

Crenarchaeota colonize terrestrial plant roots

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Summary

Microorganisms that colonize plant roots are recruited from, and in turn contribute substantially to, the vast and virtually uncharacterized phylogenetic diversity of soil microbiota. The diverse, but poorly understood, microorganisms that colonize plant roots mediate mineral transformations and nutrient cycles that are central to biosphere functioning. Here, we report the results of epifluorescence microscopy and culture-independent recovery of small subunit (SSU) ribosomal RNA (rRNA) gene sequences showing that members of a previously reported clade of soil Crenarchaeota colonize both young and senescent plant roots at an unexpectedly high frequency, and are particularly abundant on the latter. Our results indicate that non-thermophilic members of the Archaea inhabit an important terrestrial niche on earth and direct attention to the need for studies that will determine their possible roles in mediating root biology.

Introduction

Soil is a major reservoir of microbial biomass, contributing between 10% and 50% of the earth's terrestrial, prokaryotic carbon (Whitman *et al.*, 1998). The central role of plant roots in accessing nutrients and mediating chemical and physical processes in the soil is the concerted, and poorly understood, result of partnerships between the plant and diverse microorganisms. Plants stimulate microbial growth and colonization by allocating a significant proportion of their total photosynthate to their roots, up to 20% of which is deposited into the surrounding soil in a process called rhizodeposition (Whipps and Lynch, 1985). The rhizosphere, the volume extending a few millimetres into the surrounding soil from the rhizoplane (root surface), is a rich ecological niche abundantly exploited by microorganisms. Direct microscopic counts reveal that 90% of the microbial cells present on plant roots are not recovered by cultivation *in*

vitro (Goodman *et al.*, 1998). Thus, a large proportion of the microbiota of plant roots and, by inference, biological and chemical processes mediated by these organisms remains undiscovered.

We and others, using culture-independent methods, have recently reported ribosomal RNA (rRNA) gene sequences representing a clade of Crenarchaeota from mesophilic soils in disparate locations in the United States (Bintrim *et al.*, 1997), Finland (Jurgens *et al.*, 1997) and Japan (Ueda *et al.*, 1995; Kudo *et al.*, 1997). Sequences representing this group have also been recovered from freshwater lake sediments (Hershberger *et al.*, 1996; MacGregor *et al.*, 1997; Schleper *et al.*, 1997), marsh habitats (Hershberger *et al.*, 1996) and flooded rhizosphere soil (Großkopf *et al.*, 1998). Quantitative estimates of non-thermophilic crenarchaeotes have demonstrated their significant abundance in terrestrial (Buckley *et al.*, 1998), marine (DeLong *et al.*, 1994; Massana *et al.*, 1998) and freshwater sediment (MacGregor *et al.*, 1997) environments. Despite many studies documenting their presence in environmental samples, the actual ecological roles and specific physiological functions of these abundant and cosmopolitan Crenarchaeota remain a mystery. The work presented here demonstrates that non-thermophilic crenarchaeotes colonize and flourish on plant roots, and provides clues towards their possible roles in the rhizosphere.

Results and discussion

Recovery of Crenarchaeota rRNA sequences from washed roots

We used phylogenetic staining and epifluorescence microscopy on the plant rhizoplane and analysis of small subunit (SSU) rRNA gene sequences recovered directly from genomic DNA from washed root samples to test the hypothesis that non-thermophilic soil Crenarchaeota colonize plant roots. We seeded tomato (*Lycopersicon esculentum*, cultivar M82A) into field soil collected from the site where we previously recovered crenarchaeal rRNA gene sequences (Bintrim *et al.*, 1997). At various times after seeding, we isolated roots and washed them thoroughly with autoclaved water to remove most of the soil. Attached microorganisms were then dislodged by sonication, and total DNA was extracted from the sonicate. Four experiments were conducted in a growth chamber with field-collected soil from the West Madison Agricultural Research Station, Madison, WI, USA, and one was conducted in a field plot at that site. Ribosomal RNA gene sequences

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were amplified by polymerase chain reaction (PCR) with Archaea-biased primer sets from samples of the roots of 19 out of 20 plants sampled in the five experiments. No product was obtained using the Archaea-biased primer sets when PCR templates were prepared with roots from control seedlings germinated in the laboratory on moist, sterilized filter paper.

In a preliminary study, we cloned the PCR products obtained with Archaea-biased primer sets in *Escherichia coli* and sequenced representative clones. All 12 clones sequenced had highest similarity (> 90% sequence identity) to uncultured soil Crenarchaeota SCA clones (Bintrim *et al.*, 1997) over the 300–400 nucleotides examined. We next used oligonucleotide primers designed

specifically to amplify nearly full-length SSU rRNA gene sequences from either non-thermophilic soil Crenarchaeota (Bintrim *et al.*, 1997) or all Archaea (Barns *et al.*, 1994) by PCR with total DNA isolated from washed root sonicates as template. We cloned the resulting PCR products in *E. coli* and sequenced 10 representative full-length clones. Phylogenetic analysis showed that the rhizosphere clones (designated TRC clones) consistently clustered within the soil Crenarchaeota clade (Fig. 1). Phylogenetic placement also indicated that the TRC clones were distinct from uncultivated Crenarchaeota SSU rRNA sequences previously recovered from rice rhizospheres and rice paddy soil microcosms (ARR/ABS clones; Großkopf *et al.*, 1998). Direct sequence comparisons also supported

