

Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria

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SUMMARY

The majority of bacterial species do not grow on synthetic media. Many non-growers require growth factors from other bacteria, but the nature of these compounds is largely unknown. We show here that previously uncultured isolates from marine sediment biofilm grow on a Petri dish in the presence of cultured organisms from the same environment. The growth factors produced by one cultured helper strain were identified as new acyl-desferrioxamine siderophores. A panel of previously uncultured isolates exhibited a range of siderophore promiscuity for growth promotion. This siderophore-based approach has enabled the culturing of organisms only distantly related to previously cultured microbes. The lack of growth in the laboratory for many strains from this habitat stems from an inability to autonomously produce siderophores, and the resulting chemical dependence on other microorganisms regulates community establishment in the environment.

INTRODUCTION

Sampling of diverse environments, such as soil, marine sediment, or hot springs shows that only 0.01–1% of cells visible under the microscope will form colonies on a Petri dish, leaving the remaining majority “uncultured.” This paradoxical result, known as “the great plate count anomaly,” is a major unsolved problem in microbiology (Butkevich, 1932; Keller and Zengler, 2004; Rappe and Giovannoni, 2003; Staley and Konopka, 1985). Surveys of 16S rRNA gene sequences directly isolated from the environment showed that the anomaly is more than a gap in the total cell count, since >99% of species are uncultured (Barer and Harwood, 1999; Colwell and Grimes, 2000; Giovannoni, 2000; Grimes et al., 2000; Stackebrandt and Embley, 2000), although the precise percentages are hotly debated (Reeder and Knight, 2009).

We recently developed a general method to grow previously uncultured bacteria (here referred to as “uncultured”) from a variety of environments (Bollmann et al., 2007; Kaerberlein et al., 2002; Nichols et al., 2008). The method grows cells in their

natural environment in a diffusion chamber. Cells are taken from an environment such as marine sediment, diluted, mixed with agar, and sandwiched between two semipermeable membranes. The chamber is then placed back in the same environment where the sample originated. Alternatively, a porous membrane allows filamentous microbes to selectively migrate into a sterile chamber, which serves as a trap (Gavrish et al., 2008). The success of these approaches relies on the ability of small molecules from the natural environment to freely diffuse in and out of the chamber, while the movement of cells is restricted. After 1 to 2 weeks of incubation, colonies appear in the chamber, resulting in recovery of up to 40% of the bacteria in that environment (Kaerberlein et al., 2002). In contrast, recovery by standard cultivation methods from the same environment is only 0.01%–0.05% (Cifuentes et al., 2000; Keller and Zengler, 2004; Llobet-Brossa et al., 1998). Application of the method to freshwater sediment showed that the richness and variety of species cultured in the chamber was considerably greater than on a Petri dish (Bollmann et al., 2007). Growth in a natural environment was used to successfully culture *Pelagibacter*, the most abundant marine bacterial species that does not grow on synthetic media (Rappe et al., 2002). Soil organisms have also been cultured on the surface of a membrane placed on top of soil (Ferrari et al., 2005), and while quantitative recovery has not been reported for this technique, FISH analysis indicated microcolonies of representatives of the elusive domain TM7, for which no cultured representatives are known.

We noticed that some organisms forming colonies in the diffusion chamber can grow on a Petri dish, but only in the presence of other species from the same environment (Kaerberlein et al., 2002; Nichols et al., 2008). Interspecies symbiosis based on nutrient exchange (syntrophy) is well known in the bacterial world (McInerney et al., 2008). Bacteria are also known to communicate using an interspecies quorum-sensing factor [autoinducer 2 (AI-2)] that induces synthesis of proteins that are useful for a community rather than a single cell, such as toxins or polymer hydrolases (Williams et al., 2007). Uncultured bacteria, however, do not grow on rich synthetic media (such media should largely obviate the need for nutrient supply by other species), and AI-2 has not been found to act as a growth-promoting factor, raising questions about the nature of unknown growth-promoting factors in microbial communities.

We suggested that uncultured bacteria only commit to division in a familiar environment, which they recognize by the presence of growth factors released by their neighbors (Kaerberlein et al.,

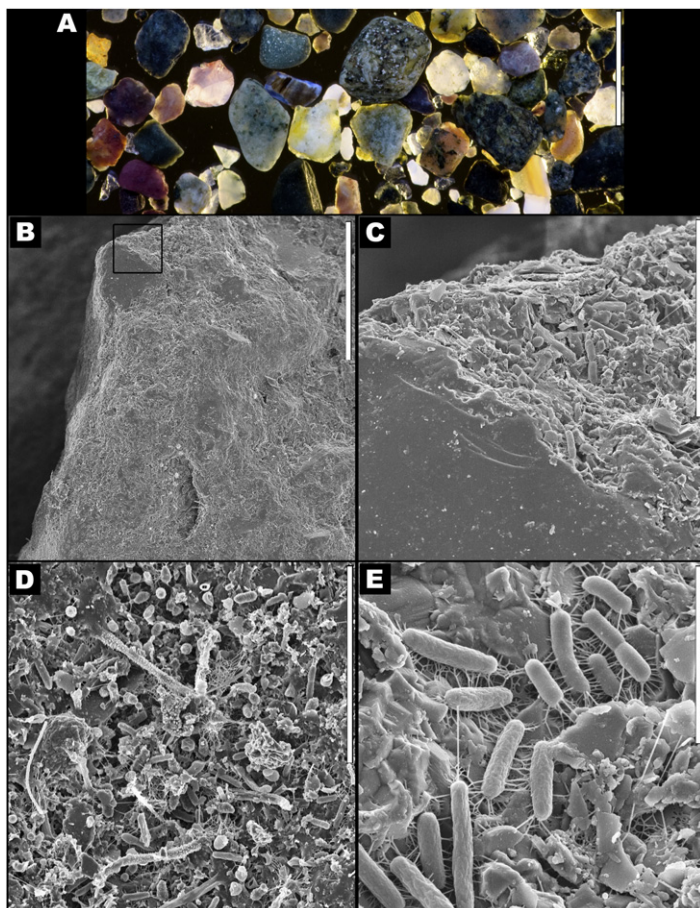


Figure 1. Intertidal Sand Grains

(A) Photograph of a collection of washed sand grains in seawater. Scale bar, 3 mm.

(B) Overview scanning electron micrograph (SEM) of a single sand grain. Scale bar, 50 μm .

(C) Higher resolution SEM of the boxed region in image B. Scale bar, 10 μm .

(D) SEM image of a biofilm that can be seen attached to the surface of the sand particle, which represents the source of the bacterial cells that were resuspended by vortexing for isolation in this study. Scale bar, 10 μm .

(E) SEM image of individual bacteria attached to the surface of the sand particle. Scale bar, 3 μm .

