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Evolutionary divergence and biogeography of sympatric niche-differentiated bacterial populations

Brian B Oakley[1], Franck Carbonero[1], Christopher J van der Gast[2], Robert J Hawkins[3] and Kevin J Purdy[1]

[1]Department of Biological Sciences, University of Warwick, Coventry, UK; [2]NERC Centre for Ecology and Hydrology, Oxford, UK and [3]Department of Biological Sciences, University of Reading, Reading, UK

Using multiple lines of evidence from denaturing gradient gel electrophoresis, environmental sequences and TaqMan quantitative PCR assays targeting a functional gene for sulfate respiration (*dsr*) affiliated with the geochemically important genus *Desulfobulbus*, we revealed strongly restricted distributions of specific genotypes and populations correlated with sampling position along an estuarine gradient free of dispersal barriers. Evidence of evolutionary divergence of populations was provided by three complementary analyses. First, analysis of molecular variance rejected the null hypothesis that genetic diversity within each sampling site was not significantly different than that of all sites pooled together ($P < 0.0001$). Second, UniFrac and Parsimony tests showed phylogenetic clustering of sampling sites was highly significant ($P < 0.001$). Third, pairwise F_{ST} statistics showed significant evolutionary divergence of populations based on the location in the estuary. To test the hypothesis that environmental niche-driven evolutionary divergence can create and maintain microbial biogeography, we used both statistical inference and an experimental manipulation to assess the independent effects of environment and geography. Significant effects of each on genotype distributions and population divergence supported the hypothesis. Our data are consistent with both sympatric and parapatric models of speciation, and suggest niche partitioning can contribute to evolutionary divergence and observable biogeographic patterns in microbial communities even among closely related taxa at limited spatial scales without significant barriers to dispersal.
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Introduction

Despite recent calls for an increased role for ecological theory in microbial ecology (Prosser *et al.*, 2007), the applicability of many ecological theories and principles to microorganisms remains relatively untested as evidenced by recent debates regarding, for example, the importance of niche differentiation and biogeography (for reviews, see Green and Bohannan 2006; Green *et al.* 2008; Martiny *et al.* 2006). There is an increasing body of evidence suggesting that various microbial communities are structured by environmental niche specialization (West and Scanlan, 1999; Gray *et al.*, 2007; Hunt *et al.*, 2008; Macalady *et al.*, 2008), which can give rise to geographic structure, but the influence of geographic distance by itself on microbial communities has generally been considered to be small

(Horner-Devine *et al.*, 2004; Yannarell and Triplett, 2005; Fierer and Jackson, 2006; Van der Gucht *et al.*, 2007), and exceptions limited to taxa with presumed barriers to dispersal (Papke *et al.*, 2003; Whitaker *et al.*, 2003) and/or geographically widely separated environments (Cho and Tiedje, 2000).

Weak biogeographic signals in microbial communities are usually explained by invoking the working hypothesis of microbial cosmopolitanism famously first formulated by Baas Becking (de Wit and Bouvier, 2006), which has intuitive appeal based on the small size and high dispersal potential of microbes, their large populations and low presumed extinction rates. However, insufficient taxonomic resolution has been shown to mask true geographic differences among strains (Cho and Tiedje, 2000), and sampling problems commonly encountered in analyzing entire communities as taxon-rich and diverse as most microbial communities may also obscure true biological patterns, particularly when observational approaches are relied upon exclusively.

As a test of the general applicability of niche partitioning and biogeography to microbial communities, we combined observational and experimental

approaches to investigate a system in which these phenomena might reasonably be expected not to occur: a single monophyletic bacterial genus distributed across a km-scale estuary strongly connected physically by tidal water flows. If, in the absence of dispersal barriers, niche differentiation and biogeography can be observed in the distributions of common microbial taxa at small spatial and phylogenetic scales then these basic ecological concepts may apply more generally to microbial communities.

As a model taxon, we targeted sulfate-reducing bacteria (SRB) belonging to the genus *Desulfobulbus*, in the class δ-Proteobacteria. Dissimilatory sulfate reduction is a globally important biogeochemical process in which sulfate-reducing prokaryotes link the carbon and sulfur cycles through their use of sulfate as a terminal electron acceptor in the oxidation of organic matter; sulfate-reducing prokaryotes are estimated to re-mineralize ca. 50% of the fixed carbon in temperate coastal marine sediments (Jorgensen, 1982). All known members of the genus *Desulfobulbus* grow anaerobically through the respiration of sulfate coupled to the oxidation of three-carbon compounds, and are also metabolically versatile with the ability to grow fermentatively or mixotrophically (Laanbroek *et al.*, 1982). Sulfite reductases of sulfate-reducing prokaryotes perform the 6 e$^-$ dissimilatory reduction of SO_3^{2-} to H_2S and occur in varying forms, but all contain iron–sulfur clusters and siroheme groups, and are highly conserved across phylogenetic lineages (Klein *et al.*, 2001; Zverlov *et al.*, 2005), and genes encoding these enzymes (*dsrAB*) have been widely used as phylogenetic markers (Wagner *et al.*, 1998; Stahl *et al.*, 2002; Geets *et al.*, 2006).

