### Title

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### Authors

Jensen, Paul R

Mafnas, Chrisy



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# Biogeography of the marine actinomycete *Salinispora*

**Paul R. Jensen* and Chrisy Mafnas**
*Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, CA, USA.*

## Summary

**Marine actinomycetes belonging to the genus *Salinispora* were cultured from marine sediments collected at six geographically distinct locations. Detailed phylogenetic analyses of both 16S rRNA and *gyrB* gene sequences reveal that this genus is comprised of three distinct but closely related clades corresponding to the species *Salinispora tropica*, *Salinispora arenicola* and a third species for which the name '*Salinispora pacifica*' is proposed. *Salinispora arenicola* was cultured from all locations sampled and provides clear evidence for the cosmopolitan distribution of an individual bacterial species. The co-occurrence of *S. arenicola* with *S. tropica* and *S. pacifica* suggests that ecological differentiation as opposed to geographical isolation is driving speciation within the genus. All *Salinispora* strains cultured to date share greater than 99% 16S rRNA gene sequence identity and thus comprise what has been described as a microdiverse ribotype cluster. The description of this cluster as a new genus, containing multiple species, provides clear evidence that fine-scale 16S rDNA sequence analysis can be used to delineate among closely related species and that more conservative operational taxonomic unit values may significantly underestimate global species diversity.**

## Introduction

Bacterial biogeography remains an unresolved issue in microbiology (Fenchel, 2003). Because of their small size, high abundance and ease of dispersal, the prevailing hypothesis in the field is that free-living bacteria are not subject to geographical isolation and, without this constraint, should exhibit a cosmopolitan distribution (reviewed by Staley and Gosink, 1999; Martiny *et al*., 2006). While it is widely accepted that bacterial genera are widely distributed in their respective habitats (Hedlund and Staley, 2004), there is currently little empirical support for the 'everything is everywhere but the environment selects' paradigm (De Wit and Bouvier, 2006) when applied at the species level. Without a better understanding of the extent to which geographical isolation affects the population structure of individual bacterial species, it will not be possible to effectively estimate global species richness or to understand the forces driving speciation among bacteria.

Little emphasis has been given to the study of microbial biogeography (Cho and Tiedje, 2000), and as a result it is not clear if similar populations occupy analogous environments on a global scale. The most outspoken support for microbial cosmopolitanism comes from studies of microeukaryotes (Findlay, 2002); however, this support is based largely on the analysis of protozoan morphospecies. As might be expected, evidence for cosmopolitanism among environmental prokaryotes includes taxa with robust survival strategies, such as the spore-forming genus *Bacillus*, for which it has been shown that migration rates are sufficiently high to prevent geographical isolation (Roberts and Cohan, 1995). Additional evidence comes from a study of fluorescent *Pseudomonas* strains where cosmopolitanism was evident by the analysis of 16S rDNA and, to a lesser extent, 16S-23S intergenic spacer regions (Cho and Tiedje, 2000). Evidence for endemism was documented at the infraspecific level among the same *Pseudomonas* strains when higher resolution genomic fingerprinting methods were applied. Additional evidence for endemism is found among prokaryotes inhabiting extreme environments where the barriers to surviving dispersal are high. This includes gas vacuolated sea ice bacteria (Staley and Gosink, 1999), the thermophilic archeon *Sulfolobus* (Whitaker *et al*., 2003) and the thermophilic cyanobacterium *Synecococcus* (Papke *et al*., 2003).

Any discussion of species-level bacterial biogeography is affected by uncertainty surrounding the species concept for bacteria (Cohan, 2002; Gevers *et al*., 2005). Recently, it has been proposed that molecular sequence data can be used to define natural units of bacterial diversity that possess the fundamental properties of species (Cohan, 2002). These units can be recognized as clusters of sequences that share greater similarity to each other than to related sequences and are believed to delineate ecologically distinct populations or ecotypes (Cohan, 2002). Ecotypes may arise through various processes (Gevers *et al*., 2005) including geographical isolation or natural selection and can be difficult to resolve using highly con-

Received 21 November, 2005; accepted 4 March, 2006. *For correspondence. E-mail pjensen@ucsd.edu; Tel. (+1) 858 534 7322; Fax (+1) 858 558 3702.

served loci such as the 16S rRNA gene (Fox *et al*., 1992; Palys *et al*., 1997; Staley and Gosink, 1999). This has led to an increased reliance on protein coding genes and, more recently, multilocus sequence analysis for the resolution of intrageneric relationships (Gevers *et al*., 2005). In several cases, it has been demonstrated that named species are comprised of multiple ecotypes (Palys *et al*., 2000), leading to the suggestion that the bacterial species generally recognized today are in fact composites of multiple ecotypes each possessing the dynamic properties of individual species (Cohan, 2002).