(Figure 1). The liquid phase containing bacterial cells was serially diluted and plated on nutrient medium containing 50% sterile seawater (R2Asea). After 5 days of incubation at 30°C, colonies of varied morphology appeared on the plates (Figure 2A). Similar results were obtained at 24°C. There were disproportionately more colonies appearing on densely inoculated plates compared with more dilute plates. We hypothesized that some of the cells that grew on the densely seeded plates were receiving growth factors from neighboring colonies and would not grow in isolation. To test the possible growth dependence of microorganisms on neighboring species, pairs of colonies growing within a 2 cm distance of each other were picked from high-density plates (50–200 colonies per plate) and restreaked in close proximity to each other. Each of the two isolates was streaked on one half of an R2Asea plate and cross-streaked through the center of the plate, resulting in regions of

proximal, distal, and overlapping inoculation (Figure 2B). Potential uncultured isolates were identified by their diminishing growth with increasing distance from the cultured “helper” strain on the cross-streak plates. In the example given in Figure 2B, colonies of the cultured organism *Micrococcus luteus* KLE1011 (a marine sand sediment isolate 99.5% identical to *Micrococcus luteus* DSM 200030^T according to 16S rRNA gene sequence) grew larger as their distance from other colonies increased. This pattern of growth is typical for cultured bacteria, with the size of colonies increasing in a less crowded, nutrient-rich environment. In contrast, colonies of the uncultured isolate *Maribacter polysiphoniae* KLE1104 (99.9% similar to *Maribacter polysiphoniae* LMG 23671^T) became barely visible as the distance from the cultured helper organism increased. Approximately 100 randomly picked pairs of colonies were restreaked from the high density plates, and 10% of these pairs showed this pattern of growth induction on cross-streaked plates.

2002). Here we use co-culture of organisms from marine sediment to determine the nature of these diffusible molecules and find that siderophores from neighboring species induce growth of uncultured marine bacteria. Siderophores are low molecular weight compounds that have a high binding affinity for insoluble Fe(III). Microorganisms release siderophores to scavenge Fe(III) and then transport the ferric form back into the cells (Crosa and Walsh, 2002; Neilands, 1995; Wandersman and Delepelaire, 2004). Soluble Fe(II) is severely limited in most aerobic environments. Siderophore biosynthesis is tightly regulated in cultured species and the chelators are produced when available iron is low. Uncultured species would be expected to have the same ability to autonomously produce siderophores since maintaining functional genes for siderophore production consumes few resources, and an inability would severely limit their ability to grow in Fe(II)-deficient but otherwise nutrient-rich environments.

RESULTS

Recovery of Environmental Isolates Depends on Growth-Inducing Helper Bacteria

Samples of intertidal coarse sand sediment were collected from Canoe Beach in Massachusetts Bay, near Nahant, MA. The sediment was separated from seawater and vortexed in sterile saltwater to disperse the biofilm covering the sand particles

Identification of Genes Coding for a Growth Factor

We reasoned that a small molecule growth factor could be readily identified if we found an uncultured isolate that was helped by *Escherichia coli*. Testing a collection of *E. coli* deletion strains for a lack of helping ability would then reveal its biosynthetic genes. Filtered spent supernatant of *E. coli* was able to induce growth of several uncultured isolates, including *M. polysiphoniae* KLE1104 (Figure 3A).

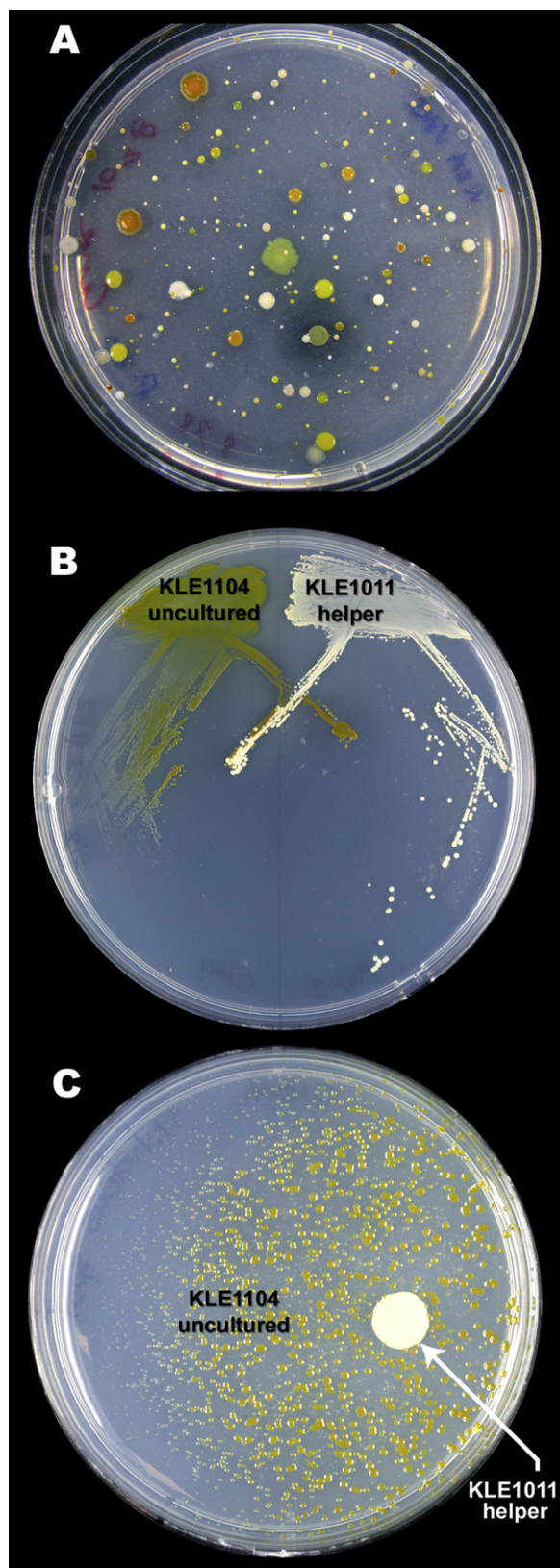


Figure 2. Growth of *M. polysiphoniae* KLE1104 Is Induced by *M. luteus* KLE1011

Bacterial cells were resuspended from sand biofilms and plated to isolate species dependent on neighboring colonies.

(A) Isolation spread plate of bacteria from sand grains.

(B) Environmental helper *M. luteus* KLE1011 cross-streaked (right side of plate) with uncultured isolate *M. polysiphoniae* KLE1104 (left side of plate). Colonies of the uncultured strain are larger when in closer proximity to the helper.

(C) Filtered spent supernatant of *M. luteus* KLE1011 induces growth of *M. polysiphoniae* KLE1104. In all cases tested, the uncultured phenotypes, including KLE1011, remained stable. See also Table S7.

