environmental microbiology reports

Environmental Microbiology Reports (2011) **3**(4), 491–499 doi:10.1111/j.1758-2229.2011.00248.x

Role of the terrestrial subsurface in shaping geothermal spring microbial communities

Sara Tin, Richard W. Bizzoco and Scott T. Kelley*
Department of Biology, 5500 Campanile Drive, San Diego State University, San Diego, CA 92182-4614, USA.

Summary

In this study, we explored the possibility that dispersal from terrestrial subsurface sources 'seeds' the development of geothermal spring microbial assemblages. We combined microscopy and culture-independent molecular approaches to survey the bacterial diversity of spring source waters in Yellowstone National Park, Lassen Volcanic National Park, and Russia's Kamchatka peninsula. Microscopic analysis uncovered clear evidence of microbial cells from spring sources in all three regions. Analysis of source water phylogenetic diversity identified members of all bacteria groups found previously in downstream sediments, as well as many other phylogenetic groups. Closely related or identical 16S sequences were determined from the source waters of geographically distant, chemically distinct springs, and we found no association between spring water chemistry and microbial diversity. In the source waters of two different Yellowstone springs, we also discovered a phylogenetic group of uncultured *Firmicutes* never before reported in geothermal habitats that were closely related to uncultured bacteria found in the hyper-arid Atacama Desert. Altogether, our results suggest geothermal features can be connected via the subsurface over long distances and that subsurface sources provide a potentially diverse source of microorganisms for downstream surface mat communities.

Introduction

The discovery of chemolithotrophic microbes thriving at 'extremes' of temperature and pressure makes it conceivable that the subsurface harbours microbial life. Studies have documented significant microbial life under the seafloor (Kormas *et al*., 2003; D'Hondt *et al*., 2004; Teske, 2005; Hubert *et al*., 2009), in petroleum reservoirs (Magot *et al*., 2000), in deep-water aquifers (Stevens and McKinley, 1996; Chapelle *et al*., 2002; Takai *et al*., 2004; Nealson *et al*., 2005), beneath ice sheets (Priscu *et al*., 1999), and in drilling samples collected below the world's deepest mineshafts (Takai *et al*., 2001; Chivian *et al*., 2008; Wanger *et al*., 2008). The subsurface may also play a central role in shaping geothermal microbial ecosystems. Specifically, the subsurface may provide a source for the microbes found in the water and sediments of hot springs and pools, and may also be a conduit for dispersion within geothermal systems. The downstream sediments of geothermal springs harbour vibrant and diverse microbial communities that change dramatically along geochemical gradients (Donahoe-Christiansen *et al*., 2004; Boyd *et al*., 2007; D'Imperio *et al*., 2007; Mathur *et al*., 2007). Although numerous researchers have studied the diversity and chemistry of many such mats, the dispersal origins of the microbes comprising these communities are poorly understood (Bonheyo *et al*., 2005). A study by Macur and colleagues (2004) showed that visible microbial mats developed *less than one day* after the redirection of a spring outflow channel, suggesting rapid seeding of these communities (Macur *et al*., 2004).

Mechanisms of dispersal discussed in the literature include aerosol dispersal from other springs and insect dispersal. A study by Bonheyo and colleagues (2005) found evidence of aerosolization and air dispersal of geothermal microbes. However, after 48- to 72-hour sampling periods, Bonheyo *et al*. collected only a small number of cells with a large net despite the fact that they sampled adjacent to a rich microbial mat. More efficient methods of geothermal steam collection have shown that geothermal steam may contain up to 10^3 cells ml^{-1} (Ellis *et al*., 2008). Nevertheless, it is difficult to imagine how aerosols might rapidly seed a microbial mat so close to the source of a rapidly flowing spring as was observed by Macur and colleagues (2004).

Another possible source of dispersal may be waters arising from the subsurface itself. Subsurface waters may carry microorganisms into a flowing spring or along an aquifer between springs. Bonheyo and colleagues (2005) dismissed the possibility of subsurface hydrothermal sources in Yellowstone connecting very distant springs because springs from different regions are often

Received 5 February, 2010; accepted 8 February, 2011. *For correspondence. Email skelley@sciences.sdsu.edu; Tel. (+1) 619 594 5371; Fax (+1) 619 594 5676.

hydraulically isolated from one another. Nevertheless, subsurface waters may connect more closely situated springs within a hydrological region. For instance, a study of subterranean hot springs in Iceland found diverse microbial communities in deep subsurface volcanic zones (1500–2000 m deep) with many organisms closely related to those found in geothermal surface features (Marteinsson *et al*., 2001). The authors suggested that thermophilic microbes within volcanic zones may disseminate from the subsurface through water conduits. Another recent study by Boomer and colleagues (2009) found evidence that waters gushing out of geysers contained the same organisms (*Roseiflexus*-like *Chloroflexi*) that formed the basis of the artificial biofilms at the air-water interface. And, in a study of CO_2 uptake and fixation, Boyd and colleagues (2009) also collected sufficient biomass for experimentation by sampling directly from Dragon Spring origin waters. These findings suggest that the subsurface, albeit hydraulically restricted, may provide an important source for geothermal mat colonization.

