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Genetic Characterization of Aerobic Photosynthetic Bacteria which were Predominant in Otsuchi Bay in the Summer of 1993*1.

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One hundred five strains of aerobic photosynthetic bacteria that were isolated from the seawater of Otsuchi Bay in July and August of 1993 were classified according to the restriction fragment length polymorphism of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer region. Of 105 strains examined, 94 were clustered into the two groups that were different from each other only in the fragment pattern of 16S rDNA digested with *AvaI*. Sequences of the spacer regions of the two clusters were identical to each other, and only three nucleotide substitutions including the restriction site of *AvaI* were found in the fragment of 16S rDNA (942 bp). Hence it is concluded that the community of the aerobic photosynthetic bacteria in Otsuchi Bay in the summer of 1993 had been comprised of very limited phylogenetic groups.

1 Introduction

Aerobic photosynthetic bacteria are a group of heterotrophic bacteria that synthesize bacteriochlorophyll *a* only under the aerobic conditions.[1] Light energy is utilized in the presence of oxygen, but cellular level of the pigment is around 1 nmol/mg dry cell or less, and far less than that in anaerobic photosynthetic bacteria.[2-4] Hence photosynthetic activity is feeble and light energy seems to be utilized as an auxiliary energy source only useful in the paucity of respiratory substrate.[5]

In summers of 1992 and 1993, we observed the prevalence of aerobic photosynthetic bacteria in the surface seawater of Otsuchi Bay, Iwate.[6] The population of the aerobic photosynthetic bacteria reached 5.6 to 54 % of viable count of aerobic heterotrophic bacteria. Since some ecological functions seemed to favor the distribution, it was examined how many different kinds of aerobic photosynthetic bacteria were present in the seawater. One hundred five strains were randomly isolated from different stations of Otsuchi Bay, in July and August of 1993. The structures of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer region were examined to elucidate the phylogenetic difference of the bacteria.

2. Materials and Methods

2.1. Bacterial strains

Bacterial strains examined in this experiment were isolated from Otsuchi Bay, with PPESS-II agar plate medium,[7] in July and August of 1993.

2.2. PCR amplification

DNA was extracted by boiling bacterial

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cells in sterilized distilled water, ethanol-precipitated and utilized as template DNA for PCR. 16S rDNA and the 16S-23S rDNA intergenic spacer region were PCR-amplified using an automatic thermal cycler, model 480 (Perkin Elmer). The PCR amplification was performed by 30 thermal cycles of 93 ℃ for 60 s, 48 ℃ for 45 s and 72 ℃ for 90 s. Hot start at 98 ℃ for 180 s, and additional extension at 72 ℃ for 300 s after the cycles were also performed. A 100 μl of the PCR mixture contained 0.1 to 1.0 μg of template DNA, 20 nmol each of dNTP, 100 pmol each of PCR primer, 1.35 unit of *Taq* DNA polymerase (Takara, Japan), 10 μl of 10-fold concentrated PCR buffer prepared by the manufacturer. PCR primers for 16S rDNA were 27F (5'- AGAG TTTGATC [C or A] TGGCTCAG-3') and 1492R(5'-TACGG[C or T] TACCTTGTTAC GACTT-3').[8] The primers for the spacer, INT-F (5'-AAGTCGTAACAAGGTA [A or G] CCG-3') and INT-R (5'-CT [G or T] [A or G] CTGCC [A or T, C] AGGCATCCA-3'), were designed based on the consensus sequence of 16S rDNA and 23S rDNA.[8] Molecular size of the PCR products was determined with an agarose-gel electrophoresis.

2.3. PCR-RFLP (restriction fragment length polymorphism)

The PCR product was concentrated with ethanol precipitation, and digested with one of the restriction enzymes, *Hae*III, *Rsa*I, *Ava*I, *Hha*I and *Taq*I, based on the protocol recommended by the manufacturer (Takara). DNA fragment was examined with an agarose gel electrophoresis. Agarose gel I (Wako, Osaka) was melted in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH7.2), at the concentration of 2 %. A 100-base pair ladder (Amersham-Pharmacia) was used as the DNA size marker. The gel length was 6 cm. Electrophoresis was performed at 100 V for 50 minutes. The DNA fragments were stained with an ethidium bromide solution (0.5 μg / ml).

2.4. Cloning of PCR-product

The PCR-amplified DNA product was applied to the agarose-gel electrophoresis. The DNA fragment, coincident in molecular size to the expected one, was excised off and re-amplified with PCR. After span off with Suprec-O2 (Takara), the PCR product was cloned by using pT7BlueT vector (Novagen) and *Escherichia coli* JM109.