E. coli has a limited repertoire of known secreted metabolites, which include the siderophore enterobactin (Braun and Braun, 2002), the universal quorum sensing factor AI-2 (Bassler and Losick, 2006; Surette et al., 1999), and the autoinducer indole (Di Martino et al., 2003). Supernatant from a strain deficient in AI-2 production ($\Delta luxS$) and from a strain that does not secrete indole ($\Delta trnA$) showed normal growth induction (Figures 3B and 3C). However, $\Delta entB$ or $\Delta entC$, strains disabled in enterobactin synthesis, were unable to induce the growth of *M. polysiphoniae* KLE1104 (Figures 3D and 3E). Enterobactin spotted onto a plate with *M. polysiphoniae* KLE1104 strongly induced growth (Figure 3F).

Structure Elucidation of Siderophores from *M. luteus* KLE1011

Spent medium from the natural helper *M. luteus* KLE1011 induced growth of the uncultured *M. polysiphoniae* KLE1104 (Figure 2C). An iron-binding assay-guided fractionation [chromazurol S (Schwyn and Neilands, 1987)] was then used to purify possible siderophores from the spent culture supernatant. Marine species survive with very low iron, 20 pM to 1 nM (Granger and Price, 1999; Johnson et al., 1997), and siderophore production in artificial media is induced under iron-limited conditions (Payne, 1994). Consequently, *M. luteus* KLE1011 was cultured in an iron-limited [100 pM Fe(III)] artificial seawater medium (Haygood et al., 1993). The spent culture supernatant was acidified (pH 2) and the metabolites were purified by solid phase extraction (Amberlite XAD-2). Five different siderophores were further purified over size exclusion chromatography (LH-20) and reversed-phase (C18) high-pressure liquid chromatography (HPLC). In addition to exhibiting iron binding properties in the chromazurol S assay, both the desferric and ferric forms of each siderophore were observed by HPLC-mass spectrometry (MS) coupled with UV-visible spectroscopy analysis, confirming their role in iron acquisition (Figure 4A).

Detailed nuclear magnetic resonance (NMR) spectroscopy allowed structural elucidation of the purified siderophores (see Figure S1 and Tables S1–S5 available online). One dimensional (^1H) chemical shifts and two dimensional homonuclear correlations (COSY), heteronuclear single quantum coherence correlations, and heteronuclear multiple bond correlations revealed that all of the siderophores consisted of a central core with alternating N-hydroxycadaverine and succinic acid units and were of the desferrioxamine class (Challis, 2005). The metabolites differed by terminal acyl attachments. High-resolution LC Fourier-transform MS (Table S6) provided further evidence supporting their molecular formulas, and subsequent fragmentation analysis (MS/MS) confirmed the amide/hydroxamate backbone linkages,

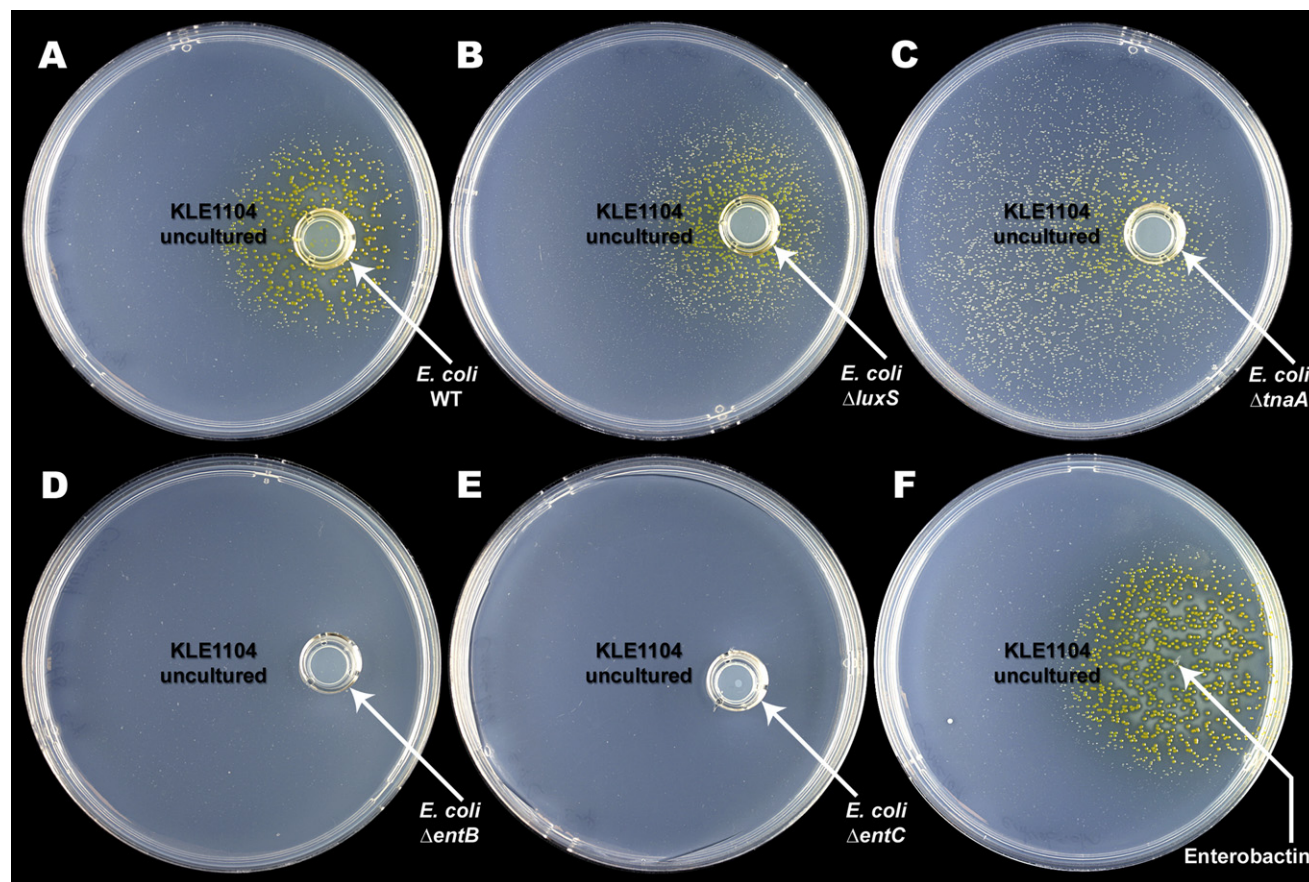


Figure 3. Growth Induction of *M. polysiphoniae* KLE1104 by Enterobactin from *E. coli*

Filtered spent supernatant of *E. coli* BW25113 induces growth of *M. polysiphoniae* KLE1104, but spent culture media from enterobactin mutant strains do not. (A) Supernatant from the parental *E. coli* BW25113 strain induces growth, as does supernatant from deficient strains, $\Delta luxS$ (B) and $\Delta tnaA$ (C). There was more growth around the *tnaA* mutant in this experiment, but subsequent examination showed that it was due to variation in this qualitative test. (D and E) Supernatant of strains deficient in enterobactin synthesis, $\Delta entB$ (D) and $\Delta entC$ (E), do not induce growth of the uncultured. Growth was not observed even at high densities of KLE1104. (F) Purified enterobactin spotted on a plate evenly spread with *M. polysiphoniae* KLE1104 induced growth of the isolate. Colony size decreased with increasing distance from the enterobactin source and no growth of the uncultured was seen further away from the source.

corroborating the proposed structures (Figure S2). *M. luteus* KLE1011 produces a series of new acyl-desferrioxamine siderophores (Figure 4B) with variable flanking acyl side chains that drastically affect the central core's aqueous solubility. Three of the siderophores (3–5) terminate with branched chain fatty acids while two (1 and 2) terminate with an atypical phenylacetyl moiety. Acyl-desferrioxamines 1 and 3 also were observed beginning with a primary amine acetyl-cap (2 and 5). The five new metabolites were able to individually induce the growth of the previously uncultured *M. polysiphoniae* KLE1104, demonstrating that these siderophores represent the growth factors responsible for the helping activity.

