Involvement of the response regulator CtrA in the extracellular DNA production of the marine bacterium Rhodovulum sulfidophilum

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The marine bacterium Rhodovulum sulfidophilum is a nonsulfur phototrophic bacterium, which is known to produce extracellular nucleic acids in soluble form in culture medium. In the present paper, constructing the response regulator ctrA-deficient mutant of R. sulfidophilum, we found that this mutation causes a significant decrease in the extracellular DNA production. However, by the introduction of a plasmid containing the wild type ctrA gene into the mutant, the amount of extracellular DNA produced was recovered. This is the first and clear evidence that the extracellular DNA production is actively controlled by the CtrA in R. sulfidophilum.

Key Words: ctrA; extracellular DNA; gene transfer agent; marine phototrophic bacterium; Rhodovulum sulfidophilum

Introduction

The marine bacterium Rhodovulum sulfidophilum is a nonsulfur phototrophic bacterium, which can grow under both aerobic-dark and anaerobic-light conditions (Hiraishi and Ueda, 1994; Kikuchi, 2010). This bacterium also is known to produce extracellular nucleic acids in soluble form in culture medium (Ando et al., 2006; Suzuki et al., 2009a, b). The term “nucleic acids in soluble form” is used here for the free nucleic acids present in the culture supernatant, but not those bound to any cells, vesicles, or particles, even those which are categorized as “extracellular”. Using this property, we developed a method for the extracellular production of homogeneous recombinant RNA molecules, such as RNA aptamers (reviewed by Kikuchi, 2010; Suzuki et al., 2010, 2011; Umekage et al., 2012) and short hairpin RNAs (Nagao et al., 2014) by the introduction of an appropriate RNA expression plasmid into the cell. This approach has been proposed as a possible method in industrial technology to produce RNA medicines in the future (Kikuchi, 2010; Kikuchi et al., 2010). For such medical applications, extensive genetical and physiological elucidation of this bacterium is important, especially from the viewpoint of the rigorous production demands of medicines. Accordingly, the complete genome sequences of several strains of this bacterium have been determined by us and others (Guzman et al., 2017; Masuda et al., 2013; Nagao et al., 2015a).

During the course of this study, we also found that R. sulfidophilum produces a gene transfer agent-like particle (GTA-like particle) (Nagao et al., 2015b). Gene transfer agents (GTAs) were first discovered in Rhodobacter capsulatus (basionym Rhodopsseudomonas capsulata) by Marrs (1974), but have now been described from various prokaryotic species (Lang and Beatty, 2007, 2010). GTAs are shaped like bacteriophage particles, but are different from bacteriophages in many ways. GTA particles do not contain their own genome coding their structure, but they have short DNA fragments randomly cut from the host genome.
phoresis, the gel was stained with ethidium bromide. Band intensities were evaluated using image gauge software (Fuji Film, Japan). The band at the position around 23 kbp on 0.7 or 1% agarose gel electropherogram was used for extracellular DNA.

**Construction of the ctrA mutant of R. sulfidophilum.** A ctrA mutant, strain No. 3 was constructed from DSM 1374 by transduction using the suicide vector containing an inactivated ctrA gene by the insertion of a Kanamycin resistant gene (Km). First, the ctrA coding region of 583 bp (base number from 2,691,164 to 2,691,746; the numbers from the complete genome sequence of Masuda et al., 2013) was inserted into SmaI site of pGEM3Z to yield pGEM-ctrA. Since the ctrA has a NruI site (base number from 2,691,412 to 2,691,417, Masuda et al., 2013) at almost the center of the gene, the Km was inserted to this site (the blunt end cutting site, 2,691,414) to disrupt the ctrA. We call this plasmid, pGEM-ctrA-Km. Using the pGEM-ctrA-Km as a template, a DNA sequence having the 5′-ctrA-Km-ctrA-3′ (disrupted ctrA gene) was amplified by PCR. This sequence was inserted into the site between XhoI and NotI of the plasmid vector pUTmini-Tn5Km (Biomedical Co.) to yield pUT-ctrA-Km. As this plasmid requires Apir for replication, this plasmid cannot be maintained in R. sulfidophilum and then function as a suicide vector in this bacterium. The plasmid pUT-ctrA-Km containing E. coli S17-Apir was co-cultivated with R. sulfidophilum, and 12 colonies of Km-resistant R. sulfidophilum were selected as candidates of the ctrA mutants. Among these 12 candidates, we finally obtained a strain (No. 3) as a ctrA mutant. The ctrA gene of this mutant was disrupted by single crossover event on the genome with pUT-ctrA-Km. This was confirmed by colony PCR, and Southern blotting analyses of the genome using probes specific for ctrA and Km. Also, with Northern analyses using the ctrA specific probes, no mature ctrA mRNA from this strain, No. 3, could be detected; instead, a longer mRNA of over 1.5 kb containing the Km sequence could be detected. We concluded that the wild type ctrA gene was completely replaced by the sequence 5′-ctrA-Km-ctrA-3′ in strain No. 3.