We recently reported the discovery of the actinomycete genus *Salinispora*, which is widely distributed in tropical and subtropical marine sediments (Mincer *et al*., 2002; Maldonado *et al*., 2005). To date, two species have been formally described (*S. arenicola* and *S. tropica*), and a third ('*S. pacifica*') is proposed based on DNA–DNA hybridization. *Salinispora* belongs to the Micromonosporaceae and is the first actinomycete genus known to require seawater for growth. As these bacteria produce resistant spores and have been cultured from worldwide locations, they represent model organisms to test hypotheses about bacterial biogeography and the processes that drive speciation. In this paper, the phylogenetic relationships of 152 strains were assessed using 16S rRNA and *gyrB* gene sequences. The results provide compelling evidence that an individual bacterial species comprised largely of 16S rDNA clones can have a cosmopolitan distribution and that speciation within the genus *Salinispora* is not due to geographical isolation.

## Results

*Salinispora* strains were cultivated from all six tropical/subtropical locations sampled. These locations included multiple collection sites within the Bahamas, where they were originally discovered (Jensen *et al*., 1991), the US Virgin Islands, the Red Sea, the Sea of Cortez, Palau and Guam. In addition, strains were recently reported from the sponge *Pseudoceratum clavata* collected from the Great Barrier Reef (Kim *et al*., 2005) and the ascidian *Polysyncraton lithostrotum* collected from Fiji (He *et al*., 2001), providing the first Southern hemisphere sites from which *Salinispora* strains have been recovered. Despite extensive effort, we have yet to cultivate *Salinispora* strains from temperate Pacific Ocean sediments collected off La Jolla, California. They also do not appear to be among the numerous *Micromonospora* strains recovered from North Sea sediments (E. Helmke, pers. comm.). Detailed 16S and *gyrB* phylogenetic analyses of 46 *Salinispora* strains clearly reveal that the genus, as we know it today, is comprised of three distinct but closely related species (Figs 1 and 2). Two of these, *S. arenicola* and *S. tropica*, were recently described (Maldonado *et al*., 2005), while