As siderophores provide bacteria with a soluble source of iron, we investigated whether drastically increased quantities of soluble elemental iron could similarly allow growth of the uncultured isolates. Iron completely oxidizes to the insoluble Fe(III) state at the pH and oxygen levels of both seawater (the intertidal surface sand is continually mixed by wave action) and the laboratory media; however, by adding soluble FeSO₄ after the media

is autoclaved, the level of bioavailable Fe(II) is temporarily increased. Incubation of *M. polysiphoniae* KLE1104 over a range of Fe(II) concentrations showed that ~40 μM FeSO₄ was necessary for optimal growth. Since this exceeds the reported picomolar levels of iron in the surface ocean (Johnson et al., 1997) by some six orders of magnitude, it is unlikely that uncomplexed iron is available to these organisms in the environment.

Siderophore Specificity of Uncultured Isolates

Given that siderophores from both *E. coli* and a neighboring species (*M. luteus* KLE1011) were able to induce growth of uncultured *M. polysiphoniae* KLE1104, it was important to determine the extent of siderophore promiscuity in growth promotion. Therefore, a screen was conducted to isolate uncultured bacteria induced by *M. luteus* KLE1011 and test their siderophore preferences. Environmental samples from Canoe Beach were spread on solid medium and then a culture of *M. luteus* KLE1011 was spotted on the plate as a potential helper. After incubation, colonies were picked and cross-streaked against

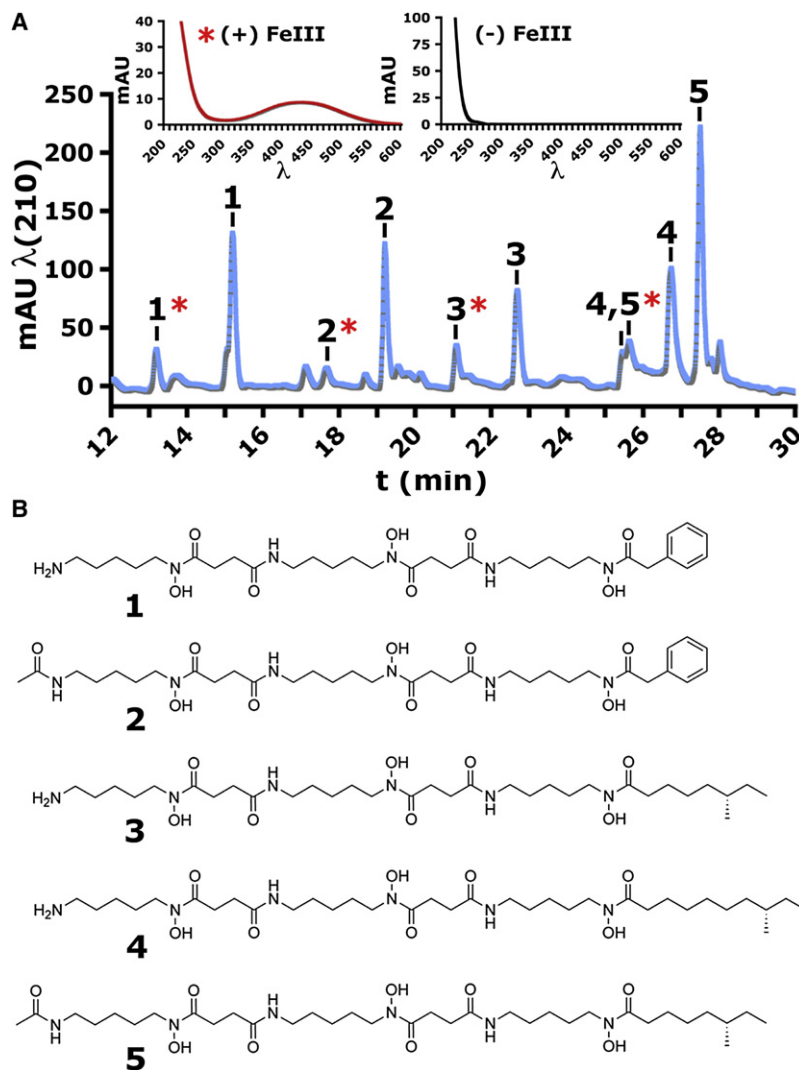


Figure 4. Active Siderophores Produced by Helper Strain *M. luteus* KLE1011

(A) Reversed-phase C18 HPLC-MS/UV-visible spectroscopic analysis. The ferric and desferric siderophores were separated over C18 HPLC, demonstrating a range of hydrophobic interactions with the nonpolar resin. The more polar ferric forms eluted faster than the desferric forms, showed masses indicative of iron complexation (MS), and exhibited characteristic iron-ligand charge transfer absorption (UV visible).

(B) Acyl-desferrioxamine siderophore structures. See also Figures S1 and S2 and Tables S1–S6.

The model uncultured *M. polysiphoniae* KLE1104 and one other strain (KLE1009) isolated from initial cross-streaks with environmental helpers were also tested with the siderophore panel. The siderophore panel consisted of linear and cyclic trihydroxamates, dihydroxamates, phenols/catechols, oxazolines/thiazolines, and carboxylic acid-type siderophores, providing a range of iron-binding structural types that target different siderophore receptors. Each of the eight isolates was induced by a particular set of siderophores. KLE1009 displayed wide siderophore promiscuity and was induced by all 26 siderophores tested, while other strains, such as KLE1123, showed greater selectivity and only grew in the presence of 7 of the siderophores. Acyl-desferrioxamine 1 identified from the model helper *M. luteus* KLE1011 was capable of inducing growth of all eight isolates tested, while the variable side chains in acyl-desferrioxamines 2–5 caused a decrease in the spectrum of growth induction. A broad range of growth induction was only seen for two of the commercial siderophores:

M. luteus KLE1011. Plates were observed for a growth dependence pattern and candidate uncultured organisms were verified by seeding a plate spotted with the helper. A total of 185 colonies were screened, of which 46 (25%) were helped by *M. luteus* KLE1011; the rest were independently fully cultured. Twenty of the *M. luteus* KLE1011-dependent isolates showed weak growth when plated alone, while the remaining 26 completely dependent isolates (14% of 185 screened) had no growth in the absence of helper. Six isolates that showed strong dependence on *M. luteus* KLE1011 and were not capable of producing colonies visible at 160 \times magnification on their own were chosen for further study. Based on 16S rRNA gene sequence comparisons, the six uncultured isolates were 96.5% to 99.3% identical to typically cultured relatives, which are typical marine bacteria (Table S7).