We hypothesized that restricted distributions of *Desulfobulbus* genotypes and populations can be observed across a continuous environmental gradient and this can be inferred as evidence of environmental niche partitioning. In addition, we hypothesized if niche partitioning exists and is truly ecologically significant, it should contribute to restricted gene flow among local populations over evolutionary time scales, which can be detected as evolutionary divergence and biogeographic patterns observable among extant taxa. To test these hypotheses, we used both observational and experimental methods to analyze the distribution of *Desulfobulbus* in triplicate sediment samples from 11 sites along a small (15.4 km), macrotidal estuary with strong gradients of salinity and sulfate (Figure 1). Using several complementary approaches, we first determined whether the distributions of *Desulfobulbus* populations and specific genotypes are significantly spatially constrained across the estuary. Next, we used two related measures, genetic distance and phylogenetic clustering, to assess population divergence and sympatric evolution. Finally, we used both statistical inference and a reciprocal transplant experiment to quantify the relative contributions

of environmental factors and geography to the distribution of *Desulfobulbus* genotypes and populations along the estuary.

## Materials and methods

Study site and sample collection
Triplicate sediment cores for pore water analyses and DNA extractions were taken at low tide from 11 sites along the Colne estuary (Figure 1) in September 2004, as described previously (Hawkins and Purdy, 2007). Samples for the slurry experiment were collected in September 2008 from sites 1 and 10 (Figure 1).

Molecular methods
PCR. Amplification of a portion of the bisulfite reductase gene targeted to *Desulfobulbus* was achieved by PCR with a newly designed forward primer, DSR458F (5′-CGCC/T/GCTGATGA/GATCTG TG-3′) and DSR4R (Wagner *et al.*, 1998). The sensitivity and specificity of this PCR was evaluated empirically using eight strains of *Desulfobulbus* available from the German collection of microorganisms and cell cultures (2908, 18378, 13871, 2032, 8777, 2058, 2033 and 13044) and several closely related non-target SRBs (*Desulfosarcina variabilis*, DSM2060; *Desulfobacter postgatei*, DSM2034). To generate a PCR product size suitable for denaturing gradient gel electrophoresis (DGGE) and to improve amplification from environmental samples, a semi-nested PCR of *dsrB* was conducted using the same reverse primer as above and 2060F′ (5′-CAACATCGTC/TCATACC/ACAGGG-3′; modified from Geets *et al.* (2006) to improve specificity) and with a 5′ GC clamp added (Muyzer *et al.*, 1993).

The PCR reactions containing 1 μl template DNA (mean concentration 30 ng μl^{-1}), 1× PCR buffer (Promega, Southampton, Hampshire, UK), 200 nM dNTPs, 0.4–1 μM each primer, 2.0 mM MgCl$_2$, 0.8 μM bovine serum albumin and 1 U Taq polymerase (Promega) were carried out using a touchdown protocol of denaturation at 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 53 °C (−0.5 °C per cycle) for 30 s, 72 °C for 2 min 15 s, followed by 20 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 2 min 15 s, and a final 10 min extension at 72 °C. Subsequently, 1 μl of these products was used with a thermal cycling regime of 94 °C for 3 min followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min, and a final 30-min extension at 72 °C.

DGGE. Denaturing gradient gel electrophoresis was performed with PCR products obtained from each of the three replicate sediment cores from the 11 sampling sites separately and also with amplicons combined in equimolar ratio. Polyacrylamide gels (8%) with a 30–80% denaturant gradient (defined with 100% as 42% w/v urea and 40% v/v formamide) were electrophoresed in 0.5× Tris
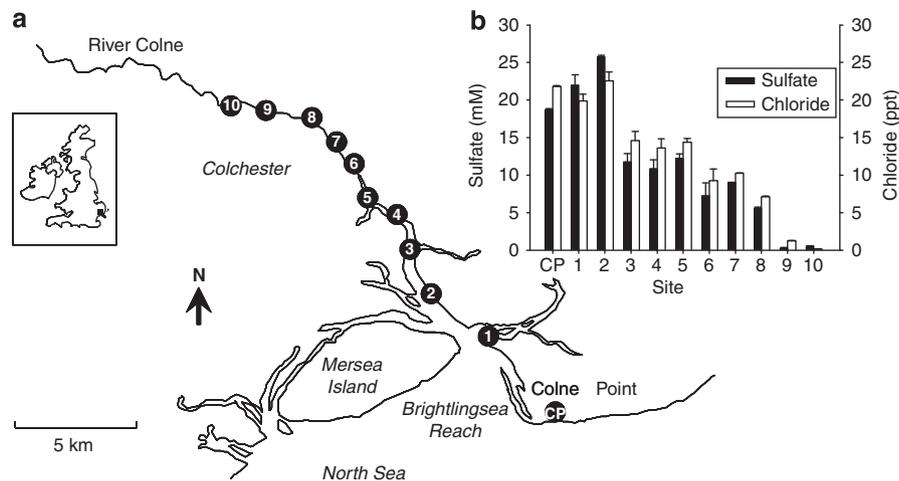
Figure 1 (**a**) Map of sampling locations in the Colne estuary, a small macrotidal (3–5 m) estuary on the east coast of England, which enters the North Sea at Brightling sea (51°45′N, 01°03′E). (**b**) Pore water concentrations of Cl^- and SO_4^{2-} ions. Data represent means (± 1 s.e.) of three sediment samples taken within a 50-cm radius at each sampling site.

acetate EDTA using a Bio-Rad (Hemel Hempstead, Hertfordshire, UK) D-Code DGGE system at 75 V for 24 h, as empirically optimized for band separation and resolution. Gels were stained with ethidium bromide, visualized under UV light and images analyzed with GelCompare 3.0 (Applied Maths, Kortrijk, Belgium). Selected bands were removed from gels with minimal exposure to UV light by excision with a pipette tip, reamplified and sequenced to confirm a single sequence type.

