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Characterization of Extracellular RNAs Produced by the Marine Photosynthetic Bacterium *Rhodovulum sulfidophilum*

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The marine photosynthetic bacterium *Rhodovulum sulfidophilum* produces extracellular nucleic acids that are involved in its flocculation. These were found to be produced concomitantly with cell growth. The RNA fraction of these extracellular nucleic acids was subjected to cDNA analysis by applying a micro RNA cloning method and found to contain mainly fully mature-sized tRNAs and fragments of 16S and 23S rRNAs. Analyses of modified bases and genes of the RNAs revealed no structural difference between the intracellular and extracellular RNAs. This is the first report of structural analyses of bacterial extracellular RNAs.

Key words: extracellular RNA, flocculation, photosynthetic bacterium, *Rhodovulum sulfidophilum*, RNA cloning.

Nucleic acids have a large variety of functions in the main system of life, gene maintenance and expression, and therefore usually perform these functions inside viable cells. However, several recent studies have revealed active production of extracellular nucleic acids in some bacteria (*1*). Some gram-negative bacteria such as *Pseudomonas aeruginosa* form membrane-derived vesicles (blebs) to release various intracellular material including chromosomal and plasmid DNA (*1–3*). *Streptococcus pneumoniae* produces extracellular nucleic acids through lysis of a subfraction of cells. This cell lysis is active, unlike cell lysis in the post-stationary phase, and is controlled by the extracellular concentration of a secreted peptide pheromone called the competence stimulation peptide (*4*). These extracellular nucleic acids are thought mainly to serve as donor material for bacterial gene transfer by natural genetic transformation in the environment (*1*). On the other hand, extracellular nucleic acids with direct physiological functions have recently been reported. *P. aeruginosa* produces extracellular DNA that is involved in bacterial biofilm formation (*5*). Bacterial biofilm consists of structured communities of cells that protects cells from antibiotics and immune attack. In *P. aeruginosa*, extracellular DNA accounts for the majority of the extracellular material in the biofilm and is required for the initial establishment of biofilm (*5*). Another bacterium that produces extracellular nucleic acids is the marine photosynthetic bacterium *Rhodovulum* sp. strain PS88 (*6*), which also forms structured communities of cells called floc. Watanabe *et al*. showed that this bacterium produced extracellular nucleic acids and that the flocculated cells were deflocculated by treatment with nucleases (*6*). The flocculating ability of bacteria generally depends on secreted capsular mucopolysaccharides or proteins (*7*, *8*), but the floc formation of this bacterium is due to produced extracellular nucleic acids (*6*). In our preliminary study, such flocculation was also observed in some bacteria of the genus *Rhodovulum* that are closely related to *Rhodovulum* sp. strain PS88.

Although production and function of extracellular nucleic acids have been reported, the nucleic acids themselves have not been well characterized. In this study, we analyzed the RNA fraction of the extracellular nucleic acids of the strain *Rhodovulum sulfidophilum* DSM 2351 (*9–11*). We prepared cDNAs of extracellular RNAs by a newly developed method and sequenced them. Here, we report the relationship between cell growth and the production of extracellular nucleic acids and characterization of the extracellular RNAs including modified base analyses. This is the first report of characterization of bacterial extracellular RNAs.

MATERIALS AND METHODS

Cultivation—A marine photosynthetic bacterium, *R. sulfidophilum* DSM 2351 (9, *11*), was cultivated essentially by the method of Hiraishi and Ueda (*10*).

Analysis of Cell Growth and Extracellular Nucleic Acid Production—Cell growth was evaluated by measuring turbidity of culture medium at 660 nm. Amounts of secreted nucleic acids were calculated from the band intensity of extracellular nucleic acids on 0.7% agarose gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide. Band intensities were evaluated using software IQ-Mac (Molecular Dynamics, Sunnyvale, USA).

Preparation of Extracellular RNAs—Cells were removed from culture medium by centrifugation, and the supernatant was transferred to another tube. The nucleic acid fraction of the supernatant was precipitated with ethanol. The extracellular nucleic acid fraction thus collected was incubated with 1% SDS (w/v) and 0.05 μg/μl proteinase K (Merk, Darmstadt, Germany) at 37°C for 60 min to remove proteins. The preparation was then phenolyzed with phenol/chloroform, and nucleic acids were collected by ethanol precipitation. This sample was treated with

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DNase I (RNase free grade, Promega, Madison, USA), and the extracellular RNAs were purified by 10% denaturing polyacrylamide gel electrophoresis.

