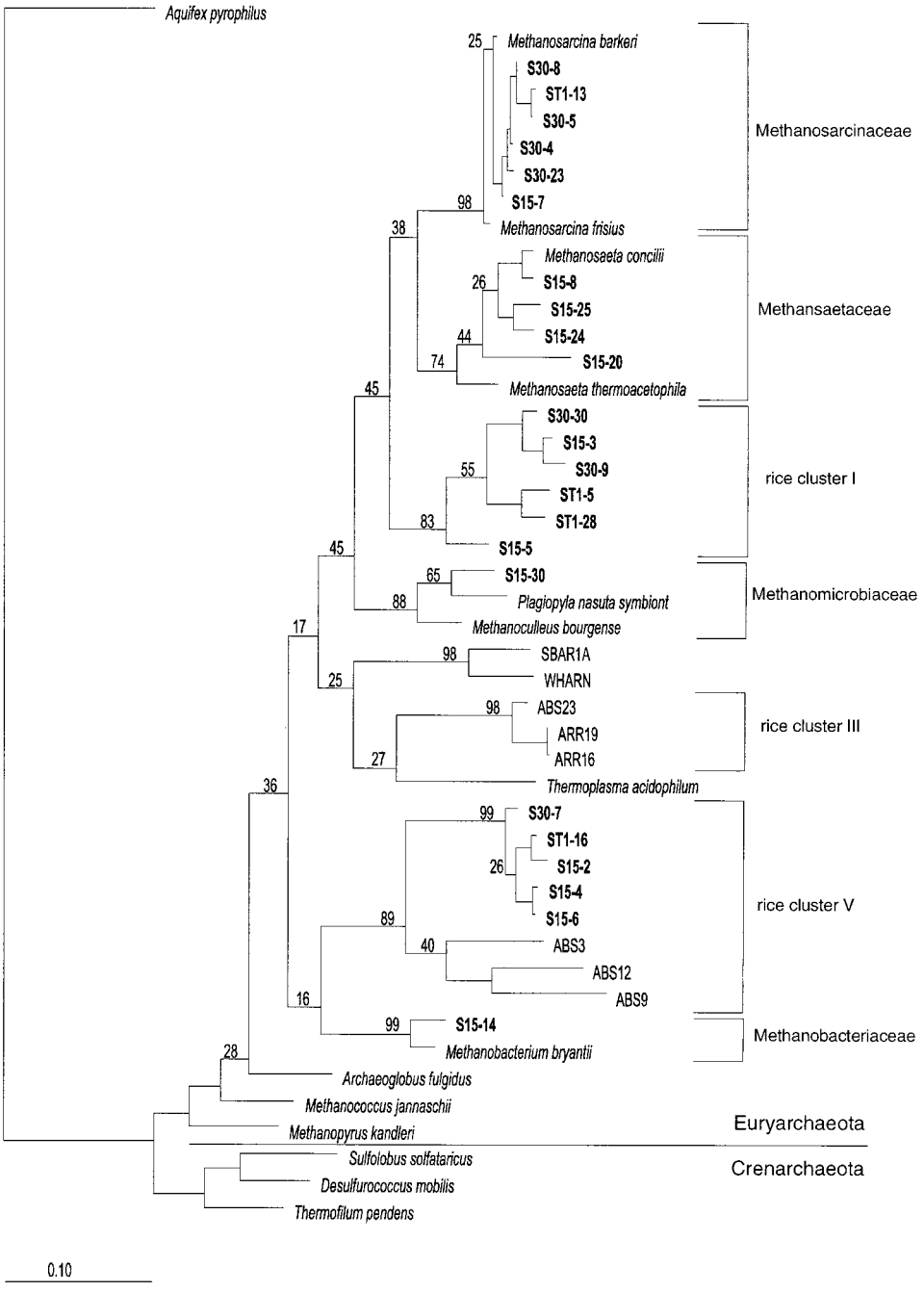

FIG. 4. Evolutionary distance dendrogram showing SSU rDNA sequences of representative soil clones (complete list in Table 1) in relation to known sequences of methanogenic *Euryarchaeota* and to environmental sequences retrieved from coastal marine environments (SBAR1A and WHAR N [15]), from rice roots (ARR19 and ARR16), and from bulk rice soil (ABS3, ABS9, ABS12, and ABS23 [17, 18]). The scale bar indicates the estimated number of base changes per nucleotide sequence position.

such a low dissimilarity would probably not justify the definition of separate species (43) and therefore combined clones with <1% dissimilarity into the same OTU. Plotting the cumulative number of OTU against the numbers of clones analyzed resulted in curves which were fitted by a hyperbolic function (χ^2 <0.27) (Fig. 7). Extrapolation of the curves to saturation indicated the presence of about 26, 26, and 50 archaeal OTU for ST1, S30, and S15, respectively. Diversity indices (equitability) were calculated from the numbers of clones per OTU and the total number of clones (n = 30) analyzed. These indices were 0.54, 0.55, and 0.85 for ST1, S30, and S15, respectively, thus indicating a higher diversity at 15°C than at 30°C.

