



## Diadenylate cyclase evaluation of ssDacA (SSU98\_1483) in *Streptococcus suis* serotype 2

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**ABSTRACT.** Cyclic diadenosine monophosphate is a recently identified signaling molecule. It has been shown to play important roles in bacterial pathogenesis. SSU98\_1483 (ssDacA), which is an ortholog of *Listeria monocytogenes* DacA, is a putative diadenylate cyclase in *Streptococcus suis* serotype 2. In this study, we determined the enzymatic activity of ssDacA *in vitro* using high-performance liquid chromatography and mass spectrometry. Our results showed that ssDacA was a diadenylate cyclase that converts ATP into cyclic diadenosine monophosphate *in vitro*. The diadenylate cyclase activity of ssDacA was dependent on divalent metal ions such as Mg<sup>2+</sup>, Mn<sup>2+</sup>, or Co<sup>2+</sup>, and it is more active under basic pH than under acidic pH. The conserved RHR motif in ssDacA was essential for its enzymatic activity, and mutation in this motif abolished the diadenylate cyclase activity of ssDacA. These results indicate that ssDacA is a diadenylate cyclase, which synthesizes cyclic diadenosine monophosphate in *Streptococcus suis* serotype 2.

**Key words:** Cyclic diadenosine monophosphate; Diadenylate cyclase; DacA; *Streptococcus suis* serotype 2

## INTRODUCTION

*Streptococcus suis* serotype 2 (SS2) is an important zoonotic pathogen in pigs and humans, associated with meningitis, arthritis, endocarditis, septicemia, and even sudden death (Staats et al., 1997; Dominguez-Punaro, 2007). Currently, effective control of SS2 infections remains limited by a lack of sufficient understanding of the mechanisms involved in the pathogenesis and virulence of the bacterium. Cyclic diadenosine monophosphate (c-di-AMP) was recently discovered and recognized as a signaling molecule utilized by many bacteria, including bacterial pathogens during infection. In *Bacillus subtilis*, bacterial c-di-AMP levels are reduced in response to DNA damage, resulting in delayed sporulation (Oppenheimer-Shaanan et al., 2011). High c-di-AMP levels result in smaller bacterial size in *Staphylococcus aureus* (Corrigan et al., 2011), c-di-AMP secreted by *Listeria monocytogenes* triggers a cytosolic pathway for innate immunity (Woodward et al., 2010), and c-di-AMP homeostasis is required for the virulence of *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Bai et al., 2013; Cho and Kang, 2013; Witte et al., 2013). c-di-AMP is synthesized by a protein containing a diadenylate cyclase domain (DAC domain). The SSU98\_1483 protein of SS2 is a putative DacA ortholog known as ssDacA. The 2 DGA and RHR motifs that are conserved in DacA proteins are also conserved in ssDacA. DacA orthologs appear to be essential for the viability of bacteria, as they cannot be deleted using traditional genetic techniques (Song et al., 2005; Glass et al., 2006; French et al., 2008; Woodward et al., 2010; Corrigan et al., 2011), and the significance of c-di-AMP in reported pathogens imply an important role of this protein in SS2 pathogenesis. In this study, we report this functional diadenylate cyclase (ssDacA) in SS2.

## MATERIAL AND METHODS

### Bacterial strains and culture conditions

The SS2 HA9801 strain was grown in Todd-Hewitt broth. *Escherichia coli* DH5 $\alpha$  and BL21 (DE3) were grown in Luria-Bertani liquid medium or plated on Luria-Bertani agar, while the plasmid-containing *E. coli* strains were grown in or on the same medium but containing 50 mg/mL kanamycin. All cultures were grown at 37°C.