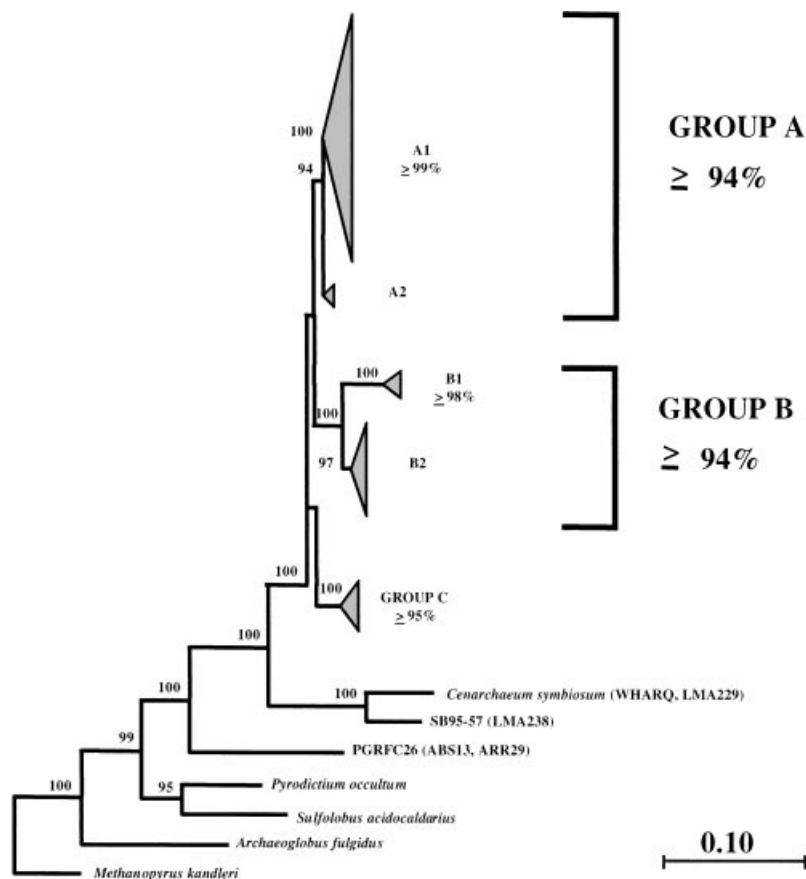


Fig. 1. Inferred, unrooted phylogenetic tree of SSU rRNA gene sequences of Crenarchaeota isolated from the tomato rhizosphere. The evolutionary tree was generated by the neighbour-joining program (TREECON Version 1.3b; Van de Peer and De Wachter 1997). Distance data were generated by neighbour joining using the Kimura (1980) two-parameter correction for multiple base changes and 1230 homologous positions of sequence from each organism or clone, with random addition of taxa. Additional trees were made using partially sequenced clones (606 homologous nucleotide positions were used), and those clones, indicated by parentheses, were added to the scaffold tree constructed from the near full-length sequences. The numbers at the bifurcations represent the proportional occurrence (only values above 50% are shown) of the respective nodes in a bootstrap analysis of 100 resamplings. The scale bar represents 10 fixed mutations per 100 nucleotides of homologous sequence positions. The clones represented by the triangles are: group A1: TRC23-28, TRC23-30, TRC23-31, TRC23-38, TRC132-6, TRC132-7, TRC132-8, SCA1145 (KBSCUL13); group A2: SCA1175; group B1: TRC132-3, SCA1154; group B2: TRC23-10, SCA1158, SCA1170, PGRFA4 (KBSNAT11); group C: TRC132-9, SCA1150, SCA1151, SCA1166, SCA1173, SCA1180 (KBSNAT4, KBSCUL9). Similarity scores are indicated for the sequences in each group. Additional clone designations: ABS/ARR, from rice soil microcosms and rice rhizospheres (Großkopf *et al.*, 1998) respectively; PGRF, from marsh and freshwater sediments (Hershberger *et al.*, 1996); KBS, from Michigan agricultural soil (Buckley *et al.*, 1998); LMA, from Lake Michigan sediments (MacGregor *et al.*, 1997); WHARQ, from North American coastal picoplankton samples (DeLong, 1992); and SB, from Pacific picoplankton samples (Massana *et al.*, 1997).

divergence of these two groups, yielding a sequence identity of between 76% and 84% between the TRC and ARR/ABS sequences, compared with a range of 91–99% sequence identity between the TRC and SCA (Bintrim *et al.*, 1997) clones (similar to the 91–99% sequence identity among TRC clones). The SSU rRNA gene sequence data for the TRC clones have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AF227635 to AF227644.

Our sequence analysis of rhizosphere archaeal clones has, thus far, only yielded evidence for members of the division *Crenarchaeota*. We may have biased our results through our choice of PCR primers, although the archaeal-biased oligonucleotide, 23FPL, has been used to generate sequences from members of the division *Euryarchaeota* in other environments (Barns *et al.*, 1994). Furthermore, only sequences that place phylogenetically with the non-thermophilic *Crenarchaeota* were obtained (Z. Cui, J. Handelsman and R. M. Goodman, in preparation) from soybean rhizospheres from two locations in south-central Wisconsin with a different set of primers used previously to amplify both *Euryarchaeota* and *Crenarchaeota* sequences from rice rhizospheres (Großkopf *et al.*, 1998). It is, however, possible that some other factor(s), such as our extraction method, may result in preferential recovery of *Crenarchaeota* over other archaeal cell types, or that we have not examined enough samples yet to detect other sequences that may be present in lower abundance or have an amplification bias.

Archaea colonize tomato roots

Washed root samples were also fixed for fluorescence *in situ* hybridization (FISH), and signals specific to an

Archaea domain probe were observed (Fig. 2A and B). Root samples that were simultaneously hybridized with oligonucleotides designed to be specific for the SSU rRNA of either *Archaea* (Arch915; Stahl and Amann, 1991) or *Bacteria* (Bact338; Amann *et al.*, 1990; and Bact927; Giovannoni *et al.*, 1990), and counterstained with 4',6-diamidino-2-phenylindole (DAPI), revealed a specific association of a small number of cells with the rhizoplane of both tap and lateral roots, as well as on root hairs. These results indicate that *Archaea* are widely distributed on these root samples. The fact that extensive washing (see *Experimental procedures*) failed to remove the cells from the root is consistent with the formation of intimate and specific, rather than casual, root associations by these microorganisms.

