ORIGINAL PAPER

IDENTIFICATION OF A *STREPTOCOCCUS PYOGENES* SF370 GENE INVOLVED IN PRODUCTION OF *C*-DI-AMP

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ABSTRACT

Here we show that bis(3'–5') cyclic diadenylic acid (*c*-di-AMP) and a diadenylate cyclase (DAC) domain protein involved in the biosynthesis of *c*-di-AMP were identified in *Streptococcus pyogenes*. The matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrum of the cell extract of *S. pyogenes*, which showed a fragment pattern very similar to that of the authentic sample of *c*-di-AMP, revealed that *S. pyogenes* produces *c*-di-AMP in the cell. Subsequently, we confirmed by an *in vitro* experiment that the production of *c*-di-AMP in the cell is due to the action of Spy1036 gene encoding a DAC domain protein named spyDAC, which is a new protein different from a well-known diadenylate cyclase. Moreover, the experiment gave a product with a molecular weight of 657.021, which is consistent with the molecular weight of *c*-di-AMP. Furthermore, the mass spectral fragment pattern of the product obtained by the *in vitro* biosynthesis is quite similar to that of the product produced by the above *in vivo* experiment. This *in vitro* production of *c*-di-AMP indicated that spyDAC in *S. pyogenes* actually catalyzes the i*n vivo* biosynthesis of *c*-di-AMP from ATP.

Key Words: *Streptococcus pyogenes*, *c*-di-AMP, Diadenylate cyclase, spyDAC

INTRODUCTION

The synthesis of *c*-di-AMP in *Bacillus subtilis* occurs in the diadenylate cyclase (DAC) domain of DNA integrity scanning A (DisA) via the condensation of two ATP molecules. The DisA scouts the chromosome for DNA double-stranded breaks, which has led to the suggestion that *c*-di-AMP is involved in the signaling of DNA damage[1]. Subsequent genomic mining has revealed that DAC domain proteins are widespread in bacteria and archaea, with many of them associated with putative sensor domains[2].

The binding of DisA to branched nucleic acids through its C-terminal helix-hairpin-helix (HhH) domain inhibits its diadenylate cyclase activity, whereas binding to the double-stranded DNA is very weak and has no effect on diadenylate cyclase activity. Branched DNA is formed as an intermediate structure during the repair of DNA double-strand breaks by homologous recombination. DNA double-strand breaks occur spontaneously during the cell cycle, for example, during segregation of chromosomes or when the replication fork is stalled. DNA double-strand breaks can also be induced by exogenous agents[3,4]. Upon introduction of a DNA double-strand break,

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DisA colonizes with the expected site of the lesion. Therefore, the creation of DNA double-strand breaks interferes with successful *c*-di-AMP production. In general terms, the regulatory role of *c*-di-AMP might also regulate the timing or amplitude of some cellular processes, some of which may be associated with chromosomal repair and damage signaling. Low *c*-di-AMP synthesis may be involved in adaptation failure caused by exceptionable stimuli such as ultra violet (UV) light, oxidative stress or DNA double-strand breaks[2].

Genomic analyses of Firmicutes have revealed that a DisA homolog is widespread in spore-forming bacteria such as *Bacillus spp.* and *Clostridium spp*. Incidentally, *S. pyogenes* is a human pathogen that causes a variety of clinical manifestations ranging from non-invasive diseases (e.g., pharyngitis and impetigo) to severe invasive infections (e.g., necrotizing fasciitis, sepsis, and toxic shock-like syndrome)[5,6]. However, *B. subtilis* is capable of sporulation, while *S. pyogenes* is not. Therefore, it is possible that DAC domain proteins, which would be responsible for different cellular processes, are present in *S. pyogenes*, and that their identification will help elucidate the survival strategy during the transition of *S. pyogenes* from persistence in the environment to survival in the human host. Thus, the goal of this study was to determine whether *c*-di-AMP is synthesized in *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions The bacterial strains used in this study are listed in Table 1. *S. pyogenes* SF370 was grown in a brain heart infusion broth (BHI, BD, Franklin Lakes, New Jersey, U.S.) or on a BHI agar plate supplemented with 0.3 % Yeast extract (YE, BD) at 37°C without shaking.