### Protein expression and purification

Genomic DNA from SS2 HA9801 was prepared using a genomic DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA). Plasmid DNA from *E. coli* was extracted using a plasmid purification kit (Omega) according to manufacturer instructions. DNA fragments encoding the cytoplasmic portion of ssDacA (99-283) were polymerase chain reaction (PCR)-amplified using the primer pair DAC-F: CG GGATCC GCTATGCTTGAAAACTCGGT (containing a *Bam*HI restriction enzyme site) and DAC-R: CG GAATTCC AGCTTTAAACTT ATCATTCATGTTTGC (containing an *Eco*RI restriction enzyme site). Mutations in the DGA (amino acids 181-183) and RHR (amino acids 213-215) motifs in ssDacA were generated using splicing by overlap PCR. Primers dga-MF: 5'-TCCCGAATACACCGCTACATGCAGCA TTAGTAATTGTCAAGGAAGACAA-3', dga-MR: 5'-TTGTCTTCCTTGACAATTACTAA TGCTGCATGTAGCGGTGTATTTCGGGA-3', DAC-F and DAC-R were used to replace DGA with AAA (ssDacA<sub>DGA</sub>). Primers rhr-MF: 5'-TTTCAAAGGAATTTGGAACAGCTGCTGCA

GCAGCCATTGGCTTATCGGA-3', rhr-MR: 5'-TCCGATAAGCCAATGGCTGCTGCAGCAGCTGTTCCAAATTCCTTTGAAA-3', DAC-F and DAC-R were used to replace RHR with AAA (ssDacA<sub>RHR</sub>). The PCR products for ssDacA<sub>99-283</sub>, ssDacA<sub>DGA</sub>, and ssDacA<sub>RHR</sub> were cloned into the pET28a(+) vector (Novagen, Madison, WI, USA) between the *Bam*HI and *Eco*RI sites. These plasmids were sequence-verified and transformed into a competent *E. coli* BL21 (DE3) strain.

Protein expression was induced with 0.1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside for 4 h at 27°C. C-His-recombinant proteins were purified using a Ni-NTA resin (Qiagen, Hilden, Germany) according to the manufacturer protocol.

### ***In vitro* enzymatic activity assays**

Determination of diadenylate cyclase activity using high-performance liquid chromatography (HPLC) was performed as reported with modifications (Oppenheimer-Shaanan et al., 2011; Bai et al., 2012). Briefly, 50- $\mu$ L reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 150 mM NaCl, and 0.1 mM ATP. The reaction was initiated by adding 2  $\mu$ M protein and was incubated at 37°C for 1 h. The reaction was then terminated by heating the reaction mixture in a boiling water bath for 10 min, and the mixture was centrifuged at 12,000 g for 10 min to remove the denatured protein. Next, 20  $\mu$ L reaction mixture was injected and separated by reversed-phase-HPLC on an RPC-18 column (250 x 4.5 mm, Kromasil, Bohus, Sweden) using the CXTH-3000 HPLC system and a 10 mM NH<sub>4</sub>OAc, pH 5.5, (Buffer A) and 100% MeOH (Buffer B) solvent system. The column temperature was set to 25°C and the flow rate was 0.7 mL/min. Samples were eluted using a linear gradient from 0 to 50% solvent B over 20 min. Nucleotides were detected at A<sub>254</sub>. c-di-AMP and pApA (BioLog, Hayward, CA, USA) standards were run in each experiment. The c-di-AMP standard was purchased from BioLog and ATP was purchased from Sigma (St. Louis, MO, USA).

Products were identified using electrospray ionization-mass spectrometry (MS). The parameters were as follows: ionization source, electrospray ionization source; ion transfer capillary temperature 350°C, ion transfer capillary voltage 50 V, spray voltage 2.8 kV. Full scan mass spectra were recorded in the negative ion mode over the range m/z = 100-1000.

### **Metal ion and pH dependence**

The assay conditions used for metal screening were: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM [metal<sup>2+</sup>], 2  $\mu$ M recombinant protein, and 2 mM ATP. To determine pH dependence, reactions consisted of 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 2  $\mu$ M recombinant protein, 2 mM ATP, and 50 mM Tris-HCl at pH 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. Reactions were incubated for 1 h at 37°C, terminated by adding 1 mL 0.5 M EDTA and analyzed by HPLC. Relative diadenylate cyclase activity in the presence of Mg<sup>2+</sup> and pH at 8.0 was considered to be 100%.