Cloning. Same amplicons used for DGGE were cloned using the TOPO TA cloning kit (Invitrogen, Paisley, Renfrewshire, UK) after purification using Qiaquick spin columns and subsequent addition of A overhangs by a 30-min incubation at 72 °C in the presence of 200 nM dATP and 1 U Taq polymerase. Recombinant clones were screened for insert presence and size by PCR with M13 primers that were also used for sequencing of PCR products.

Quantitative PCR. Sequence data from clone libraries were used to design new quantitative PCR (qPCR) assays using Applied Biosystems' (Warrington, Cheshire, UK) TaqMan gene expression master mix with minor groove binder (MGB) probes. For each assay, the sensitivity and specificity of 1000–5000 candidate assays were evaluated using a custom software tool we developed for this purpose (http://go.warwick.ac.uk/thermophyl/). The PCR conditions were as per manufacturer recommendations and the reactions were carried out on an ABI 7000 or ABI 7500. Each assay was optimized with titrations of primer and probe, and sensitivity and specificity were validated with plasmids from representative clones of target operational taxonomic units and, at least, four of the most closely related non-target sequences as determined from alignments of primer and probe sequence

regions. Optimized assays contained 300 nM (600 nM for FW2; FW, freshwater) each of the forward primer and reverse primers, 200 nM MGB probe and $1 \times$ ABI GeneExpression Master Mix in 25-μl reactions. Primer and probe sequences for assays M, Mh, FW1 and FW2 are listed in Supplementary Table S3. Assays were considered valid when all no-template controls were negative, calibration curve R^2 values were $>98\%$, and amplification efficiency was between 90% and 115%. All replicates from each sampling site were run on a single plate for consistency.

To check for inhibition of PCR reactions both $1 \times$ and $0.1 \times$ dilutions of environmental preps, shown to contain the target sequence, were amplified. To confirm qPCR specificity, amplicons from each assay were cloned and 10–12 clones sequenced.

Slurry experiment
Sediment from sites 1 and 10 was incubated in a factorial design in which sulfate and salinity were manipulated independently. For each site, three replicate sediment cores (0–5 cm depth) were taken within 50 cm of each other, and maintained at 8 °C for <12 h before initiating the experiment. Each core was homogenized and 0.4–0.5 g of sediment added to anaerobic Balch tubes containing 5 ml of medium in one of four formulations (S+/Cl+, S+/Cl−, S−/Cl+ and S−/Cl−), with sulfate and chloride concentrations based on pore water concentrations at sites 1 and 10. DSMZ Medium 194 was prepared anaerobically without Na_2SO_4, NaCl, and $MgCl_2$ and with the following amounts of each added to the treatments (g l^{-1}): 3.0, 20 and 3.1 (S+/Cl+); 3.0, 1.0 and 0.4 (S+/Cl−); 0.075, 1.0 and 3.1 (S−/Cl+); and 0.075, 1.0 and 0.4 (S−/Cl−). Carbon was provided to all treatments as sodium propionate per standard medium formulation (1.5 g l^{-1}; 15.6 mM). The DNA

was extracted according to the procedure used by Purdy et al. (1996) from sediment samples at the initiation of the experiment and after incubation at 23 °C for 7 days. Data were analyzed using a three-factor analysis of variance after confirming they met the appropriate assumptions.

Phylogenetic and statistical analyses

Raw sequence data were edited and contigs assembled using SeqMan (DNASTAR, Madison, WI, USA). Sequences were incorporated into ARB (Ludwig et al., 2004) for translation with amino-acid alignments, manually adjusted according to a curated seed database (http://fungene.cme.msu.edu/) and used as the basis for DNA alignments. Trees were built using maximum-likelihood and neighbor-joining algorithms in ARB and with MrBayes (Huelsenbeck et al., 2001) with the following modifications to default parameters: GTR model of evolution with gamma-distributed rate variation, number of chains set to 6, numbers of swaps to 3, and temperature to 0.1; 200 000 generations produced stable convergence. Two approaches were used to restrict analyses to those sequences that were clearly affiliated with Desulfobulbus. First, sequences were grouped into operational taxonomic units using the furthest-neighbor method of the DOTUR software package (Schloss and Handelsman, 2005), and trees built as above. In addition, blastn and blastp were run locally against a representative seed database as above. The intersection of these two approaches produced a set that contained 82% (270/331) of the sequences retrieved from the environment.