Escherichia coli XL-1 blue was used for the host of plasmids. Transformants containing plasmids were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates. When required, LB broth and LB agar plates were supplemented with ampicillin and chloramphenicol to final concentrations of 100 and 50 μg/ml, respectively.

Table 1 Bacterial strains, plasmids, and primers used in this study

Bacterial strain	Characteristic / genotype	Reference/source
S. pyogenes strain		
SF370	*emm1*, φSF370.4, *mutS⁺*, *mutL*, *ruvA*	20
Other strains		
E. coli XL-1 blue	endA1 gyrA96(nalᴿ) thi-1 recA1 re1A1 lac glnV44 F'[::Tn10 proAB⁺lacIᵃ Δ(lacZ)M15] hsdR17(rₖ⁻ mₖ⁺)	laboratory strain
E. coli Rosetta 2	F⁻ *ompT hsdS*ᵦ(rᵦ⁻ mᵦ⁻) *gal dcm* pRARE2 (Camᴿ)	laboratory strain
XL-pCold-spy1036	*E. coli* XL-1 blue with pCold-spy1036 vector	this study
Rosetta2-pCold-spy1036	*E. coli* Rosetta 2 with pCold-spy1036 vector	this study
Rosetta2-pCold	*E. coli* Rosetta 2 with pCold TF vector (Negative control)	this study
plasmid		
pCold TF vector	Cold-shock expression vector	11,14
Primers	Sequenceᵃ	
exSpy1036F	GCCG<u>CATATG</u>AATAATTTATCTAGTATCGATATTAAATTTTTATTAAG	this study
exSpy1036R	GCCG<u>CTCGAG</u>TCATTTAGATTTCCCTCCTAG	this study
pCold-TF-F2	GCGAAAGTGACTGAAAAAG	Takaraᵇ
pCold-R	GGCAGGGATCTTAGATTCTG	Takaraᵇ

ᵃ Newly introduced restriction sites are underlined.
ᵇ Primer was referred to manufacturer's protocol (http://catalog.takara-bio.co.jp/product/manual_info.asp?unitid=U100004634).

DNA manipulation A DNA ligation reaction mixture was introduced by electroporation into *E. coli* Rosetta 2 (Merck, Whitehouse Station, New Jersey, U. S.). Electroporation for plasmids into bacterial cells was carried out according to the manufacturer's instructions. PCRs were performed using KOD plus DNA polymerase (TOYOBO, Osaka, Osaka). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, Massachusetts, U.S.) and Promega (Madison, Wisconsin, U. S.) respectively. All enzymes were used as recommended by suppliers. Purification of DNA fragments was carried out with a QIAquick PCR purification kit and/or a QIAquick gel extraction kit (QIAGEN, Strasse, Hilden, Germany), with the extraction of plasmid DNA performed using a QIAprep Spin Miniprep Kit (QIAGEN). The primers used in PCRs and sequencing reactions are listed in Table 1. Selected transformants were confirmed using PCR and sequence analysis as recommended by suppliers.

Isolation and detection of c-di-AMP from S. pyogenes The Isolation and detection of *c*-di-AMP was carried out as described previously[7,8], replacing bis (3'–5') cyclic diguanylic acid (*c*-di-GMP) to *c*-di-AMP. *S. pyogenes* overnight cultures were inoculated on BHI-YE agar plates. The bacterial cells were harvested the next day from the BHI-YE plates. For ethanol extraction, the bacterial cells (approximately 100 mg wet WT) were washed with water and resuspended in 300 μl of water. The suspension was heated at 100°C for 10 min and nucleotides were extracted twice with 700 μl of 70 % ice-cold ethanol. The extract was lyophilized and saved in a freezer. The extract equivalent to 100 mg cells was adjusted to 500 μl in 0.1 M ammonium acetate buffer pH 7.0. (Nacalai Tesque, Kyoto, Kyoto)

High-performance liquid chromatography (HPLC) was performed on a 250×3.0 mm reverse phase column (5C$_{18}$-AR-II cosmosil/cosmogel packed column; Nacalai Tesque). Running conditions were optimized using synthetic *c*-di-GMP. Runs were carried out in 0.1 M ammonium acetate buffer pH 7.0 at 0.4 ml min^{-1} using a linear gradient. Fractions containing *c*-di-AMP of 800 μl were collected and lyophilized.