### **ATP binding assay**

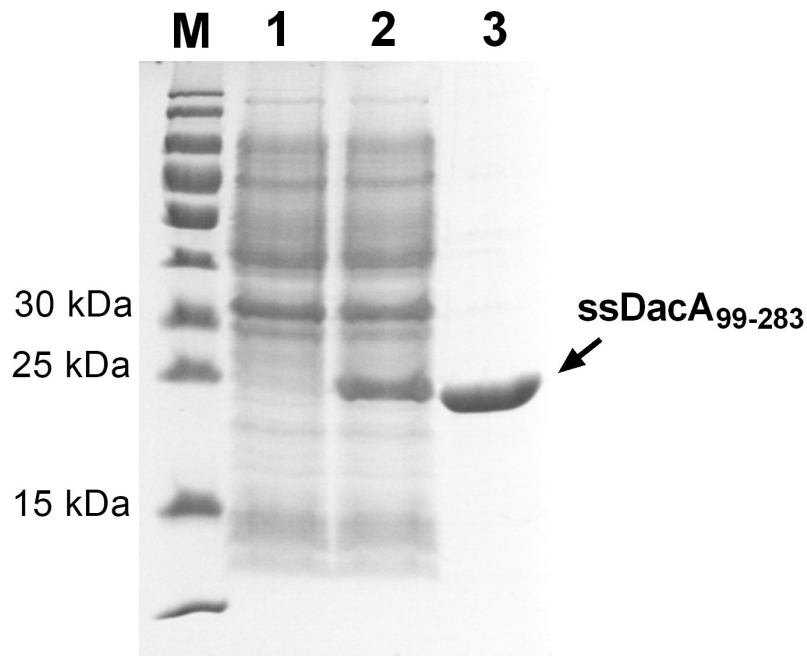
To analyze the ATP binding to ssDacA<sub>99-283</sub>, ssDacA<sub>DGA</sub>, and ssDacA<sub>RHR</sub>, an ATP-Sepharose binding assay was used in a binding procedure previously described with minor modifications (Dias et al., 2009; Reese and Boothroyd, 2011; Bai et al., 2012; Salter et al., 2012). Briefly, purified recombinant proteins and bovine serum albumin, used as negative control,

were incubated at 4°C for 1 h with a 50- $\mu$ L bed volume of  $\gamma$ -linked ATP-Sepharose (Innova Biosciences, Cambridge, UK) in 50 mM Tris, pH 7.5, and 150 mM NaCl, respectively. The beads were sedimented and washed 3 times with 1 mL ice-cold reaction buffer before elution of proteins in sodium dodecyl sulfate loading buffer. The pulled down protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie blue.

## RESULTS

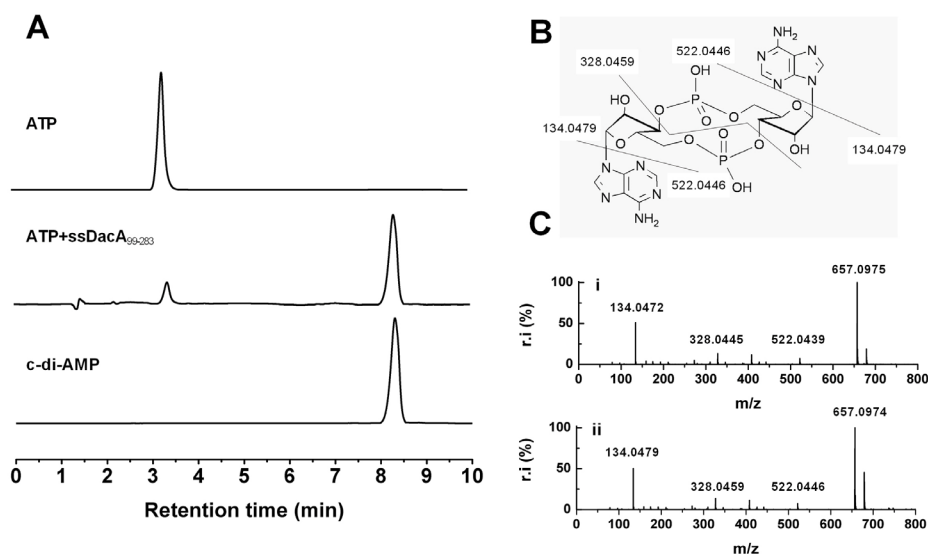
### SS2 SSU98\_1483 (ssDacA) is function as a diadenylate cyclase