To investigate the role of the subsurface in geothermal spring mat formation, we collected source-origin waters arising from 10 geothermal springs in three geothermal regions: two in the USA and one in Russia. We purposefully selected artesian springs with rapid flow rates and easy access to the spring origins. The positive pressure of the water assured that microbes sampled in the spring waters came from underground. The waters of flowing springs are not recycled underground, unlike pools (Brock and Mosser, 1975), and the positive pressure excluded airborne microorganisms from moving into the subsurface. Because we sampled source waters of flowing springs before their emergence point, we were confident that the organisms found came from below ground. Having obtained water samples, we used microscopic analysis to detect intact microbial cells and estimated cell abundance, and culture-independent methods based on 16S ribosomal RNA (16S rRNA) gene sequences to reveal the phylogenetic diversity of source-water organisms.

Results and discussion

Our results allow us to make a number of important inferences about the microbial diversity of subsurface waters in geothermal systems. Below-ground source waters of every geothermal spring tested contained intact microbial cells (Fig. 1). Phase-contrast, DAPI and SEM images provided clear evidence of microorganisms in the spring origin waters (Fig. 2, Figs S1–S3). While cell counts were relatively low on a per millilitre basis (Fig. 1), they were significant given the estimated flow rates of the various springs. Calculating from cell counts and flow rates of the sites tested, outflow channel seeding rates would range from 7.5×10^4 cells s^{-1} (spring AS101) to 1.8×10^6 cells s^{-1} (spring RM), indicating that spring source waters could be an abundant source of intact microbial cells for downstream communities. We also note that the cell counts were very conservative estimates since we counted 'clusters' of cells (see examples in Fig. 2) as a single cell because we could not reliably count the number of cells in these groups.

Culture-independent molecular analysis of bacterial 16S ribosomal RNA gene sequences also uncovered considerable diversity of organisms in the subsurface origin waters, including many *Aquificales* and a number of other groups commonly discovered in geothermal habitats (Fig. 3, Figs S4–S6). Not only did the subsurface waters contain a broad diversity of thermophilic organisms, but we also found substantial phylogenetic relatedness between organisms arising from source waters and those collected in a previous study from downstream sediments. For example, we uncovered a large number of *Hydrogenobaculum* sequences closely related, though not identical, to those found abundantly in AS101 and AS102 sediments. We also discovered organisms related to *Acidicaldus* and *Deinococcus* found previously in sediments (Fig. 3, Fig. S4). Our results are similar to those of a recent study of biofilm formation in a geyser run-off channel that found the same organisms in geothermal waters that were also present on slow-growing artificial biofilms (Boomer *et al*., 2009).

The overall diversity found in the subsurface waters, as measured by numbers of discreet phylogenetic groups, equalled or exceeded the combined diversity previously found in downstream sediments using the same PCR primer and cloning methods. A phylogenetic diversity test (PD test) using a jackknife re-sampling approach (see Appendix S1) found no significant difference between AS102 source and sediments, but found AS101 and RM source waters to be significantly more diverse than the combined diversity in the sediments (PD test; $P \leq 0.0001$). Indeed, a number of the sequences determined from the AS101 waters were closely related to sequences found in the Roaring Mountain sediments (Fig. S4). This is quite surprising given the stark chemical differences between the AS and RM (sulfur versus non-sulfur) springs and the fact that the phylogenetic diversity of the microbial communities found in the AS101 and RM sediments was essentially non-overlapping (Mathur *et al*., 2007). Our study of the origin waters (Fig. S7) and their relation to the sediments does come with a time-lapse liability. However, even if they were done concurrently there is no way to be certain about how long the sediment organisms had remained in place. The organisms in the origin waters are not identical, but closely related to those previously uncovered in our earlier sediment study (Mathur *et al*., 2007), suggesting some continuity of