Mass spectrometric analysis of fractions containing c-di-AMP The collected fraction of 800 μl or synthetic *c*-di-AMP was applied on a stainless-steel target by fast evaporation method (matrx: α-cyano 4-hydroxycinnamic acid). After drying, MALDI-TOF mass spectrometric analysis was performed on an UltraflexIII (Bruker Daltonics, Billerica, Massachusetts, U. S.) mass spectrometer using a negative ion mode[8].

Plasmid construction and introduction into E. coli Rosetta 2 competent cells Spy1036 gene of *S. pyogenes* SF370 was amplified using primers exSpy1036F(5'-GCCGCATATGAATAATT TATCTAGTATCGATATTAAATTTTTATTAAG-3') and exSpy1036R(5'-GCCGCTCGAGTCATTTA GATTTCCCTCCTAG-3') for cloning. The purified fragment was ligated into Nde I and Xho I sites at pCold TF vector (TaKaRa, Otsu, Shiga)[9,10] multi-cloning site, and the product was introduced into *E. coli* XL-1 blue strain with electroporation using a Bio-Rad gene pulser at 1.75 kV, 200 Ω, and 25 μF. After selection, the transformant was confirmed by PCR, and pCold exSpy1036 was finally generated.

After 1 μg of pCold exSpy1036 was added to 20 μl of *E. coli* Rosetta 2 competent cells, the mixture was incubated on ice for 5 min, after which it was heated for 30 sec in a water bath, and heated mixture was incubated on ice for 2 min. Then, 250 μl of SOC medium was added to the mixture, and incubated at 37°C for 1 hour. Adequate cells were inoculated into the selective medium, and transformants were selected.

Expression and purification of spyDAC. *E. coli* Rosetta 2 carrying pCold exSpy1036 was grown in the medium with ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml), and the expression was induced by cold shock (15°C) at OD$_{660}$ 0.4 for 30 min. Isopropyl 1-thio-β-D-galactopyranoside was then added to the culture at a final concentration of 0.5 mM and incubated at 15°C for 3 hours with shaking. After collection with centrifugation, the cells were resuspended

in 15 ml lysis buffer with an Ni-NTA fast start kit (QIAGEN). The suspension was incubated on ice for 30 min, centrifuged at 14,000 ×g, and the supernatant was collected. 0.01 g of imidazole was added, it and the solution was loaded onto Ni-NTA column. Which was then washed twice with wash buffer. The His$_6$ protein was eluted with 1 ml of the buffer containing 250 mM imidazole. The expression and purification of the protein were carried out as recommended by suppliers. The eluted protein fraction was dialyzed overnight in Slide-A-Lyzer 10,000 molecular weight cut-off dialysis cassettes (Thermo Fisher Scientific, Waltham, Massachusetts, U. S.) against a reaction buffer (100 mM NaCl, 40 mM Tris-HCl, pH 7.8, 10 mM MgCl$_2$). The extracted His$_6$ protein was examined for purify with SDS-PAGE, and the concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

Synthesis of c-di-AMP with enzyme assay In vitro enzymatic synthesis of *c*-di-AMP was carried out with the method of Witte G *et al.*[1] in dependence upon the enzymatic assay[11,12,13,14,15]. A reaction mixture containing the purified 5 μM His$_6$ protein in 100 mM NaCl, 40 mM Tris-HCl, pH 7.8, 10 mM MgCl$_2$ and 100 μM ATP was prepared, and the resulting mixture was then incubated at 37°C for 1 hour. After the incubation, the mixture was immediately precipitated by heating for 2 min at 100°C followed by centrifugation for 1 min at 14,000 rpm. One hundred μl of the suspension was analyzed with HPLC as described above. An equivalent to the reaction mixture of 600 μl was applied to MALDI-TOF mass spectrometric analysis.