Arlequin (Excoffier et al., 2005) was used to calculate analysis of molecular variance and F_{ST} statistics. Phylogenetic clustering was assessed with UniFrac (Lozupone and Knight, 2005) and Parsimony tests (Martin, 2002), which compare observed phylogenies with a null model derived from random permutations. Ecologically coherent clusters of sequences were defined phylogenetically using AdaptML (Hunt et al., 2008). AdaptML uses inputs of a phylogenetic tree and habitats from which samples were derived to build a hidden-Markov model to determine the probability that adjacent nodes in the tree share the same 'projected habitat' that is used to define ecologically meaningful phylogenetic groups. We ran the model with default parameters except for a more precise numerical optimization of the habitat transition rate parameter.

To assess the significance of taxonomic turnover across the estuary, we used distance decay of similarity of three measures: curve-based profiles of DGGE banding patterns, relative intensities of sequenced DGGE bands, and variation in dsrB gene copy number determined by qPCR assays. The first approach measures the turnover of all Desulfobulbus-affiliated populations as reflected by DGGE, and the latter two metrics assess turnover of specific genotypes. Distance decay calculations and partial

Mantel tests were carried out as described previously (Green et al., 2004).

Results

Population-level distributions of Desulfobulbus genotypes

Denaturing gradient gel electrophoresis analysis of Desulfobulbus-specific dsrB amplicons indicated significant differences among populations associated with location in the estuary. Unweighted Pair Group Method with Arithmetic mean (UPGMA) and neighbor-joining clustering of DGGE intensity profiles showed sites nearer to each other were generally more similar to each other than to sites
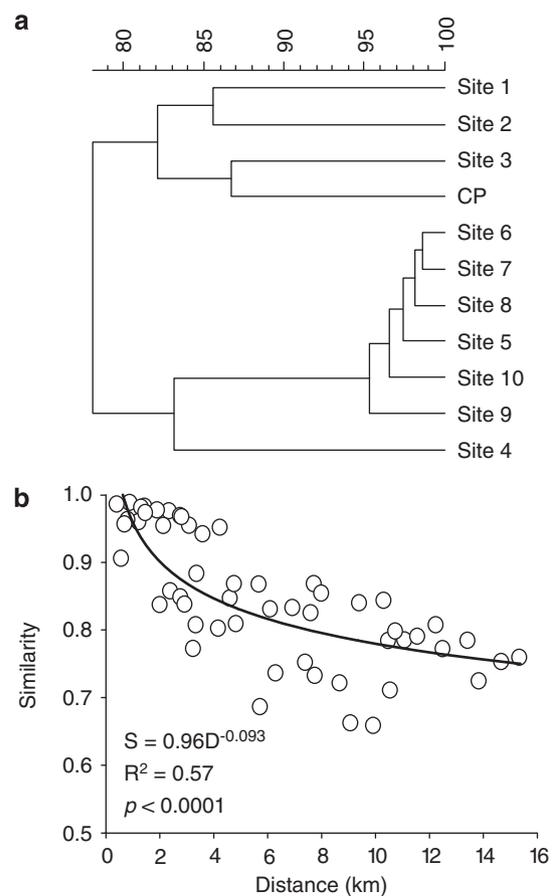
Figure 2 Turnover and structuring of Desulfobulbus populations across the estuary based on analyses of curve-based profiles of denaturing gradient gel electrophoresis (DGGE) fingerprints from equimolar mixtures of PCR amplicons from each of three sediment replicates per site. (**a**) UPGMA cluster analysis of curve-based DGGE profiles; horizontal scale represents % similarity. Neighbor-joining (NJ) methods produced an identical topology, consistent with the ordination of the same data matrix shown in Supplementary Figure S1c. Nearly identical tree topologies resulted from each sediment replicate run separately with DGGE (data not shown). (**b**) Distance-decay of Pearson similarity showing significant turnover of Desulfobulbus-like genotypes. The distance decay power law equation is of the form $S = cD^d$, where S is similarity between any two samples separated by distance (D), c is a constant and d is the rate of decay in similarity.

distant from one another, with the major distinction between *Desulfobulbus* populations at the marine and freshwater ends of the estuary (Figure 2a). This result was confirmed by a distance decay analysis that showed a significant decline in DGGE profile similarity with increasing spatial distance between samples ($P < 0.0001$; Figure 2b).

Distributions of specific Desulfobulbus *genotypes*
Two lines of evidence revealed restricted distributions of specific *Desulfobulbus* genotypes with relative abundance strongly correlated to the location in the estuary. First, DGGE bands, which were sequenced to confirm they represented a single *Desulfobulbus* sequence type (Figures 3a and b), showed variations in relative intensity strongly

coupled to location along the estuarine gradient. Specifically, Band M was only detected at sites 1 and 2 at the marine end of the estuary, Band Mh was only detected at sites 3–5 with maximal intensity at site 4 and, and bands FW1 and FW2 were associated with the freshwater end of the estuary and were not detected at the marine end (Figure 3c).