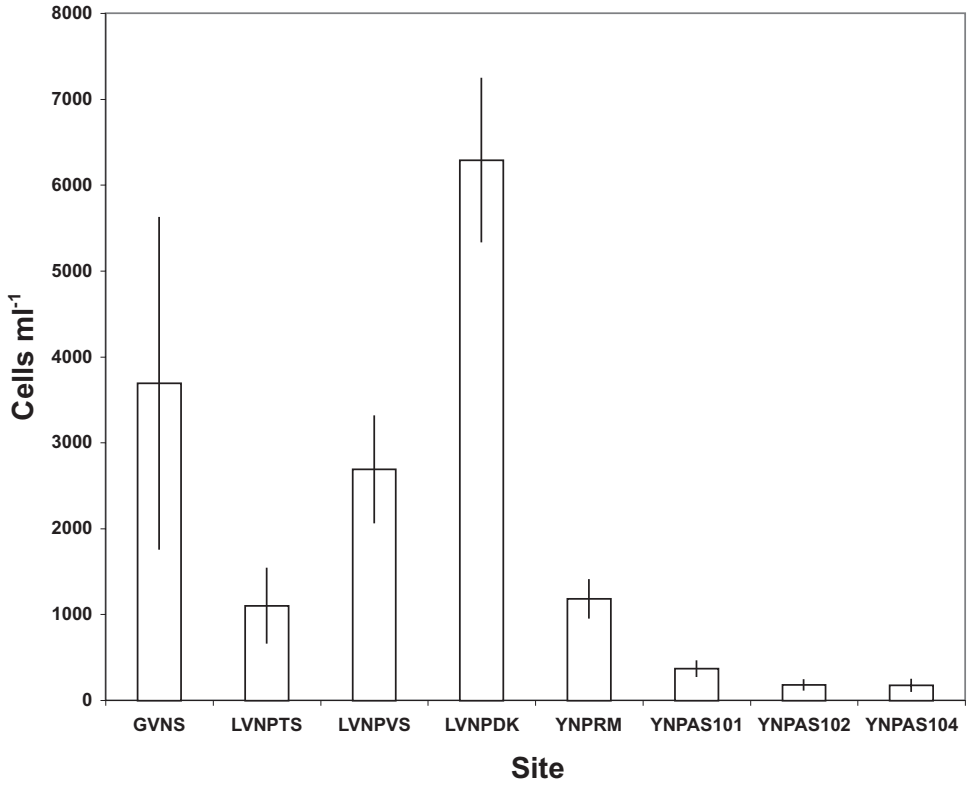

Fig. 1. Origin water cell counts of DAPI-stained cells. Counts of cells were estimated by counting at least 500 cells or 100 fields. GVNS, Kamchatka, Geyser Valley Neutral Spring; LVNPTS, Lassen Volcanic National Park, Toto Spring; LVNPVS, Lassen Volcanic National Park, Voldemort Spring; LVNPDK, Lassen Volcanic National Park, Devil's Kitchen; YNPRM, Yellowstone National Park, Roaring Mountain; YNPAS101,Yellowstone National Park, Amphitheater Springs 101; YNPAS102, Yellowstone National Park, Amphitheater Springs 102; YNPAS104, Yellowstone National Park, Amphitheater Springs 104. Cells and micro-colonies were also observed in Kamchatka samples, but cell concentrations were not estimable due to the excessive mineral fluorescence. Error bars represent 95% confidence intervals constructed using standard error and *t*-values (0.05) based on $n > 100$. A correction factor was applied to account for sample volume and area counted.

potential colonists available in the source waters. In support of this, the chemistry (Table 1) shows that there was little change over the elapsed yearlong time (Mathur *et al*., 2007), but that certainly does not take into consideration physical factors, such as meteoric conditions, that might have altered the sediment diversity.

Although we do not have downstream sediment comparisons for the Lassen and Kamchatka springs, these source waters contained even greater phylogenetic diversity than that found in the Yellowstone subsurface waters (Figs S5 and S6; PD test; $P \leq 0.0001$). Moreover, we found distantly situated source waters contained nearly identical types of organisms. The phylogenetic analysis of Lassen spring waters reveals many instances in which distant springs contained overlapping phylogenetic groups of organisms (Fig. S5). The pattern was even more dramatic in the Kamchatka springs. For example, the Geyser Valley and Uzon Caldera springs are located 24 km apart, yet both contained many sequences from the same phylogenetic groups. For example, two related 16S sequences in the *Sulfurihydrogenibium* clade, B07KNSYR05 and E12KUCYR05 came from Geyser Valley and Uzon Caldera samples, respectively, as did the *Thermus*-related clones B02KNSYR05 and F04KUCYR05 (Fig. S6). The same held for the Mutnovsky Volcano and Geyser Valley subsurface waters, situated 160 km apart (Fig. S6).

The lack of association between phylogenetic diversity and spring origins is particularly intriguing given the stark chemical and physical differences among springs. For example, the Mutnovsky Volcano spring waters had a pH of 3.5, while both the Uzon Caldera and Geyser Valley spring had neutral pH. Geyser Valley also contained very high levels of arsenic, known to be an important factor in determining sediment diversity (Clingenpeel *et al*., 2009), while Mutnovsky Volcano had high levels of sulfur resulting in a low pH (Table S1). The Lassen springs were also each very distinctive for both pH and temperature. The pH ranged from 1.5 in Devil's Kitchen to 6.7 in Toto spring, and the temperature ranged from a low of 73°C in Toto spring to 91°C in Voldemort spring (Table 1). Yet, we found many of the same phylogenetic groups in these springs.

