

A σ^D -dependent antisense transcript modulates expression of the cyclic-di-AMP hydrolase GdpP in *Bacillus subtilis*

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Received 16 July 2012
Revised 29 August 2012
Accepted 6 September 2012

Cyclic-di-AMP (c-di-AMP) is an essential second messenger in *Bacillus subtilis*, and depletion leads to defects in the integrity of the cell wall. Levels of c-di-AMP are regulated by both the rates of synthesis (by diadenylate cyclases) and the rates of degradation (by the GdpP phosphodiesterase, formerly YybT). Little is known about the regulation of *gdpP* expression or GdpP activity, but mutations that inactivate GdpP lead to high-level resistance to β -lactam antibiotics. Here we demonstrate that expression of *gdpP* is regulated by a *cis*-acting antisense RNA (*gdpP_{as}*) *in vivo*. Transcription of this antisense RNA is initiated in the middle of the *gdp* gene and is dependent on an alternative sigma factor, σ^D , previously associated with the expression of late flagellar genes, chemotaxis proteins and cell wall autolytic enzymes. Changes in σ^D activity can modulate GdpP protein levels by ~2.5-fold, which may provide a mechanism for the cell to upregulate c-di-AMP levels in coordination with the activation of autolytic enzymes.

INTRODUCTION

Cyclic-di-AMP (c-di-AMP) is a recently recognized second messenger molecule in bacteria. It was first identified as an endogenous metabolite in the crystal structure of DisA, which catalyses its synthesis at its DAC domain (diadenylate cyclase domain; previously DUF147 domain) (Witte *et al.*, 2008). DAC domain-containing proteins can be found in many other (predominantly Gram-positive) bacteria and archaea (Römling, 2008). Many species only harbour one DAC domain protein, and null mutation of the sole DAC appears to be lethal in *Listeria monocytogenes*, *Staphylococcus (Staph.) aureus*, *Streptococcus pneumoniae*, *Mycoplasma pulmonis* and *Mycoplasma genitalium* (Chaudhuri *et al.*, 2009; Corrigan *et al.*, 2011; French *et al.*, 2008; Glass *et al.*, 2006; Song *et al.*, 2005; Woodward *et al.*, 2010). The genome of *Bacillus subtilis* encodes three DAC-containing proteins, DisA, YbbP and YojJ. Although single mutants of these DAC proteins are viable, the double mutant lacking both DisA and YbbP is non-viable (Luo & Helmann, 2012). The essential roles of c-di-AMP are not well understood, but recent results suggest that it is, directly or indirectly, involved in peptidoglycan (PG) homeostasis (Corrigan *et al.*, 2011; Luo & Helmann, 2012).

Cellular levels of c-di-AMP are regulated by rates of synthesis and degradation. The DAC domain in the cyclase catalyses the synthesis of c-di-AMP from two ATP molecules, while the DHH domain in the hydrolase

catalyses cleavage of c-di-AMP to linear 5'-pApA. In *B. subtilis*, the three c-di-AMP cyclases (DisA, YbbP and YojJ) and one hydrolase (GdpP) are subject to both transcriptional and post-transcriptional regulation. Expression of *disA* is regulated by both σ^A and σ^M (Eiamphungporn & Helmann, 2008), and the cyclase activity of DisA is influenced by DNA integrity. DisA forms a large octamer that moves along intact chromosomal DNA. Upon encountering a DNA double-strand break, the DisA complex pauses at the lesion site and ceases c-di-AMP synthesis, thus delaying sporulation (Bejano-Sagie *et al.*, 2006; Oppenheimer-Shaanan *et al.*, 2011; Witte *et al.*, 2008). The second cyclase, YbbP, is orthologous to the essential DAC proteins of *L. monocytogenes* and *Staph. aureus*. Its transcript is mainly dependent on σ^A , with possible read through from an upstream σ^W promoter (Cao *et al.*, 2002; Luo & Helmann, 2012). YbbP is a membrane-localized protein that responds to cell envelope stress. Mutation of *ybbP* results in increased susceptibility to β -lactam antibiotics such as cefuroxime (Luo & Helmann, 2012). The third enzyme, YojJ, is a cytosolic protein. In vegetatively growing cells, YojJ is able to restore growth to a normally lethal double *disA ybbP* mutant only if artificially overexpressed (Luo & Helmann, 2012).