Cloning and Sequencing Analysis of Extracellular RNAs—To clone extracellular RNAs, we modified a micro RNA cloning method (*12*). First, the extracellular RNAs obtained were dephosphorylated. A reaction mixture containing 20 μg of extracellular RNAs, 300 units of calf intestine alkaline phosphatase (Takara, Kyoto, Japan), 50 mM Tris-HCl (pH 9.0), and 1 mM $MgCl_2$ in a total volume of 300 μl was incubated at 50°C for 60 min. The reaction was stopped by phenol/chloroform extraction, and the RNA was collected by ethanol precipitation. Dephosphorylated extracellular RNAs were ligated with the 3′-adapter synthetic DNA (5′-pTTTAACCGCATCCTTCTCx-3′: p, phosphate; x, biotin). The 3′-biotinyl group was used as a blocking group. Ligation reaction mixture contained 20 μg of dephosphorylated extracellular RNAs, 5 μM 3′-adapter, 150 units of T4 RNA ligase (Takara, Kyoto, Japan), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.006% BSA (w/v), 25% PEG#6000 (w/v) in a total volume of 300 μl. The mixture was incubated at 4°C for 16 h. The ligation reaction was stopped by ethanol precipitation. The ligation product was purified by 10% denaturing polyacrylamide gel electrophoresis and subjected to 5′-phosphorylation [100 μl reaction, 37°C, 60 min, 100 units of T4 polynucleotide kinase (Takara, Kyoto, Japan), 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM ATP, 5 mM DTT]. The phosphorylation reaction was stopped by phenol/chloroform extraction; the sample was recovered by ethanol precipitation. Then, the 5′-adapter RNA (5′-UACUAAUACGACUCACUAAA-3′) was ligated to the phosphorylated product by T4 RNA ligase as essentially described above. The new ligation product was gel-purified. Products were reverse-transcribed with 3′-RT primer (5′-GACTAGCTG**AAGCTT**AAGGATGCGGTTAAA-3′:bold letters denote *Hin*dIII site). The cDNAs were amplified by PCR using a 5′-primer (5′-CAGCCAACG**GAATTC**ATACGACTCACTAAA-3′: bold, *Eco*RI site) and the 3′-RT primer. The PCR products were purified by phenol/chloroform extraction and collected by ethanol precipitation. The PCR products were then digested with *Eco*RI and *Hin*dIII and inserted into the appropriate sites of pGEM-3Z vector using T4 DNA ligase (Takara, Kyoto, Japan). Clones were sequenced by use of a Thermo sequenase cycle sequencing kit (USB Corporation, Cleveland, USA). Sequences were analyzed by BLAST searches.

Southern Blotting—*R. sulfidophilum* DSM 2351 genomic DNA and *Escherichia coli* genomic DNA (as a control) were prepared by the CTAB method (*13*). These genomic DNAs were digested with restriction endonucleases, *Eco*RI, *Bam*HI and *Pst*I. The products were electrophoresed on 0.7% agarose gel. Then developed DNA fragments were transferred from the agarose gel to positive charge membrane filter (Hybond^{TM}-N+, Amersham Bioscience, Tokyo, Japan). Probes were synthesized by transcription from obtained clones using DIG RNA Labeling Kit (Roche, Tokyo, Japan). Hybridizations were done at 50°C for 12 h in a solution of DIG Easy Hyb (Roche, Tokyo, Japan). After hybridization, filters were washed two times for 5 min at room temperature with 2× SSC and 0.1% SDS, then two times for 15 min at 60°C with 0.1× SSC and 0.1% SDS (w/v). Hybridized probe was detected as recommended by the supplier (DIG Nucleic Acid Detection Kit, Roche, Tokyo, Japan).