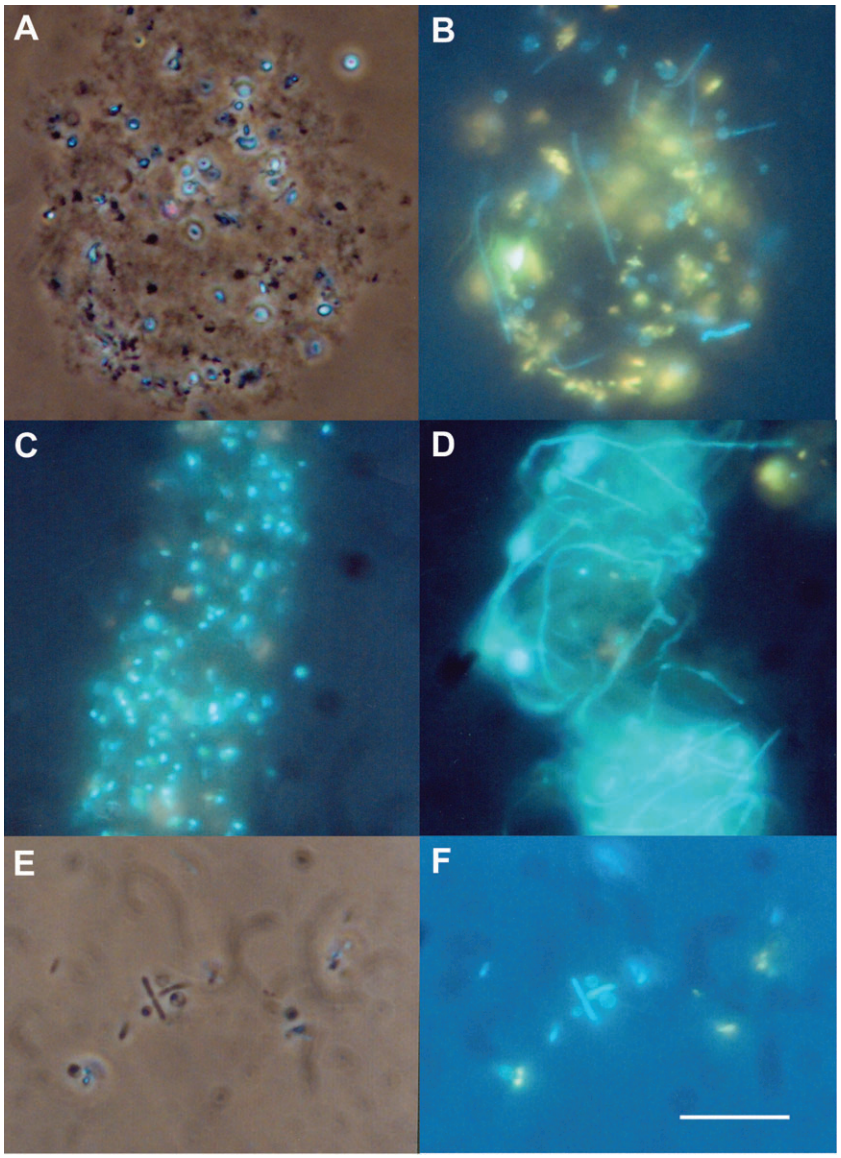

Fig. 2. Origin waters from Lassen Volcanic National Park flowing springs.
A and B. Devil's Kitchen. (A) Phase contrast; (B) DAPI.
C and D. Toto Spring, Little Hot Springs Valley. (C) DAPI; (D) DAPI.
E and F. Voldemort Spring, Little Hot Springs Valley. (E) Phase contrast; (F) DAPI. Bar, 10 μm.

Table 1. Chemical and physical characteristics of sampled springs along with GPS co-ordinates.

Spring name	Location	Region	GPS co-ordinates		pH	Source temperature (°C)	Eh (mV)	Flow rate (l s^{-1})
			Latitude	Longitude				
AS101	Amphitheater Springs	YNP	44°48′4.14″N	110°43′43.50″W	1.08	75.8	31.6	0.25
AS102	Amphitheater Springs	YNP	44°48′3.90″N	110°43′43.70″W	1.14	77	16	1.46
AS104	Amphitheater Springs	YNP	44°48′2.00″N	110°43′43.0″W	1.66	71.6	5.8	1.29
Roaring Mountain	Roaring Mountain	YNP	44°46′46.80″N	110°44′19.20″W	1.19	93.5	21.4	1.82
Devil's Kitchen	Devil's Kitchen	LVNP	40°26′27.90″N	121°25′59.50″W	1.5	88	101	0.34
Voldemort Spring	Little Hot Spring Area	LVNP	40°27′22.80″N	121°31′04.40″W	3.57	81.7	–13	NA
Toto Spring	Little Hot Spring Area	LVNP	40°27′21.60″N	121°31′04.50″W	6.7	76.9	–270	NA
Uzon Caldera	Uzon Caldera	Kamchatka	53°59′55.06″N	159°26′50.75″E	7	91	NA	NA
Mutnovsky Volcano	Mutnovsky Volcano	Kamchatka	52°28′00.74″N	158°09′26.16″E	3.5	89	NA	NA
Geyser Valley	Valley of the Geysers	Kamchatka	53°57′55.25″N	159°04′41.95″E	7	96	NA	NA

No Eh measurements were taken in Kamchatka due to lack of instrumentation.
YNP, Yellowstone National Park; LVNP, Lassen Volcanic National Park. NA, not available.