GdpP (formerly YybT) is the only known c-di-AMP phosphodiesterase (PDE) in *B. subtilis*. This transmembrane protein contains three functional domains: a haem-binding PAS domain, a degenerate GGDEF domain and a DHH/DHHA1 PDE domain (Rao *et al.*, 2010, 2011). The PDE activity of GdpP can be inhibited by the alarmone ppGpp and by haem *in vitro*. The haem-dependent PAS

Abbreviations: c-di-AMP, cyclic-di-AMP; DAC, diadenylate cyclase; MLS, macrolide-lincomycin-streptogramin; PG, peptidoglycan; 5'-RACE, 5' rapid amplification of cDNA ends; WT, wild-type.

inhibition can be partially relieved by nitric oxide (NO). However, the biological relevance of these haem, NO and ppGpp effects on PDE activity has not been studied *in vivo*. In this work, we characterize an antisense RNA transcribed from within *gdpP*. This *cis*-acting RNA is dependent on σ^D and it can modulate the cellular levels of GdpP.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. *B. subtilis* strains are derivatives of strain 168 or NCIB 3610. *Escherichia coli* strain DH5 α was used for standard cloning procedures. Unless noted otherwise, all cultures were grown in Luria–Bertani (LB) broth at 37 °C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μ g ampicillin ml⁻¹ for *E. coli*, and 1 μ g erythromycin ml⁻¹ plus 25 μ g lincomycin ml⁻¹ (MLS; macrolide-lincomycin-streptogramin B resistance), 10 μ g chloramphenicol ml⁻¹, 100 μ g spectinomycin ml⁻¹, 5 μ g tetracycline ml⁻¹ and 10 μ g kanamycin ml⁻¹ for *B. subtilis*. OD₆₀₀ readings were taken on a Spectronic 21 spectrophotometer.

Strain construction. All strains were generated by transformation of strain 168 to antibiotic resistance using chromosomal DNA, PCR products or plasmids as described elsewhere (Harwood & Cutting, 1990). When required, mutations in the strain 168 background were transferred to the strain NCIB 3610 background by SPP1-mediated generalized transduction, as described elsewhere (Kearns & Losick, 2005). Unless stated otherwise, all PCR products were generated using chromosomal DNA from strain 168 as a template and all constructs were verified by sequence analysis (Cornell University Life Sciences Core Laboratories Center). The oligonucleotides used in this study are listed in Table 2.

To generate promoter–*lacZ* fusions, a DNA fragment containing P_{yybs} or P_{gdpPas} was PCR-amplified with primer pairs 5565/5566 or 5598/5619, respectively, and cloned into vector pDG1661 (Guérout-Fleury *et al.*, 1996). The resulting plasmids were linearized by digestion with *ScaI* and integrated into strain 168 at the *amyE* locus. To create the P_D*-*lacZ* fusion, the same protocol was used except that the DNA fragment was amplified using strain HB15837 (168 P_D*) as template.

To generate the at-locus marker-less mutation P_D* (HB15837), we utilized an unstable integrative plasmid pMUTIN4, which harbours MLS resistance and *lacZ* genes (Vagner *et al.*, 1998). A DNA fragment containing P_D* was first generated using overlap-extension PCR as described previously (Gaballa *et al.*, 1998; Ho *et al.*, 1989) with some modifications. Briefly, the up-fragment (including 620 bp upstream of the mutation site) and down-fragment (including 900 bp downstream of the mutation) were amplified using PCR with primer pairs 5620/5621 and 5622/5623, respectively. Primers 5621 and 5622 introduce the desired P_D* mutation and are complementary to each other. These up- and down-fragments were then joined using PCR with primers 5620/5623. The resulting PCR product was cloned into pMUTIN4, which was transformed into strain 168. The transformants were plated on LB agar plates supplemented with MLS and X-Gal (50 μ g ml⁻¹). The resulting strain with plasmid pMUTIN4 integrated at the *gdpP* locus (strain HB15836) was resistant to MLS and blue on X-Gal plates. This at-locus integration of pMUTIN4 is not stable, and is capable of looping out from the chromosome, leaving behind either the wild-type (WT) or mutant sequence in the chromosome. To loop out pMUTIN4, cells of strain HB15836 were grown overnight in LB broth (without antibiotic selection), re-inoculated into LB diluted to 1:100, grown to OD₆₀₀ 0.4 and diluted to 1:10 000, and 100 μ l of cells was plated on LB agar supplemented with X-Gal. Cells that had