However, many more cells were observed hybridizing to both Arch915 and the bacterial domain probes, suggesting either previously unknown phylogenetic groups of organisms or non-specific binding by the Arch915 probe. Although Arch915 did not hybridize to the majority of bacterial control strains tested, we observed its hybridization with two cultured *Bacillus* species. This result indicated that Arch915 might be hybridizing to the SSU rRNA sequences of certain bacteria on the roots. To evaluate this possibility by another approach, we performed PCR reactions using each of two different oligonucleotides specific for the SSU rRNA gene sequences of the domain bacteria (27F; Giovannoni, 1991; and Bact338) as forward primers and Arch915 as a reverse primer, with root sonicate DNA as template. We obtained DNA products of the predicted size with both sets of primers (data not shown). Cloning, sequence and phylogenetic analysis (to be reported elsewhere) revealed that these products were bacterial in origin. This is consistent with the idea that

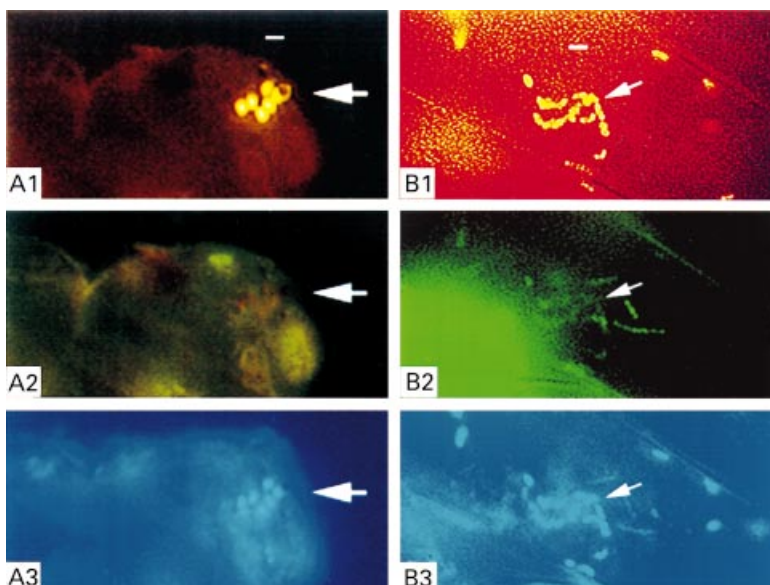


Fig. 2. *In situ* hybridization of the tomato rhizoplane for *Archaea*. Fluorescent micrographs of tomato (A) root hair and (B) rhizoplane hybridized with the oligonucleotide probes Arch915 (1), Bact338 and Bact927 (2) and stained with DAPI (3). Arrows indicate the same position on the root surface in each series of images. The scale bar represents 5 μ m.

Arch915 hybridizes to a number of different bacterial phylotypes under the conditions of the assay, and that only those cells that hybridized to Arch915, but not to either Bact338 or Bact927, are Archaea.

Design of Crenarchaeota-specific probes for FISH

Because rRNA gene sequence data indicated the presence of members of the non-thermophilic soil clade of Crenarchaeota, we next used those sequences recovered from both soil and washed root samples to design a specific probe to detect members of this clade by FISH. Upon examining fixed root samples, we were unable to find, reproducibly, cells that hybridized to the probe. Others have only detected non-thermophilic crenarchaeotes *in situ* using a mixture of specific probes (Preston *et al.*, 1996). We therefore designed additional probes, designated Cren113a, Cren745a and Cren1209 (referred to as Crenarchaeota-specific probes hereafter), and used these in combination for FISH. We observed specific FISH signals on the roots of all plants examined with these Crenarchaeota-specific probes, using the domain-specific bacterial probes Bact338 and Bact927 as negative controls. No FISH signal was observed when the Crenarchaeota-specific probes were tested against diverse cultured bacterial strains, including the *Bacillus* strains that gave a positive signal with Arch915.

In another control, PCR using either an archaeal or a bacterial domain-specific oligonucleotide as a forward primer, each of the Crenarchaeota-specific oligonucleotides as reverse primers and root sonicate DNA as template only produced DNA product with the archaeal forward primer (data not shown). This result, in addition to the fact that no hybridization was seen with control bacterial strains, suggested that the Crenarchaeota probes had higher specificity than that observed for Arch915 in our assays.

We also developed a novel expression system for use as a positive control and to optimize hybridization conditions for these probes, based on inducible expression of a nearly full-length rRNA gene from an uncultured Crenarchaeota clone in *E. coli*. We constructed a heterologous rRNA gene expression vector by cloning the SSU rRNA gene sequence from an environmental Crenarchaeota clone (SCA1175, GenBank accession no. U62819) downstream of an inducible promoter (Lanzer and Bujard, 1988). *E. coli* cells expressing this construct hybridized with each of the Crenarchaeota-specific probes and with Bact927 (to be reported in detail elsewhere).

Detection and abundance of Crenarchaeota on roots

We observed a number of different morphological cell types hybridizing to the Crenarchaeota-specific probes on

both young and senescent rootlets (Fig. 3A–D) and in rhizosphere soil (Fig. 3E); the most commonly observed morphology was coccoid but highly irregular, similar to that described for most members of the order Sulfolobales. In two experiments measuring cells on young, secondary lateral rootlets from four plants ranging from 7 to 8 weeks old, the abundance of Crenarchaeota was about 3.3% relative to the sum total of cells hybridizing to either Bacteria- or Crenarchaeota-specific probes (Crenarchaeota mean 6.6 ± 3.6 cells; Bacteria mean 194 ± 83.9 cells per plant, total cells counted in six microscopic fields \times two rootlets per plant) (Table 1). On senescent rootlets, the abundance of crenarchaeal cells was about 16% relative to total probe-positive cells (Crenarchaeota mean 69.5 ± 36.3 cells; Bacteria mean 372 ± 202 cells per plant). We observed a highly significant difference between the higher frequency of crenarchaeal signal on senescent, compared with that on younger, rootlets ($P < 0.0001$) from these plants. The frequency with which we observed pairs of cells and microcolonies (defined as four or more cells touching or in very close proximity, in order to appear to be non-randomly grouped) was also significantly higher on senescent rootlets ($P < 0.001$ and 0.02 respectively), as was the total crenarchaeal cell number ($P < 0.002$). Microcolonies containing crenarchaeotes on senescent rootlets also frequently contained higher cell numbers compared with those on non-senescent rootlets of the same age or with those in rhizosphere soil (although rhizosphere soil was only examined from two plants in one experiment).