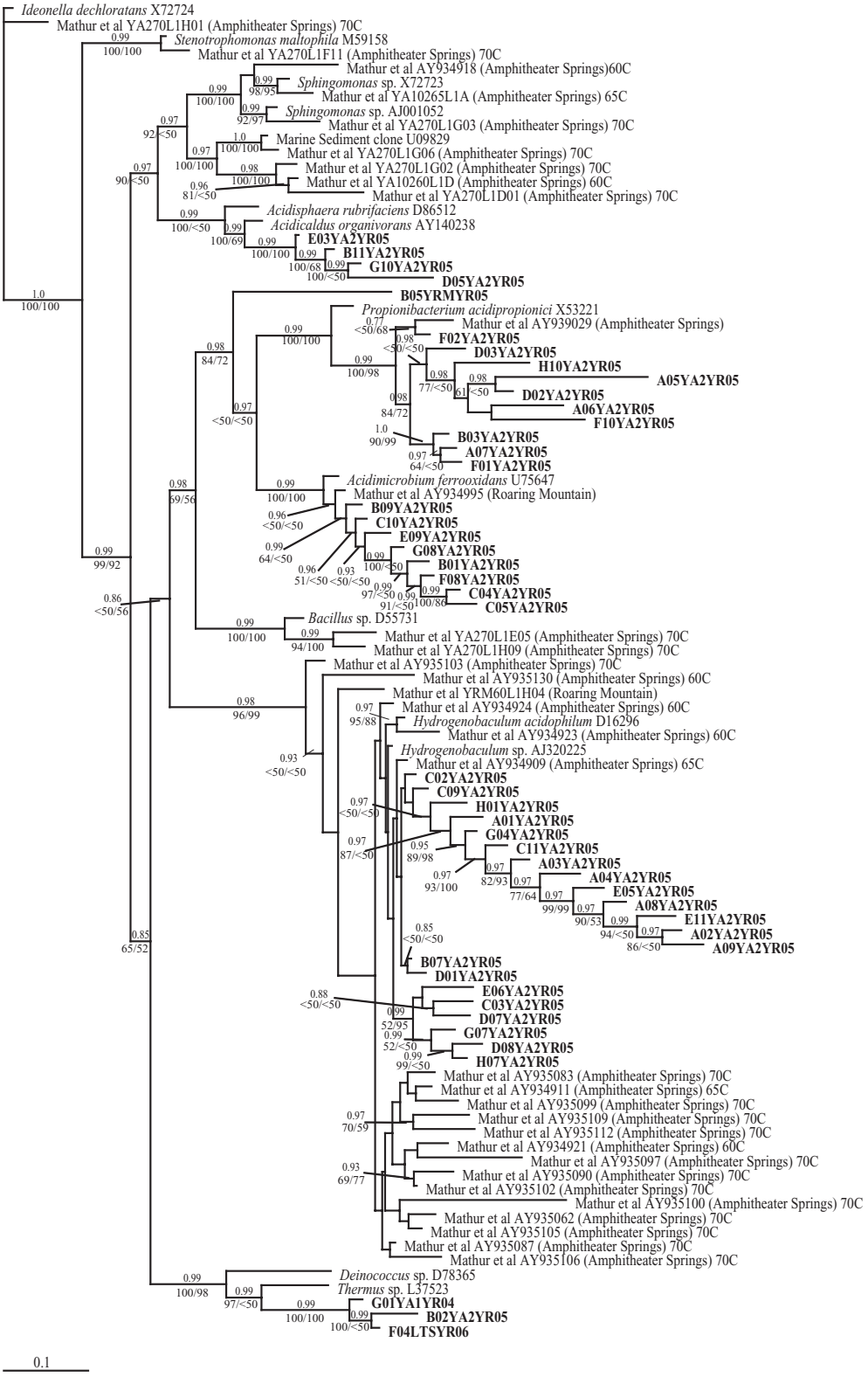

Fig. 3. Phylogenetic tree representing 16S rRNA gene sequences determined from AS102 source waters (boldface) and sediments from a previous study (Mathur *et al.*, 2007) of the same spring along with sequences from cultured and uncultured relatives obtained from GenBank (accession numbers included). Numbers above the branches indicate Bayesian posterior probabilities, while MP and NJ bootstrap values are presented below the branches. Bootstrap support values below 50% were not included in the figure.

In regards to the origins of the microbes found in the spring source waters, our phylogenetic trees reveal a 'potpourri' of microbial lineages related to bacteria isolated from hot springs and sediment, volcanic soils, and other sources. Members of the *Aquificales* were common in our samples. However, we also found many organisms, particularly in the Kamchatka samples, that were related to bacterial genera found in geothermally heated soils, including *Acetobacter*, *Alicyclobacillus*, *Bacillus*, *Methylobacterium*, *Paenibacillus* and *Thiobacillus* (Norris *et al*., 2002; Botero *et al*., 2005), as well as many known soil bacterial genera (e.g. *Bradyrhizobium*). The considerable phylogenetic diversity found in every spring we studied, and the dissociation between this diversity and the chemistry of the spring waters, both indicate that the organisms we discovered arose from a wide variety of habitats.

In fact, a number of previous studies have isolated microbes from habitats whose physical parameters lie clearly outside their temperature and/or pH optimum (e.g. Hankinson and Schmidt, 1988; Zakalyukina *et al*., 2004; Ellis *et al*., 2008). These findings support the idea that hardy microbes can persist in unsuitable environments for long periods of time, perhaps in a dormant state. In the case of the spring source waters, we suggest that microbes are transported via subsurface waters into new habitats where a subset thrive and grow. This would explain both the high diversity of the spring source waters and the fact that so much of the diversity, based on the types of organisms found, appears adapted to very different types of environments.

Further emphasizing this point, we also discovered at least one bacterial group in the spring source waters never before reported from geothermal habitats: a putative new genus of *Firmicutes* with no cultured representative. These sequences were found abundantly in samples of AS101 and RM source water collected in 2005, and all were nearly identical (< 1% divergent) to uncultured bacterial sequences. The GenBank matches included four sequences of uncultured bacteria found in the hyper-arid Atacama desert (Maximum bootstrap and Bayesian posterior probability support; Fig. 4). These sequences formed a strongly supported monophyletic group of uncultured bacteria within the *Firmicutes* from arid or hyper-arid soils (Fig. 4). This result appears quite surprising, given that all our sequences were obtained from water sources rather than soils. To our knowledge, this would be the first discovery of sequences from this group in Yellowstone springs or any geothermal habitats. We suggest that a shift in the hydrology, or perhaps the spring run-off, may have dislodged these organisms from a soil or rock habitat and transported them in the subsurface waters. The hydrology of the Yellowstone ecosystem is constantly in flux and undergoes particularly dramatic alterations during the spring run-off period (Kharaka *et al*., 2000). A hydrological shift would also explain why we found these organisms so abundantly in two different springs 2 km apart on back to back days in 2005, but at no other time.