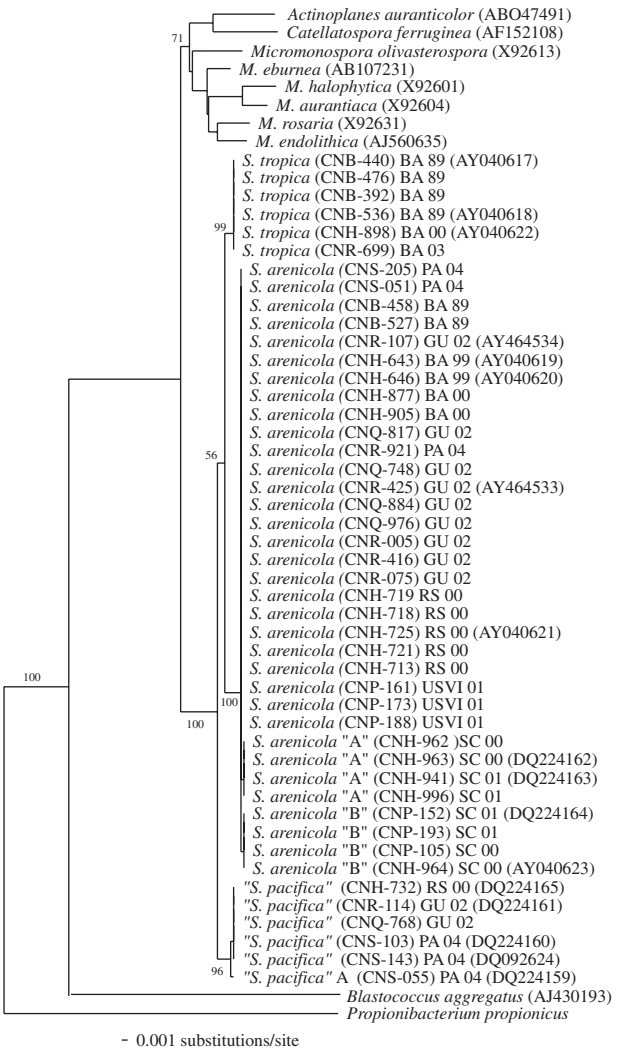


**Fig. 1.** Neighbour-joining phylogenetic tree created from 46 nearly complete (1449 nucleotides) 16S rRNA gene sequences from *Salinispora* strains cultured from worldwide locations. The three major *Salinispora* phylotypes, consisting of the two formally described species *S. tropica* and *S. arenicola* and the proposed species '*S. pacifica*', are clearly delineated. Type strains representing the five *Micromonospora* species most closely related to *Salinispora*, along with *Micromonospora halophytica*, are included. Species names are followed by strain number, strain source (BA, Bahamas; RS, Red Sea; GU, Guam; PA, Palau; USVI, US Virgin Islands; SC, Sea of Cortez), year of collection (89 = 1989, etc.) and accession number (for representative sequences). *Propionibacterium propionicus* and *Blastococcus aggregatus* were used as outgroups.

'*S. pacifica*' is proposed based on ≤ 60% interspecies DNA–DNA hybridization (performed by the DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig).

### *Biogeographical distribution*

The three *Salinispora* species vary in their biogeographical distributions (Figs 1 and 2). *Salinispora arenicola* has

 

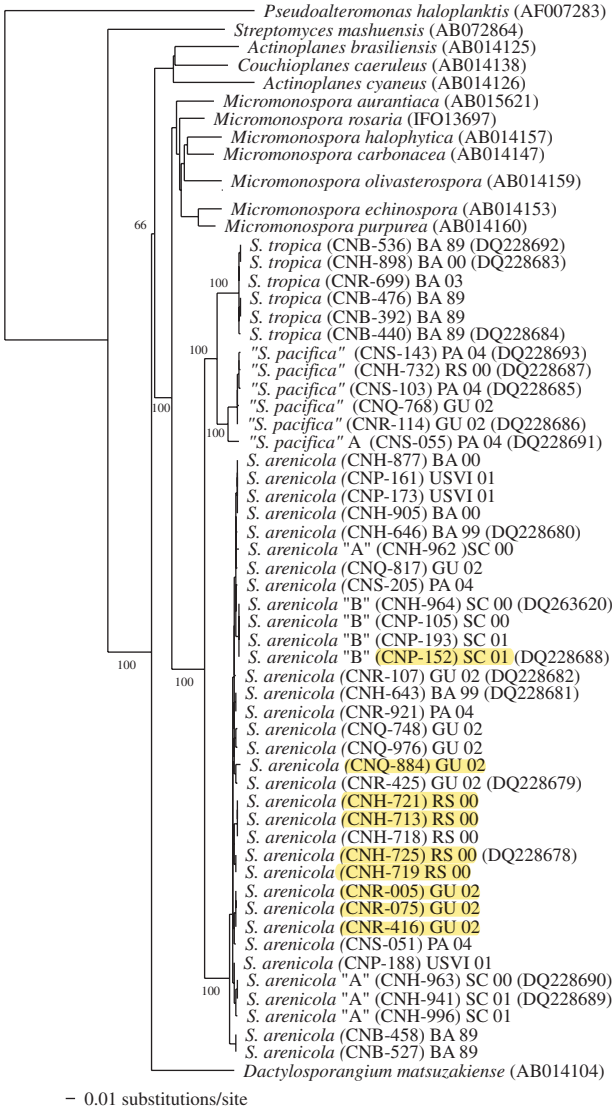


**Fig. 2.** Neighbour-joining phylogenetic tree created from 46 nearly complete (1164 nucleotides) *gyrB* gene sequences from *Salinispora* strains cultured from worldwide locations. Labelling is similar to Fig. 1.*Pseudoalteromonas haloplanktis* was used as an outgroup.