2.5. Sequencing

Partial sequence (942 bp) of 16S rDNA was determined using internal sequence primers, 517R (5'-GTATTACCGCGGCTG CTGGC-3'), 357F (5'-ACTCCTACGGGAG GCAGCAG-3'), and 536F (5'-GCCAGCAG CCGCGGTAATAC-3'). Clones for 16S-23S rDNA spacer region were sequenced using INT-F and INT-R primers. Nucleotide sequence was performed by Dye-terminator Cycle Sequencing Ready Reaction Kit and DNA sequencer model ABI373 (Perkin Elmer).

2.6. Copy number of ribosomal RNA operon

Bacterial DNA was digested with 6-base recognizing restriction enzyme, *Pst*I, *Sal*I, *Bam*HI *Hind*III and *Kpn*I that have no restriction site in 16S rDNA of the strains examined. The digest was electrophoresed on 1% agarose gel (length, 14 cm), at 50 V for 5 h, and Southern-blotted to a nylon membrane (Boehringer). A 1435-bp fragment of the 16S rDNA of the strain C1, which was isolated at station 1 and included in the group A (see the text), was obtained by the PCR and labeled with fluorescent dye using Random Prime Labeling and Detection

Kit version II (Amersham-Pharmacia). Hybridization was performed based on a protocol recommended by a manufacturer (Amersham-Pharmacia).

3. Results and Discussions

3.1. Analysis of 16S rDNA

Table 1 listed the distribution of strains examined in this experiment. The strains were isolated from 7 different stations of Otsuchi Bay, and percent occurrences of aerobic photosynthetic bacteria at each station were also indicated. Stations 1 through 4 were located along with the axis from the mouth of the bay to the bottom, while stations 6 and 7 were located near the mouth of rivers (Fig. 1). The strains examined were not evenly distributed among the stations, but randomly selected from each station.

Difference in fragment pattern of the 16S rDNA digested with three different restriction enzymes was summarized in Table 2. Among the isolated strains, two different fragment patterns were observed in the digests of *Hae*III, five patterns in *Rsa*I and two in *Ava*I. According to the difference in the pattern, the bacterial strains were clas-

Table 1. List of the strains

Station	Number of strains	% Occurrence of aerobic phototrophs
July, 1993		
1, 0 m	16	52.2
10 m	18	24
2, 0 m	3	28.6
10 m	4	40.6
3, 0 m	4	5
10 m	4	56.5
4, 0 m	7	26.3
10 m	8	20.2
5, 0 m	1	7.6
6, 0 m	5	4.4
7, 0 m	6	23.1
August, 1993		
1, 0 m	7	6.4
3, 0 m	7	3.6
5, 0 m	3	9.7
6, 0 m	7	9.6
7, 0 m	5	6.2
Total Number of Strains	105	6

The percent occurrence of July was referred to the reference "6".

Table 2. Clustering of bacterial strains depending on fragment pattern

Cluster	Restriction enzyme		
	*Hae*III	*Rsa*I	*Ava*I
	Fragment pattern		
A	α	a	I
B	α	a	II
C	α	b	II
D	β	c	II
E	α	d	II
F	α	e	ND

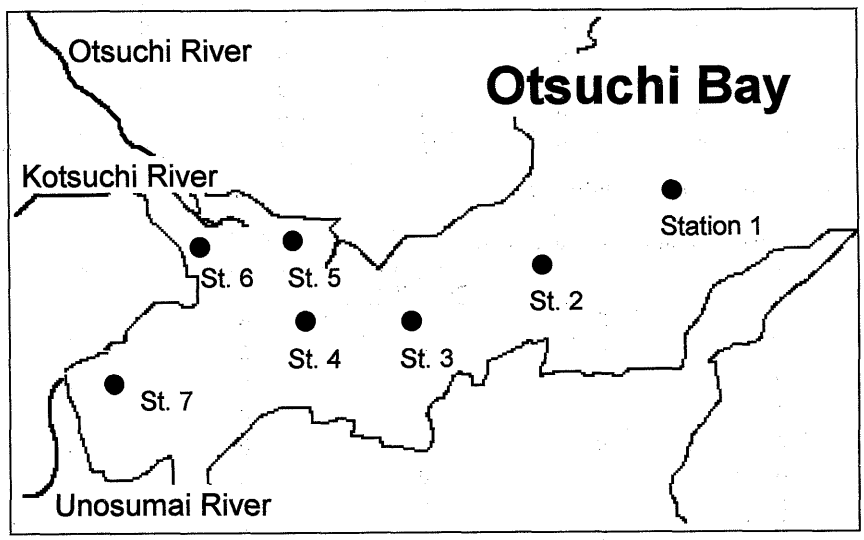

Fig. 1. Locations of sampling stations in Otsuchi Bay.