Thin Layer Chromatography—To test whether the extracellular RNAs contain modified bases, nucleotide analysis by thin layer chromatography (*14*, *15*) was performed. The reaction mixture (total volume 15 μl) contained 5 μg of extracellular RNAs, 42 units of RNase T_2 (Gibco, Rockville, USA), and 6.7 mM sodium acetate (pH 4.5). The mixture was incubated at 37°C for 24 h. The digested product was labeled with [γ-^{32}P] ATP (Amersham Bioscience, Tokyo, Japan) and T4 polynucleotide kinase (Takara, Kyoto, Japan). After labeling, the reaction mixture was treated with glucose and hexokinase (Roche, Tokyo, Japan) to remove excess [γ-^{32}P] ATP. To complete 3′-dephospholylation, the labeled products were treated by nuclease P1 (Yamasa, Choshi, Japan). Products were developed by two-dimensional thin layer chromatography. For the first dimension, the solvent was isobutyric acid/29% ammonia water/dH_2O (66:1:33). Two different solvents were used for the second dimension. The first one was 2-propanol/HCl/dH_2O (70:15:15). The second was ammonium sulfate/0.1 M sodium phosphate buffer (pH 6.8)/*n*-propanol (60 g:100 ml:2 ml). The plate was autoradiographed by BAS-1800 (Fuji film).

Accession Numbers—The nucleotide sequence data reported are available in the DDBJ database. These accession numbers of the RNAs sequenced in this study are shown in Table 1.

RESULTS AND DISCUSSION

Cell Growth and Extracellular Nucleic Acid Production—It has been reported that *R. sulfidophilum* can be cultivated under both anaerobic light conditions and aerobic dark conditions (*9*). We observed that extracellular nucleic acids were produced in both case. For the present study, *R. sulfidophilum* DSM 2351 was chosen (*9*–*11*). In preliminary experiments, this strain showed relatively rapid growth and the highest production of the extracellular nucleic acids among the strains of *Rhodovulum* species tested. To clarify the relationship between cell growth and the production of extracellular nucleic acids, these were measured during cultivation under anaerobic dark conditions. Cell growth was estimated by measuring the optical density of culture medium at 660 nm after dispersing cell flocs by vortexing (Fig. 1A). Extracellular nucleic acids were estimated by agarose gel electrophoresis after precipitation with ethanol, since direct photometric measurement of extracellular nucleic acids in culture supernatant gave unreliable results, probably because of co-production of extracellular proteins and polysaccharides. The electropherogram showed a relatively sharp band of large size (more than 23 kbp) and smaller, broader bands (around 2.3 kbp) (Fig. 1B). From the experiment using DNase and RNase (Fig. 1B, right panel), we concluded that the band of large size and the smaller bands are the DNA and RNA, respectively. The time course of DNA and RNA productions were determined by quantitative analysis of the electropherogram using IQ Mac (Fig. 1C). Using the intensity values of the bands (Fig. 1B), the amounts of DNA and RNA released into the culture medium were calculated (Fig. 1C, ordinate). For the quantification of RNA, we used only the

Table 1. **Extracellular RNA sequences.** Inserted sequences are shown as RNA sequences. These sequence data have been submitted to DDBJ.

