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Bacterial response to siderophore and quorum-sensing chemical signals in the seawater microbial community

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Abstract

Background: Oceans are iron-deficient and nutrient-poor environments. These conditions impart limitations on our understanding of and our ability to identify microorganisms from the marine environment. However, less of knowledge on the influence of siderophores and *N*-acyl homoserinelactone as interspecies communication signals on the bacterial diversity of seawater has been understood.

Results: In the presence of 0.1 nM of the commercial siderophore desferroixamine and the known quorum-sensing chemical signals, synthetic *N*-(3-oxo)-hexanoylhomoserine lactone (0.1 nM) or *N*-octanoylhomoserine lactone (0.1 nM), the total numbers of bacteria in S9905 seawater increased nearly three-fold, and nearly eight-fold in S0011 seawater as determined by DAPI staining and counting, and increased three-fold by counting colony forming units in S9905 seawater after 7 days of incubation. Similar bacterial changes in bacterial abundance were observed when high concentration of desferroixamine (1 μM) and each of homoserine lactone compounds (1 μM) were presented in seawater samples. The number of cultivable bacterial species observed was also found to increase from 3 (without addition) to 8 (with additions) including three unknown species which were identified by phylogenetic analysis of 16S rDNA sequences. The growth of unknown species was found to be related to their siderophore production with response to the addition of desferroixamine and *N*-acyl homoserine lactones under iron-limited conditions.

Conclusion: Artificial addition of siderophores and HSLs may be a possible method to aid in the identification and isolation of marine bacterial species which are thought to be unknown.

Background

It is well known that prokaryotes are major primary producers [4] and heterotrophic consumers [9] in most marine waters. In contrast to the comparative wealth of knowledge available regarding the patterns of diversity among plants and animals, very little is known about the patterns of microbial diversity in the ocean ecosystem. Recently, molecular methods such as denaturing gradient gel electrophoresis (DGGE) [8,25], terminal restriction fragment analysis [3,22], and 16S rDNA cloning [6,11] which rely on direct amplification and analysis of 16S rRNA (rDNA) sequences, have been developed to compared the composition, richness, and structure of microbial communities. Recent development of DNA based techniques has expanded our knowledge of microbial ecology; however it is still a challenge to isolate and

culture bacteria from nutrient-limited environments and to compare, in parallel, such efforts to methods for assessing similarities between communities. Uncultured microorganisms still may not be recovered and characterized though they have been shown to exist in the seawater by molecular methods.

Limited concentration of iron in surface seawater combined with its biogenic significance suggests that iron is one of the factors those affect the growth of bacteria in the ocean [14,28,29]. To survive under iron-limited conditions, microorganisms are known to biosynthesize siderophores, which are small molecules with high binding affinity for Fe (III) [24]. Recently, some marine bacteria have been found to acquire iron through the siderophore desferroixamine [16]. Also some marine bacteria were found to have stimulated growth under iron-limited nutrient-poor conditions similar to the marine environment in the presence of an exogenous siderophore [18], or an exogenous siderophore plus an *N*-acyl homoserine lactone (HSL) [17]. HSLs are known to be chemical signals for the quorum-sensing activity in many gram negative bacteria [12,13]. All of the well-known quorum-sensing systems are based on symbiotic or pathogenic conditions for bacteria in "closed" or "semi-closed" environments [30]. Both siderophores and HSLs have been suggested to play roles as chemical signals for interspecies communication between bacteria [17]. However, little is known on interspecies communication in the nature microglobal ecosystem and no research has been done on the relationship between chemical signals and bacterial diversity in natural seawater.

The goal of this study is to understand the influence of co-dependency of siderophores and HSL as interspecies chemical signals on a bacterial community in the natural marine ecosystem, and to test for the possibility that they stimulate the growth of uncultivable species. In the current work, seawater from two locations was investigated for their total bacterial number and cultivable species with or without the addition of commercial siderophore desferroixamine (DEF) and synthetic HSLs. DEF, a trihydroxamate, is the main siderophore of *Streptomyces pilosus* [2] and was also found to be produced by *Nocardia* and *Micromonospora* [33]. HSLs used in these experiments were chosen as follows: (i) The 3OC6-HSL related system from *Vibrio fisheri,* a bioluminescent bacterium which lives symbiotically with the squid [7] in the aquatic environment, and (ii) C8-HSL, which has been observed to stimulate the bacterial growth and siderophore production of non-siderophore-producing marine bacteria with an exogenous siderophore [17]. DEF was also used because some marine bacteria have been reported to utilize DEF as iron chelators the same as their own siderophores [16]. Differences in total bacterial numbers and cultivable species were observed under the above conditions and siderophore production may be one of factors which influenced the bacterial community structure change.