sified into 6 groups, as summarized in Tables 3 and 4. of 105 strains examined, 94 strains were clustered into the groups A and B, while the other 4 groups included only 4 to 1 strains. The groups A and B were prevalent in both of July and August samples. No clear difference was found in the distribution pattern between July and August. Also no difference was found in the distribution at each different depth (data not shown). The groups A and B were different from each other only in the digests of *Ava*I (Fig. 2). Sequence analysis of representative strains, C1 (group A) and C6

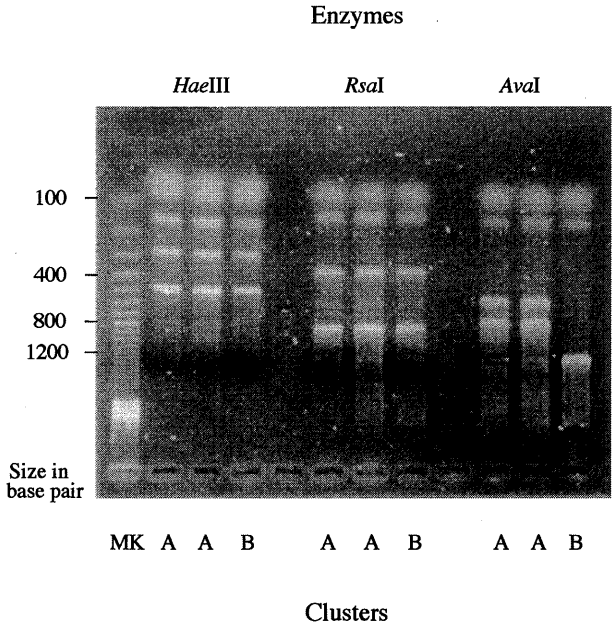

Fig. 2. Restriction fragment polymorphism of PCR-amplified 16S rDNA. MK, molecular marker; the strains C1 (group A), C36 (group A), and C6 (group B) were plotted from the left to the right.

Table 3. Number of bacteria included in each cluster

Cluster	Stations							Total
	1	2	3	4	5	6	7	
A	17	4	5	4	1	3	4	38
B	9	3	3	9	0	1	2	27
C	4	0	0	0	0	0	0	4
D	0	0	0	0	0	1	0	1
E	1	0	0	1	0	0	0	2
F	3	0	0	1	0	0	0	4
	34	7	8	15	1	5	6	76

The data indicates the distribution in July, 1993.

Table 4. Number of bacteria included in each cluster

Cluster	Stations					Total
	1	3	5	6	7	
A	5	6	2	5	1	19
B	2	1	1	2	4	10
C	0	0	0	0	0	0
D	0	0	0	0	0	0
E	0	0	0	0	0	0
F	0	0	0	0	0	0
Total	7	7	3	7	5	29

The data indicates the distribution in August, 1993.

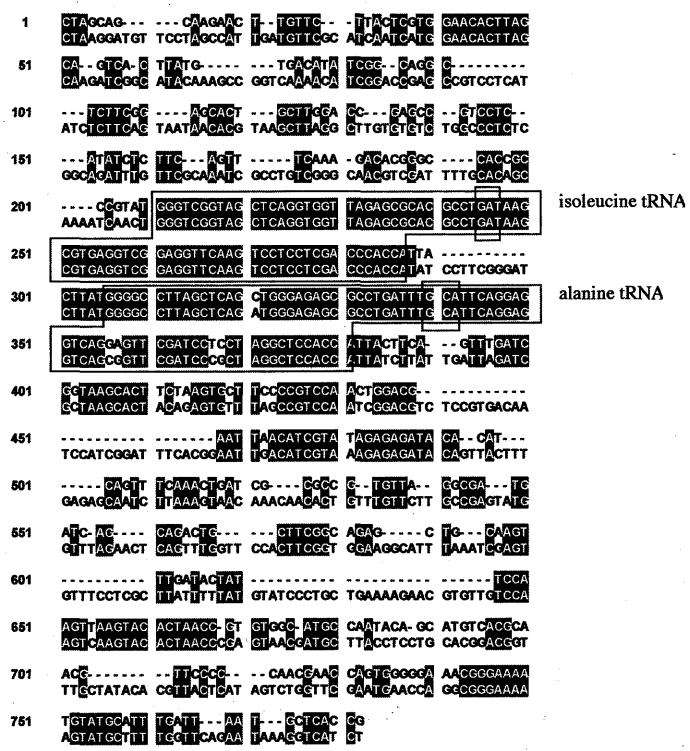

Fig. 3. The structures of 16S-23S rDNA intergenic spacer region. The upper sequence indicates the sequence of the strain C1, the lower one *Roseobacter litoralis*. Each box indicates the anticodon for isoleucine and alanine, respectively.