Clone No.	Predicted RNA type*	Accession No.	Sequence (5′ to 3′)
9	16S rRNA frag (933–1007)	D16423	GAAUUCCUUCAGUUCGGCUGGAUCAGUGACAGGUGCUGCAUGGC-UGUCGUCAGCUCGUGUCGUGAGAUGUUCG
15	16S rRNA frag (1058–1206)	D16423	UUGGGCACUCUAGGAGAACUGCCGAUGAUAAGUCGGAGGAAGGU-GUGGAUGACGUCAAGUCCUCAUGGCCCUUACGGGUUGGGCUACA-CACGUGCUACAAUGGCAGUGACAAUGGGUUAAUCCCUAAAAACUG-UCUCAGUUCGGAUUGU
19	16S rRNA frag (304–380)	D16423	AAUCUUGGACAAUGGGGGAAACCCUGAUCCAGCCAUGCCGCGUG-AGCGAUGAAGGCCUUAGGGUUGUAAAGCUCUUU
59	16S rRNA frag (1306–1386)	D16423	ACCAUGGGAGUUGGGUUUACCCGAAGACGGUGCGCCAACCCUU-ACGGGGGGCAGCUGGCCACGGUAAGCUCAGCGACUGGGCUGC
1	tRNA (Met)	AB201391	GGCGGAGUAGCUCAGUUGGUUAGAGCAGAGGAAUCAUAAUCCU-UGUGUCGGGGGUUCAAGUCCCUCCUCCGCUACCA
3	tRNA (fMet)	AB201392	CGCGGGGUGGAGCAGCCCGGUAGCUCGUCAGGCUCAUAACCUG-AAGGUCGUAGGUUCAAAUCCUACCCCCGCAACCA
4	tRNA (Ala,GGC)	AB201393	GGGGCCAUAGCUCAGUUGGGAGAGCGCUUGCAUGGCAUGCAAG-AGGUCAGGGGUUCGACCCCCCUUGGCUCCAC
20	tRNA (Ala,UGC)	AB201394	GGGGCCAUAGCUCAGUUGGGAGAGCACCUGCUUUGCAAGCAGG-GGGUCAUCGGUUCGAUCCCGAUACGCUCCACCA
23	tRNA (Ala,CGC)	AB201395	GGGGCCGUAGCUCAGUUGGGAGAGCGCGUCGUUCGCAAUGACG-AGGUCAGGGGUUCGAUCCCCCUCGGCUCCACCA
40	tRNA (Glu,UUC)	AB201396	GGCCCGUUCGUCUAUCGGUUAGGACGCCAGGUUUUCAACCUGG-AAAGAGGGGUUCGACUCCCCUACGGGCUGCCA
25	tRNA (Thr) fragment	AB201397	CUGGUAGAGCAGGUCCUUCGUAAGGACAAGGUCGGGGGUUCGA-GUCCCUCUUGAGGCACCAA
11	tRNA (Arg,GCG) fragment	AB201398	UUGGAUAGAGUACUUGACUGCGAAUCAAGGGGUCGGGGGUUCG-AAUCCUCCCCAGCGCGCU
17	tRNA (Arg,GCG) fragment	AB201399	GCGAAUCAAGGGGUCGGGGGUUCGAAUCCUCCCCAGCGCGCCA
22	tRNA (Arg,GCG) fragment	AB201400	UGGUUAGAGCGCACGCCUGAUAAGCGUGAGGUCGGAGGUUCAA-GUCCUCCUCGACCCACCA
12	tRNA (Arg,CCU) fragment	AB201401	UUAGAGCAAUCCCCUCCUAAGGGAUAGGUUGCAGGUUCGAGUCC-UGCCGGGGUCACCA
24	tRNA (Arg,CCU) fragment	AB201402	CGUAUCACACGAGCCUUCCAAGCUCUUGGuGCGGGUUCGAUUCCC-GCUACCCGCUCCA
13	tRNA fragment	AB201403	UGCCAAGGUAAGGGUCGUGAGUUCGAAUCUCAUCGCCCGCUCCA
39	23S rRNA frag	AB201404	UAAUGGGUCAUCGACUUGGUCUCACGUGCAAGCUUAAGCCGAUA-GGUGUAGGCGCAGCGAAAGCGAGUCUUAAAUGGGCGUCGAGUUC-GUGGGAUCAGACCCGAAACCGA
27	23S rRNA frag	AB201406	AUGCCUGUCAAGAUGCAGGCUUCCCGCGGUUAGACGGAAAGACCC-CGUGCACC
75	23S rRNA frag	AB201407	AAACUCCGAAUACCCGGGAGUACUAGAUGGCAGACACACGGCGGG-UGCUAACGUCCGUCGUGGAG

*Numbers (304–1386) are nucleotide numbers of 16S rRNA. frag, fragment. In tRNAs, amino acids and anticodon sequences are shown in parentheses.

sharp RNA bands of about 2.3 kbp (Fig. 1B). We omitted smaller bands (smeared probably by salt) from the data, because of the difficulty of reliable quantification. Therefore, the true production of total RNAs by this cultivation may be underestimated. Most importantly, however, this experiment revealed the time course curve of RNA production.

Fig. 1A shows an initial lag phase of 24 h, followed by a rapid increase in the growth rates. The production of extracellular nucleic acids showed similar pattern to bacterial cell growth except that the rapid increase of nucleic acids production began from 30 h (Fig. 1, B and C). Both extracellular DNA and RNA productions increased until 48 h, after which a significant decrease in the RNA fraction was observed (Fig. 1C). Overall, Fig. 1 reveals that the production of extracellular nucleic acids closely followed cell growth. Because the production was observed in the log phase but not in the stationary or the post-stationary phase, it appears that the nucleic acids do not originate from the usual cell autolysis such as is observed in the post-stationary phase, but from active production.

Size Distribution and Sequences of Extracellular RNAs—The molecular size distribution of extracellular RNAs was compared with that of intracellular RNAs using denatured polyacrylamide gel electrophoresis. The patterns of RNA bands of both samples were very similar to each other (Fig. 2). From the size estimation, the extracellular RNAs seem to be tRNAs and rRNAs.