Results

Analysis of total bacterial number in seawater

The total number of bacteria in S9905 seawater was analyzed by DAPI staining and counting during 7 days. The iron content in S9905 seawater was determined to be 7.8 nM by ICP analysis. Seawater was amended with trace amount of DEF (1 nM) plus 3OC6-HSL (1 nM) or C8-HSL (1 nM), DEF(1 nM) only, each of HSL(1 nM) only. Total bacterial numbers were shown to increase after the addition of DEF, HSL or DEF plus an HSL in comparison with those obtained from seawater without any addition (Fig. 1A). However, the total bacterial number in the seawater with the addition of DEF or HSL only was observed to reach to a maximum value and then began to decrease after the sixth day (Fig. 1A), after which low amounts were recorded during the remaining time of the four-week incubation period (data not shown). Total bacterial numbers in seawater treated with DEF plus an HSL increased during seven days and the value was found to start decreasing after day 14 during four-weeks incubation period (data not shown). Comparing the maximum values of the total bacterial numbers during the first week, the values for DEF, 3OC6-HSL, C8-HSL, DEF plus 3OC6-HSL, and DEF plus C8-HSL treated seawater were 2.50-fold, 2.69-fold, 1.60-fold 3.14-fold, and 2.62-fold respectively higher than plain seawater. The increase in total bacterial number in the seawater control was unclear but such bacterial changes in bacterial abundance have also been observed in several iron enrichment experiments [5,21].

To confirm the effects of such chemical compounds as a nutrient or not, high concentrations of DEF, HSL, DEF plus HSL were amended to the same seawater, and the total bacterial number was determined by DAPI counting (Fig. 1B). The total bacterial number was also stimulated by the addition of high concentrations of DEF, HSL, DEF plus HSL. The maximum value of S9905 seawater was 5.07×10^5 cells/ml, and 4.22×10^5 cells/ml for 1 μM of DEF plus 1 μM of 3OC6-HSL, and 1 μM of DEF plus 1 μM of C8-HSL amended seawater samples respectively, which was similar to 0.1 nM of DEF plus 0.1 nM of 3OC6-HSL (4.72×10^5 cells/ml), and 0.1 nM of DEF plus 0.1 nM of C8-HSL (3.95×10^5 cells/ml) amended seawater samples. Also, the trend of bacterial number increase was very similar for the seawater with the amended with HSL under high or low concentration. However, the maximum value of S9905 1 μM of DEF amended seawater was 1.42×10^5 cells/ml, which was lower than the control seawater (Fig. 1B).