DISCUSSION

The analysis of the archaeal community structure in anoxic rice soil slurries showed most of the major phylogenetic groups that had previously been detected in Italian rice field soil, i.e.,

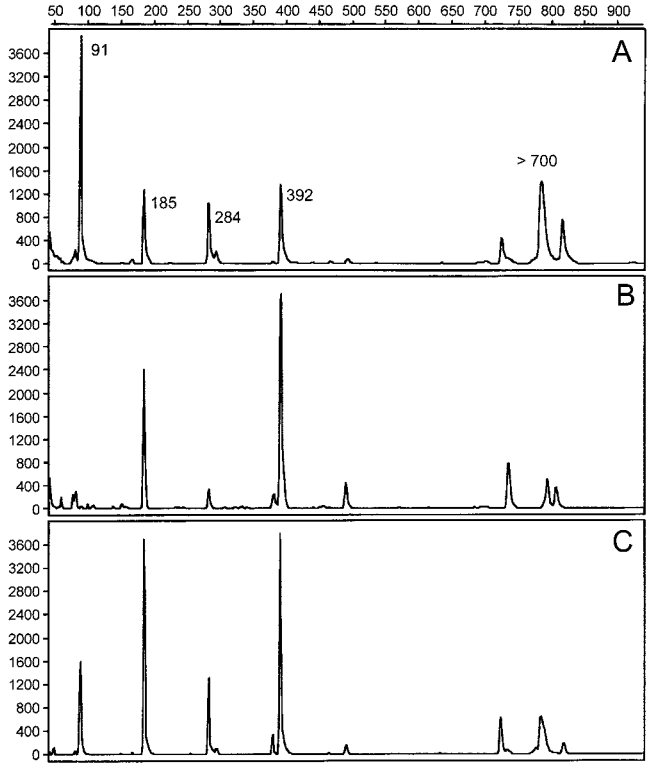

FIG. 5. T-RFLP patterns of archaeal SSU rDNA amplified from DNA extracts of anoxic rice soil slurries (A) before the temperature shift (ST1), (B) after further incubation at 30°C (S30), and (C) after further incubation at 15°C (S15). The *x* axis shows the length (base pairs) of the terminal restriction fragment, and the *y* axis shows the intensity of the bands in arbitrary units.

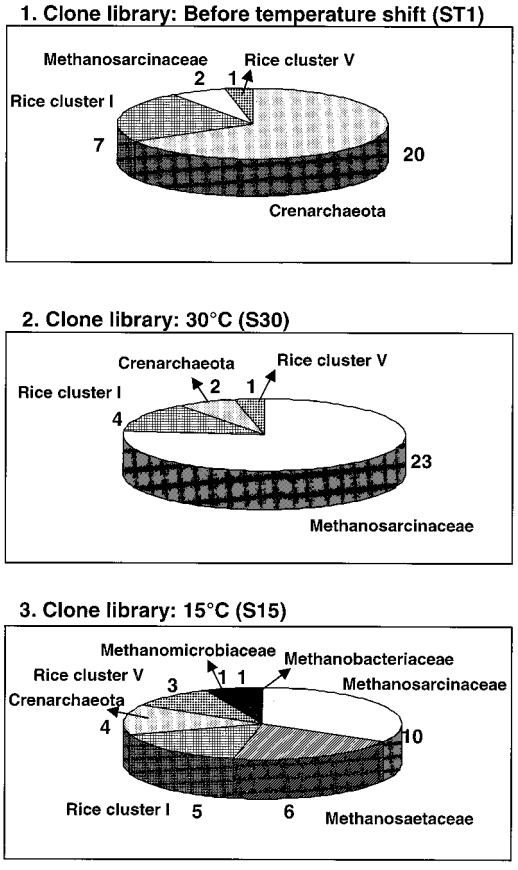

FIG. 6. Diversity of the major phylogenetic archaeal lineages in the soil samples ST1, S30, and S15 as represented by the numbers of SSU rDNA clones with characteristic sequence information.