Second, novel qPCR TaqMan assays were designed to specifically and sensitively target each of these four *dsrB* sequence types (M, Mh, FW1 and FW2). These assays confirmed the DGGE results with only minor differences between the two methods (Figure 3d). Sequence type M was again detected at the marine-dominated sites 1 and 2 and also in low abundance ($< 10^2$ copies per μg extracted DNA) in single sediment replicates from two additional sites (3 and 7). Genotype Mh was
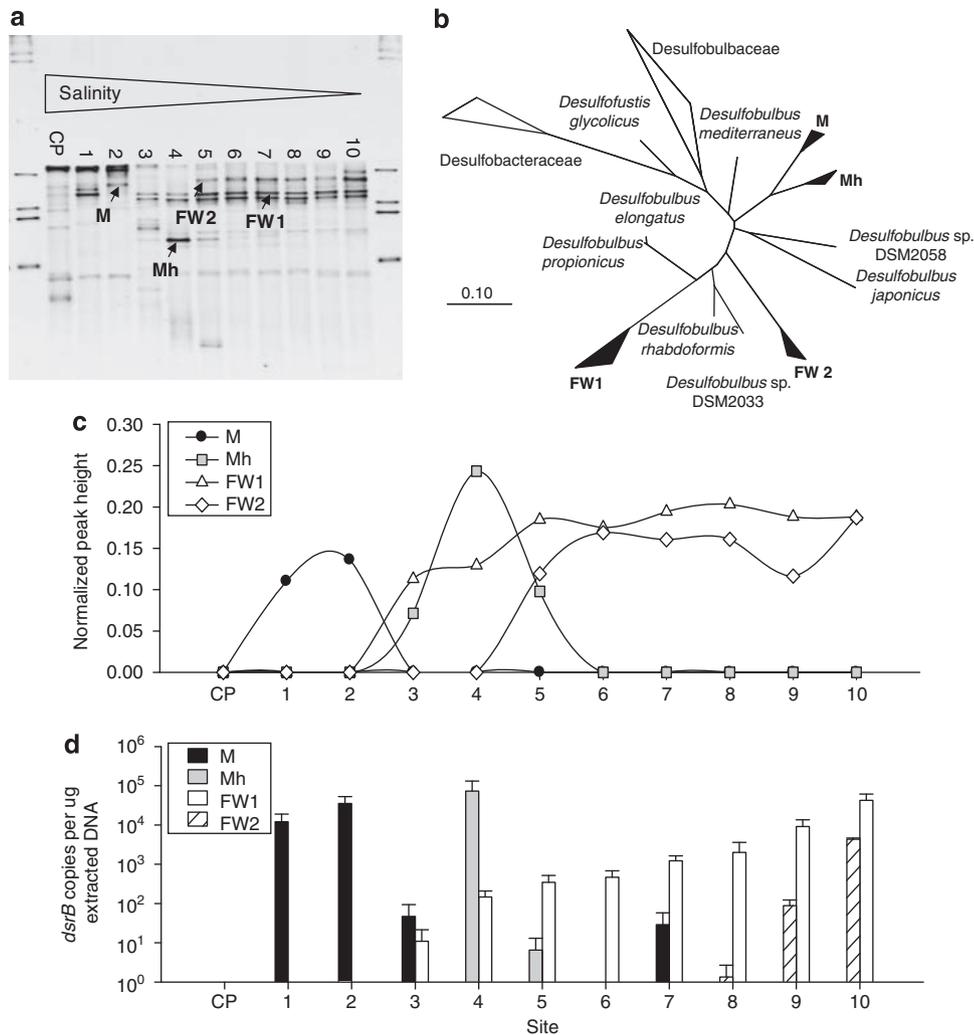
Figure 3 Identity and distributions of *Desulfobulbus*-affiliated *dsrB* ecotypes. (**a**) Bands sequenced from denaturing gradient gel with (**b**) phylogenetic positions shown within *Desulfobulbus*. Tree was reconstructed from amino acid informed DNA alignment using the maximum-likelihood algorithm AxML. (**c**) Distributions of these four genotypes across the estuary as assessed by denaturing gradient gel electrophoresis (DGGE). Values represent peak heights normalized within each lane to control for loading differences of same gel shown in (**a**), which was run with equimolar mixtures of PCR amplicons from three sediment replicates per site. (**d**) Distributions of four genotypes as assessed by clade-specific quantitative PCR (qPCR) assays. Values represent means of three biological replicates ± 1 s.e. New assays were developed for each sequence type based on a library of sequence data constructed from clones and DGGE band sequences.

restricted to the middle of the estuary, genotype FW1 had a relatively wide range from sites 3–10, whereas FW2 had a more narrow distribution restricted to the freshwater end of the estuary (Figure 3d). Cloning and sequencing of qPCR products from all four assays confirmed affiliations with the appropriate clades shown in Figure 3b. Distance decay measures confirmed the significance of the restricted distributions of these genotypes; the decline in similarity with increasing distance was highly significant ($P < 0.0001$) for both DGGE- and qPCR-based assessments of genotype distributions.

Evolutionary divergence of populations

To assess the evolutionary divergence of *Desulfobulbus* populations in the Colne estuary, we used two related measures: genetic distance and phylogenetic clustering. First, analysis of molecular variance rejected the null hypothesis that genetic diversity within each site was not significantly different than that of all sites pooled together ($P < 0.001$). As an initial test of the hypothesis that sites near in space and with similar environmental parameters should be more similar to each other than to more distant sites, we grouped the seven sites for which we had sequence data from clone libraries into three clusters (sites 1 and 2; sites 3 and 4; and sites 5, 8 and 10) as suggested by the DGGE and qPCR results shown in Figures 2 and 3. Analysis of molecular variance confirmed that genetic variability was significantly different among these three groups ($P = 0.005$; Supplementary Table S1).