Bacteria were also observed more frequently ($P < 0.05$), their microcolony formation was more frequent ($P < 0.006$), and their overall abundance was higher ($P < 0.05$) on senescent rootlets compared with younger rootlets (Table 1). However, the number of crenarchaeal cells was 10-fold higher on senescent rootlets compared with the number on younger roots, although we estimated that the number of bacterial cells only differed by two- to a fewfold in the same samples. The greater microbial cell density and decay of the senescent rootlets interfered to some degree with cell counts and made it difficult to assess the bacterial cell numbers with higher confidence. Nevertheless, there is a clear difference in the magnitude of the increase in crenarchaeotes compared with that of total bacteria on the senescent rootlets.

We also attempted to quantify total DAPI-stained cells, but root fluorescence in most fields examined with the UV filter set prohibited an assessment of the entire field before photobleaching occurred. The greater UV fluorescence also interfered with image quality; thus, counts could not typically be taken from micrograph images. We observed occasional regions of the root with less background fluorescence and, over the course of several experiments, quantified DAPI-stained cells in those regions.

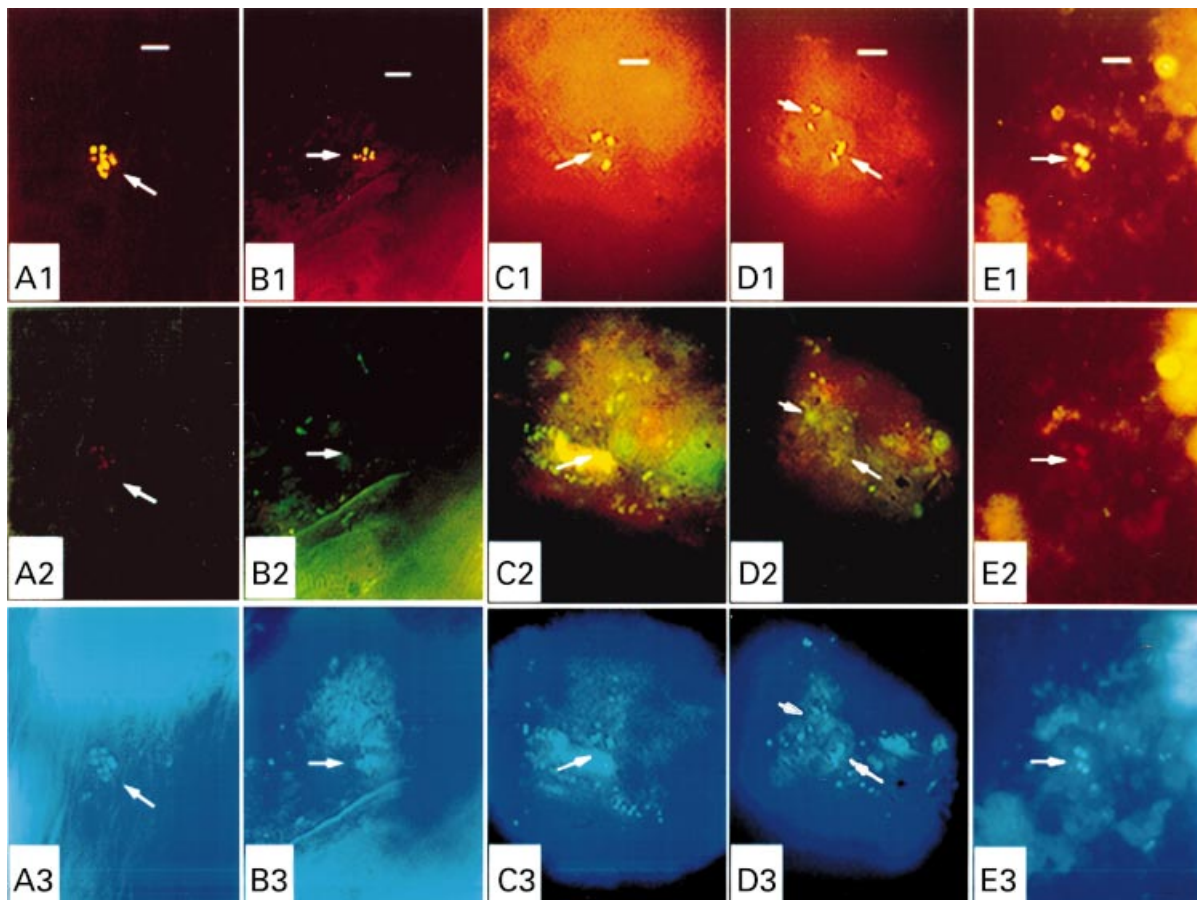


Fig. 3. *In situ* hybridization of the tomato rhizosphere for Crenarchaeota. Fluorescent micrographs of the tomato rhizoplane of 1-week-old (A) tap and (B) lateral rootlets; (C and D) senescent rootlets; and (E) rhizosphere soil hybridized with the oligonucleotide probes specific for non-thermophilic Crenarchaeota (see text) (1), with the oligonucleotide probes Bact338 and Bact927 specific for Bacteria (2) and stained with DAPI (3). Arrows indicate the same position on the root surface in each series of images. The scale bar represents 5 μm .