members of the families *Methanosarcinaceae*, *Methanomicrobiaceae* and *Methanobacteriaceae* and of the novel *Euryarchaeota* and *Crenarchaeota* (17, 18, 25). The members of the novel *Euryarchaeota* have so far mainly been detected on the roots of rice, but some clones have also been retrieved from bulk soil of 90-day-old rice microcosms (17). These bulk soil clones belonged to rice clusters I, III, and V (18). Rice cluster II has so far only been detected on the roots. Interestingly, most of the novel euryarchaeotal clones obtained by us belonged to rice clusters I and V which were found in each of the soil samples analyzed. Rice cluster III, on the other hand, was not detected, and rice cluster II was found only in one tentatively assigned clone (S30-29). Possibly, members of these clusters are preferentially present on rice roots rather than in soil slurries.

A striking difference between our results obtained on soil slurries and the results of Großkopf et al. (17) obtained with bulk soil from 90-day old rice microcosms is that they observed a high abundance of *Methanosaetaceae*, whereas we observed this phylogenetic group only in slurries that had been incubated for more than 20 days and furthermore at a low (15°C) rather than high (30°C) temperature. This difference may be explained by the slow growth rates of *Methanosaeta* species, which required a longer time (>20 days) to establish themselves in soil after it became anoxic. Furthermore, we speculate that the extant *Methanosaeta* species may be more tolerant of low temperatures than the *Methanosarcina* species. Both methanogenic genera utilize acetate. However, the acetate concentration itself was not restrictive for either genus, since acetate was well above 1 mM at the 15°C incubation temperature and thus above the threshold of both *Methanosarcina* sp. (typically <1,000 μM) and *Methanosaeta* sp. (typically <100 μM) (20).

Our results indicate that the archaeal community structure changed during the course of incubation of anoxic rice field soil dependent on the incubation temperature. Sequence analysis of clone libraries showed that the relative abundance of clones

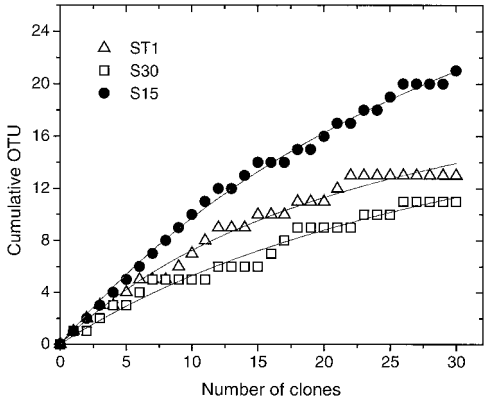

FIG. 7. Cumulative operational taxonomic units represented by the SSU rDNA clones obtained from the soil samples ST1, S30, and S15. The plots were fitted to a hyperbolic function [$y = ax/(b + x)$] with $a = 25.9 \pm 1.6$, 26.4 ± 2.8, and 49.6 ± 2.3, and $b = 25.7 \pm 2.7$, 40.0 ± 6.5, and 40.9 ± 2.8, respectively.

belonging to *Methanosarcinaceae* further increased when the soil was incubated at 30°C for another 2 weeks (a total of 3 weeks). Clones of *Crenarchaeota*, on the other hand, were relatively abundant in sample ST1, which was obtained after only 1 week of incubation, but decreased with further incubation, at both 30°C (S30) and 15°C (S15). Furthermore, the crenarchaeotal clones present in sample ST1 all belonged to rice cluster VI, whereas those in sample S30 or S15 mostly belonged to rice cluster IV. This result is plausible, if we assume that members of the *Crenarchaeota*-rice cluster VI were preferentially active in the first phase of soil flooding when inorganic electron acceptors such as Fe(III) and sulfate were still available (typically until days 5 to 7 in this particular soil) and that methanogenic archaea increased in relative abundance when methanogenesis was left as the exclusive terminal redox process. The ecophysiological role of members of the *Crenarchaeota* (especially of rice cluster VI) during the first phase of soil flooding is unknown. The same crenarchaeotal cluster has been found in other soils (5, 7). The relative change in the abundance of *Crenarchaeota* versus methanogenic archaea unfortunately could not be confirmed by the patterns of the T-RFLP analysis, since the relevant fragment lengths could not unambiguously differentiate between members of the *Methanosarcinales* and the *Crenarchaeota*.