To further characterize the extracellular RNAs, we attempted direct RNA sequencing, but the purification of analyzable RNA molecules was unsuccessful. We therefore prepared cDNAs of the RNAs, then cloned and sequenced them. First, we prepared extracellular RNAs by 10% denaturing polyacrylamide gel electrophoresis. Although the main RNA band seemed to be about 2.3-kb

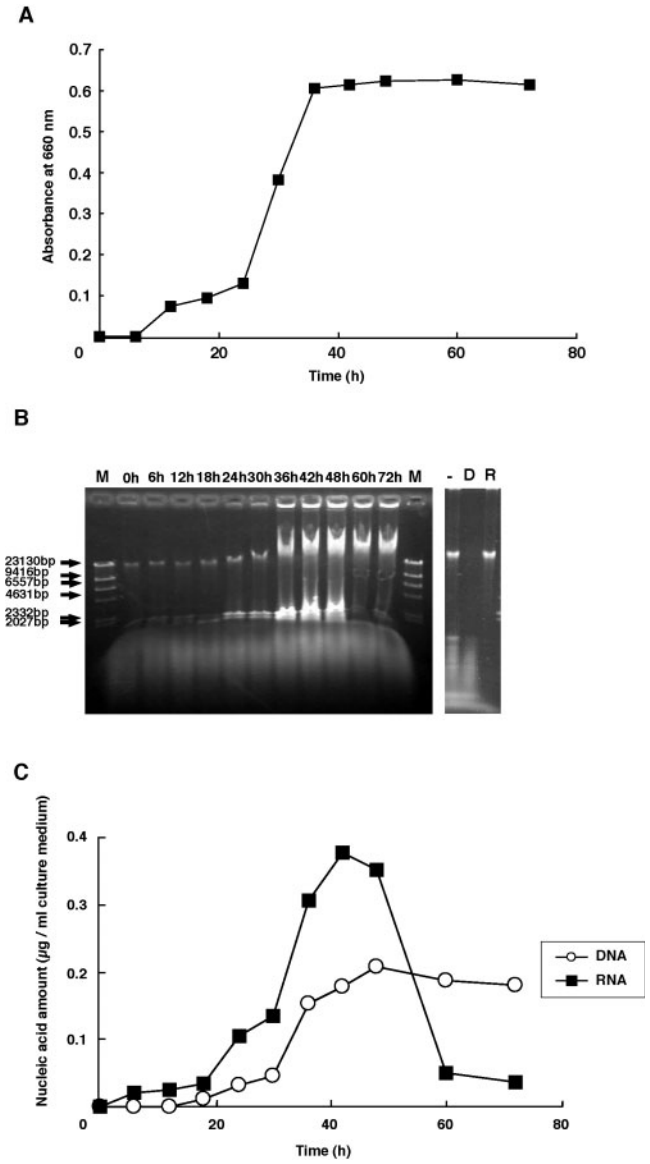

Fig. 1. **Growth of *R. sulfidophilum* DSM2351 and production of the extracellular nucleic acids.** (A) Cell growth. (B) Electrophoretic analysis of extracellular nucleic acids. Lanes 0 h–72 h, cultivation times. M, size markers, phage λ DNA *Hin*dIII fragments. Right panel, DNase or RNase treatment of the nucleic acids from 48 h culture. Lanes D, DNase treated. R, RNase treated. –, no enzyme control. (C) Production of extracellular nucleic acids. See text for full description.