In these cases, the number of DAPI-stained cells typically agreed fairly closely with the number of fluorescently labelled cells (in 149 fields, 88.6% were the same or within a few cells using bacterial and archaeal domain probes and DAPI, 8% had two or three times higher counts with DAPI, and 3.4% had 10 times higher counts with DAPI).

It is not surprising that the majority of cells observed by DAPI are metabolically active and therefore can be observed by FISH, given that microbial activity of the rhizosphere is greatly enhanced compared with, for example, bulk soil. These data suggest that counts normalized to total probe-positive cells are similar to (within reasonable error) counts normalized to total DAPI-stained cells, although obviously some metabolically active cells may not be detected.

Colonization of roots by Crenarchaeota over time

To pursue the idea that Crenarchaeota are particularly good colonizers of senescent roots, we performed a time course experiment. We seeded tomatoes into field-collected soil in the growth chamber and harvested

tomato roots each week, over a period of 6 weeks. Although the abundance and frequency of crenarchaeal cells observed were relatively high on both tap roots and primary lateral rootlets 1 week after planting, they decreased significantly with the age of the plant ($P < 0.0001$ for frequency of detection on tap roots, and $P < 0.04$ for primary lateral rootlets at week 1 compared with weeks 2–5) (Table 2). The decrease in the frequency of pairs of crenarchaeal cells after 1 week was also significant on both tap ($P < 0.0001$) and lateral ($P < 0.05$) rootlets, but the frequency of microcolonies ($P = 0.07$ and $P = 0.29$ respectively) and number of cells ($P = 0.12$ and $P = 0.068$ respectively) were not. In these two cases, high sample variation may have affected the statistical analysis disproportionately, for even though the frequency of microcolonies on tap roots did not decrease significantly after week 1, it is noteworthy that we observed microcolonies at a fairly high frequency (mean $42 \pm 12\%$) on both of the 1-week-old tap roots, but at a much lower frequency (mean $2.1 \pm 6.0\%$) and on only one of the eight older tap roots. Furthermore, the decrease in

Table 1. Enumeration of Crenarchaeota and Bacteria on tomato roots.

Expt	Rootlet condition ^a	Plant ^b	Crenarchaeota				Bacteria		
			% Fields ^c	% Fields ^c with pairs	% Fields ^c with Mcol ^d	Total cell no. ^e	% Fields ^c	% Fields ^c with Mcol ^d	Total cell no. ^{ef}
1	Young	A	33	0	0	8	92	50	189
1	Young	B	8.3	8.3	0	4	92	25	95
1	Young	C	25	8.3	0	4	92	58	228
1	Young	D	67	0	0	10	92	33	275
		Mean	33	4.2	0 ^g	6.5 (3) ^h	92	35	197 (76.4)
1	Senescent	A	75	33	0	28	100	100	848
1	Senescent	B	100	67	25	107	92	67	250
1	Senescent	C	75	33	0	26	92	42	292
1	Senescent	D	100	92	42	128	100	75	296
		Mean	88	42	17	72 (53)	96	71	422 (285)
2	Young	E	8.3	0	0	1	100	42	136
2	Young	F	17	8.3	0	8	92	50	157
2	Young	G	42	17	0	12	100	83	345
2	Young	H	17	17	0	6	83	42	129
		Mean	21	10	0	6.8 (4.6)	94	54	192 (103)
2	Senescent	E	92	25	25	58	100	92	269
2	Senescent	F	100	58	8.3	59	100	75	271
2	Senescent	G	83	50	8.3	60	100	92	300
2	Senescent	H	92	50	33	91	100	75	447
		Mean	92	46	19	67 (16)	100	84	322 (84.7)

a. At the time of harvest.

b. Letters indicate individual plants.

c. Frequency of microscope fields examined that contained Crenarchaeota or Bacteria; six fields per rootlet were examined for each of two rootlets per plant, for both young and senescent rootlets.

d. Microcolonies.

e. Enumerated in six microscope fields per rootlet, and for two rootlets per plant.

f. Minimum estimate.

g. A frequency of 0.85% was observed for microcolonies on young rootlets, examining 10 whole rootlets from four different plants.

h. Numbers in parentheses indicate standard deviations.

crenarchaeal cell numbers after 2 weeks on tap and 1 week on lateral rootlets is not significant at $\alpha = 0.05$, but is significant at $\alpha = 0.1$.

These data suggest that, although Crenarchaeota do colonize young rootlets, as shown by their relatively high abundance and high frequency of pair and microcolony formation on 1-week-old tap and lateral rootlets, they may not compete well there against other rhizosphere microorganisms. These data demonstrate that Crenarchaeota either persist on, or colonize anew, rootlets of up to several weeks old. Their abundance is much greater, however, on senescent rootlets of a similar age. Senescent rootlets were present in our samples after 21 days in this experiment and, in all cases, these supported high cell numbers and a high frequency of microcolony formation by Crenarchaeota (Table 2). These results corroborate those from our experiments on 7- to 8-week-old plants.

Taken together, our data suggest that function(s) of Crenarchaeota may relate to late stages of root development. It is also possible that their abundance on very young rootlets has important implications for root biology and archaeal ecology. Observed differences in colonization

on young and senescent rootlets may reflect differences in the composition or amount of root exudation, as these are known to be affected by plant age (Rovira, 1956; 1959; Lugtenberg *et al.*, 1999). The occurrence of microcolonies containing relatively small cell numbers and our failure to detect cells using a single fluorescent probe are consistent with the hypothesis that the apparent relative abundance of crenarchaeotes on these roots is a result of their slow growth rates. This may also be a factor in determining the colonization patterns of these microorganisms.