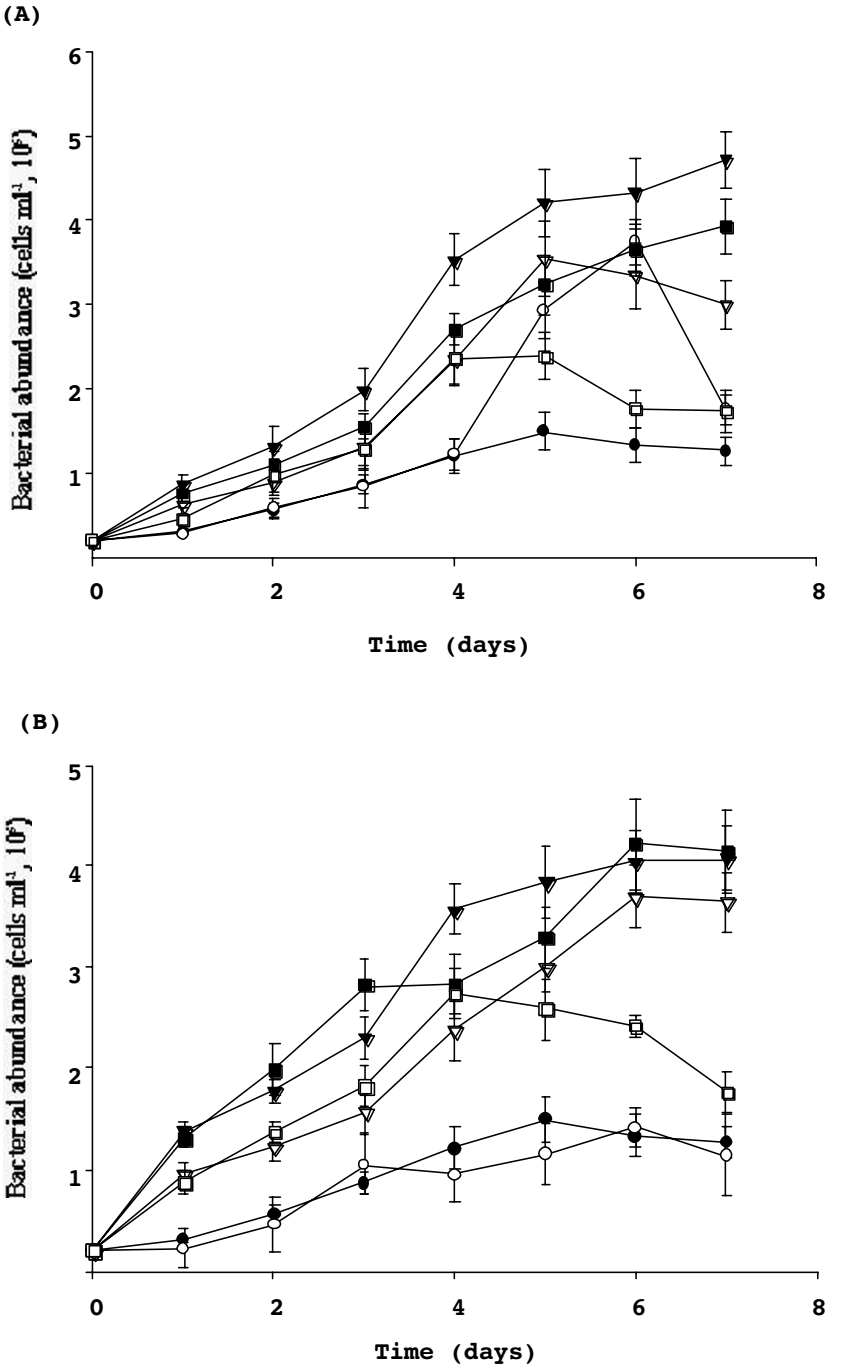

Figure 1
Analysis of total bacterial numbers (cells/ml) in S9905 seawater in the presence of 0.1 nM (FIG 1A) or 1000 nM (FIG 1B) of siderophore DEF siderophore DEF, synthetic HSLs, DEF plus HSL respectively with DAPI staining counting method during 7 days incubation at 30°C. (filled circle) without addition; (circle) with the addition of 0.1 nM DEF, (triangle) with the addition of 0.1 nM 3OC6-HSL, (filled triangle) with the addition of 0.1 nM DEF plus 0.1 nM 3OC6-HSL (Fig 1A), (square) with the addition of 0.1 nM C8-HSL (Fig 1B), (filled square) with the addition of 0.1 nM DEF plus 0.1 nM C8-HSL. Each points represents the mean coaggregration value from three separate measurements.

Repeat experiments were performed with S0011 seawater, which was collected from a different location. The iron content in S0011 seawater was 11.2 nM and total bacterial number was shown in Fig. 2. S0011 seawater was observed to contain more bacteria (2.1×10^5 cells/ml) than that in S9905 seawater (2.07×10^4 cells/ml). The total bacteria number in 0.1 nM DEF plus 3OC6-HSL or C8-HSL (Fig 2A) showed a similar pattern with that in Fig 1A, where an increasing trend in 7 days incubation occurred except for a drop in values for 2 nd-day samples. Comparing the maximum values of the total bacterial numbers, the values for DEF, 3OC6-HSL, C8-HSL, DEF plus 3OC6-HSL, and DEF plus C8-HSL treated seawater were 3.64-fold, 2.73-fold, 2.73-fold, 4.84-fold, and 7.92-fold respectively higher than plain seawater. In the meantime, the bacterial counting were performed for high concentration amendments S0011 seawater (Fig. 2B), 3.80-fold, 3.66-fold 5.11-fold, and 8.18-fold respectively of bacterial number increase were observed in 1 μM of 3OC6-HSL, 1 μM of C8-HSL, 1 μM of DEF plus 1 μM of 3OC6-HSL, 1 μM of DEF plus 1 μM of C8-HSL treated seawater. There was not a large difference in the maximum values in the low (0.1 nM) and high concentration (1 μM) DEF and HSL amended seawater, but a slight change of bacterial number was also observed from 1 μM of DEF treated seawater.

Analysis of cultivable bacterial amount

To understand bacterial diversity, the cultivable species from the above seawater samples was further investigated. Samples were collected every 24 hr during one week and were evaluated by investigation of colony formation units (CFU) of different phenotypes on marine broth or IDSM agar plates. The phenotypes of observed strains obtained are summarized in table 1. Total numbers of cultivable bacteria in seawater samples as enumerated by CFU were found to occur as follows: without addition < with DEF < with 3OC6-HSL < with C8-HSL < with DEF plus 3OC6-HSL < with DEF plus C8-HSL. By comparison three phenotypes were obtained from the untreated seawater, seven, four and five phenotypes were obtained from the seawater with the addition of 0.1 nM DEF, 0.1 nM 3OC6-HSL or 0.1 nM C8-HSL respectively. Nine and eight phenotypes were isolated from seawater with the addition of 0.1 nM DEF plus 0.1 nM 3OC6-HSL, 0.1 nM DEF plus 0.1 nM C8-HSL respectively. CFU counting of each phenotype indicated that the relative contribution of each strain changed even though the same strains were detected in all samples. For example, as shown in Table 1: Strain GMO4-1 was detected in all six seawater samples; it was the preferentially cultivable strain in unamended seawater only. The relative percent contribution of other organisms increased (as calculated by CFU values) depending upon which amendment was used. The preferentially cultivable species from each amendment were: GMO4-4 and GMO4-5 (the same CFU value), GMO4-2, GMO4-12, GMO4-2, GMO4-8 from DEF, 3OC6-HSL, C8-HSL, DEF plus 3OC6-HSL, DEF plus C8-HSL amended sea water respectively (Table 1).

Identification of bacterial species by 16S rDNA sequences

To identify whether the eighteen total isolated phenotypes were different species or not, 16S rDNA analysis of all the strains was performed. Table 1 shows the 16S rDNA sequence homology of the strains. Each isolated strain obtained from the agar plates was identified to be unrelated.

Table 1 showed that the cultivable microorganism diversity obtained in the same seawater was affected by the addition of DEF and HSL. *Erwinia nigrifluens* GMO4-1 and *Shewanella putrefaciens* GMO4-2 were identified from all seawater samples, and indicates that these two species were not influenced by either DEF and HSL. *Pseudomonas doudoroffii*. GMO4-3 was isolated from the unamended seawater and seawater amended with DEF, or DEF plus C8-HSL. Some strains could only be isolated from the seawater after the additions. For example, *Cytophaga* sp. GMO4-6, *Sphingomonas* sp. GMO4-11, and Beta proteobacterium strain GMO 4-10 were isolated from the seawater with DEF plus 3OC6-HSL; *Cytophaga* sp. GMO4-6 was also obtained from the community that was treated with DEF plus C8-HSL. Furthermore, GMO4-13, GMO4-14, GMO4-15, GMO4-16, GMO4-17 and GMO4-18 were found to be unknown species cultivated only from seawater that was amended with DEF, DEF plus 3OC6-HSL, C8-HSL, or DEF plus C8-HSL respectively.