To further examine siderophore specificity in growth induction, a panel of 20 commercially available siderophores (Figure 5) from both bacterial and fungal sources, desferrivulnibactin purified from *Vibrio vulnificus*, and the five siderophores from *M. luteus* KLE1011 were tested for their ability to induce growth of the six uncultured organisms identified in the screen (Table 1).

the linear trihydroxamates desferrioxamine B (equivalent to the hydrophilic core of the *M. luteus* KLE1011 siderophores) and desferricoprogen. Isolates belonging to the division *Bacteroidetes* were able to use a wider variety than the *Proteobacteria* tested. The *Bacteroidetes* isolates were induced by an average of 22 of the siderophores, while the *Proteobacteria* were induced by an average of 10. Taken together, these data show a considerable variation in siderophore preferences among the uncultured isolates.

Isolation of Distantly Related Uncultured Microorganisms

The screening for uncultured microorganisms that require *M. luteus* KLE1011 produced a variety of isolates, but all of them are closely related to known cultured species. This may have resulted either from the preponderance of common microorganisms in this environment, both previously cultured and uncultured, or from the bias in the screen. Given the preference for particular siderophores, we sought to obtain uncultured isolates by an unbiased approach. For this purpose, unphysiologically high concentrations of soluble Fe(II) were used as a

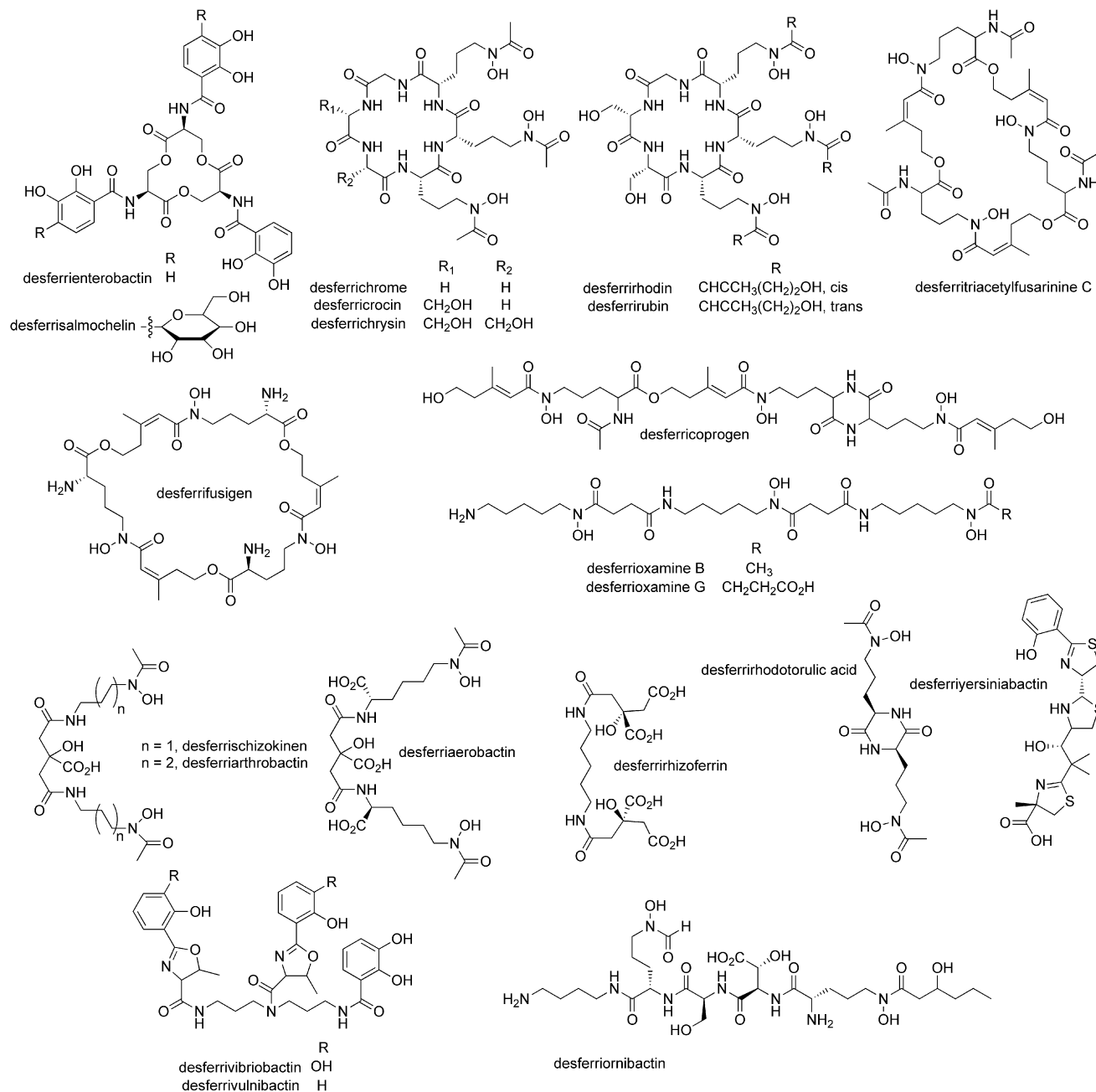


Figure 5. Siderophores Tested for Growth Induction Ability

The structures of 21 representative siderophores are shown, which were each tested for growth induction of eight uncultured species. The siderophores include cyclic and linear trihydroxamates, dihydroxamates, carboxylic acid types, phenols, catechols, oxazolines, and thiazolines that provide a variety of iron-binding structural types that would target various siderophore receptors.

surrogate for siderophores in an attempt to culture distant uncultured microorganisms and circumvent the natural systems that would likely require new siderophore classes.