SS2 ssDacA possesses 3 transmembrane helices at the N-terminus, and we were unable to obtain the full-length ssDacA through expressing in *E. coli*. Thus, we expressed its cytoplasmic portion ssDacA (99-283), and purified it to homogeneity. As seen in Figure 1, the apparent molecular weight of this protein was nearly 25 kDa on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, which is consistent with the calculated molecular weight of 23.2 kDa.



**Figure 1.** Protein detection by SDS-PAGE 12/5 % (w/v). Lane M = standard protein size marker (kDa); lane 1 = *BL-21*(DE3) with pET-28a(+); lane 2 = induced ssDacA<sub>99-283</sub> with 0.1 mM IPTG; lane 3 = purified ssDacA<sub>99-283</sub>.

The purified protein was then tested for the ability to synthesize c-di-AMP *in vitro* by using HPLC. The results showed that ssDacA converted ATP into a major product with the same retention time as that of the c-di-AMP standard (Figure 2A).



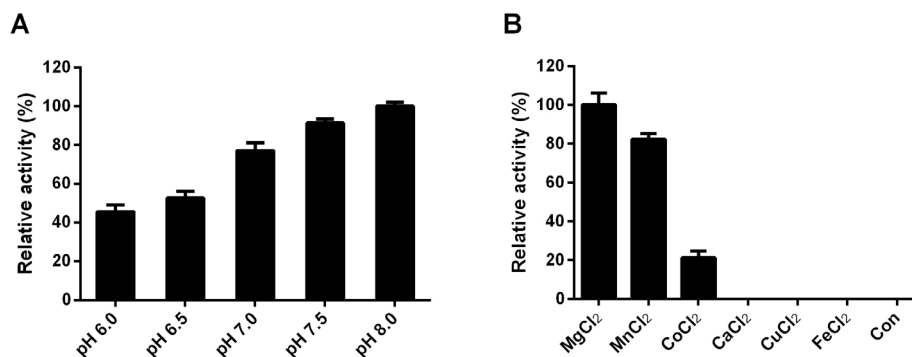
**Figure 2.** Determination of DacA<sub>99-283</sub> activities using HPLC and ESI-MS. **A.** Enzymatic activity of ssDacA<sub>99-283</sub>. ATP was incubated with purified ssDacA<sub>99-283</sub>, and the reaction was separated using HPLC. ATP and c-di-AMP standards were used as controls. **B.** Chemical structure of c-di-AMP. Black lines indicate ion fragmentation by single and double bond cleavage as detected by MS/MS displayed in (C). **C.** ESI-MS analysis of relevant HPLC fractions of c-di-AMP by ssDacA<sub>99-283</sub> synthesis (i) and commercial c-di-AMP (ii) were performed in the negative-ion detection mode. c-di-AMP was detected at an  $m/z$  of 657 [M-2H+Na]<sup>-</sup>. Three major ions were visible in the fragmentation pattern, whereby  $m/z$  134 and 522 corresponded to products from single bond fragmentation, and 328 originated from double bond fragmentations, as indicated in (B).