Although 16S methods provide considerably greater insight into environmental microbiological diversity than culture-based approaches, we cannot rule out any biasing effects of primer selection or cycling conditions on our results (Suzuki and Giovannoni, 1996). However, by using the same primer combination that was used in our earlier studies (Mathur *et al*., 2007), we assured that the bias would at least be consistent for comparative purposes. Also, our interpretations do not rely on abundance data, per se, but rather on patterns of phylogenetic diversification. Collectively, our results support the hypothesis that the subsurface provides at least a partial source, in addition to potential wind or insect dispersal, of the microbial diversity observed in surface communities, though the precise origins of the microbes remain unclear. The underground water appears to provide a conduit for dispersion of these organisms, rather than a habitat in and of itself similar to the steam vent waters of fumaroles (Ellis *et al*., 2008). However, it is not understood whether microbes are originating from distant mats and simply travelling through the subsurface, coming from nearby geothermal soils, or actually persisting in underground reservoirs. Regardless, our findings have important ramifications for understanding the formation and dispersal patterns of geothermal communities.

Acknowledgements

We thank Lisa Thurn for her help with the water chemistry analysis and Dr David Lipson for assistance with Systat, the PCoA analyses and for helpful comments on the manuscript. We thank Dr Aaron Pietruska for his advice and insights into the geochemistry of our subterranean systems. We thank Dr Roger Sabbadini and the International Programs Office for awarding a travel grant that allowed S. Tin to collect samples in Kamchatka and attend the National Science Foundation Microbial Observatories workshop. We thank Dr Matt Kane for arranging the international workshop, 'Biodiversity, molecular biology and biogeochemistry' through which we obtained our Kamchatka samples. Mark J. Young helped collect samples in Russia. P. Hugenholtz graciously spent time helping us align and double-check our novel Firmicute sequences. We appreciate the assistance of the National Park Service, Mike Magnusen in Lassen Volcanic National Park, and Christie L. Hendrix and Stacie M. Gunther in Yellowstone National Park. The generous contributions of Schering-Plough Biopharma are gratefully acknowledged. We extend our gratitude to former members of the Kelley lab for their support, advice and help over the course of the research: Eric Alegre, Dean Ellis, Jason Holzman, Lesley Lee, Jayanti Mathur, Collin McMannus, Alex Poole, and Sujata Sovani. We also thank three anonymous reviewers for their helpful comments. This work was supported by the

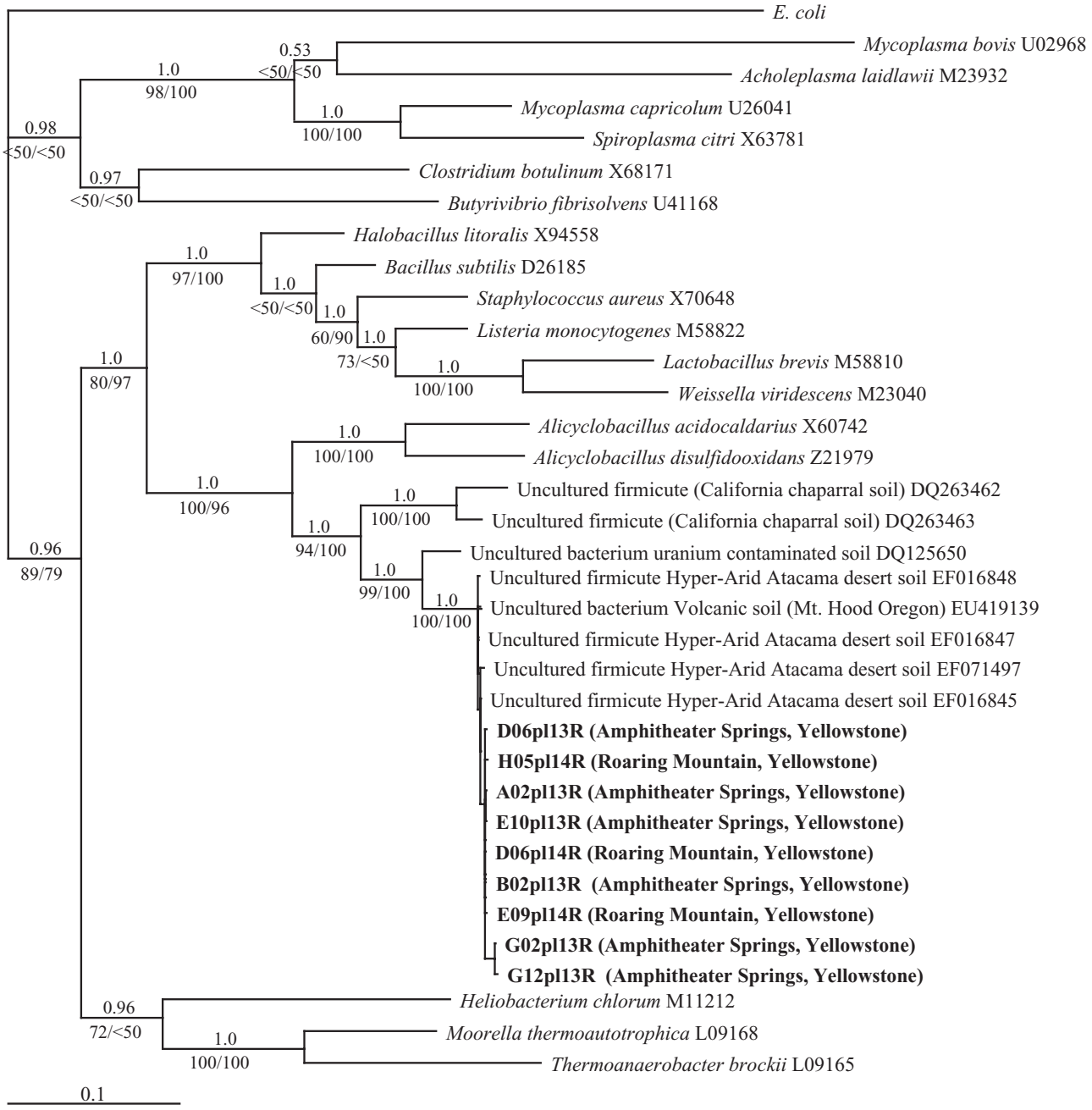

Fig. 4. Phylogenetic relationships of Firmicute-related clones found abundantly in two Yellowstone spring source waters in 2005 along with sequences from cultured and uncultured relatives obtained from GenBank (accession numbers included). Numbers above the branches indicate Bayesian posterior probabilities, while MP and NJ bootstrap values are presented below the branches. Bootstrap support values below 50% were not included in the figure.