As an additional test, we performed pairwise comparisons of genetic distance between sites. Pairwise F_{ST} statistics, a commonly used metric of genetic distance between populations (Excoffier *et al.*, 1992), were highly significant ($P < 0.0001$; $P < 0.004$ for site 5 versus 8) for all comparisons except one, demonstrating that these populations have indeed diverged significantly from one another. The only non-significant pairwise comparison was between the two closest sites, 8 versus 10 ($P = 0.540$).

As evolutionary divergence should be reflected in phylogeny, we next performed several statistical analyses to test whether the *Desulfobulbus dsrB* phylogeny was significantly clustered by site. For both UniFrac and Parsimony test statistics (Martin, 2002; Lozupone and Knight, 2005) based on branch lengths or branch positions unique to each site, the null hypothesis of random phylogenetic
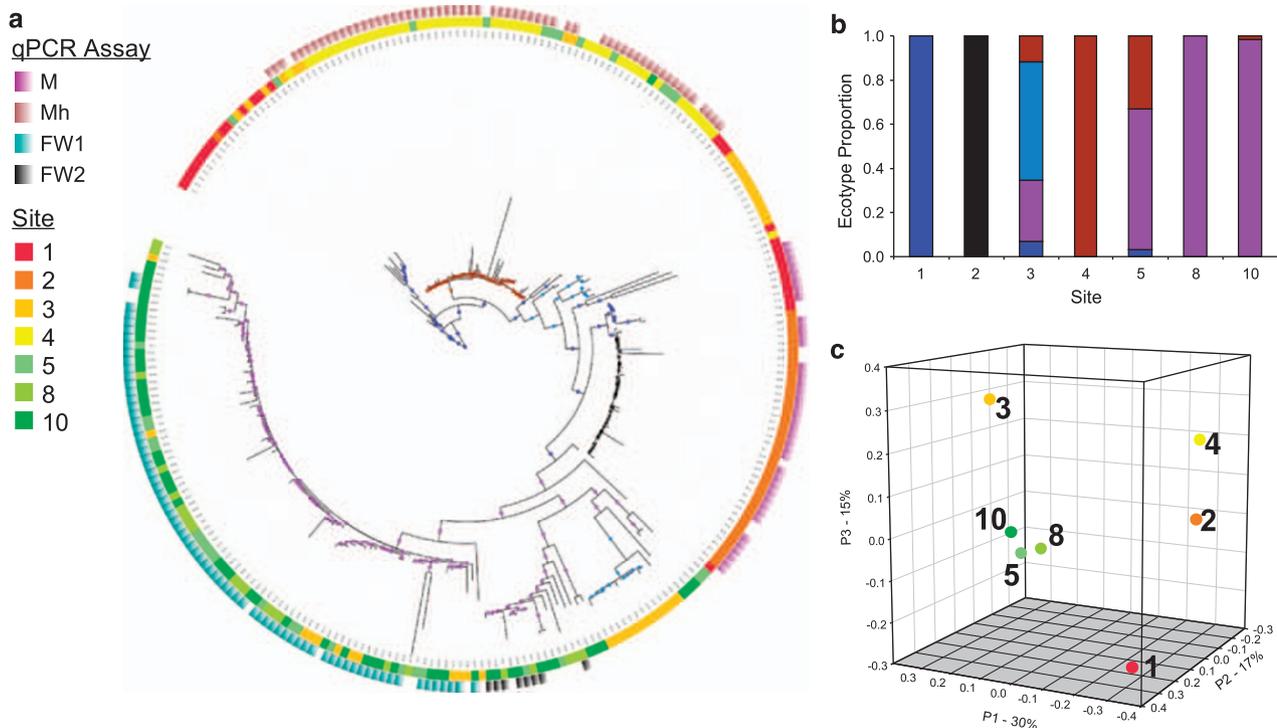

Figure 4 Phylogenetic clustering of *Desulfobulbus*-affiliated sequences and ecotype definitions and distributions. (**a**) Phylogenetic placement of sequences on a maximum-likelihood reconstruction based on amino acid-informed DNA alignment with a 50% minimum homology filter. Sampling sites are depicted by color on the first outer ring for the seven sites for which sequence data was obtained. The null hypothesis of random clustering of sequences by site was rejected with both UniFrac and Parsimony tests ($P < 0.001$). The second outer ring indicates the sequences in the tree with exact matches to each of the four clade-specific quantitative PCR (qPCR) assays. Five phylogenetic clusters (ecotypes) predicted by the AdaptML model as described in the text are shown at the nodes of the tree. The relative proportions of these five predicted ecotypes for each sampling site are shown in (**b**). The model converged on five clusters regardless of the number of initial habitats initialized. (**c**) Position of each site on three most significant eigenvectors of a principal component analysis applied to phylogenetic positions from (**a**). Analyses of neighbor-joining and Bayesian trees gave essentially the same results.

clustering by site was rejected ($P<0.001$; Figure 4a). We next defined ecologically coherent populations (ecotypes) as recently advocated (Koeppel *et al.*, 2008) by using the AdaptML model that, briefly, uses a hidden-Markov model to assign a 'projected habitat' or ecotype to each node based only on the habitat from which each sample was derived and the observed phylogeny (Hunt *et al.*, 2008). Regardless of the number of initial habitats defined, the model consistently grouped sequences into five ecotypes that were clearly associated with sampling site (Figures 4a and b). Clustering of phylogenetic position by site with principal component analysis (PCA) also showed distinct groupings of the sampling sites (Figure 4c), regardless of the tree reconstruction method and consistent with the F_{ST} results.