A sample from marine sediment was cultured on R2Asea with a high concentration of Fe(II), and then screened for isolates that would only grow in the presence of Fe(II). Of a total of 121 isolates, 31 showed strict dependence on Fe(II). 16S rRNA genes were sequenced for fifteen of these, identifying 13 separate

isolates and two potential duplicates. The 13 separate isolates varied in their sequence identity to typed species between 91.7% and 99.8% (Table S7), and the large proportion of strains isolated only once suggests that this environment has a considerable diversity of uncultured organisms. Importantly, three organisms in this small set of 15 were highly different from any typed strains by 16S rRNA gene sequence. One is a *Verrucomicrobia* sp. (KLE1210), a member of a phylum that is widespread

Table 1. Growth Induction of Uncultured Isolates by Different Siderophores

Strain	Desferricoprogen	KLE1011 Sid1	Desferrioxamine B	KLE1011 Sid2	KLE1011 Sid3	Desferrioxamine G	Desferrirhodotorulic acid	Desferrischizokinen	Desferrifusigen	Desferrienterobactin	KLE1011 Sid4	KLE1011 Sid5	Desferriarthrobactin	Desferriaerobactin	Desferritriacetylfusarinine C	Desferriyersiniabactin	Desferrisalmoachelin	Desferriornibactin-C6	Desferrichrysin	Desferrirhodin	Desferriuinibactin	Desferricrocin	Desferriurubin	Desferrichrome	Desferrirhizoferrin	Desferritribriobactin	Siderophores Inducing Growth	
KLE1009	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	26	
KLE1063	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24
KLE1104	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	20
KLE1078	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	19
KLE1080	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	18
KLE1125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8
KLE1111	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7
KLE1123	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7

Strains *Maribacter* sp. KLE1063, *Winogradskyella* sp. KLE1078, *Hyphomonas* sp. KLE1080, *Reinekea* sp. KLE1125, *Simidiua* sp. KLE1111, and *Sulfitobacter* sp. KLE1123 were isolated by screening for environmental bacteria helped by *M. luteus* KLE1011. Strains *Cyclobacterium* sp. KLE1009 and *M. polysiphoniae* KLE1104 were isolated from earlier cross-streak plates with environmental helpers. The five *M. luteus* KLE1011 siderophores and 21 representative siderophores were spotted on plates seeded with each uncultured and observed for growth induction after 1 week of incubation at 30°C. Siderophores that induced growth are indicated in blue while siderophores that failed to induce growth are indicated in yellow.

in nature but is known mainly from uncultured isolates based on direct determination of 16S rRNA gene sequences from environmental DNA. *Verrucomicrobia* bacterium KLE1210 is only 91.7% identical to the closest cultured species, *Rubritalea spongiae* YM21-132^T (Hedlund et al., 1996; Wagner and Horn, 2006) (Figure S3).

A sample from the same environment was screened for cultured organisms able to support growth of *Verrucomicrobia* sp. KLE1210, as the model helper *M. luteus* KLE1011 was incapable of inducing growth. The intertidal sediment sample was added to the surface of ten plates seeded with *Verrucomicrobia* sp. KLE1210 and observed for growth promotion activity. A candidate helper was identified, isolated from the mix, and verified to induce growth of KLE1210. This led to the identification of an isolate with 97.1% 16S sequence identity to *Lacinutrix mariniflava* AKS432^T (KLE1211), which is capable of inducing growth of KLE1210 (Figures 6A–6C). Assay-guided fractionation and MS analysis of spent supernatant resulted in the detection of the siderophore growth induction factor (Figure S4). While this siderophore induces growth, none of the 26 tested siderophores from the panel induced growth, revealing the high siderophore selectivity of KLE1210.

The second distant isolate, KLE1250, belongs to a recently described α -proteobacterial order, “*Parvularculales*” (Lee et al., 2005). *Parvularculaceae* KLE1250 shows 93.7% identity by 16S rRNA gene sequence to *Parvularcula bermudensis* KCTC 1208^T, one of only two initially described isolates (Cho and Giovannoni, 2003) that established this putative order and the only examples reported to be cultured. The apparent rarity of this order may

be due to most species being uncultured, rather than an actual scarcity in the environment. Isolate KLE1250 may represent a new genus within this putative order (Figures 6D–6F; Figure S5).

The third unusual isolate, KLE1212, is a member of the γ -proteobacteria and shows only 90.4% 16S identity to the nearest typed strain, *Kangiella aquimarina* SW-154T (Figure S6). The isolate was cultured by plating cells resuspended from a single sand grain on media supplemented with Fe(II) and screening for species strongly dependent on high concentrations of Fe(II). Cultured isolates from the same sand biofilm were screened for their ability to induce growth of KLE1212, resulting in the identification of a helper (KLE1213) with 99.7% 16S identity to *Vibrio tasmaniensis* LMG 21574T (Figures 6G–6I). Interestingly, none of the characterized isolates from Fe(II) plates could be induced to grow by the model helper *M. luteus* KLE1011, indicating both altered specificity in the growth dependence and high diversity of uncultured species.

DISCUSSION

Uncultured bacteria from many environments, including intertidal sediment, do not form colonies on select synthetic media and could account for up to 99.9% of the cells present in a sample (Keller and Zengler, 2004). Our results indicate that many of these bacterial strains will not commit to division unless they are provided with a small molecule growth-inducing factor from a neighboring microbe from that community.

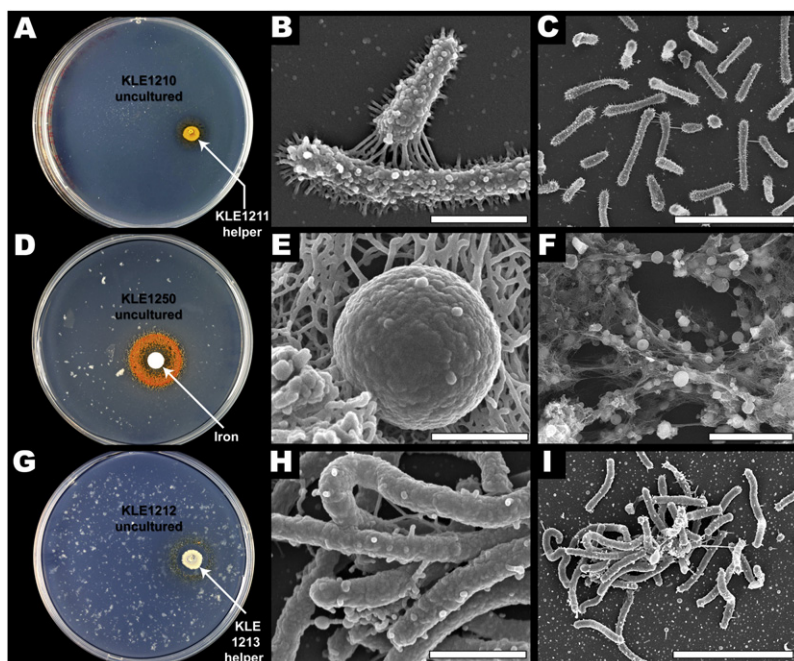


Figure 6. Isolation of Distantly Related Uncultured Microorganisms

Three organisms only distantly related to typed strains were isolated on high [Fe(II)] media.

(A) The cultured *Lacinutrix* sp. KLE1211 (yellow spot) induces growth of the uncultured *Verrucomicrobia* isolate, KLE1210 (orange ring).

(B and C) SEM images of *Verrucomicrobia* KLE1210. Scale bars: 1 μ m (B) and 5 μ m (C).

(D) *Parvularculaceae* KLE1250 (orange ring), growing near a filter disk (white spot) containing 10 μ l of 1% iron sulfate.

(E and F) SEM images of *Parvularculaceae* KLE1250. Scale bars: 1 μ m (E) and 10 μ m (F).