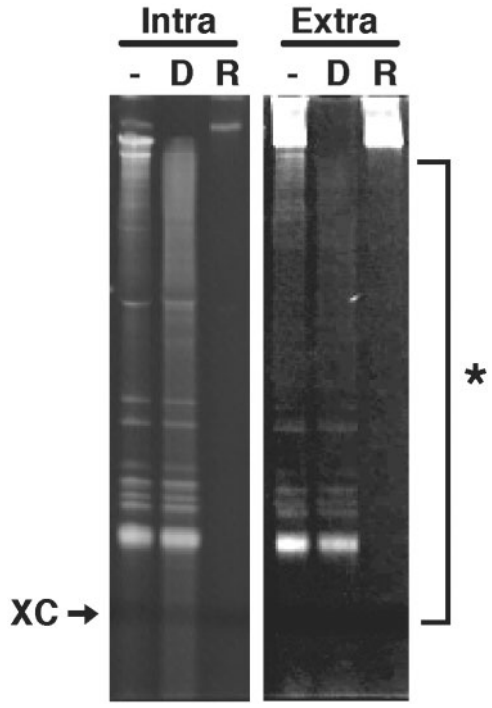

Fig. 2. **Electrophoretic analysis of intracellular RNAs and extracellular RNAs.** The extracellular and intracellular RNA preparations were subjected to denaturing 10% polyacrylamide gel electrophoresis. 'Intra' and 'Extra' represent the intracellular RNA and extracellular RNA, respectively. '–', 'D' and 'R' represent minus enzymatic treatment as a control reaction, DNase treated product and RNase treated product, respectively. 'XC' represents xylene cyanol dye marker. Extracellular RNAs in the size range indicated by the symbol '*' were used for cDNA cloning.

long in agarose gel electrophoresis (for example, Fig. 1B), in the electropherogram of 10% denaturing polyacrylamide gel (Fig. 2), major RNA bands were observed in the region with the size from about 70 to 300 nucleotides. The RNAs in this range were eluted from the gel and analyzed by the micro RNA cloning method (*12*). Briefly, two different oligonucleotides of defined sequences were added to 3′ and 5′-ends of RNAs. Using these defined sequences, reverse-transcription and the polymerase chain reaction (RT-PCR) were performed. In the micro RNA cloning method developed by Elbashir *et al.*, they used 4-hydroxymethylbenzyl group as a protecting group at the 3′-end of an oligonucleotide, but we found that the commercially available biotinyl group can be used instead for this purpose. The cDNAs were ligated to pGEM-3Z vector and *E. coli* JM109 was transformed. We analyzed 100 clones. Almost all inserts cloned had lengths of about 100 bp (including primer regions), which reflected the size distribution of the region of the electropherogram (Fig. 2). Among these clones, we arbitrarily chose 30 clones and sequenced them. The sequences of inserted DNAs are shown in Table 1. From BLAST analysis, some sequences (clone numbers 9, 15, 19 and 59) were identical to parts of *R. sulfidophilum* DSM 2351 16S rRNA sequence. Some inserted parts of different clones originated from distantly separated regions on the 16S rRNA sequence. Therefore, it is possible that whole 16S rRNA might have been produced, and then degraded during production or cultivation before being cloned, although the possibility remains that the degradation occurred during our manipulation. Other sequences (clone numbers 27, 39 and 75) seem to be from 23S rRNA, because these were homologous to part of the 23S rRNA (*rrn* operon; ribosomal RNA operon) of related species (the sequence of *R. sulfidophilum* 23S rRNA has not been published). Another group of clones (clone numbers 1, 3, 4, 11, 12, 13, 17, 20, 22, 23, 24, 25 and 40) were tRNAs. The sequences of the inserts were determined to be formyl-methionine tRNA ($tRNA_f^{Met}$), methionine (elongator) tRNA ($tRNA^{Met}$), alanine tRNA