**Construction of the plasmid for ctrA expression.** To confirm the CtrA function for extracellular DNA production, we planned to test whether the amount of the extracellular DNA can be recovered by introduction of a plasmid containing the wild type ctrA gene into the strain No. 3. For this experiment, the plasmid containing the wild type ctrA gene was constructed. First the wild type ctrA gene including the promoter region was amplified from the genome DNA of R. sulfidophilum by the PCR technique using the forward primer, 5′-GAATTCGCCAGT-TGGAAGAAGCGCG-3′, and the reverse primer, 5′-TTATTAGGCGCCAGCGCGAAGGCCG-3′. The 5′ end of the forward primer corresponds to the base number 2,690,877 of the genome and the 5′ end of the reverse primer does to the base number 2,691,820 of the genome. The base numbers are from Masuda et al., 2013. The amplified DNA fragment of 944 bp contains a long 5′ UTR of the ctrA gene (244 bp) which is thought to include the promoter region of this gene. This fragment was inserted

**Materials and Methods**

**Bacterial strains and growth conditions.** The purple phototrophic marine alphaproteobacterium, *Rhodovulum sulfidophilum* DSM 1374 and a ctrA mutant, strain No. 3, were used throughout this study. Strain No. 3 was constructed from DSM 1374 by displacement of the ctrA gene with a disrupted version of ctrA using a suicide vector (see below). Cultivation was performed essentially as described (Suzuki et al., 2010). The strains were grown anaerobically at 25°C in a 1.5-ml or 50-ml tube filled with PYS medium (Nagashima et al., 1997) containing 2% (wt/vol) NaCl under incandescent illumination (about 3,000 lux). Kanamycin was used at a concentration of 30 μg/ml for the strains containing the Kanamycin resistant gene. Transformation of *R. sulfidophilum* was carried out by the method described previously (Suzuki et al., 2011).

**Analysis of cell growth and extracellular nucleic acid production.** Cell growth and extracellular nucleic acid production were analyzed as previously described (Ando et al., 2006). Cell growth was evaluated by measuring the turbidity of the culture medium at 600 nm. Amounts of extracellular DNA and RNA produced was calculated from the band intensity of gel electrophoresis. After electro-

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into the BglII site of the plasmid vector pBBR RESO (MoBiTec). The cleaved BglII site of this plasmid was treated with a Klenow fragment to obtain the blunt ends, then the insertion of the wild type ctrA gene DNA was achieved by blunt end ligation with T4 DNA ligase. We call this plasmid, pBBR RESO-ctrA which contains a full-length of the wild type ctrA gene.

Assay for the recovery of extracellular nucleic acid-producing function in ctrA mutant by transformation of the wild type ctrA gene. Three strains, wild type DSM 1374, the ctrA mutant, and the ctrA mutant harboring the wild type ctrA gene containing plasmid, were cultivated for 48 h. After centrifugation of each culture to remove the cells, 1 ml of supernatant was transferred to the new tube. The extracellular nucleic acids from each strain were precipitated by isopropanol. Each precipitate was quantitatively solubilized with sterilized water. These samples were applied to 1% agarose gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide. Band intensities were evaluated as described above.

Results and Discussion

Mutation of the ctrA gene causes a significant decrease in the extracellular soluble DNA production

First, we compared the amounts of extracellular soluble DNA of the two strains, R. sulfidophilum DSM 1374 (wild type) and its ctrA-deficient mutant, strain No. 3. Figures 1A and B show electrophoretic analyses of the time course of the extracellular soluble DNA production. Error bars indicate the respective standard deviations which were calculated from the results of three independent experiments.

The extracellular DNA sample was prepared from 1 ml of supernatant of each 48 h culture, and subjected to 1% agarose gel electrophoresis. DNA was detected by ethidium bromide staining. Lanes WT, the DNA sample from the wild type culture; delta c, sample from the culture of the ctrA mutant; T, sample from the culture of the mutant strain transformed by the ctrA containing plasmid pBBR RESO-ctrA; M, size marker, phage lambda DNA digested by HindIII. The sizes (in kbp) are shown to the left of the panel.
Similar effects of CtrA have been noted in the growth curves of the two strains (Fig. 1C). The wild type strain maximally produced DNA of about 0.12 μg/ml, whereas the ctrA mutant produced 0.05 μg/ml. These values may be underestimated because the values are derived from only the bands of the electropherograms as described above, but these values may be useful to show reliable time course curves. Most importantly, these data clearly showed that the ctrA gene is necessary for the regular yield of the extracellular soluble DNA production. Also, it is revealed that CtrA in R. sulfidophilum may not be essential for viability, as there is not a significant difference between the growth curves of the two strains (Fig. 1C). Similar effects of CtrA have been noted in Rhodobacter capsulatus. Mercer et al. (2010) reported that a loss of the CtrA did not affect growth phase regulation in Rba. capsulatus. Functions of the ctrA gene of R. sulfidophilum may be similar to that of Rba. capsulatus.