(G) *Gammaproteobacterium* KLE1212 (yellow ring) is induced by a cultured species, *Vibrio tasmaniensis* KLE1213 (beige spot), isolated from the same sand biofilm.

(H and I) SEM images of the uncultured isolate, *Gammaproteobacterium* KLE1212. Scale bars: 1 μ m (H) and 5 μ m (I). See also Figures S3–S6.

Based on previous observations of bacteria growing only in the presence of other strains from the same environment (Kaerberlein et al., 2002; Morris et al., 2008; Nichols et al., 2008), we isolated pairs of colonies growing in close proximity on a nutrient-rich solid medium and plated them in a cross-streak to observe a helper-dependent pattern. This allowed us to isolate a number of organisms that were unable to grow in isolation, as well as the organisms that induced their growth. In addition to the environmental helper *M. luteus* KLE1011, wild-type *E. coli* was able to induce the growth of a particular uncultured isolate, *M. polysiphoniae* KLE1104, which then allowed us to identify mutant strains lacking the helping activity. *E. coli* *entB* and *entC* mutants defective in enterobactin synthesis failed to induce growth of *M. polysiphoniae* KLE1104.

We then examined the ability of the natural helper organism *M. luteus* KLE1011 to produce siderophore growth factors. While marine isolates of *M. luteus* have been previously reported to show iron-binding activity, the identity of its siderophores has been unknown (Cabaj and Kosakowska, 2007). Filtered spent supernatant of *M. luteus* KLE1011 induced growth of *M. polysiphoniae* KLE1104. Subsequent iron-binding assay-guided fractionation followed by MS and NMR characterization led to the isolation and characterization of *M. luteus* siderophores. Five new acyl-desferrioxamine siderophores were identified and each was able to induce growth of *M. polysiphoniae* KLE1104.

The five siderophores isolated from *M. luteus* KLE1011 harbor terminal hydrophobic modifications that alter their aqueous solubility. It has been proposed that marine bacteria produce siderophores with a range of hydrophobicities that could avoid significant losses to the open ocean. For example, the aquachelin and marinobactin class of marine siderophores have a long fatty acyl side chain of varying length to increase lipophilicity, leading to varying degrees of membrane association (Martinez

et al., 2003, 2000). Similarly, the acyl-desferrioxamines discovered here show a variety of hydrophobicities in contrast to their hydrophilic terrestrial counterpart, desferrioxamine B, suggesting that this is a common strategy in

marine siderophore biosynthesis. Synthetic N-acyl derivatives of desferrioxamine B drastically reduced aqueous solubility and enhanced membrane permeability (Ihnat et al., 2000). *M. luteus* KLE1011 uses the same strategy of capping the primary amine with an acetyl group (acyl-desferrioxamines **2** and **5**). Unlike the single fatty acid modification in aquachelins and marinobactins, *M. luteus* KLE1011 uses a two-component modification strategy to tailor both ends of the central core, including an atypical aromatic phenylacetyl side chain.

Using *M. luteus* KLE1011 as bait, a substantial number of uncultured bacteria were isolated in this study. Fourteen percent of colonies from a marine sediment sample growing in the vicinity of *M. luteus* KLE1011 appeared to depend on this isolate for growth. The relative ease of identifying strains dependent on *M. luteus* KLE1011 illustrates the commonality of siderophore dependence in the environment. Six of the uncultured strains from the screen along with two previously isolated organisms from the same environment were tested for their ability to grow in the presence of a variety of siderophores, including the five *M. luteus* KLE1011 acyl-desferrioxamines and 21 representative siderophores. Each uncultured isolate showed a particular pattern of siderophore dependence. *Cyclobacterium* sp. KLE1009 appeared the most promiscuous and grew in the presence of all 26 siderophores tested, while *Sulfitobacter* sp. KLE1123 was the most restrictive and was only induced by 7 of the siderophores. The *Bacteroidetes* strains on average were capable of using a larger array of siderophores than the *Proteobacteria*.

In an unbiased screen for uncultured organisms, high levels of soluble Fe(II) were used as a surrogate for siderophores, and a small sampling of candidates resulted in the discovery of three organisms highly divergent from known species. These organisms were not induced to grow by *M. luteus* KLE1011, illustrating the specificity and diversity of dependent species in the sand biofilm.

Perhaps the most puzzling aspect of the “uncultured” phenomenon is that many of the uncultured microorganisms are closely related to known and well-studied cultured species by their 16S rRNA gene sequence signatures. Indeed, why would bacteria closely related to organisms that thrive on a large variety of nutrients *in vitro* fail to grow similarly well? We find that a common theme among uncultured bacteria in the marine sediment environment is their dependence on siderophores from neighboring species. Early studies of the function of siderophores revealed the growth-promoting ability of these compounds. The lag phase of *Bacillus megaterium*, for example, was shown to be reduced by the addition of schizokinen, a siderophore it is capable of producing (Lankford et al., 1966). The strains in these earlier studies were capable of growth in isolation in contrast to the strains isolated in this study. The results imply that the microorganisms examined in this study, which belong to unrelated groups, have lost the ability to autonomously produce siderophores or fail to upregulate production on synthetic media without assistance (Reissbrodt et al., 2000, 2002).

The results are surprising and counterintuitive from what we know about siderophore production by cultured microorganisms. Many species can acquire siderophores they do not produce, a phenomenon known as “siderophore piracy” (Luckey et al., 1972; Schubert et al., 1999). For example, mycobactin and carboxymycobactin produced by *Mycobacterium* species induce the FemIRA siderophore transport system in *P. aeruginosa* (Llamas et al., 2006). It appears that it is a common phenomenon for bacterial genomes to contain more siderophore uptake genes than synthesis genes. For example, genome and biochemical analyses on *Pseudomonas fluorescens* SBW25 showed the presence of 24 putative siderophore receptors, allowing the cell to acquire a range of heterologous pyoverdine siderophores in addition to its own (Moon et al., 2008). Siderophore synthesis is typically upregulated when available iron is low (Winkelman, 2002), but some uncultured bacteria do not seem to follow this strategy. The cost of maintaining siderophore biosynthetic genes is probably very low; in contrast, the cost of losing the ability to autonomously produce siderophores and inability to grow in any environment that lacks suitable siderophores seems imposing. Due to the widespread nature of siderophore synthesis in aerobes (Kraemer, 2004) and the considerable level of horizontal transfer of these systems (Challis and Hopwood, 2003), it is unlikely that there is an aerobic lineage that has never possessed the ability to make them, suggesting a functional loss or altered siderophore regulation in uncultured organisms. A possible advantage to not being able to autonomously produce siderophores may be the security of initiating growth only in an environment populated by compatible neighbors. In this model, the specificity of siderophore uptake determines the range of environments suitable for growth. Growing populations are vulnerable to noxious conditions, while non-growing stationary and dormant persister cells are very resilient (Lewis, 2007), which may provide a distinct advantage for cells that only grow in familiar environments.