Colonization patterns of Crenarchaeota on lateral roots

We compared the colonization patterns of Crenarchaeota with those of total rhizoplane-colonizing bacteria on young, secondary lateral rootlets of 7- to 8-week-old plants. We analysed the abundance of both crenarchaeotes and bacteria relative to their distance from the point of lateral root emergence (Fig. 4). These data indicate that crenarchaeal abundance was greatest in the older regions (within 0.42 mm from the point of emergence) of the rootlets, whereas bacterial abundance was lowest there.

Table 2. Time course of Crenarchaeota colonization on tap and lateral tomato roots.

Plant age (days) ^a	Rootlet condition ^b	Rootlet type	% Fields ^c	% Fields ^c with pairs	% Fields ^c with Mcol ^d	Total cell no. ^e	Mean cell no. ^f	% Fields with bacteria
7	Young	Tap	100	50	42	118	59 (25) ^g	100
14	Young	Tap	75	25	0	43	22 (7.8)	100
21	Young	Tap	66	8.5	0	24	12 (13)	100
28	Young	Tap	58	33	8.5	24	12 (0)	100
35	Young	Tap	58	25	0	19	9.5 (7.8)	100
7	Young	Lateral	84	38	4.2	94	47 (9.9)	100
14	Young	Lateral	71	8.5	0	38	19 (9.9)	100
21	Young	Lateral	42	4.2	4.2	19	9.5 (6.4)	100
28	Young	Lateral	42	4.2	0	17	8.5 (2.1)	100
35	Young	Lateral	62	13	0	36	18 (7.1)	100
42	Young	Lateral	38	4.2	0	20	10 (5.6)	100
21 ^h	Senescent	Lateral	100	50	33	Nc ⁱ	–	100
28	Senescent	Lateral	96	42	8.5	130	65 (35)	100
35	Senescent	Lateral	96	62	8.5	257	128 (79.9)	100
42	Senescent	Lateral	96	54	17	193	96.5 (61.5)	100

a. Age is calculated from time of planting. Two plants were sampled at each time point.

b. At the time of harvest.

c. Frequency of microscope fields examined that contained Crenarchaeota; six fields were examined for each rootlet, and either one tap or two lateral rootlets were examined per plant.

d. Microcolonies.

e. Of Crenarchaeota; enumerated in six microscope fields per rootlet for one tap or two lateral rootlets per plant.

f. Of Crenarchaeota, per plant examined (enumerated in six microscope fields per rootlet for one tap or two lateral rootlets).

g. Numbers in parentheses indicate standard deviation.

h. Only one senescent rootlet was present in one of the plants sampled.

i. Not comparable.

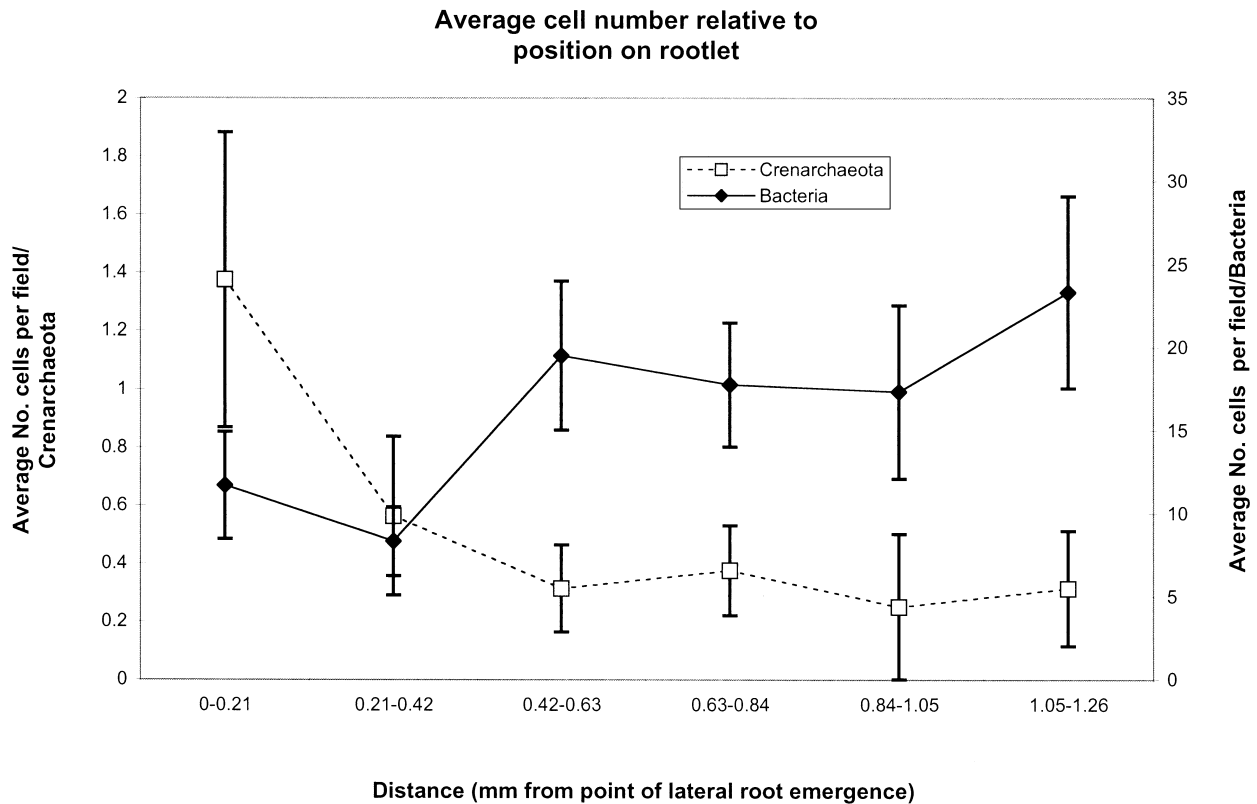


Fig. 4. Enumeration of Crenarchaeota and Bacteria relative to position on rootlet. Combined data from two experiments enumerating crenarchaeotes and total bacteria on secondary lateral rootlets from plants 7–8 weeks of age. Six microscopic fields were observed on two rootlets from each plant, starting at, and observing consecutive fields away from, the point of lateral root emergence. Each data point represents the mean of cells counted in 16 microscopic fields. Error bars represent the standard error of the mean.