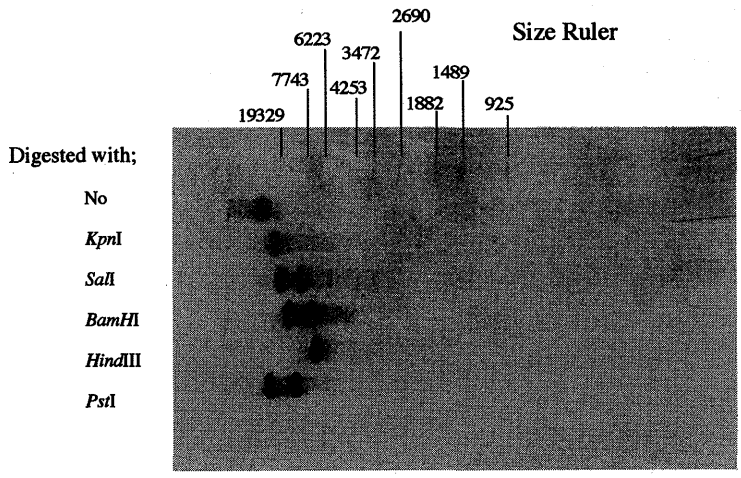

Fig. 4. Southern blot hybridization of genomic DNA with PCR-amplified 16S rDNA

(group B), indicated only three nucleotide substitutions in PCR-amplified 16S rDNA (942 bp), including the restriction site of *Ava*I (data not shown).

3.2. Analysis of Intergenic Spacer

Then we analyzed the structures of 16S-23S rDNA intergenic spacer regions, to elucidate the phylogenetic relationship between the group A and B. Since heterogeneity of intergenic spacer in genome has been reported to affect structure analysis,[9,10] copy difference in the structures of 16S-23S rDNA spacer region was examined. The PCR amplified 16S-23S rDNA spacer region of C1 strain, which was included in the group A, was ligated with pT7Blue T vector and the vector was introduced into *Escherichia coli* JM109. As a result of sequencing, no difference was found in the structures of the 573-bp DNA fragment of the spacer region between nine different clones. Alignment of the sequence with the intergenic spacer region of the aerobic photosynthetic bacterium, *Roseobacter litoralis*, indicated the presence of isoleucine-tRNA and alanine-tRNA regions (Fig. 3).[11]

Further we analyzed the heterogeneity of the spacer region in the other strains. Although Southern hybridization suggested the presence of at least 2 ribosomal RNA operons in the strain C6 (Fig. 4), the sum of each fragment size found in the digests of *Hae*III, *Rsa*I, *Ava*I, *Hha*I or *Taq*I did not exceed the sizes of the non-digested PCR amplified products. Hence it is clear that no effect on PCR-RFLP analysis is present, even though heterogeneity is reported in the spacer region of many bacteria.[9,10]

When the PCR amplified intergenic spacer of the other strains was digested with *Hae* III, *Rsa*I, *Ava*I, *Hind*III, or *Taq*I, only one fragment pattern was observed in the electrophoretic pattern of the digest of each enzyme. No difference was found between the groups A and B. Sequence analysis also indicated no difference between the strains C1 (group A) and C6 (group B). Since it is well known that the spacer regions are variable between the strains included in the same species,[12,13] it is probable that the strains of groups A and B are intimately correlated with each other and the three nucleotide substitutions in 16S rDNA was caused by a recent mutation.

3.3. Classification of the isolated strains

As the community of the aerobic photosynthetic bacteria in the surface layer of Otsuchi Bay in the summer of 1993 was comprised of very limited phylogenetic groups, a few limited environmental factor seems to favor the distribution of the aerobic photosynthetic bacteria. Since water mass was stratified in the summer and the surface was affected by the run off of river water,[6] two explanations are possible for the distribution of the aerobic photosynthetic bacteria. One is the supply of the aerobic photosynthetic bacteria from the bottom region of the bay, and another is the physiological adaptation of the aerobic photosynthetic bacteria to shiny and low salinity environment. Sequence analysis of 16S rDNA indicated that both strains C1 and C6 were closely related to *Roseobacter*.[14] The similarities to *Roseobacter litoralis*, in 16S rDNA, were from 95.2% and 95.5%, respectively.

4. References

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1993年夏の大槌湾で優占になった好気性光合成細菌の遺伝学的特徴

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1993年夏の大槌湾で優占になった好気性光合成細菌の16S rDNAおよび16S rDNA-23S rDNA間のインタージェニックスペーサーの構造を，PCR-RFLP法および塩基配列から調べた。その結果，7月および8月に大槌湾から分離された105株の内，94株は2つのグループ，AおよびB，に属することがわかった。AおよびB間では，16S rDNAで*Aval*制限サイトに1塩基，その他の場所に2塩基の違いが見られるのみであり，インタージェニックスペーサーの構造は完全に一致した。このことから，1993年夏の大槌湾で優占になった好気性光合成細菌は，遺伝学的に均一な細菌群により構成されていることがわかった。