a cosmopolitan distribution having been recovered from all six of the locations sampled. It is also consistently the most abundant species observed, representing 86% of the 152 strains examined in this study. *Salinispora tropica* has the most restricted distribution having thus far only been detected from the Bahamas, where it has been consistently recovered over a 15 year period and represents seven of the 19 strains examined. Surprisingly, this species was not recovered from the US Virgin Islands, despite the examination of 20 strains from this site and its proximity to the Bahamas. '*Salinispora pacifica*' has been recovered from Guam, Palau and the Red Sea, with only one strain being recovered from the latter. This species is also considerably less common than *S. arenicola* (three of 59 strains from Guam, seven of 23 strains from Palau, one of 18 strains from the Red Sea). Although more widely distributed than *S. tropica*, '*S. pacifica*' was absent or remained below the detection limit in the Caribbean and the Sea of Cortez and represents a second species that, at present, appears to be geographically restricted relative to *S. arenicola*. The *Salinispora* strains recently reported from the Great Barrier Reef (Kim *et al*., 2005) fall within both *S. arenicola* and '*S. pacifica*', while the single strain reported from Fiji (He *et al*., 2001) is identical (based on 16S rRNA gene sequence) to '*S. pacifica*'.

### *SSU rRNA gene diversity*

There is a remarkable lack of intraclade diversity within the three *Salinispora* species. Despite the inclusion of strains isolated over a 15 year period from multiple collections sites throughout the Bahamas, all *S. tropica* strains cultured to date share 100% sequence identity throughout the 1479 base pairs examined (Table 1). This absence of sequence variation could only be detected once careful corrections were made for polymerase chain reaction (PCR) and sequencing errors, including corrections to previously reported data (Mincer *et al*., 2002; Jensen *et al*., 2005). The 34 *S. arenicola* strains exam-

**Table 1.** *Salinispora* intra- and interspecific genetic similarity (number of strains in parentheses after species identifier).

| | % Similarity | | | *gyrB* (DNA) | | |
|---|---|---|---|---|---|---|
| Species | 16S (rDNA) | *gyrB* (DNA) | *gyrB* (aa) | $d_N$ | $d_S$ | $d_N/d_S$ |
| St (6) | 100 (1479) | 99.57 (1159) | 99.23 (385) | 3 | 2 | 1.50 |
| Sa (34) | 99.86 (1477) | 96.13 (1119) | 98.45 (382) | 6 | 39 | 0.15 |
| Sp (6) | 99.86 (1477) | 97.16 (1131) | 99.23 (385) | 5 | 30 | 0.17 |
| St : Sp | 99.59 (1473) | 95.10 (1107) | 98.71 (383) | 5 | 52 | 0.10 |
| Sa : Sp | 99.26 (1468) | 92.87 (1081) | 96.65 (375) | 13 | 70 | 0.19 |
| St : Sa | 99.53 (1472) | 92.87 (1081) | 96.39 (374) | 14 | 69 | 0.20 |

Sa, *S. arenicola*; Sp, '*S. pacifica*'; St, *S. tropica*. 16S similarities generated from 1479 nucleotide positions, *gyrB* DNA similarities generated from 1164 nucleotide positions, *gyrB* amino acid (aa) similarities generated from 388 positions. Number of invariant positions in parentheses after per cent similarities. $d_N$, non-synonymous nucleotide substitution; $d_S$, synonymous nucleotide substitution. Interspecific comparisons were made using the type strains for each species.

 

ined in detail possessed nearly identical sequences (99.86% similarity) with the only variations arising from strains cultured from the Sea of Cortez, all of which contained one of two possible single nucleotide polymorphisms (Fig. 1) resulting in the subclades *S. arenicola* 'A' (12 strains observed) and *S. arenicola* 'B' (five strains observed). None of the Sea of Cortez strains were a perfect sequence match with the *S. arenicola* type strain (CNH-643) providing extremely fine scale (one nucleotide) biogeographical resolution of these two Sea of Cortez populations. Despite analysing partial sequence data for an additional 96 *S. arenicola* strains, including multiple representatives from all locations, no new intraclade sequence diversity was detected. As with *S. arenicola*, '*S. pacifica*' intraclade similarity was 99.86% (two variable nucleotide positions out of 1479 examined). Both of these nucleotide variations occurred in strain CNS-055 relative to the proposed type strain (CNS-143) and delineate the '*S. pacifica*' 'A' clade.