Interestingly, this strategy seems very similar to the germination of spores. Recently, it was reported that germination of *B. subtilis* spores is strongly induced by mucopeptides, breakdown products of peptidoglycan derived from other bacteria

(Shah et al., 2008). Apparently, the presence of nutrients is not enough and there must be other factors indicating the presence of an environment favorable for growth.

SIGNIFICANCE

The results of this study indicate a widespread occurrence of siderophore-dependent uncultured bacteria in marine sediment and point toward siderophores being key components in establishing microbial communities in this environment. It is likely that many uncultured microorganisms require as yet undiscovered siderophore classes for growth promotion. The diversity of uncultured organisms should vary widely based on siderophore receptor specificity, providing a general strategy to access new microorganisms. Their growth can be induced by the addition of exogenously supplied helper siderophores or artificially high concentrations of Fe(II), and these approaches could expand drug discovery efforts, as microbial metabolites have afforded some of our most useful small molecule therapeutic agents (Newman and Cragg, 2007).

EXPERIMENTAL PROCEDURES

Isolation of Uncultured Bacteria and Helpers

Samples of intertidal sediment were collected from Canoe Beach in Nahant, MA. Sediment (5 g) was resuspended in 5 ml of filtered, autoclaved seawater and vortexed for 1 min. Samples were diluted and plated on solid R2Asea medium [R2A (Difco) diluted in 50% filtered, autoclaved seawater] in 10-fold dilutions to obtain plates of varying colony density. Plates were incubated at 30°C for 5 days. Pairs of colonies growing within a 2 cm distance were cross-streaked in the pattern shown in Figure 2B on fresh plates to screen for helper-dependent patterns. Potential uncultured and helper isolates were resuspended in sterile seawater with 15% glycerol and frozen at –80°C. Dependence on a helper was verified by spreading a plate with 10³ to 10⁴ cells of the potential uncultured and spotting the potential helper species. Plates were incubated at 30°C for 3 to 7 days and observed for growth of the potential uncultured near the helper spot. To isolate species dependent on *M. luteus* KLE1011, environmental samples were plated and spotted with KLE1011. After incubation for 5 days at 30°C, colonies were screened that grew within 2 cm of KLE1011.

Isolation of Distantly Related Uncultured Bacteria

R2Asea agar medium (R2A diluted in 50% filtered, autoclaved seawater) was supplemented with 0.001% Iron(II) Sulfate after autoclaving. Sediment was resuspended in 5 ml of filtered, autoclaved seawater, vortexed for 1 min, and plated. After incubation at 30°C for 5 days, colonies were picked and restreaked on R2Asea media with and without Fe(II) supplementation to screen for isolates with Fe(II) growth dependence.

Identification of Isolates by 16S rRNA Gene Sequencing

16S rRNA gene fragment (~1400–1500 bp) was amplified using universal bacterial primers 27F and 1492R (*E. coli* numbering system; Weisburg et al., 1991). The identification of phylogenetic neighbors and the calculation of pairwise sequence similarity were carried out using the EzTaxon server (<http://www.eztaxon.org/>; Chun et al., 2007). The obtained sequences were aligned with phylogenetically related type strains obtained from the Ribosomal Database Project (Cole et al., 2003). Phylogenetic tree was obtained by neighbor-joining (Saitou and Nei, 1987) method using MEGA 4 (Tamura et al., 2007). An evolutionary distance matrix for neighbor-joining method was generated according to the model of Jukes and Cantor (1969). The robustness of the tree topologies was assessed by bootstrap analyses based on 1000 replications. Nucleotide sequences obtained in this study have been deposited in the GenBank database (see Table S7 for accession numbers).

Testing Spent Media of *M. luteus* KLE1011 and *E. coli* Strains

To test the helping ability of *M. luteus* KLE1011 spent media, 5 ml cultures of liquid R2NP media (0.5 g yeast extract, 0.5 g casamino acids, 0.5 g dextrose, 0.3 g sodium pyruvate, and 0.3 g dipotassium phosphate dissolved in 50% filtered, autoclaved seawater) were inoculated from a glycerol stock and incubated at 30°C with shaking for 2 days. Cultures were then centrifuged and filtered (0.22 μm). *M. polysiphoniae* KLE1104 was seeded on R2Asea plates followed by the addition of a tissue insert (Nunc) with a 0.02 μm bottom filter. 500 μl of spent media was added to the insert and plates were incubated at 30°C for 5 days. *E. coli* BW25113 and the Δ*luxS*, Δ*trnA*, Δ*entB*, and Δ*entC* knockouts from the Keio Collection knockout library (Baba et al., 2006) were tested using the same protocol.

Structure Elucidation of KLE1011 Acyl-desferrioxamine Siderophores

NMR experiments were performed on desferric siderophores in deuterated dimethylsulfoxide with a symmetrical NMR microtube susceptibility matched with dimethylsulfoxide (Shigemi, Inc.) on a Varian INOVA 600 MHz NMR. The ferric and desferric forms of each purified siderophore were also separated over HPLC and detected using either a linear quadrupole ion trap/Fourier transform ion cyclotron resonance hybrid MS (Thermo Electron) or a linear quadrupole MS (Agilent 6130). The +1 ion of the desferric and ferric siderophores were selected for fragmentation analysis by MS/MS.

Growth Induction by Siderophores

The commercial siderophores include desferrifusigen, desferriaerobactin, desferricrocin, desferriarthrobactin, desferrischizokinen, desferrichrome, desferrirhizoferrin, desferrirhodin, desferrirhodotorulic acid, desferriacetyl-fusarinine C, desferrioxamine G, desferriornibactin-C6, desferrichrysin, desferrioprogen, desferriurbin, desferrioxamine B, desferriyersiniabactin, desferri-salmochelin, desferriivibriobactin (EMC Microcollections), and enterobactin (Genaxxon Bioscience). Vulnibactin was isolated from *Vibrio vulnificus* grown in iron-deplete conditions similarly to as previously described (Okujo et al., 1994). To test growth induction, 10 μl of siderophore was spotted on R2Asea plates seeded with an uncultured isolate. All commercial siderophores were tested at 0.01 mg/ml except desferrienterobactin, desferriyersiniabactin, desferri-salmochelin, and desferriivibriobactin, which were tested at 1 mg/ml. Plates were incubated for 3 to 7 days at 30°C and observed for growth around the spotted siderophore.

ACCESSION NUMBERS

16S rRNA gene sequences reported in this study have been deposited in the GenBank database under accession numbers FJ229465, FJ229461, FJ229467, FJ229459, FJ229460, FJ229466, FJ229462, GU644354, FJ229463, GU644361, GU644355, GU644363, GU644367, GU644356, GU644360, GU644358, GU644357, GU644359, GU644362, GU644368, GU644365, GU644366, GU644364, GQ262724, GU262724, GU812862, and GU812863.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and seven tables and can be found with this article online at doi:10.1016/j.chembiol.2010.02.010.

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