Relationship between bacterial growth and siderophore production under iron-limited conditions

To investigate whether the isolated strains grew under iron-limited conditions, all strains were inoculated on seawater-based IDSM agar plates containing 0.01 μM Fe(III) which is similar to the iron content in of seawater. Iron content in the seawater was detected to be 7.8 nM, which dissolved iron would be more less than 7.8 nM. Thus this seawater may be classified as an iron-limited environment for most microorganisms.

From eighteen isolates, only three strains were found to grow on iron-limited IDSM agar plates. Bacterial growth and siderophore production of all isolated strains were investigated with chrome azurol (CAS) assay [27] and the cross-feeding assay [17] with the addition of 0.1 nM each of DEF, 3OC6-HSL, C8-HSL and DEF plus 3OC6-HSL or C8-HSL respectively. Table 2 shows that the strains GMO4-11 *Sphingomonas* sp. and GMO4-14 did not grow on iron-limited IDSM agar medium and did not produce siderophores on CAS agar plates. However, their colo-

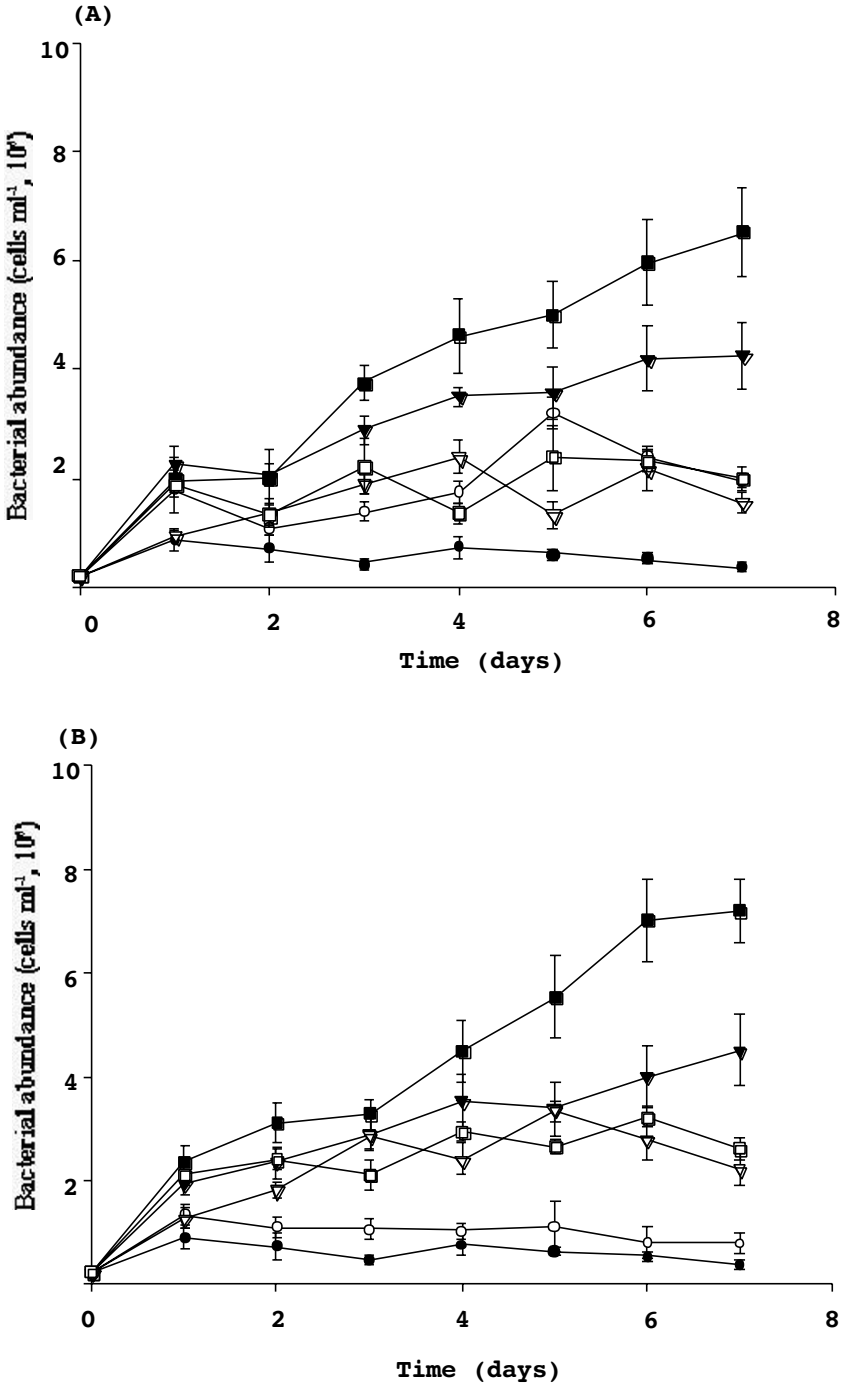

Figure 2
Analysis of total bacterial numbers (cells/ml) in S0011 seawater in the presence of 0.1 nM (FIG 2A) or 1000 nM (FIG 2B) of siderophore DEF, synthetic HSLs, DEF plus HSL respectively with DAPI staining counting method during 7 days incubation at 30°C. (filled square) without addition; (circle) with the addition of 0.1 nM DEF, (triangle) with the addition of 0.1 nM 3OC6-HSL, (filled triangle) with the addition of 0.1 nM DEF plus 0.1 nM 3OC6-HSL (Fig 2A), (square) with the addition of 0.1 nM C8-HSL (Fig 2B), (filled square) with the addition of 0.1 nM DEF plus 0.1 nM C8-HSL. Each points represents the mean coaggregration value from three separate measurements.