Interclade diversity among the three *Salinispora* species was also low and places the entire genus into what has been described as a microdiverse sequence cluster (Acinas *et al*., 2004). Pairwise similarities (BLAST bl2seq, NCBI) reveal that *S. tropica* and *S. arenicola* share 99.53% 16S rRNA gene sequence identity (Table 1). This is a difference of seven nucleotides of 1479 examined. *Salinispora tropica* was found to differ from *S. arenicola* subclades 'A' and 'B' by one additional nucleotide (99.46% similarity). The greatest sequence differences occurred between *S. arenicola* and '*S. pacifica*' (11 nucleotides, 99.26%) and between *S. arenicola* subclade 'A' or 'B' and '*S. pacifica*' (12 nucleotides, 99.19% similarity). The most similar species were *S. tropica* and '*S. pacifica*', which differed by only six nucleotides (99.59% similarity). Despite the high level of sequence identity, *S. tropica* and *S. arenicola* have been classified as distinct species (Maldonado *et al*., 2005), while the classification of '*S. pacifica*' as a third species is supported by DNA–DNA hybridization experiments (Wayne *et al*., 1987) in which the proposed type strain shared < 60% genomic similarity to *S. tropica* and *S. arenicola* (data provided by the DSMZ). *Salinispora* species share 96.50–96.60% similarity with *Micromonospora chalcea*, the type strain for the genus *Micromonospora*, and 97.28–97.56% similarity with *Micromonospora rosaria*, the most closely related *Micromonospora* species.

The majority of the *Salinispora* sequence diversity thus far detected occurs in appropriately variable regions of the SSU rRNA gene and, with the exception of CNS-055, in multiple strains, providing strong evidence that these changes are not due to PCR or sequencing errors. Nine of 15 variable nucleotide positions occur in the V2 variable region (Rijik *et al*., 1992) with all but one of these occurring in non-conserved helixes. Of the remaining substitutions, only a G⇔A hairpin loop transition (*Escherichia coli* position 262) occurs in a conserved region (90–98% among all bacteria).

### gyrB *phylogeny*

The phylogenetic tree based on nearly complete *gyrB* DNA sequences (1164 nucleotides) re-affirms the monophyletic nature of the *Salinispora* clade and its separation from other genera within the Micromonosporaceae (Fig. 2). The three *Salinispora* phylotypes, corresponding to *S. tropica*, *S. arenicola* and '*S. pacifica*' are clearly delineated providing additional phylogenetic support for the separation of these taxa. No cryptic species were detected from the analysis of this protein-coding gene, and no variations were detected among any of the 46 strains in terms of species-specific 16S rDNA and *gyrB* cladding patterns, although there is a difference in the branching patterns in the two trees with the *gyrB* pattern being better supported by bootstrap analysis.

As with the 16S rRNA gene sequence data, there was a remarkably high level of *gyrB* sequence similarity within the three *Salinispora* species (Table 1), with *S. tropica* strains sharing 99.57% sequence identity, *S. arenicola* strains sharing 96.13% sequence identity and '*S. pacifica*' strains sharing 97.16% sequence identity. The interspecies similarity was greatest between *S. tropica* and '*S. pacifica*' (95.10%) and least between *S. arenicola* and the other two phylotypes (92.87%). The closest Blastn *gyrB* sequence match for all three of the *Salinispora* phylotypes was *M. rosaria* (BAA89737) for which the sequence identity was 89–90%. The *Salinispora gyrB* sequence data were translated into 388 amino acids and, as expected, both the intra- and interspecies amino acid similarities are high (98.45–99.23% and 96.39–98.71% respectively). *Salinispora* species share 90–92% amino acid sequence identity with *M. rosaria*, the closest BLASTp (NCBI) match. The $d_N/d_S$ ratio for *S. tropica* was approximately 10-fold greater than for the other two species; however, this may be due to small sample size.