Influences of environment and geography

To assess the relative contributions of environmental factors versus geography to the distributional patterns we documented, we used both statistical inference and an experimental manipulation. First, constrained ordinations of community composition and dissimilarity showed significant associations between clustering of sites and geographic distance, sulfate and chloride (Supplementary Figure S1). Partial constrained ordinations generally showed no significant relationship with environment or geography when the effect of the other was removed, but this result seemed to depend on the form of the geographic distance data (Supplementary Figure S1). Second, partial Mantel tests showed sulfate was significantly correlated with the distribution of the four genotypes (M, Mh, FW1 and FW2), as well as that of the entire *Desulfobulbus* community when the effect of distance between sites was removed (Supplementary Table S2). In the converse analysis, after controlling for the influence of sulfate and chloride concentrations, distance between sites alone was also highly significant (Supplementary Table S2). Partial Mantel tests with pairwise F_{ST} as the dependent variable showed a highly significant effect of geographic distance between sites on genetic distance after controlling for both sulfate and chloride, whereas, when the effect of geographic distance between sites was removed, only sulfate was significant ($P<0.03$; Supplementary Table S2).

To test these statistical inferences experimentally, we collected fresh sediment from two locations in the estuary (sites 1 and 10) and performed incubations in media in which salinity and sulfate were varied independently. After laboratory incubation for 7 days, changes in *dsrB* copy number of three genotypes, FW1, FW2 and M, were determined by qPCR. The response of the two freshwater genotypes (FW1 and FW2) suggested that location (geography) is the primary determinant of abundance for these taxa—both genotypes were significantly more abundant in sediment from the freshwater site 10 than
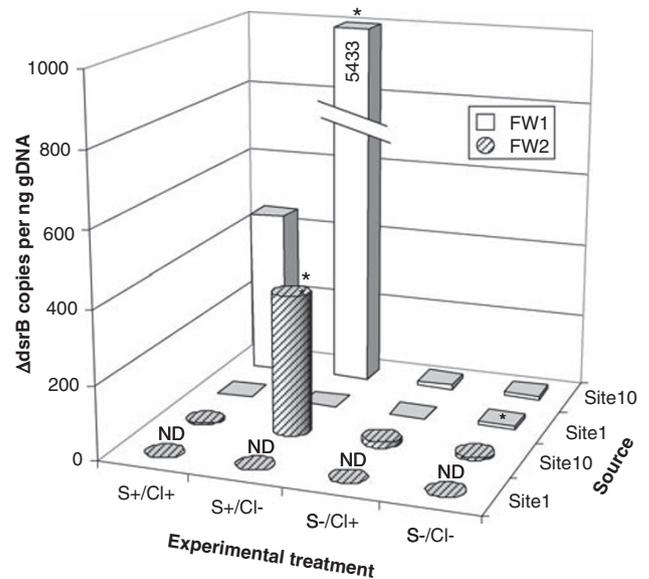
Figure 5 Growth responses of FW1 and FW2 (freshwater) genotypes to manipulations of sulfate and chloride concentrations in a sediment slurry. Media formulations were based on *in situ* conditions at sites 1 and 10 as described in the text. The *y*-axis represents increase in *dsrB* gene copy number after 1 week (mean of three biological replicates). Between-site means are significantly different ($P<0.05$) for both genotypes. Significant treatment differences within each site for each genotype are marked by *. No signal was detected for any replicate before or after treatment for bars labelled 'ND'. Natural distributions of the two genotypes across the estuary are shown in Figure 3.

that from the marine site 1 regardless of experimental manipulation of environment (Figure 5). The more widely distributed genotype FW1 (natural distribution shown in Figure 3) was detected in all site 1 experimental replicates, but only at very low abundance (maximum 9.8 ± 6.0 gene copies per ng genomic DNA; Figure 5), whereas genotype FW2 was not detected in any sample from site 1 regardless of treatment (Figure 5).

Both genotypes also clearly have different environmental tolerances—both responded most positively to the addition of sulfate and preferred their native low-salinity media with the addition of sulfate ($S+/Cl-$ treatment; Figure 5). Interestingly, FW1 seemed to be able to tolerate increased salinity ($S+/Cl+$ treatment), whereas FW2 did not (Figure 5). This result is consistent with the distributions we measured of these two genotypes in the environment, in which FW1 is relatively widely distributed and FW2 is restricted to the freshwater end of the estuary (Figure 3). Taken together, results of this multifactorial slurry experiment confirmed that patterns of distribution for these *Desulfobulbus* genotypes reflect both environment and geography.

Given the clear site-specific differences among *Desulfobulbus* populations and the relatively narrow distributions of several genotypes, we expected to find some temporal variability in the community

composition at any given location, and indeed, genotype M was not detectable in any samples collected in 2008 regardless of treatment or location. As site 1 was at the marine limit of the distribution of genotype M in 2004 (Figure 3), we assume a shift in environmental conditions rendered site 1 unfavorable to genotype M.