**Recovery of the amount of extracellular DNA in the ctrA mutant by the introduction of the wild type ctrA gene**

To further confirm the involvement of the ctrA gene in the extracellular DNA production, the effect of the introduction of the wild type ctrA gene into the mutant strain No. 3 was tested. The wild type ctrA gene containing plasmid, pBBR RESO-ctrA, was constructed as described in Materials and Methods. The plasmid pBBR RESO-ctrA contains a full-length of the wild type ctrA gene. This plasmid was introduced into the mutant strain No. 3 by a heat-shock transformation method (Suzuki et al., 2011). As shown in Fig. 2, the amount of extracellular DNA was recovered by the introduction of the plasmid into the mutant (Fig. 2, lane T). The intensity of the band around 23 kb of lane T of Fig. 2 is similar to that of the band of the wild type (Fig. 2, lane WT). The amounts of extracellular soluble DNAs were calculated from the intensities of the bands around 23 kb, as described in Materials and Methods. The values of the wild type (Fig. 2, lane WT), the mutant (lane delta c), and the transformant by pBBR RESO-ctrA (lane T), were determined to be 0.11 μg/ml, 0.02 μg/ml, and 0.10 μg/ml, respectively. From these values, the recovery of the function can be estimated to be almost 90%. The copy number of the plasmid pBBR, the original vector of pBBR RESO-ctrA, in E. coli has been reported to be about 30 (Antoine and Locht, 1992). Although we do not know the copy number of pBBR RESO-ctrA in R. sulfidophilum at present, it is presumed that the transformant of this experiment has multiple genes of the wild type ctrA. It may be supposed that there is no gene dosage effect in this case. It probably indicates that the ctrA gene does not directly affect the extracellular DNA production but indirectly controls it through downstream gene(s).

The ctrA mutant still produces small amounts of extracellular DNA as visible in Fig 2 lane delta c and Fig. 1B. It may be natural to suppose that these are derived from some unknown basal mechanism for extracellular DNA production. It is possible that this mechanism may be stimulated by the CtrA.

**Relationship between the 4.5 kb DNA band and the gene transfer agent-like particle**

In a previous paper (Nagao et al., 2015b), we reported that R. sulfidophilum produces the GTA-like particle which is thought to be involved in genetic exchange between host cells. We also mentioned that the particle production is also controlled by the gene ctrA (Nagao et al., 2015b). Although the relationship between the extracellular soluble DNA and the GTA-like particle of this organism is not clear at present, both may be used for genetic exchange between cells. R. sulfidophilum also produces the DNA fragment of 4.5 kb as an extracellular soluble DNA (Fig. 1A, lanes 9–13 and Fig. 2, lane WT). These may be the genome fragments which would be incorporated in, or have leaked from, the GTA-like particles (Nagao et al., 2015b). The band of 4.5 kb is visible in lane T of Fig. 2, although it is faint. This means that the production of this fragment of 4.5 kb is also controlled by the gene ctrA, even if this controlling process is not exactly the same as that of the extracellular DNA production.

**Future application and environmental nucleic acids**

Previously, we developed a method for extracellular production of artificially designed, functional RNAs (RNA aptamers and short hairpin RNAs) in the culture medium using R. sulfidophilum (Kikuchi, 2010; Kikuchi et al., 2010; Nagao et al., 2014; Suzuki et al., 2010, 2011; Umekage et al., 2012). This method has been proposed for the industrial production of RNA medicines. Elucidation of the control mechanism, especially the up-regulation mechanism of extracellular nucleic acids production by the ctrA gene may now be important to obtain a high yield of the artificial RNA products. In the present paper, we concentrate mainly on DNA production: the behavior of RNA production is almost the same as that of DNA.

Extracellular DNA is detected ubiquitously throughout the environment, such as in seawater and soil (Lorenz and Wackernagel, 1994; Tani and Nasu, 2010; Vlassov et al., 2007). Environmental DNAs, especially high molecular weight DNAs of bacterial origin, are, at least in part, derived from the active production by a similar stimulation mechanism as shown in the present paper. This extracellular DNA production is an important phenomenon for genetic exchange, not only within species, but also may contribute to dynamic and global genetic diversity.

**Conclusions**

We have demonstrated that the amount of the extracellular soluble DNA of R. sulfidophilum is controlled by the gene ctrA. Although the precise mechanism for the extracellular nucleic acid production of this bacterium is not yet known, we have provided a clue for its elucidation.

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