National Science Foundation and the International Society for Microbial Ecology.

References

Bonheyo, G.T., Frias-Lopez, J., and Fouke, B.W. (2005) A test for airborne dispersal of thermophilic bacteria from hot springs. In *Geothermal Biology and Geochemistry in Yellowstone National Park*. Inskeep, W.P., and McDermott, T.R. (eds). Bozeman, MT, USA: Montana State University Publications, pp. 327–340.

Boomer, S.M., Noll, K.L., Geesey, G.G., and Dutton, B.E. (2009) Formation of multilayered photosynthetic biofilms in an alkaline thermal spring in Yellowstone National Park, Wyoming. *Appl Environ Microbiol* **75:** 2464–2475.

Botero, L.M., D'Imperio, S., Burr, M., McDermott, T.R., Young, M., and Hassett, D.J. (2005) Poly(A) polymerase modification and reverse transcriptase PCR amplification

of environmental RNA. *Appl Environ Microbiol* **71:** 1267–1275.

Boyd, E.S., Jackson, R.A., Encarnacion, G., Zahn, J.A., Beard, T., Leavitt, W.D., *et al*. (2007) Isolation, characterization, and ecology of sulfur-respiring crenarchaea inhabiting acid-sulfate-chloride-containing geothermal springs in Yellowstone National Park. *Appl Environ Microbiol* **73:** 6669–6677.

Boyd, E.S., Leavitt, W.D., and Geesey, G.G. (2009) CO2 uptake and fixation by a thermoacidophilic microbial community attached to precipitated sulfur in a geothermal spring. *Appl Environ Microbiol* **75:** 4289–4296.

Brock, T.D., and Mosser, J.L. (1975) Rate of sulfuric-acid production in Yellowstone National Park. *Geol Soc Am Bull* **86:** 194–198.

Chapelle, F.H., O'Neill, K., Bradley, P.M., Methé, B.A., Ciufo, S.A., Knobel, L.L., and Lovley, D.R. (2002) A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* **415:** 312–315.

Chivian, D., Brodie, E.L., Alm, E.J., Culley, D.E., Dehal, P.S., DeSantis, T.Z., *et al*. (2008) Environmental genomics reveals a single-species ecosystem deep within Earth. *Science* **322:** 275–278.

Clingenpeel, S.R., D'Imperio, S., Oduro, H., Druschel, G.K., and McDermott, T.R. (2009) Cloning and in situ expression studies of the arsenite oxidase genes. *Appl Environ Microbiol* **75:** 3362–3365.

D'Hondt, S., Jørgensen, B.B., Miller, D.J., Batzke, A., Blake, R., Cragg, B.A., *et al*. (2004) Distributions of microbial activities in deep subseafloor sediments. *Science* **306:** 2216–2221.

D'Imperio, S., Lehr, C.R., Breary, M., and McDermott, T.R. (2007) Autecology of an arsenite chemolithotroph: sulfide constraints on function and distribution in a geothermal spring. *Appl Environ Microbiol* **73:** 7067–7074.

Donahoe-Christiansen, J., D'Imperio, S., Jackson, C.R., Inskeep, W.P., and McDermott, T.R. (2004) Arsenite-oxidizing *Hydrogenobaculum* strain isolated from an acid-sulfate-chloride geothermal spring in Yellowstone National Park. *Appl Environ Microbiol* **70:** 1865–1868.

Ellis, D.G., Bizzoco, R.W., and Kelley, S.T. (2008) Halophilic *Archaea* determined from geothermal steam vent aerosols. *Environ Microbiol* **10:** 1582–1590.

Hankinson, T.R., and Schmidt, E.L. (1988) An acidophilic and a neutrophilic *Nitrobacter* strain isolated from the numerically predominant nitrite-oxidizing population of an acid forest soil. *Appl Environ Microbiol* **54:** 1536–1540.

Hubert, C., Loy, A., Nickel, M., Arnosti, C., Baranyi, C., Brüchert, V., *et al*. (2009) A constant flux of diverse thermophilic bacteria into the cold Arctic seabed. *Science* **325:** 1541–1544.

Kharaka, Y.K., Sorey, M.L., and Thordsen, J.J. (2000) Large-scale hydrothermal fluid discharges in the Norris-Mammoth corridor, Yellowstone National Park, USA. *J Geochem Explor* **69–70:** 201–205.

Kormas, K.A., Smith, D.C., Edgcomb, V., and Teske, A. (2003) Molecular analysis of deep subsurface microbial communities in Nankai Trough sediments (ODP Leg 190, Site 1176). *FEMS Microbiol Ecol* **45:** 115–125.

Macur, R.E., Langner, H.W., Kocar, B.D., and Inskeep, W.P. (2004) Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulphate-cloride geothermal spring. *Geobiology* **2:** 163–177.