Discussion

The relative influence of geography and environment on the distributions of microbes has been a source of debate ever since Baas Becking (de Wit and Bouvier, 2006). Here, we argue for an evolutionary perspective that reconciles environment and geography by providing evidence for a microbial biogeography like that of macro-organisms, whose distributions represent both contemporary environmental conditions and an evolutionary history reflected in their geography. Our data support niche partitioning on the basis of environment (environmental selection or habitat filtering), which over evolutionary time scales can support the divergence of local populations from their neighbors as a distance-dependent function. We found strong, independent associations between genetic distance and both environmental distance and geographic distance. On the basis of multiple lines of evidence from DGGE, sequencing and targeted qPCR assays, we concluded that several *Desulfobulbus* genotypes have strongly restricted distributions that contribute to significant spatial autocorrelation of *Desulfobulbus* populations across the estuary. Each of the genotypes we investigated seems to be restricted to particular locations in the estuary, and in other locations is either absent or present only at very low abundance. These distributions are strong evidence for ecologically meaningful niche partitioning and, as we demonstrate using a variety of statistical inferences and an experimental manipulation, for evolutionary divergence of locally adapted populations and clearly observable biogeographic patterns.

The conclusion that both environment and geography have a role in structuring microbial communities supports the prediction that community composition at a given site in the estuary fluctuates slightly with changing environmental conditions (for example, the site 2 community may periodically resemble that of site 1 or site 3), but sites distant from one another should have little overlap (for example, site 1 should always be different than site 10), regardless of changes in environment, at least over ecological time scales. This prediction was borne out by the slurry experiment—genotype M was non-detectable, presumably because site 1 was at the marine edge of its distribution, and a change in environmental conditions between sample collections has led to its local absence. In addition, for the two freshwater genotypes, location (site 1 versus 10; the two estuarine end points) was the primary

determinant of abundance, regardless of experimental treatment.

Convincing evidence exists for the evolutionary divergence and biogeography of allopatric microbial populations (Papke et al., 2003; Whitaker et al., 2003) and ecological specialization of sympatric populations (West and Scanlan, 1999; Gray et al., 2004; Hunt et al., 2008; Hesselsoe et al., 2009). In contrast, evidence in support of microbial cosmopolitanism (Fenchel and Finlay, 2004) and a persistent dogma that 'everything is everywhere' (Baas Becking, 1934) have led to the suggestion that biogeographic patterns and niche differentiation only apply to microbial communities spanning major environmental gradients or separated by large geographic distances, such as those encountered in global ocean surveys. Our data demonstrate that significant biogeographical patterns can be clearly observed even at relatively small spatial scales with no significant barriers to dispersal.

Whether these data represent distributions of endemic taxa is an interesting question, but one whose answer does not alter our conclusions. Definitive proof of strict endemism requires conclusive evidence of absence, which of course is difficult if not impossible to obtain for microbes, and thus the statement 'everything is everywhere' is a non-falsifiable hypothesis. On the basis of our own calculations of detection thresholds for our qPCR assays (ca. 1000 gene copies per g sediment) and estimates for DGGE (ca. 1% of total DNA; (Muyzer et al., 1993)), we are confident that true distributions of the genotypes we measured do in fact have longer tails than we are currently able to detect. However, this does not affect the conclusion that particular sequence types are preferentially associated with certain portions of the estuary, or the conclusion that locally adapted populations show evidence of evolutionary divergence.

Our data are consistent with both a sympatric model of speciation, in which ranges of daughter taxa overlap (for example, FW1 and FW2), and a parapatric model, in which populations evolve in a niche space adjacent and connected to the original (for example, FW2 and Mh), but exact mechanisms of evolution are still unknown. Homologous recombination rates are known to decrease log-linearly with genetic distance (Majewski, 2001) and so may act as a positive feedback for divergence within populations with some genetic basis for ecological specialization. Ecologically (and spatially) more distant populations should accumulate more mutations, reducing the potential for homologous recombination, in effect experiencing a degree of sexual isolation correlated with geographic distance.

We are not yet able to correlate function with phylogeny or speculate about genetic divergence of genes other than sulfite reductase. We are investigating this subject by analyzing new *Desulfobulbus* strains we have isolated from the Colne estuary, which can also help identify mechanisms for

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population divergence. The fact that we have observed strong signals defining differentiated populations using both *dsr* (this study) and 16S ribosomal RNA genes (Hawkins and Purdy, 2007), and developed sensitive, clade-specific environmental assays for a single gene suggests that our measures of niche differentiation and evolutionary divergence may in fact prove to be conservative. Concatenated multilocus analyses are required in many cases to resolve closely related taxa (Hanage *et al.*, 2006), but this was not the case for our study.

The distributions of the taxa we measured here reflect current environmental conditions and, over evolutionary time, have resulted in significant divergence of populations seemingly free of significant dispersal barriers. Indeed, because of the strong bidirectional physical connections inherent to an estuarine system and the relatively small spatial and phylogenetic scale of this study, the evidence we present for niche differentiation, evolutionary divergence of sympatric populations and biogeography may indicate that these phenomena commonly structure microbial communities.

Acknowledgements

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