Table 1: Summary of cultivable bacteria counting by CFU on agar plates and contribution of each phenotype.

Seawater sample	Strain (collection no.)	Identification (homology)	CFU 10^4colonies/ml	Contribution
None	GMO4-1	*Erwinia nigrifluen*	0.4	43.96%
	GMO4-3	*Pseudomonas doudoroffi*	0.28	30.77%
	GMO4-2	*Shewanella putrefaciens*	0.23	25.27%
			Total: 0.91	
DEF	GMO4-3	*Pseudomonas doudoroffi*	0.3	22.39%
	GMO4-4	*Caulobactezsp.*	0.3	22.39%
	GMO4-1	*Erwinia nigrifluen*	0.2	14.93%
	GMO4-2	*Shewanella putrefaciens*	0.2	14.93%
	GMO4-7	*Rhodobacfesp.*	0.14	10.44%
	GMO4-5	*Roseobactesp.*	0.1	7.46%
	GMO4-13	Unidentified*	0.1	7.46%
			Total: 1.34	
3OC6-HSL	GMO4-2	*Shewanella putrefaciens*	0.7	36.65%
	GMO4-1	*Erwinia nigrifluen*	0.6	31.41%
	GMO4-9	*Bartonella bacilliforms*	0.4	20.94%
	GMO4-8	*Flavobacteriunsp*	0.21	11.00%
			Total: 1.91	
DEF + 3OC6-HSL	GMO4-12	Oligotrophic bacteria	0.5	21.65%
	GMO4-1	*Erwinia nigrifluen*	0.4	17.32%
	GMO4-10	Beta ptroteobacterium	0.3	12.98%
	GMO4-11	*Sphingomonasp.*	0.3	12.98%
	GMO4-9	*Bartonella bacilliforms*	0.2	10.39%
	GMO4-2	*Shewanella putrefaciens*	0.24	8.66%
	GMO4-6	*Cytophagsp*	0.18	7.79%
	GMO4-4	*Caulobactesp.*	0.12	5.2%
	GMO4-14	Unidentified*	0.07	3.03%
			Total: 2.31	
C8-HSL	GMO4-2	*Shewanella putrefaciens*	0.74	37.56%
	GMO4-1	*Erwinia nigrifluen*	0.4	20.3%
	GMO4-4	*Caulobactesp.*	0.3	15.24%
	GMO4-15	Unidentified*	0.3	15.24%
	GMO4-5	*Roseobactesp.*	0.23	11.66%
			Total: 1.97	
DEF + C8-HSL	GMO4-6	*Cytophagsp.*	1.0	35.71%
	GMO4-3	*Pseudomonas doudoroffi*	0.4	14.28%
	GMO4-17	*Erwinia nigrifluen*	0.4	14.28%
	GMO4-16	Unidentified*	0.3	10.71%
	GMO4-1	Unidentified*	0.2	7.15%
	GMO4-2	*Shewanella putrefaciens*	0.2	7.15%
	GMO4-8	*Flavobacteriunsp.*	0.2	7.15%
	GMO4-18	Unidentified*	0.1	3.57%
			Total: 2.8	

* presents the stains whose 16S rDNA sequences have low matching scores in DDBJ data

Table 2: Influence of DEF and HSL on bacterial growth and siderophore production.

Strains	Bacterial growth and Siderophore production on CAS agar plates					
	Without addition	0.1 nM DEF	0.1 nM 3OC6-HSL	0.1 nM DEF+ 0.1 nM 3OC6-HSL	0.1 nM C8-HSL	0.1 nM DEF+ 0.1 nM C8-HSL
GMO4-1	-	-	-	-	-	-
GMO4-2	+	+	+	+	+	+
GMO4-3	+	+	+	+	+	+
GMO4-4	-	-	-	-	-	-
GMO4-5	-	-	-	-	+	-
GMO4-6	-	-	-	-	-	-
GMO4-7	-	-	-	-	-	-
GMO4-8	-	-	-	-	-	-
GMO4-9	-	-	-	-	-	-
GMO4-10	-	-	-	-	-	-
GMO4-11	-	-	-	+	-	-
GMO4-12	-	-	-	-	-	-
GMO4-13	-	-	-	-	-	-
GMO4-14	-	-	-	+	-	-
GMO4-15	-	-	-	-	-	-
GMO4-16	-	-	-	-	-	+
GMO4-17	-	-	-	-	-	-
GMO4-18	-	-	-	-	-	+
*Pelagiobacte*sp. V0110	-	-	-	-	-	+

"+" represents the colony and siderophore halo formation on the CAS agar plates which was the same with the control strain *Pelagiobacter sp.* V0110 (17).

Table 3: Concentration of iron ligands in GMO4-14, GMO4-16, GMO4-18 culture supernatant extracts.