RESULTS

To determine whether *c*-di-AMP is produced in *S. pyogenes* SF370 cell, nucleotides were extracted with ethanol[8] and the cell extract was analyzed with reversed-phase HPLC coupled with MALDI-TOF analysis using the conditions optimized for separation and detection of *c*-di-AMP (Fig. 1B). The retention time (t_r) of synthetic *c*-di-AMP (Biolog, Flughafendamm, Bremen, Germany) represents 20.6 min under these conditions. The same retention time was observed in the extract from *S. pyogenes* SF370 (Fig. 1A).

The relevant fraction was collected and subjected to MALDI-TOF analysis to verify the presence of *c*-di-AMP. The mass/charge (m/z) ratio of *c*-di-AMP (m/z = 657.001) was detected in fractions from the extract of *S. pyogenes* SF370. In addition, the MS/MS spectra of the [M-H]$^-$ ion at m/z 657 yielded identical fragmentation products (Fig. 2 C and D) for both of the isolated and synthetic *c*-di-AMP compounds (Fig. 2 A and B).

Consequently, these experiments suggested that *S. pyogenes* SF370 synthesizes *c*-di-AMP *in vivo*. Genomic analysis revealed that a gene encoding a protein comparable to DisA in *B. subtilis* was not present in *S. pyogenes* SF370. However, a protein, which was encoded by spy1036, containing a sequence with high scores of identity and similarity to the DAC domain was found with a BLAST search (Fig. 3). We therefore speculated that the spy1036 protein belonged to the protein family containing a DAC domain and designated it as spyDAC. Römling[2] has reported that spyDAC is composed of a trans-membrane domain (TM-DAC domain) and a DAC domain without a DNA-binding domain (HhH domain). Thus, spyDAC has a considerably different domain structure compared with that of DisA. Moreover, it seems likely that spyDAC possesses the DAC activity since the protein contains the Asp- Gly- Ara (DGA) and Arg- His-Arg (RHR) motifs responsible for the cyclase reaction (Fig. 3).