Subsequently, the product peak was collected and identified by electrospray ionization-MS. The mass/charge ( $m/z$ ) ratio of c-di-AMP ( $m/z = 657.0980$ ) was detected. In addition, the MS/MS spectra of the [M-2H+Na]<sup>-</sup> ion at  $m/z$  657 yielded identical fragmentation products (Figure 2B and C) for both the product and c-di-AMP standard. These results indicated that SS2 ssDacA possesses diadenylate cyclase activity and converts ATP into c-di-AMP.

### Metal and pH dependence of diadenylate cyclase activity

The effect of pH on diadenylate cyclase activity of ssDacA<sub>99-283</sub> was analyzed at pH 6.0, 6.5, 7.0, 7.5, and 8.0. The results showed that ssDacA synthesized c-di-AMP more effectively at pH 8.0 compared to at pH 6.0 (Figure 3A). This indicated a strong preference of alkaline condition for the diadenylate cyclase activity of ssDacA.

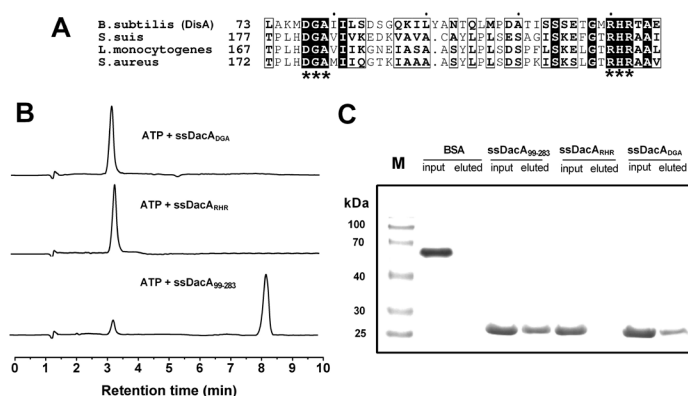
Diadenylate cyclase activity is strictly dependent on divalent cations, which has been reported in previous studies (Witte et al., 2008; Bai et al., 2012). In this study, the effect of metal ions on the diadenylate cyclase activity of ssDacA<sub>99-283</sub> was analyzed using 6 divalent ions: Mg<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup>, and Fe<sup>2+</sup> at pH 7.5. The production of c-di-AMP was detected in the reactions with Mg<sup>2+</sup>, Mn<sup>2+</sup>, and Co<sup>2+</sup> in the presence of ATP, and ssDacA<sub>99-283</sub> preferred Mg<sup>2+</sup> > Mn<sup>2+</sup> > Co<sup>2+</sup> as co-factors under our testing conditions (Figure 3B). Additionally, as a control, ssDacA<sub>99-283</sub> showed no enzymatic activity in the absence of divalent metal ion.



**Figure 3.** Effect of divalent metal ions and pH on ssDacA<sub>99-283</sub> activities. **A.** Effect of metal ions on c-di-AMP diadenylate cyclase activity of ssDacA<sub>99-283</sub>. **B.** Effect of pH on diadenylate cyclase activity of ssDacA<sub>99-283</sub>.

### RHR motif is essential for ATP binding, and both DGA and RHR motifs are required for ssDacA activity

Previous studies showed that the DGA and RHR motifs are essential for diadenylate cyclase activity (Witte et al., 2008; Bai et al., 2012), and these 2 motifs are conserved in ssDacA (Figure 4A). To determine the function of these 2 motifs in ATP binding and diadenylate cyclase activity, we substituted the residues within the DGA and RHR motifs of ssDacA<sub>99-283</sub>. We mutated DGA to AAA (ssDacA<sub>DGA</sub>) and RHR to AAA (ssDacA<sub>RHR</sub>). An ATP-Sepharose binding assay was used to analyze the interaction with ATP. Identical to native ssDacA<sub>99-283</sub>, ssDacA<sub>DGA</sub> was efficiently pulled-down by the ATP resin, whereas ssDacA<sub>RHR</sub> was not eluted after incubation with ATP-Sepharose. This indicated that the RHR motif is the ATP binding site and essential in the interaction with ATP (Figure 4B). The diadenylate cyclase activity assay using HPLC showed that, as expected, both mutations of DGA to AAA and RHR to AAA completely abolished the production of c-di-AMP (Figure 4C), suggesting that these motifs are essential for ssDacA diadenylate cyclase activity.