Strain	Supplement compound in liquid IDSM medium	Mean siderophore content (SDα) as detected by Csaky test	Arnow reaction
GMO4-11	DEF+3OC6-HSL	ND	0.14(0.01)
GMO4-14	DEF+3OC6-HSL	ND	0.08 (0.02)
GMO4-16	DEF+C8-HSL	ND	ND
GMO4-18	DEF+C8-HSL	ND	ND

SDα values are given as micromoles of ligand per gram (dry weight) of cells and represent the mean of three experiments. ND, not detected (i.e., the concentration was below the detection limit of the assay.)

nies and siderophore halo formation were observed on the IDSM and CAS agar plates after the addition of 0.1 nM DEF plus 0.1 nM 3OC6-HSL. Similarly, GMO4-16, and GMO4-18 only grew on the IDSM and CAS agar plates with the addition of DEF plus C8-HSL. Strains GMO4-11, GMO4-14, GMO4-16, GMO4-18 were further investigated in the A *tumefaciens* A136 reporter strain assay [34] using the synthetic HSLs as standards. However, 3OC6- and C8-HSLs were not detected in the supernatant extracts of these strains by this reporter strain assay. Also siderophore production by GMO4-11, GMO4-14, GMO4-16 and GMO4-18 in IDSM medium containing 0.01 μM Fe(III) with the addition of 0.1 nM DEF plus 0.1 nM of 3OC6- or C8-HSL were partially extracted and investigated by the Csaky test and Arnow reaction (Table 3). These two assays are well known for the detection of hydroxamate (Csaky test) or catechol groups (Arnow reaction) which are typical functional groups that bind iron. The induced siderophores from the above four strains were most likely to be different types as shown by these assays. Furthermore, all of the siderophore components were observed to have different reactivities in the Csaky test and Arnow reaction with exogenous DEF. Siderophore components from GMO4-11, GMO4-14 were positive in the Arnow test and indicated the existence of catechol groups while those from GMO4-16 and GMO4-18 showed negative reactivities in both assays. Artificially added DEF is a hydroxamate and was positive in the Casky test. The added DEF (0.1 nM) to the cultivation

medium of the four strains was at a lower concentration than the detection limit for both assays. Thus, the Arnow test positive components from GMO4-11 and GMO4-14, and siderophore components from GMO4-16 and GMO4-18 were siderophores produced by these strains only in response to the existence of DEF plus 3OC6-HSL or C8-HSL.

Discussion

Marine bacterial content in seawater may range between 10^{9-10} cells per milliliter of seawater or more in the ocean [10]. However, a lack of knowledge regarding marine bacteria and the nutrient limiting conditions of the ocean restrict our abilities to obtain and isolate many marine bacteria using the usual protocols such as enrichment and selective culture methods. Most marine bacteria have been classified as uncultivable species due to their ambiguous survival ability and limited cell amounts under low nutrient conditions. In this study, we found that total bacterial numbers in seawater was stimulated by artificially amending such seawater with trace amounts of siderophore DEF, quorum sensing signal HSL or DEF plus an HSL (Fig. 1 & Fig. 2). In addition, colony number and morphology of cultivable species were found to increase after the addition of trace amounts of DEF and HSL (Table 1). These phenomena suggest two possibilities. (a) Nutrient levels were increased, e.g., some organisms may utilize DEF or HSL as a nutrient for their growth improvement and the increase of such organisms may affect the balance between all organisms present, or (b) DEF and HSL may work not only as a nutrient enrichment but also as interspecies chemical signals which affect the bacterial community structure in this water ecosystem. That is to say, the growth of some species may be stimulated by the addition of DEF plus HSLs as we have reported for the growth of the marine bacterium *Pelagiobacter sp*. The growth of this organism was stimulated by the addition of an exogenous siderophore and C8-HSL when grown in iron-limiting seawater conditions [17] and suggested that in the natural seawater environment some bacteria like this *Pelagiobacter sp*. may exist such that their growth and detection could only be stimulated after the addition of exogenous siderophores and quorum-sensing chemical signals such as DEF and HSL. To confirm the possibility of (a), DAPI counting of bacteria after the addition of 1 μM of DEF or HSL, or DEF plus HSL (10000 times higher than the low concentration) were performed. In both S9905 and S0011 seawater, the results showed that nearly the same total bacterial cell counts as those obtained from 0.1 nM HSL, and 0.1 nM DEF plus HSL amended seawater, and reduced cell counts compared to 0.1 nM DEF added seawater occurred (Fig. 1 & Fig. 2). Recent reports on amended dissolved free amino acid (DFAA) (1 μM) in seawater experiment showed that bacterial abundance significantly increased related to the control seawater [5]. However, three of their experiments showed the abundance started to increase after 2 days incubation and they did not show any results for low concentration DAFF addition. Our results indicated that bacterial response to DEF and HSL started right after the addition of such chemical compounds. On the other hand, similar cell counting results obtained from the addition of heated-DEF and HSL to the seawater indicates that DEF may not function as degraded amino acid supplement. Also, recent research on DEF enrichments in seawater showed that inhibition of bacterial activity was due to the chelation of necessary dissolved iron in the seawater [19,32]. Our results from experiments which used high concentration of DEF addition to seawater also showed a decrease in cell density in S9905 seawater, and a slight bacterial number change in S0011 seawater (Fig 1B, & Fig 2B). These results support that DEF may chelate the dissolved iron in high concentration level and suggest that the addition of DEF to seawater may not function as a dissolved bacterial growth-stimulating nutrient. Recently we have also reported that the growth of marine bacteria could be stimulated via a novel pathway in response to exogenous siderophore [18]. Although no obvious activity of DEF was observed in above experiment, it is well-known bacteria biosynthesize iron chelators with different structural features. It suggests that marine bacteria may have multiple respond to siderophores and to survive under the iron-limited conditions like we have reported [18] and this may stimulate the growth of certain species which we have not known.