Magot, M., Ollivier, B., and Patel, B.K.C. (2000) Microbiology of petroleum reservoirs. *Antonie Van Leeuwenhoek* **77:** 103–116.

Marteinsson, V.T., Hauksdóttir, S., Hobel, C.F.V., Kristmannsdóttir, H., Hreggvidsson, G.O., and Kristjánsson, J.K. (2001) Phylogenetic diversity analysis of subterranean hot springs in Iceland. *Appl Environ Microbiol* **67:** 4242–4248.

Mathur, J., Bizzoco, R.W., Ellis, D.G., Lipson, D.A., Poole, A.W., Levine, R., and Kelley, S.T. (2007) Effects of abiotic factors on the phylogenetic diversity of bacterial communities in acidic thermal springs. *Appl Environ Microbiol* **73:** 2612–2623.

Nealson, K.H., Inagaki, F., and Takai, K. (2005) Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): do they exist and why should we care? *Trends Microbiol* **13:** 405–410.

Norris, T.B., Wraith, J.M., Castenholz, R.W., and McDermott, T.R. (2002) Soil microbial community structure across a thermal gradient following a geothermal heating event. *Appl Environ Microbiol* **68:** 6300–6309.

Priscu, J.C., Adams, E.E., Lyons, W.B., Voytek, M.A., Mogk, D.W., Brown, R.L., *et al*. (1999) Geomicrobiology of subglacial ice above Lake Vostok, Antarctica. *Science* **286:** 2141–2144.

Stevens, T., and McKinley, J. (1996) Hydrogen-Based Microbial Ecosystems in the Earth. *Science* **272:** 896c–897c.

Suzuki, M.T., and Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62:** 625–630.

Takai, K., Moser, D.P., DeFlaun, M., Onstott, T.C., and Fredrickson, J.K. (2001) Archaeal diversity in waters from deep South African gold mines. *Appl Environ Microbiol* **67:** 5750–5760.

Takai, K., Gamo, T., Tsunogai, U., Nakayama, N., Hirayama, H., Nealson, K.H., and Horikoshi, K. (2004) Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field. *Extremophiles* **8:** 269–282.

Teske, A.P. (2005) The deep subsurface biosphere is alive and well. *Trends Microbiol* **13:** 402–404.

Wanger, G., Onstott, T.C., and Southam, G. (2008) Stars of the terrestrial deep subsurface: a novel 'star-shaped' bacterial morphotype from a South African platinum mine. *Geobiology* **6:** 325–330.

Zakalyukina, Y.V., Zenova, G.M., and Zvyagintsev, D.G. (2004) Peculiarities of growth and morphological differentiation of acidophilic and neutrophilic soil streptomycetes. *Microbiology* **73:** 74–78.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Origin waters from Kamchatka peninsula flowing spring sites.

A and B. Neutral Spring, Geyser Valley. (A) Phase contrast; (B) Sybr Green.
C and D. UC1A Uzon Caldera, spring 1A. (C) Phase contrast; (D) DAPI.
E and F. Yellowstone, Roaring Mountain, Norris Geyser Basin. (E) Phase contrast; (F) DAPI. Bar, 10 μm.

Fig. S2. Origin Waters from Yellowstone National Park flowing springs.
A and B. Amphitheater Springs (AS) 101. (A) Phase contrast; (B) DAPI.
C and D. AS 102. (C) Phase contrast; (D) DAPI.
E and F. AS 104. (E) Phase contrast; (F) DAPI. Bar, 10 μm.

Fig. S3. Scanning electron microscope image of cells from origin waters in three collecting areas: (A and B) Lassen Volcanic National Park, CA, USA; (C) Yellowstone National Park, WY, USA; (D) Kamchatka, Russia.
A. Toto Spring, filamentous cluster of cells embedded in matrix material. Background, cover glass. Bar, 50 μm.
B. Toto Spring, group of matrix-associated filaments-spheres, with distinctive morphology, overlying crystal resembling sulfur (s). Background, cover glass. Compare (A) and (B) with DAPI-stained cells (C) and (D). Bar, 20 μm.
C. Roaring Mountain, spore forming cell and thin filament (arrows). Debris and particles appear on 0.22 μm Millipore filter along with cells. Bar, 10 μm.
D. Al's Spring, Mutnovsky Volcano, thin filament (arrows) lying on debris and mineral particles on 0.22 μm Millipore filter. Bar, 10 μm.

Fig. S4. Phylogenetic tree of source water determined 16S rRNA gene sequences (boldface) and sediment sequences from AS101. (See Fig. 3 legend for details concerning sequence names and statistical support values.)

Fig. S5. Community level phylogenetic analysis of springs located in Lassen Volcanic National Park, California. Both Toto Spring and Voldemort Spring were in the Little Hot Spring Area separated from each other by about 3.2 m. Devil's Kitchen was a spring about 7 km from Little Hot Spring Area.

Fig. S6. Community level phylogenetic analysis of 16S gene sequences determined from springs along the Kamchatka peninsula in Eastern Russia. Geyser Valley and the Uzon Caldera are located ~ 24 km apart. The Mutnovsky Volcano and Geyser Valley are located ~ 160 km apart.

Fig. S7. Turbulence at the spring origin point suggests high flow rates (see Table 1). (A) AS102, horizontal outlet and (B) AS104, vertical outlet. Long arrow indicates downstream direction; arrowhead marks sampling site. (A) Bar, 20 cm; (B) Bar, 5 cm.

Table S1. Chemical and physical properties of origin water samples collected from three geographic locations.

Appendix S1. Experimental procedures.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.