Another interesting result was that the archaeal community structure changed in a different way when the soil slurries were further incubated at 15°C instead of 30°C. Under these conditions, clones belonging to *Methanosaetaceae* and, to a lesser extent, clones belonging to *Methanomicrobiaceae* and *Methanobacteriaceae* appeared in addition to those of *Methanosarcinaceae*, novel *Euryarchaeota*, and *Crenarchaeota*. We would like to emphasize that both the sequence analysis of clones and the T-RFLP patterns obtained with environmental DNA are not strictly quantitative, but can only indicate a general trend. Both types of analysis are based on PCR, which under the conditions used does not allow a quantification of the target DNA (30, 46). However, since we did all the analyses with the same protocol and also used the same soil, differing only in incubation time and incubation temperature, we should be able to compare the results obtained from soil samples ST1, S15, and S30 relative to each other. These data indicate that at 30°C, the methanogenic community in soil consisted mainly of *Methanosarcinaceae*, whereas at 15°C, the diversity of methanogenic archaea was larger, in particular also comprising members of *Methanosaetaceae*. The prevalence of *Methanosaetaceae* at 15°C and of *Methanosarcinaceae* at 30°C has recently been confirmed by preparing microbial enrichment cultures from rice field soil on cellulose as substrate and incubating them at either temperature (unpublished results).

The different archaeal community structures at low and high temperatures are fairly consistent with the different physiological characteristics of the CH_4-generating microbial communities at these two temperatures. Previous experiments have shown that the contribution of acetate to CH_4 production increases with decreasing temperature (8, 12–14). This relative increase in acetate-dependent methanogenesis is in agreement with the relatively high abundance of *Methanosaetaceae* at 15°C versus that at 30°C. The *Methanosaetaceae* members that have so far been isolated utilize only acetate but not H_2. In contrast, *Methanosarcinaceae* can utilize H_2 as well as acetate, and, indeed, members of this phylogenetic group were especially abundant at 30°C.

It was striking that members of *Methanobacteriaceae* were not detected at 30°C, although in particular *Methanobacterium bryantii* can easily be isolated from paddy soil by using H_2-CO_2 as a substrate (13, 16, 17). We have to assume that this group occurred in the soil only in relatively low numbers, but was able to outgrow other methanogens when transferred into media with high H_2 concentrations. It was also unexpected that clones related to *M. bryantii* were only detected at 15°C, a temperature at which typically H_2-CO_2-utilizing homoacetogens are outcompeting H_2-CO_2-utilizing methanogens (8, 13). Although the detection of *Methanobacterium* at 15°C was based on only one clone, this result was consistent with the T-RFLP patterns which showed a major peak at a fragment length of 91 bp only at 15°C. Nevertheless, this result should not be overemphasized without further research.

Our observation that the archaeal community structure changes upon incubation of soil slurries implies significant dynamics of archaeal populations, methanogens in particular. Thus, methanogens must multiply with growth rates on the order of days, since otherwise, the changes in the relative abundance of populations would not have become apparent. On the other hand, enumeration of methanogens by cultivation techniques has shown a relatively constant population size over the season in the field, over time in slurry incubations, and even over the stage when rice field soils are drained and oxic (1, 16, 29, 39). However, it is quite possible that the cultivation-based enumeration studies have underestimated the true methanogenic population size. In particular, the counting of acetate-utilizing methanogens requires very long incubation times (>40 weeks) to avoid missing abundant *Methanosaeta* species (17). Furthermore, methanogens seem to form aggregates with syntrophic bacteria and thus are probably underestimated by usual enumeration techniques (10). Hence, the population size of this methanogenic group may change more dynamically in soil as suggested by previous enumeration studies (1, 29, 39).

The archaeal diversity was highest in soil samples incubated at 15°C, consisting of about 50 OTU. The diversity was 24 OTU lower in the soil samples ST1 and S30 than in S15. Obviously, the archaeal diversity was higher at 15°C than at 30°C. This observation is consistent with that of Sekiguchi et al. (41), who recently determined in thermophilic granular sludge a lower diversity of prokaryotes (30 OTU) than in mesophilic granular sludge (53 OTU). We think that our estimation of diversity is conservative and that more archaeal genotypes and phenotypes may be present in soil which would only be detected by more refined techniques. For example, Sass et al. (36) recently detected a much higher diversity of sulfate-reducing bacteria in lake sediments when using genomic fingerprinting and established cultivation techniques than when using phylogenetic analysis of 16S rDNA clones.

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