Dissolved organic carbon was found to stimulate bacterial growth in several field experiments [5,21]. To test whether synthetic HSLs stimulated bacterial growth only as dissolved carbon source, other synthetic HSLs with different acyl chain with an even number of carbon atoms ranging from 4 to 12 in length [17] have also been investigated. The influence pattern of these HSLs (0.1 nM) or 0.1 nM of DEF plus one of these HSLs were observed as the follows: (1) *N*-butanoyl-HSL (C4-HSL), *N*-3-oxooctanoyl-HSL (3OC8-HSL), *N*-decanoyl-HSL (C10-HSL): Control < with the addition of an HSL < with the addition of DEF plus an HSL, (Similar results as shown in Fig 1A & 2A); (2) *N*-3-hydroxybutanoyl-HSL (3OHC4-HSL), *N*-3-oxododecanoyl-HSL (3OC12-HSL): with the addition of an HSL < control < with the addition of DEF plus an HSL; (3) *N*-hexanoyl-HSL (C6-HSL), *N*-3-oxodecanoyl-HSL (3OC10-HSL): control < with the addition of DEF plus an HSL < with the addition of an HSL (data not shown). Further more, siderophore production of strain GMO4-5 was observed to be in respond to the addition of C8-HSL under iron-limited condition (Table 2). These results suggest that HSLs may influence the bacterial numbers in the seawater as interspecies communica-

tion signals because they show different patterns than nutrient supplementation. Combining the above results, we suggest the possibility that DEF and HSL may work in the capacity (b), not only as nutrient but also chemical signals in the seawater.

Investigation of bacterial growth and siderophore production by all of the isolated strains showed that some strains indeed required supplementation with DEF plus HSL for their growth on the iron-deficient IDSM and CAS agar plates (Table 2). Such stimulated growth under iron-limited conditions occurred due to the biosynthesis of their own siderophores in response to exogenous DEF and HSL to acquire the iron. As shown in table 3, the induced catechol and unknown siderophores from the four strains GMO4-14, GMO4-16, GMO4-17 and GMO4-18 in response to a hydroxamate DEF plus a quorum sensing chemical signal compound, either 3OC6-HSL or C8-HSL, indicated that biofunctions activated by interspecies communications via siderophores and HSLs may exist among bacteria. These data suggest that bacterial growth of these species, which have no detectable siderophores and/or HSLs, were stimulated by siderophore and HSL signals produced by other species in the nutrient-limited seawater. The stimulated bacterial growth improved the cell density of bacteria to a level at which colonies were formed and detected on the agar plates and subsequently isolated. Thus, addition of DEF and HSL also suggests that interspecies communication through siderophores and HSLs may occur in natural aquatic environments and that such communication may cause changes in the bacterial community structure. These results suggest that marine bacteria in the ocean ecosystem responds to siderophore and quorum-sensing chemical signals and has enabled us to detect more diversity and also to enhance our ability to isolate and cultivate heterotrophic marine bacteria in the laboratory.

In addition, 16S rDNA analysis indicated that six isolates GMO4-13 to GMO4-18 (Table 1) obtained from the seawater with amendment by DEF, C8-HSL, DEF plus 3OC6-HSL or C8-HSL are most likely novel marine bacteria. These strains were found to lose their ability to be cultivated in the Marine broth or IDSM medium without the addition of DEF or HSL or simultaneous supplementation with DEF and HSL and are currently under further investigation to ascertain their physiological characteristics. These results indicate that the species which were isolated after the addition of DEF or HSL, or DEF plus HSL were probably in less abundance in nature due to physiological stresses caused by the seawater. However these stresses were alleviated by adding trace amounts of siderophore and HSL which stimulated their ability to form colonies on plates and which therefore led to possibility of their isolation from seawater directly using standard protocols.

Conclusion

Although large numbers of organisms and species of bacteria are thought to inhabit the ocean, less than 0.1% of them have been identified and studied. This is perhaps due to the limitations imparted on organisms living in the ocean environment which is known to contain fewer nutrients from organic and inorganic sources as compared to the terrestrial environment. Our results indicate that it was possible to detect more species in a seawater community and isolate more strains if we used different siderophores and HSLs. Until now more than a few hundreds siderophores and ten HSLs have been isolated, it suggests that it may be possible to apply this technique to isolate unculturable species directly from the ocean by artificial addition of specific siderophores and HSLs.

Materials and Methods

Strains and Culture conditions

Seawater S9905 was collected from Higashishina Kai 10 km from the coast of Yiriomote Island, Okinawa, Japan in May 1999 and seawater S0011 was collected from the coast of Miho, Shimizu City, Japan in November 2000. Seawater was treated by filtration with a 1.0 μm membrane filter to remove other microorganisms such as phytoplankton or flagellates before experimentation. All seawater samples were incubated in the dark. Bacteria were isolated from seawater sample after serial dilution in sterilized seawater (10^{-1} to 10^{-4}), followed by spreading of 100 μl of each solution onto 1/10-diluted marine broth (Marine Broth 2216; Difco) or seawater based IDSM [17] agar plates. The plates were incubated at 30°C for up to 2 months, and marine bacteria were identified by growth on the plates containing 3% Nad in comparison with no growth on the agar plates containing 0.15% Nad for the same strain.