We therefore tested whether spyDAC synthesizes *c*-di-AMP from ATP *in vitro*. *In vitro* enzymatic activitiy of spyDAC was investigated as follows; spy1036 gene was cloned into pCold exSpy1036 and spyDAC was overexpressed in *E. coli* Rosetta 2. spyDAC was purified and assayed for various nucleotides. The assay buffer and reaction conditions were essentially as described
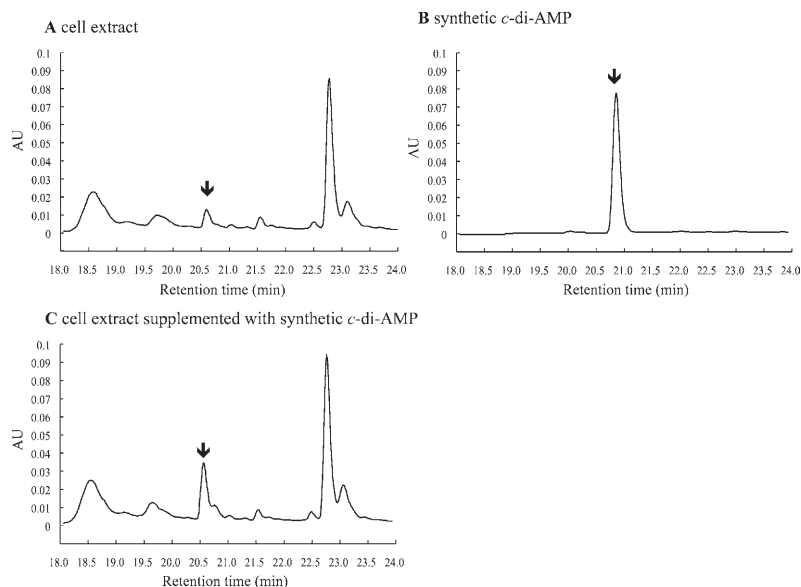
Fig. 1 Detection of *c*-di-AMP in nucleotide extracts from *S. pyogenes* SF370 by HPLC analysis. An overlay of the *A*254 curves from reverse-phase HPLC analysis of extracts from *S. pyogenes* SF370 grown on BHI-YE agar plates at 37°C overnight was shown (A). The peak at 20.6 min represents *c*-di-AMP, as determined from its correspondence to the retention time of chemically synthetic *c*-di-AMP (B). For the assessment of DAC activity, synthetic *c*-di-AMP was added to nucleotide extract and represented traces from the HPLC analysis of the *S. pyogenes* extract containing synthetic *c*-di-AMP (C).
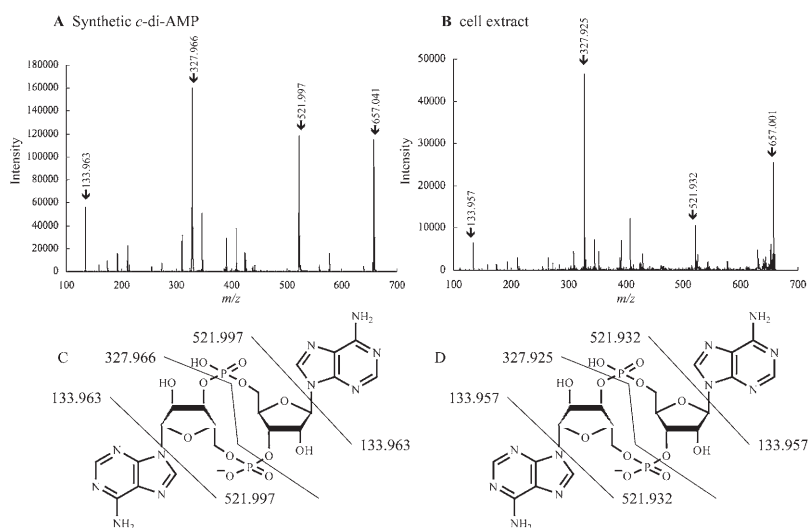

Fig. 2 Detection of *c*-di-AMP *in vivo*. MALDI-TOF analysis of relevant HPLC fractions was performed in the negative-ion detection mode. *c*-di-AMP was detected at a mass-to-charge ratio (*m/z*) of 657 [M-H]. Fractions were derived from synthetic *c*-di-AMP (positive control) (A) and *S. pyogenes* SF370 (B). Three major ions were visible in the fragmentation pattern, whereby *m/z* 133 and 521 corresponded to products from single bond fragmentation. Black lines indicate ion fragmentation by single- and double-bond cleavage as detected by MS/MS displayed in C and D.
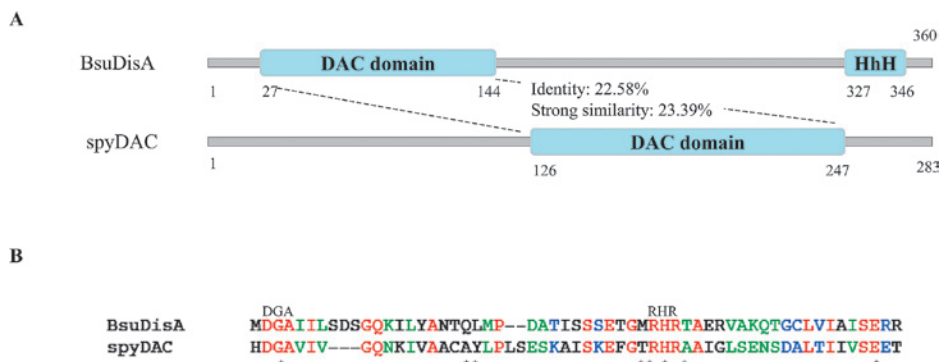
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Fig. 3 Domain structure of *S. pyogenes* SF370 spyDAC. Schematic representation of the domain structures of DisA in *B. subtilis* (BsuDisA, Accession No. P37573) and spyDAC in *S. pyogenes* (spyDAC, Accession No. Q99ZX0), respectively (A). The number represents amino acid residues. The percentages in parentheses between the two DAC domains indicate sequence identity and strong similarity. Sequence alignment of DisA and spyDAC (B). Notable functional motifs were annotated as DGA and RHR. Residues that bound the *c*-di-AMP or 3'-deoxyATP ligands were indicated (*). Colored residues were indicated in red (identical), green (strongly similar) and purple (somewhat similar).
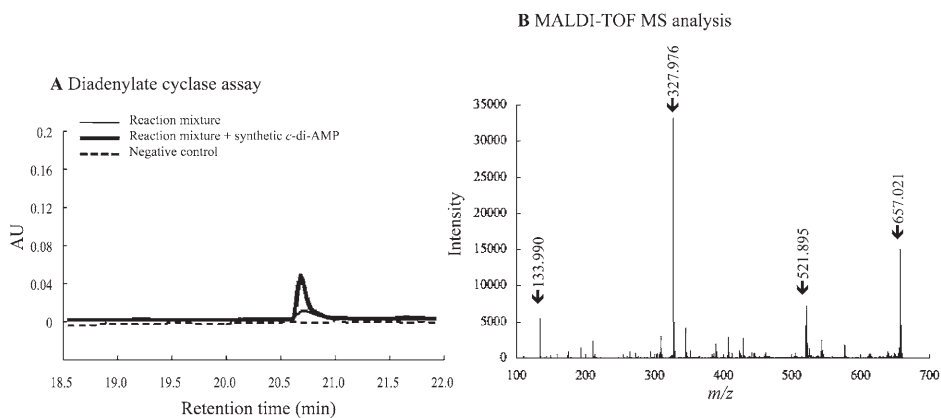