The differences observed are significant for both the decrease in crenarchaeotes ($P < 0.05$) and the increase in bacteria ($P < 0.002$) in the younger regions (> 0.42 mm from the point of emergence) of the rootlets, as determined by a standard t -test.

We also analysed the data for relationships between crenarchaeal and bacterial abundance by constructing X–Y scatter plots from their abundance on both young and senescent rootlets, independently of position on the rootlet. An inverse relationship between crenarchaeal and bacterial cell numbers on young rootlets (of the 7- to 8-week-old plants) was observed (data not shown). This relationship was determined to be significant in one of the two experiments, using Kendall's Tau statistic test. No significant relationship was observed upon analysing the senescent rootlet data in this manner.

These results clearly demonstrate differences in the colonization of tomato roots by crenarchaeotes compared with total bacteria, indicating a specific preference by crenarchaeotes for the older regions of the rootlets they colonize. This inference provides further evidence for their colonization of tomato roots. It is possible that crenarchaeotes grow more slowly than most bacteria, and therefore it takes them longer to colonize younger portions of rootlets. However, the inference of their niche specialization on older root regions, revealed by these analyses, is corroborated by our data demonstrating their significantly higher abundance on senescent rootlets. Furthermore, the abundance and frequency of crenarchaeotes is relatively high on 1-week-old rootlets, compared with that on non-senescent rootlets that are 2–8 weeks old, indicating that growth rate is not the limiting factor for colonization.

Ecological significance

Rhizosphere communities are among the most microbially active terrestrial habitats. Rhizosphere microorganisms contribute to plant health and disease, decomposition of soil organic matter, recycling of crucial inorganic nutrients such as N, S and P, and temperature regulation of the earth through CO₂ turnover and CH₄ cycling. The discovery that Crenarchaeota, until recently believed exclusively to comprise extremely thermophilic archaea, make up a significant component of the root microbiota of tomato plants highlights the deficit of knowledge that exists concerning root, as well as soil, microbial communities. This discovery also raises many questions concerning the roles of these little-studied organisms in this ecologically important habitat and warrants further research that synthesizes molecular and classical techniques for their investigation. Studying the roles of rhizosphere Crenarchaeota should also provide clues for their growth in culture, which, in turn, will lead to a better understanding of their potential ecological functions.

Experimental procedures

Soil collection, plant growth and root harvest

Soil was collected from between 2 and 8 cm below the surface from a fallow site at the West Madison Agricultural Research Station using sterile implements. Tomato (*Lycopersicon esculentum* cultivar M82A) was used in our study because we are currently developing a genetic system in tomato for the study of plant–microbe interactions. Tomato seed was placed in the soil in 12-cell plastic inserts in trays (Hummert International), and the experiments were conducted in a growth chamber at 24°C, 40% relative humidity, with 12 h of continuous light (415 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by 40 W cool white fluorescent bulbs) in a 24 h period. Avoiding contamination by non-rhizosphere microorganisms, roots were harvested by removing most of the soil through gentle washing in sterile distilled H₂O. Evidence from other research suggests that the density of bacteria in the rhizosphere is greatest nearest (1–100 μm) the root (Foster, 1978; Parke, 1990), and gentle washing has been shown to remove no more than 3–4% of the total culturable bacteria from the root (Rovira, 1974).

Separation of rhizosphere soil from root-associated microorganisms

We followed the approach of Louw and Webley (1959), with modifications, to separate the rhizosphere soil from the root surface microorganisms. Instead of allowing the soil to fall passively from the roots, it was necessary, on account of the tenacity with which the soil clung to the roots, to soak, gently agitate and rinse them extensively to remove the soil.

DNA extraction

After washing, whole tomato roots were sonicated for 30 s in sterile, ultrapure water to dislodge microorganisms. Centrifugation at 15 000 g for 1 min was performed on the samples, and DNA was extracted from the resulting pellet using the FastDNA Spin kit for soil (BIO101).

PCR and library construction

The forward primers 23FPL (Barns *et al.*, 1994) or 133F, individually in combination with the reverse primer 1492R (Lane, 1991), were used in PCR, and clone libraries were constructed in *E. coli* as described previously (Bintrim *et al.*, 1997). PCR amplification was performed with a RoboCycler 40 temperature cyler (Stratagene). The cycling parameters were: 1 min denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 1.5 min and 72°C for 2.5 min. The sequence of the Crenarchaeota-specific SSU rRNA oligonucleotide forward primer 133F is 5'-TGTTGACTACGTGTTACTGAG-3'.

Sequence and phylogenetic analysis

Sequence was generated in two experiments from a total of 22 clones representing several different operational taxonomic units (OTUs) as determined by amplified ribosomal

DNA restriction analysis (ARDRA; Moyer *et al.*, 1994). These data were analysed using BLAST (Altschul *et al.*, 1997) and software provided by the Ribosomal Database Project-II (Maidak *et al.*, 1999) and the Wisconsin Package (Genetics Computing Group, Version 10.0). Alignment of the cloned sequences to representative SSU rRNA gene sequences revealed secondary structure features consistent with the proposed structure of SSU rRNA of Archaea. We did not detect chimeras using the CHECK_CHIMERA program at the RDP or examination of secondary structure. Evolutionary trees were generated by two methods, the neighbour-joining program (TREECON, Version 1.3; Van de Peer and De Wachter, 1997) and the maximum parsimony program DNAPARS (PHYLP 3.55; Felsenstein, 1993), and both these methods gave results that were in close agreement.