**Figure 4.** Function of the DGA and RHR motifs in ssDacA. **A.** Partial sequence alignment of DisA of *Bacillus subtilis* and DacA of *Streptococcus suis*, *Listeria monocytogenes*, and *Staphylococcus aureus* showing the conserved DGA and RHR motifs. **B.** Analysis of diadenylate cyclase activity of 2  $\mu$ M ssDacA<sub>99-283</sub>, ssDacA<sub>DGA</sub> and ssDacA<sub>RHR</sub> using HPLC. **C.** ATP binding by ssDacA<sub>99-283</sub>, DacA<sub>RHR</sub> and DacA<sub>DGA</sub>. Proteins incubated with  $\gamma$ -linked ATP-Sepharose and separated by SDS-PAGE and visualized with Coomassie blue. Bovine serum albumin was used as a negative control.

## DISCUSSION

c-di-AMP is synthesized by proteins containing a DisA\_N domain, which was first identified as a DAC domain in the *Thermotoga maritima* protein DisA for DNA integrity scanning protein A (Witte et al., 2008). DAC domain-containing proteins exist across eubacteria and archaea (Corrigan and Grundling, 2013). Most of these bacteria possess 1 diadenylate cyclase, except for *B. subtilis*, in which 3 diadenylate cyclases, DisA, DacA, and DacB, were identified (Mehne et al., 2013). DacA of *B. subtilis*, *S. pneumoniae*, *L. monocytogenes*, and *S. aureus* all possess 3 transmembrane helices at the N-terminus. The localization of this protein may be responsible for sensing specific changes within the membrane or cell wall and adjusting c-di-AMP production accordingly. This prediction agrees with the reports that c-di-AMP is an essential signaling molecule in cell wall homeostasis and antibiotic resistance. Analysis of the genome of SS2 HA9801 revealed that ssDacA is the only DAC-related protein in this strain. In this study, we described the presence of this functional diadenylate cyclase in SS2.

As predicted, we identified ssDacA as a diadenylate cyclase that converted ATP into c-di-AMP in SS2 HA9801. However, ssDacA did not exhibit either ATPase or ADPase activities as previously reported for DacA in *M. tuberculosis* (Bai et al., 2012). This may be because of the different structure of *M. tuberculosis* DacA, which possesses an HhH domain, and because *M. tuberculosis* DacA is localized in the cytosol while ssDacA is in the plasma membrane. This implies that DAC domain proteins are responsible for different cellular processes in various bacteria. Future studies should further characterize whether the transmembrane domain of ssDacA plays a role in diadenylate cyclase activity or in the biological function *in vivo*.

The activities of DacA are strictly dependent on divalent metal ions. In this study, ssDacA showed catalytic activity in the presence of Mg<sup>2+</sup> and Mn<sup>2+</sup>. These divalent ions are also cofactors for the c-di-AMP phosphodiesterase GdpP in *B. subtilis* (Rao et al., 2010). Furthermore, the diadenylate cyclase activity of ssDacA is higher at a basic pH rather than under acidic or neutral conditions, which is similar to GdpP in *B. subtilis*. These results suggest that such cations and the basic condition play important roles in the c-di-AMP signaling pathway.

DGA and RHR motifs are conserved in DAC domain-containing proteins and are essential for c-di-AMP synthesis activity. In this study, we found that mutating these 2 motifs completely abolished the diadenylate cyclase activity of ssDacA. Furthermore, mutating the RHR motif inhibited the ATP-binding activity, indicating that the RHR motif is the ATP-binding site of ssDacA.

c-di-AMP has been identified as a signaling molecule that participates in regulating bacterial physiology and pathogenesis. Our results will be useful in future studies examining the role of c-di-AMP in the biology and pathogenesis of SS2.

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