Fig. 4 HPLC analysis of the DAC reaction products. Purified spyDAC (5 μM) was incubated for 1 hour with 100 μM ATP. Nucleotides were separated on a 250×3.0 mm reverse-phase column. For the confirmation of DAC activity, synthetic *c*-di-AMP was added to the reaction mixture, and represented traces from the HPLC analysis of reaction mixtures containing synthetic *c*-di-AMP (A). Fractions were analyzed by MALDI-TOF analysis (B).

above. The reaction mixture was analyzed by reversed-phase high-pressure liquid chromatography (HPLC) coupled with MALDI-TOF analysis using the condition optimized for the separation and detection of *c*-di-AMP. A weak signal at the same retention time as that of synthetic *c*-di-AMP was detected in the reaction mixture containing spyDAC, whereas no relevant signal was present in the negative control (Fig. 4A). We also confirmed that the reaction product fraction subjected to MALDI-TOF analysis revealed a prominent [M-H]⁻ ion at mass/charge (*m/z*) ratio of *c*-di-AMP (*m/z* = 657.021). Moreover, the MS/MS spectra of the [M+H]⁺ ion *m/z* 657 yielded identical fragmentation products for the isolated compound and chemically synthetic *c*-di-AMP (Fig. 4B).

Consequently, we concluded that spyDAC synthesizes *c*-di-AMP from ATP *in vitro*. Therefore, we attempted to generate spy1036 mutant strain to reveal the function of the protein. However, the mutant strain could never have generated because spy1036 gene is the lethal gene in *S. pyogenes* SF370.

DISCUSSION

Protein analysis of *Streptococcus pneumoniae* SP6-BS73 revealed that streptococci possess DAC-ybbR (ybbR: a domain of unknown function found in *B. subtilis* and other gram-positive bacteria), another DAC domain protein that is distinct from spyDAC. For this reason, *c*-di-AMP biosynthesis in *S. pyogenes* was hypothesized to be due to the cooperative activity of both spyDAC and DAC-ybbR. Thus, we searched for a DAC-ybbR protein homolog in the *S. pyogenes* SF 370 genome database. Our search showed that *S. pyogenes* had a protein with a ybbR domain. However, this protein contained no amino acid sequence equivalent of the DAC domain. We thus speculate that instead of a cooperative activity, spyDAC independently synthesizes *c*-di-AMP from ATP in *S. pyogenes*. Analysis of the genome of *S. pyogenes* SF370 also revealed that spyDAC is the only DAC-related protein in this strain.