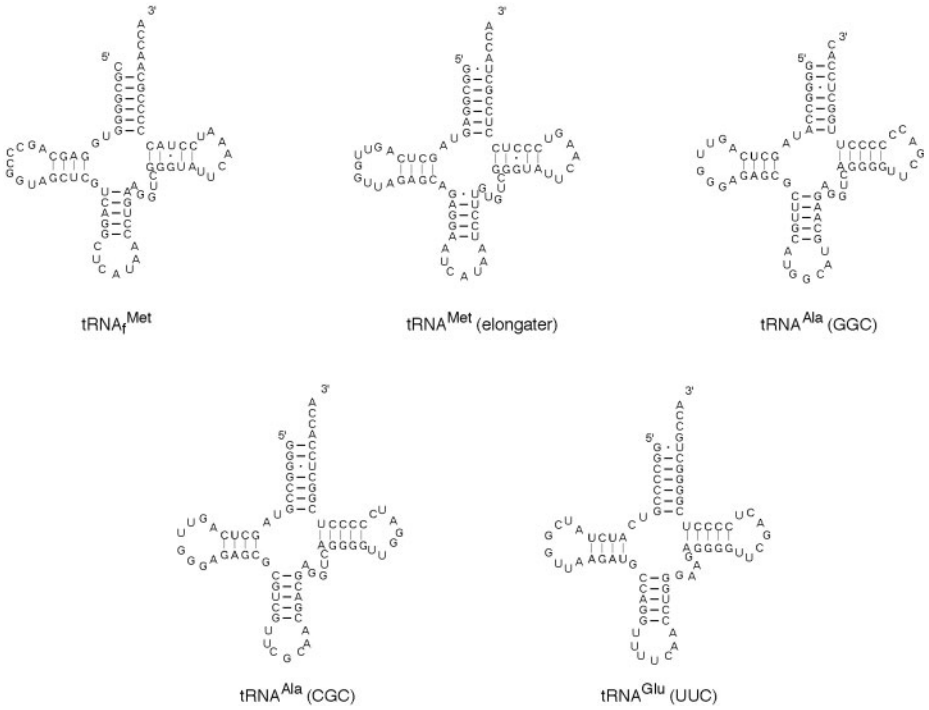

Fig. 3. **Examples of the extracellular tRNAs.** The secondary structures predicted from nucleotides sequences of cDNAs are shown.

(GGC, tRNA^{Ala}), alanine tRNA (CGC, tRNA^{Ala}), alanine tRNA (UGC, tRNA^{Ala}) and glutamate tRNA (UUC, tRNA^{Glu}). Fragments of arginine tRNA (tRNA^{Arg}) and threonine tRNA (tRNA^{Thr}) were also detected (Table 1). The amino acid specificities of these tRNAs were predicted only from the anticodon sequence. Since the conserved sequences in the bacterial tRNAs were found in these tRNAs sequenced here, these predictions of amino acid specificities may be correct. Figure 3 shows five examples of extracellular tRNA secondary structures. Interestingly, almost all clones containing full-sized tRNA had both 3′ and 5′ ends of mature tRNAs. Most of the tRNAs (or tRNA fragments) cloned had 3′-terminal CCA sequences (Table 1). This result indicates that extracellular RNAs may have already been correctly processed intracellularly, and then released into the culture medium. Therefore, extracellular nucleic acids may originate from intracellular nucleic acids.

Southern Analysis of Extracellular RNA Genes—To confirm that these sequences are coded on the genome as usual, we performed Southern blot analysis. We chose four sequences [tRNA$_f^{Met}$, tRNA^{Ala} (GGC), tRNA^{Glu} (UUC) and a fragment of 23S rRNA] as sample probes. These sequences showed clear hybridization signals as expected (Fig. 4). Since the sequence of bacterial tRNA$_f^{Met}$ is usually highly conserved throughout bacterial species, clear hybridization signals of this sequence can also be seen in the *E. coli* genome (Fig. 4, left panel). This result suggests that the sequenced extracellular RNAs are coded on the genome.

Modified Nucleotides in the Extracellular RNAs—To test whether base modifications occur in the extracellular RNAs, we performed modified nucleotide analysis using two-dimensional thin-layer chromatography. Intracellular RNAs were also analyzed for comparison. Figure 5 shows the autoradiogram of this analysis. In both intracellular and extracellular RNAs, we found many modified nucleotides. From the developed positions, eight nucleotides were identified as 2′-*O*-methyladenosine, 2-methyladenosine, 6-methyladenosine, 7-methylguanosine, 2′-*O*-methylcytidine, 5-methyluridine, pseudouridine and dihydrouridine. Most importantly, the patterns of intracellular and extracellular RNAs were very similar to each other (Fig. 5). In cloning of extracellular RNA sequences, we detected some specific tRNA clones (Table 1). Initially, we thought that the composition of specific extracellular tRNAs cloned was related to the production mechanism, but the analysis of modified nucleotides indicates that the results of composition of cloned tRNAs are probably incidental. Since, as described above, we detected same modified bases with same intensities (Fig. 5), we conclude that intracellular and extracellular RNAs are the same. This result supports the idea that extracellular RNAs are released after being fully processed including base modification.

How Does This Bacterium Produce Nucleic Acids Extracellularly?—In this study, we showed that extracellular RNAs have the same characters as intracellular RNAs. We could not detect specific extracellular RNAs or specific signals of RNA such as base modification. We are also analyzing extracellular DNA from this bacterium. Extracellular DNA is of similar size to genomic DNA in the preparative form and exhibits almost identical sequence with genomic DNA by hybridization analysis (data not shown). Therefore, it seems likely that extracellular nucleic acids originate from intracellular nucleic acids.