### *Effects of temperature on growth*

In previous studies, we have observed that *Salinispora* strains are capable of growth at 10°C but not at 4°C. To test the effects of exposure to 4°C on *Salinispora* growth and viability, seven strains were maintained at 4°C for 2, 4, 6, or 8 weeks then incubated at 25°C for 2 months. All strains incubated at 4°C for 2 or 4 weeks showed no reduction in growth relative to controls upon transfer to 25°C. After 6 weeks at 4°C however, all strains exhibited reduced growth at 25°C with two strains (CNS-103 and CNR-114) remaining reduced even after 2 months at this temperature. After 8 weeks at 4°C, these same two strains

lost viability while the remaining five strains all displayed a reduction in growth relative to controls. Both of the strains that lost viability belong to '*S. pacifica*'.

## Discussion

The extent to which individual bacterial species are globally distributed in all environments capable of supporting their growth remains a fundamental question in microbiology. Although this question is mired in uncertainty over how to apply the species concept to bacteria (Rosselló-Mora and Amann, 2001), the analysis of molecular sequence data is providing new insight into the biogeographical distributions of specific bacterial types. Recent examples of sequence-based analyses have included clear evidence for species-level endemism among bacteria inhabiting extreme environments (Staley and Gosink, 1999; Papke *et al*., 2003; Whitaker *et al*., 2003). At the infraspecific level, endemism has also been documented among free-living bacteria (Cho and Tiedje, 2000). Based on these results, it is becoming increasingly clear that all bacteria are not cosmopolitan in distribution and that the ability to detect bacterial endemism is a function of the bacterial populations studied and the resolution of the analytical techniques applied.

The detailed phylogenetic characterization of the actinomycete genus *Salinispora* provides clear evidence that an individual bacterial species can be globally distributed among environments in which its growth requirements are met. This evidence comes from *S. arenicola*, which to the best of our knowledge represents the first free-living bacterial species within which large numbers of strains possessing 100% 16S rRNA gene sequence identity have been cultured from worldwide locations. Because the species-level resolution of the 16S gene has been questioned (Rosselló-Mora and Amann, 2001), a protein-coding gene that has been used successfully to resolve species diversity within a related actinomycete genus (Kasai *et al*., 2000) was also studied. Analysis of *Salinispora gyrB* gene sequences further supports the cosmopolitan distribution of *S. arenicola*, as no 'cryptic' species were resolved. If infraspecific biogeographical patterns are to be resolved within this species, it will require the higher resolution afforded by genomic fingerprinting (e.g. Cho and Tiedje, 2000) or sequence analysis of a less conserved gene. At this time, it cannot be determined if the relative regional endemism detected for '*S. pacifica*' and *S. tropica* is due to insufficient sampling, reduced fitness, less effective dispersal, recent speciation, or limited niche availability. Of these, insufficient sampling seems least likely, as culture-independent studies of sediments collected in the Bahamas did not reveal the presence of '*S. pacifica*' or any previously uncultured species (Mincer *et al*., 2005). However, *S. tropica* and '*S. pacifica*' were recovered less frequently than *S. arenicola* so this possibility cannot be ruled out.

There is a remarkable lack of 16S rDNA sequence diversity within the genus *Salinispora* suggesting relatively recent divergence among the three species. The co-occurrence of *S. arenicola* with *S. tropica* and '*S. pacifica*' suggests ecological differentiation as opposed to geographical isolation as the force driving speciation, although other mechanisms for the formation of sequence clusters have been discussed (Gevers *et al*., 2005). That geographical isolation is not driving speciation is further supported by low global species diversity (despite the examination of more than 150 strains collected over 15 years from six worldwide locations), rapid coalescence in the phylogenetic trees (Figs 1 and 2) and the lack of correlation between genetic and geographic distances (i.e. *S. tropica* and '*S. pacifica*' are not the most dissimilar by 16S or *gyrB* gene sequence comparisons). One potential example of genetic divergence due to geographical isolation occurs at the infraspecific level among *S. arenicola* strains cultured from the Sea of Cortez (phylotypes 'A' and 'B'), which differ at one of two nucleotide positions from all other *S. arenicola* strains. This drift remains apparent (with the exception of CNH-962), yet subtle, in the *gyrB* sequence data where it is restricted to synonymous nucleotide changes.