Seawater based IDSM medium was prepared using the following components (in grams/liter): NH_4NO_3, 1.0; NaCl, 30.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.3; K_2HPO_4 1.5; $C_8H_{18}N_2O_4S$ (HEPES), 2.38; $CaCl_2$, 0.2. It also contained 10% glucose (10 ml) and 0.1 ml of 1 mM $FeCl_3$.

Commercial siderophore desferroixamine (DEF, CIBA GEIGY) and synthetic 3OC6-HSL and C8-HSL [17] were filtered by 0.2 μm membrane before use. All containers were treated by 10%HCl more than 24 h before use.

Measurement of bacterial numbers

Seawater (30 L each) was incubated at 30°C for up to four weeks with the addition of DEF, plus 3OC6-HSL or plus C8-HSL; DEF only; HSL only; or without any addi-

tion. The concentration in seawater of each of the additions was adjusted to 0.1 nM.

Seawater samples were collected after 24 h, 48 h, 72 h, 96 h, and 1 week of incubation. Total numbers of the microorganisms (cells/ml) in the seawater were determined by direct bacterial counting with 4', 6-diamino-2-phenylidole (DAPI) staining using epifluorescence microscopy [26]. Triplicate samples of seawater (1.35 ml) were collected and fixed with an equal volume of Mildform 10 NM (10% formalin neutral buffer). Three hundred microliters of 0.5 μg/ml DAPI solution was added and the solution was incubated for 5 min at room temperature. The DAPI reacted solution was filtered through a 0.2 μm GTBP membrane filter (MILLIPORE), after which the filter was fixed on a slide and was observed using an epifluorescence microscope (Nikon, EFD2). Total bacterial counts were obtained by calculation using the following formula: Bacterial number (cells/ml) = [Nave × Am] / [V × As]. Nave: Average number of cells counted from 20 views per sample; Am: Area of the filtration membrane (132.7 mm in this study); V: total filtered volume of seawater (3 ml); As: Area of counting view (64×10^{-6} mm in this study).

Cultivable species were enumerated by counting the number of colony forming units (CFU) on 1/10 Marine Broth or IDSM agar plates at 10^{-1} to 10^{-3} diluted seawater solutions.

Identification of bacteria by 16S rDNA sequences

Bacterial DNA were obtained by extracting pure cultivation cells with PureGene Kit (Gentra Systems). A 16S rDNA gene fragment (169 to 194 bp in length) was amplified by PCR using a universal primer complementary to position 517 to 534 (5'-ATTACCGCGGCTGCTGG-3') and a bacterial primer complementary to position 341 to 358 (5'-CCTACGGGAGGCAGCAG-3') [23]. The total 16S rDNA fragment was amplified by using two oligonucleotide primers, fD (5'-AGAGTTTGATCCTGGCTCAG-3') and rD (5'-AAGGAGGTGATCCAGCC-3') [31]. The PCR products were sequenced by using a 373 DNA sequencer (PE Biosystems) and analyzed in DDBJ databases.

Siderophore production and cross-feeding assay

Siderophore production and cross-feeding assays were performed as reported previously [17]. The chrome azurol S (CAS) assay [27] was used to detect siderophores. On CAS agar plates, the formation of colony and siderophore halos were evaluated following 7 days of colony incubation at 30°C. All isolated strains were inoculated on four different CAS plates: (i) without any addition, (ii) with the addition of 0.1 nM DEF, (iii) with the addition of 0.1 nM of 3OC6-HSL or C8-HSL, (iv) and with the addition of 0.1 nM DEF plus 0.1 nM of 3OC6-HSL or C8-HSL.

The induced siderophores of unknown strains were partially isolated and compared with desferroixamine by examination with the Csaky test [15] and the Arnow reaction [1] for their hydroxamate and catechol functionality respectively. In these assays, hydroxylamine and 2,3-dihydroxybenzoic acid, respectively, were used as the standards.

Determination of iron content in seawater

Collected sea water 100 ml, was concentrated by lyophilization and resuspended in 2 ml of acid-treated distilled water. The salt component in seawater was removed by centrifugation. The concentration of iron in the seawater was measured with an inductively coupled plasma spectrometry (ICPS-1000IV) sequential plasma spectrometer (Shimazu) at the absorbance of iron atom (259.940 nm, 239.562 nm respectively). Iron standard solution (Fe100, WAKO Chemical Ltd) ($Fe(NO_3)_3$ in 0.1 mol/l • HNO_3: 99 mg/L) was used for the determination of a calibration curve.

Nucleotide sequence accession number

The DDBJ GenBank accession numbers of the sequences for *Erwinia nigrifluens, Shewanella putrefaciens, Pseudomonas doudoroffii., Caulobacter* sp, *Roseobacter* sp., *Cytophaga* sp., *Rhodobacter* sp., *Flavobacterim* sp., *Bartonella bacilliforms.* Beta proteobacterium, *Sphingomonas* sp., and *Gelidibacter* sp. is Z96095, AF005255, AB019390, AB025196, Y15339, AB015545, U63949, U63955, M65249, AF026392, AB025720, AB001369, respectively.

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