As shown in Fig. 1, the production of extracellular nucleic acids occurs simultaneously with cell growth. Therefore, it seems unlikely that the release of extracellular nucleic acids is due to the usual cell autolysis in the post-stationary phase. Some active production mechanisms should exist. It is unlikely that just half of the

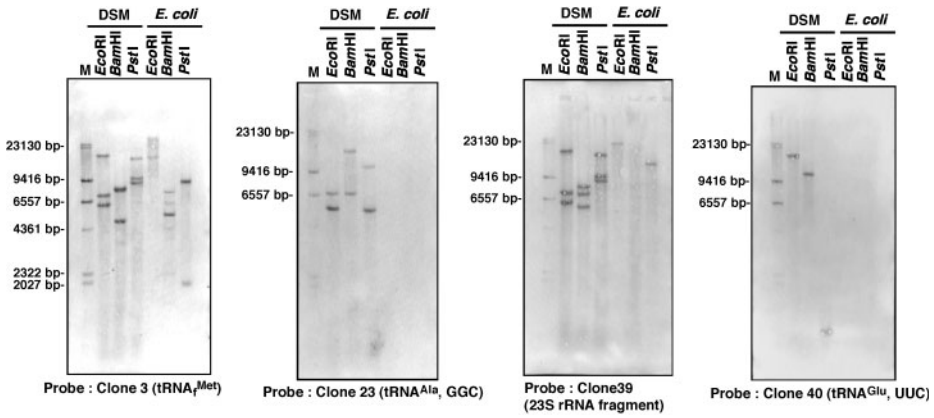

Fig. 4. **Southern blot analysis of each clone.** 'M', 'DSM' and '*E. coli*' represent molecular marker, *R. sulfidophilum* DSM 2351 genome and *E. coli* genome, respectively. '*Eco*RI', '*Bam*HI' and '*Pst*I' indicate enzymes used for genome digestions. Probes are indicated at the bottom of each panel.

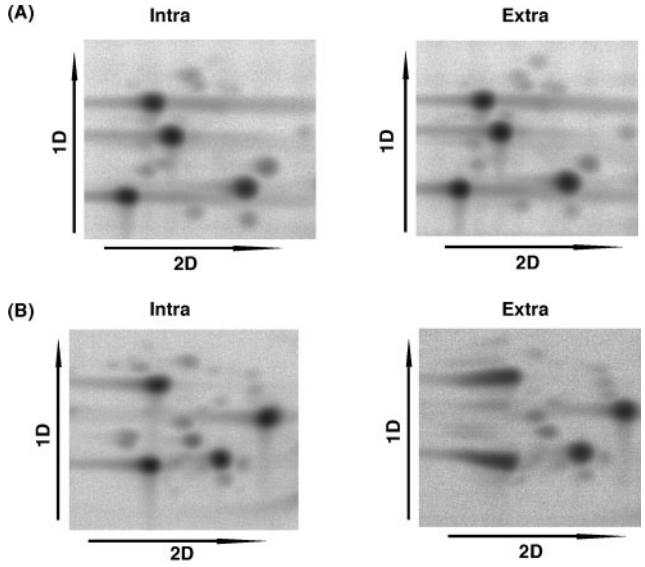

Fig. 5. **Comparison of nucleotides of intracellular RNAs and extracellular RNAs by thin layer chromatography.** 'Intra' and 'Extra' represent intracellular RNAs and extracellular RNAs, respectively. '1D' and '2D' indicate directions of separation. Solvent for 1D was isobutyric acid/29% ammonia water/dH_2O (66:1:33). Solvents for 2D were 2-propanol/HCl/dH_2O (70:15:15) for A, and ammonium sulfate/0.1 M sodium phosphate buffer (pH 6.8)/*n*-propanol (60 g:100 ml:2 ml) for B.

duplicated nucleic acids in the cell would be secreted. Another possibility is that a kind of programmed cell death might occur: partial cell death might be induced by a high level of cell density. In fact, such a mechanism has been found in *S. pneumoniae* (*4*). The cell lysis of *S. pneumoniae* is induced by an extracellular concentration of a secreted peptide pheromone called the competence stimulation peptide (CSP) (*16–18*) by a quorum sensing (*19*) mechanism. CSP also induces a competent state of this bacterium, and the extracellular nucleic acids produced are used for genetic transformation. The competent state of this bacterium is triggered when CSP reaches an extracellular concentration of 1–10 ng/ml, which corresponds to a population density of about 10^7 cells per ml (*16*). Similar cell concentration (CSP concentration) induces the cell lysis of *S. pneumoniae*. It is possible that a similar mechanism occurs in *R. sulfidophilum*. Several features of the hypothesis for the nucleic acids production are being tested.

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