Members of the genus *Salinispora* share > 99% sequence identity and thus exemplify what has been described as a microdiverse ribotype cluster (Acinas *et al*., 2004). Molecular analyses of environmental samples reveal that most bacterioplankton fall into such clusters; however, it has not been possible to determine if they represent ecologically distinct populations (Acinas *et al*., 2004). The classification of the *Salinispora* sequence cluster as a new actinomycete genus provides culture-based evidence that a microdiverse sequence cluster can represent a clearly defined unit of bacterial diversity. The characterization of three species within this genus further demonstrates that sequence clusters can be more complex than previously believed, with taxonomic significance extending to consensus groups that share > 99% sequence identity. Although it cannot be determined if the lack of 16S rRNA gene sequence diversity within individual *Salinispora* species is maintained by periodic selection or the result of recent ecological differentiation, it has been possible to use fine-scale phylogenetic analyses to resolve closely related species within a single ribotype cluster thus affecting 16S rDNA-based estimates of global species diversity.

The negative effects of exposure to 4°C on growth and survival may explain why we have not successfully cultured *Salinispora* strains from temperate waters off San Diego. To date, the deepest sediment we have examined was collected at 1100 m off the Bahamas, and *Salinispora*

 

strains were successfully recovered from this sample (Mincer *et al*., 2005). Given that the vast majority of the world's ocean temperatures at 1500 m are below 4°C, it will be important to determine if *Salinispora* strains can be recovered from samples collected at greater depths and, if so, how they are related to the strains recovered from near-shore sediments.

Ocean sediments cover 70% of the earth's surface, yet little is known about the bacterial diversity within this vast environment. The present study provides evidence that an individual bacterial species within the spore-forming actinomycete genus *Salinispora* is globally distributed in tropical and subtropical ocean sediments and that speciation within this genus is driven by ecological selection not geographical isolation. Although the ecological characteristics that distinguish these species are presently unknown, the results provide evidence that commonly applied operational taxonomic unit criteria (e.g. 97% sequence identity) underestimate global species diversity. These results support the occurrence of species-level cosmopolitanism among free-living bacteria that possess robust survival strategies and the use of 16S rRNA gene sequences to delineate among closely related species.

## Experimental procedures

### *Strain isolation*

Marine sediments were collected from multiple sites within six geographically distinct locations (the Bahamas, the US Virgin Islands, Guam, the Sea of Cortez, the Republic of Palau and the Red Sea) and processed for the cultivation of actinomycetes using previously described methods (Jensen *et al*., 1991; 2005; Mincer *et al*., 2002; 2005). In general, these methods consisted of pretreatment, usually either drying in a laminar flow hood or diluting in seawater and heating, prior to inoculation onto various types of agar media prepared with natural seawater and selective antibiotics. *Salinispora* strains were initially recognized on primary isolation plates by colony morphology. Morphologically diverse strains were selected and the taxonomic assignment of pure cultures was subsequently confirmed by partial 16S rRNA gene sequence analysis.

One hundred and fifty-two strains from various locations and sample depths were examined. These strains consisted of 19 from the Bahamas, 20 from the US Virgin Islands, 56 from Guam, 17 from the Sea of Cortez, 23 from Palau and 17 from the Red Sea. Of these strains, 36 were selected for nearly complete 16S rRNA gene sequence analysis. These strains were obtained from independent sediment samples and include multiple representatives of each phylotype (based on partial 16S rRNA gene sequence analysis) observed from each collection site and year. An additional 10 strains (five from the Bahamas, three from Guam, one from the Red Sea and one from the Sea of Cortez) from previous studies (Mincer *et al*., 2002; Jensen *et al*., 2005) were also included in the analyses.