We speculated about the function of spyDAC and *c*-di-AMP. *In vivo* observations from a previous study showed that DisA diffuses through the bacterial cell as a single focus that pauses at the site of DNA double strand breakage, thereby delaying sporulation[3]. Based on biochemical studies, it is presumed that octameric DisA produces *c*-di-AMP while scanning the DNA with its outwardly oriented HhH domains, and that DisA binding to branched nucleic acids subsequently inhibits *c*-di-AMP production. Consequently, it is likely that the decrease in *c*-di-AMP concentrations is associated with damage signaling. When DisA and spyDAC are compared, there is a significant difference in the intracellular location between DisA and spyDAC. As reported by Witte *et al.*[1], DisA is localized in the cytosol. However, it is likely that spyDAC would be associated with the cell membrane, since spyDAC contains a TM domain at its N-terminal. It is also likely that the difference in the localization of DisA and spyDAC in a cell reflect receptor stimuli. spyDAC which localizes at the cell membrane may be responsible for the detection of a signal of damaged cell membrane or cell wall.

We investigated whether other Firmicutes bacteria also possess proteins similar to spyDAC and found that spyDAC-like proteins were identified in numerous species of bacteria. For example, *Streptococcus thermophilus* CNRZ368 has an analogous protein encoded by *ossG* (Accession No. AY386239), which is essential for defense against oxidative stress. Since *ossG* mutant shows a significant decrease in survival when exposed to methyl viologen, it is possible that the *ossG* gene is specific to such a stress[15]. We formulated a hypothesis based on the cellular localization of spyDAC and the reported function of DisA. spyDAC might use *c*-di-AMP as a messenger for damage signal in order to detect a damaged cell wall or cell membrane, and would thus induce the expression of genes for the repair of the damage in *S. pyogenes*.

Using a database search, we also identified homologues of spyDAC in other genera such as Cyanobacteria, Acidobacteria, Spirochaetes, Aquificales, and Deinococcus-Thermus. As the only oxygenic photosynthetic bacteria in this group, Cyanobacteria, produce oxygen that eventually generates oxygen radical species. Although Cyanobacteria are necessary to form a protective mechanism against oxygen radicals[16,17], it is strongly suggested that they utilize a spyDAC homologue as a means of detecting oxidative stress at the sites of cell walls and cell membranes.

In another respect, the role of *c*-di-AMP is extremely interesting. *c*-di-AMP is a chemical belonging to 2', 5'-phosphodiester-linked oligoadenylates (2-5A), which is a structurally unique

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and biologically important class of compounds. One of those compounds, which is detected in the cytoplasmic extracts of interferon-treated cells, is thought to play a major role in the antiviral and antiproliferative actions of interferons[18]. Structural differences between *c*-di-AMP and 2-5A, such as their binding sites and presence or absence of cyclization are easily understandable, and cyclization takes advantage of their structural stability. Thus, it is expected that *c*-di-AMP would express antiviral activity similar to that of 2-5A, and that *c*-di-AMP would be involved in the defense system such as phage shock protein (PSP) against bacteriophage infection[19]. This putative physiological activity might be applicable as an antiviral agent. Consequently, this paper may serve as a stepping stone for further development.

In conclusion, it is possible that a regulatory system based on spyDAC would detect not only a process of chromosome segregation, but would also recognize various stimuli such as UV and radicals that can damage growth in natural and/or host environments. Bacteria are almost ubiquitous throughout the biosphere and have adapted to various environments. In the long course of evolution and differentiation, bacterial growth would have been protected through the elimination of the unfavorable effects of environmental factors by various mechanisms such as DAC domain proteins. Further research will reveal not only the function of DAC domain proteins, but also the physiological activities of *c*-di-AMP, thus uncovering the survival strategy of bacteria in considerable detail.

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