In situ hybridization

Root fixation, hybridization (with 35% formamide) and washing was performed as described previously (Li *et al.*, 1997). Briefly, washed roots were fixed overnight at 4°C in a mixture of 3:1 methanol–acetic acid. After washing in 70% ethanol, rootlets were incubated for 1 h with a prehybridization solution (Sigma) that was $5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate), $1 \times$ Denhardt's solution, $100 \mu\text{g ml}^{-1}$ sheared DNA and deionized formamide to 35%. After 1 h, the prehybridization solution was replaced by hybridization solution, which is prehybridization solution to which 10% dextran sulphate and $5 \text{ ng } \mu\text{l}^{-1}$ of the appropriate probe was added, and the samples were incubated for 12–16 h in the dark at 37°C. After hybridizations, samples were washed three times (10 min) at 24°C in $0.1 \times \text{SSC}$. Samples were mounted on glass slides in Fluorguard antifade medium (Bio-Rad) containing $2 \mu\text{g ml}^{-1}$ DAPI and observed by microscopy.

Probes

The following probes (Amann *et al.*, 1990; Giovannoni *et al.*, 1990; Stahl and Amann, 1991), with designations in accordance with the Oligonucleotide Probe Database (Alm *et al.*, 1996), were used in hybridizations: S-D-Arch-0915-a-A-20 (Arch915), designed to be specific for the SSU rRNA of Archaea, was conjugated with CY3; S-D-Bact-0338-a-A-18 (Bact338) and S-D-Bact-0927-a-A-18 (Bact927), designed to be specific for the SSU rRNA of Bacteria, were conjugated with Oregon green 488. All probes were purchased from Synthege and were high-performance liquid chromatography (HPLC) purified. Before microscopy, the root samples were stained for DNA with DAPI. Prior to performing FISH on roots, these probes were hybridized on Teflon-coated glass slides, as described previously (Li *et al.*, 1997), with control organisms from the three domains (see below) to determine that hybridizations with 35% formamide resulted in specific signals for the probes used, with the exception of a positive signal obtained with Arch915 for *Bacillus* spp. (see text).

The sequences of the Crenarchaeota-specific SSU rRNA oligonucleotide probes are as follows: Cren113, 5'-ATGTT GACTACGTGTTACTGA-3', used alone for FISH; Cren113a,

5'-ATGTTGACTACGTGTTACTGAG-3'; Cren745a, 5'-CCC AGCTTTCATCCCTCACC-3' (modified from Buckley *et al.*, 1998); and Cren1209, 5'-GTGGCCCGAGGGTTTCGG-3', combined for use in FISH.

Control strains for FISH

Strains used for controls included a number of organisms from the Eucarya, Bacteria and Archaea. These were received as gifts of cultures or were grown to saturation using standard growth medium as described previously (Atlas, 1993), or as indicated, and harvested for fixation. Strains from the Department of Bacteriology strain collection (DBSC), University of Wisconsin-Madison were grown in Difco nutrient broth (Becton Dickinson). The control strains included: *Aureobasidium pullulans*; *Bacillus cereus* UW85; *Bacillus megaterium* 7A1 (Bacillus Genetic Stock Center, Ohio State University); *Erwinia herbicola* 005; *Escherichia coli* 8009 (DBSC); *Leptospirillum ferrooxidans*; *Methanobacterium formicicum* (medium II; Kim *et al.*, 1996); *Methylosinus trichosporium*; *Pseudomonas fluorescens* 2-79; *Rhizobium etli* CE3 (Noel *et al.*, 1984); *Rhodobacter sphaeroides* 9502 (DBSC); *Rhodospirillum rubrum* 9405 (DBSC); *Salmonella typhimurium* LT2 (Lilleengen, 1948); *Staphylococcus aureus* 3001 (DBSC); *Sulfolobus shibatae*; *Sulfolobus solfataricus*; *Thermococcus litoralis*; *Thiobacillus ferrooxidans*; *Vibrio cholerae* F115A (DBSC); and *Yersinia enterocolitica* WA.

Microscopy

Specimens were examined with a BX-60 microscope (Olympus America) equipped for epifluorescence with an HBO 100 W mercury arc lamp. An Olympus UPlanFI 100 \times objective/1.3 NA, with a field area of $0.035 \pm 0.002 \text{ mm}^2$, was used to visualize cells. Filters used were a narrow-excitation DAPI (82360X), fluorescein isothiocyanate (FITC; 82485X) and a CY3/CY5 (51007) dual-filter set (Chroma Technology). A small-diameter aperture of the light beam was used to decrease the background fluorescence, improving the contrast between the microbial signal and root fluorescence. Images were recorded with a cooled charge-coupled device video camera (DEI-750; Optronics Engineering) and were converted from an analogue to a digital format (digitized) with a NuVista+ Videographics card (Truevision) controlled by IP Lab Spectrum 3.0 software (Signal Analytics). Final image adjustment for colour printing was done with Adobe Photoshop software version 4.0 (Adobe Systems), and printing was performed using a colour video printer, model UP-5600MD (Sony).

Quantification of frequency and abundance of Crenarchaeota

Tap and randomly chosen lateral root samples of approximately 30 mm in length were examined. Adjacent microscope fields were observed on tap roots starting 2.5 cm below the stem–root junction and proceeding away from it. Adjacent fields were observed on lateral rootlets, beginning at the root–rootlet junction and proceeding towards the tip. As senescent rootlets (judged to be senescent by their dark

brown colour and non-turgid nature) were not attached at the root–rootlet junction, a starting point was chosen randomly, and adjacent fields were examined. A standard *t*-test was performed on the results from expts 1 and 2, and it was determined that, in each category examined (field, pair and microcolony frequency, and cellular abundance), the results were not significantly different ($P > 0.05$). *t*-tests were therefore performed on the combined data from expts 1 and 2, and the resulting *P*-values are reported in the text.

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