### *DNA extraction, 16S rRNA gene amplification and sequencing*

Genomic DNA template was prepared as previously described (Mincer *et al*., 2002) using a method modified from the study by Marmur (1961). Nearly complete 16S rRNA genes were PCR-amplified in 50 μl reactions using 10–50 ng of genomic DNA template, 0.5 μM of the forward FC27 (5′ to 3′ AGAGTTTGATCCTGGCTCAG) and reverse RC1492 (5′ to 3′ TACGGCTACCTTGTTACGACTT) primers, 100 μM (each) dATP, dCTP, dGTP and dTTP, 2.5 U *Taq* polymerase (New England Biolabs) and 1× PCR buffer. The PCR condition were 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, followed by 72°C for 7 min. The PCR products were purified using a Qiagen QIAquick PCR clean-up kit following the manufacturer's protocols (Qiagen, Chatsworth, CA), quantified and submitted for sequencing to the UCSD Cancer Center DNA Sequencing Shared Resource (3100 Genetic Analyzer, PE-Applied Biosystems, USA). Partial sequences were obtained using the forward primer FC27 while nearly complete gene sequences were obtained for top and bottom strands using the additional forward primers F514 (5′ to 3′ GTGCCAGCAGCCGCGG TAA) and F1114 (5′ to 3′ GCAACGAGCGCAACCC) and the reverse primers R530 (5′ to 3′ CCGCGGCTGCTGGC ACGTA), R936 (5′ to 3′ GTGCGGGCCCCCGTCAATT) and RC1492.

### gyrB *gene amplification and sequencing*

Two sets of primers were designed to amplify partially overlapping, double-stranded contigs encompassing the nearly complete *gyrB* gene (1164 bp). These primer sets were: (1) F33NT and R662T (5′ to 3′ TGTAAAACGACGGCCA GTgtctccggcggyctgcaccg and CAGGAAACAGCTATGACC cctcgtgggtrccgccctc) and (2) F611T and R1300T (5′ to 3′ TGTAAAACGACGGCCAGTcgartcstayggcgagtcggtctacacc and CAGGAAACAGCTATGACCcagcacsaycttgtggtascgcagctt). M13 forward and reverse sequencing tags (capitalized) were added to the 5′ ends. Polymerase chain reactions were performed in a total volume of 50 μl that contained for primer set 1, 50–100 ng DNA template, 1.0 μM each F33NT and R662T, 200 μM (each) dATP, dCTP, dGTP and dTTP, 5.0 U AmpliTaq Gold (Applied Biosystems), 1× $MgCl_2$, 1× PCR buffer. For primer set 2, all reagent concentrations were halved except for the template and DNA polymerase. Polymerase chain reaction products were purified and sequenced as described above using M13 forward and reverse primers. The PCR conditions were as follows: 95°C for 10 min followed by 35 cycles of 94°C for 1 min, 65°C for 1 min (primer set 1) or 68°C for 1 min (primer set 2), 72°C for 1 min, followed by 72°C for 7 min.

### *Phylogenetic analyses*

16S rRNA gene sequence contigs were assembled and checked for accurate base calling using Sequencher (version 4.5, Gene Codes, Ann Arbor, MI), aligned using Clustal X, and imported into MacClade (version 4.07, Sinauer Associates, Sunderland, MA) for manual alignment and masking.

Single nucleotide changes that were observed in only one strain and occurred in areas of ≥ 98% conservation among all bacteria (Cannone *et al*., 2002) were confirmed by performing a new PCR. Secondary structure analyses were performed using the ARB software package (Ludwig *et al*., 2004). Neighbour-joining, parsimony and bootstrap analyses were performed using PAUP (version 4.0b10, Sinauer Associates, Sunderland, MA). *gyrB* DNA sequences were aligned, translated and analysed using MacClade. Sequence similarities were calculated using various NCBI (National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) functions. Sequence data have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) under accession numbers AY040617–AY040623, AY464533–AY464534, DQ224159–DQ224165, DQ092624 for 16S rRNA genes and DQ228678–DQ228693 for *gyrB* genes.

### *Effects of temperature on growth*

Seven strains were tested for the effects of storage at 4°C on growth and viability. The strains tested were CNB-440, CNR-699 (*S. tropica*), CNR-114, CNS-103, CNS-143 ('*S. pacifica*'), CNB-527, CNR-425 (*S. arenicola*). Strains were started in 25 ml of liquid cultures (A1 medium, 1.0% starch, 0.4% peptone, 0.2% yeast extract, 100% seawater) then inoculated by dilution streaking onto five replicate A1 agar plates. One replicate for each strain was immediately incubated at 25°C (positive control). All of the positive controls reached maximum visible growth within 2 weeks at 25°C. The remaining replicate plates were incubated at 4°C with one replicate per strain being transferred to 25°C after 2, 4, 6 and 8 weeks at reduced temperature. Following transfer to 25°C, plates were monitored for 2 months and growth recorded as equal, reduced, or no growth relative to controls.

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