lost the pMUTIN4 plasmid appeared as white colonies on X-Gal plates, and were sensitive to MLS. The strain harbouring the P_D* mutation was verified by PCR amplification using primers 5298/5598 and DNA sequencing. To generate P_D* in the strain NCIB 3610 background, the *gdpP::pMUTIN4-P_D** construct in strain HB15836 was transferred to 3610 by SPP1 transduction, followed by the pMUTIN4 loop-out assay as above.

To generate an at-locus, marker-less N-terminal FLAG-tagged *gdpP* strain (HB15857), a similar protocol was used as for P_D* construction. A DNA fragment containing *gdpP-flag* was constructed by overlap extension using up-fragment primers 5684/5685 and down-fragment primers 5246/5686. The *flag* sequence harbours a *PsiI* restriction site. After looping out plasmid pMUTIN4, colonies that were white on X-Gal plates and sensitive to MLS were screened by PCR using primers 5684/5686 followed by *PsiI* digestion. The correct construct was verified by DNA sequencing.

Gene deletions (including constructs of *sigD::kan*, *flgM::spc*, *flgM::cat*) were generated by replacing the coding region with an antibiotic resistance cassette using long flanking homology PCR (LFH-PCR) followed by DNA transformation as described previously (Mascher *et al.*, 2003).

β -Galactosidase activity measurements. Strains harbouring promoter–*lacZ* fusions were grown overnight in 5 ml LB broth at 30 °C with vigorous shaking. Cells from 0.5 ml culture were harvested and β -galactosidase assays were performed as described by Miller (1972). Each strain was tested in biological triplicates and repeated three times. Data are reported as the mean and SEM.

5' Rapid amplification of cDNA ends (5'-RACE). The transcriptional start site of antisense *gdpP* was determined using 5'-RACE. Five pairs of primers (5586/5587, 5588/5589, 5590/5591, 5592/5593, 5594/5595) were used to map antisense transcripts initiated at the +800 to 1980 bp region relative to the *gdpP* start codon. For each 5'-RACE, 5 μ l total RNA from a mid-exponential-phase LB culture of strain 168 cells was reverse-transcribed to cDNA using TaqMan reverse transcription reagents (Roche) and the first primer (5586, 5588, 5590, 5592 or 5594). The 3' end of cDNA was tailed with poly-dCTP using terminal deoxynucleotidyltransferase (New England Biolabs). The tailed cDNAs were then amplified by PCR with primer AAP (4549) and the second primer (5587, 5589, 5591, 5593 or 5595, respectively). The PCR products were subject to DNA sequencing.

Immunoprecipitation and Western blotting. The FLAG-tagged GdpP and FabF were purified using immunoprecipitation with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) and quantified by Western blotting analysis. An overnight LB culture of *B. subtilis* cells harbouring both *gdpP-flag* and/or *fabF-flag* constructs was inoculated into 300 ml LB broth at an initial OD₆₀₀ of 0.01, and grown to OD₆₀₀ 0.5–0.6 at 37 °C with vigorous shaking. Cells were harvested by centrifugation and resuspended in 4 ml lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 2 mg lysozyme ml⁻¹]. The cell suspension was incubated at 37 °C for 20 min, followed by sonication and centrifugation at 9000 g for 5 min at 4 °C. Anti-FLAG resins were added to the supernatant, and incubated at 4 °C overnight with gentle agitation. The anti-FLAG resins were washed three times with TBS [50 mM Tris/HCl (pH 7.4), 150 mM NaCl], and proteins bound to the resins were eluted by resuspending in 50 μ l SDS-sample buffer [62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 50 mM DTT and 0.002% bromophenol blue] and boiled for 5 min. The samples were briefly centrifuged, and proteins in the supernatant were resolved on a 10% SDS-PAGE gel and electrophoretically transferred to an Immoblot PVDF membrane (Bio-Rad). Membranes were blocked in TBS with 0.5% Tween 20 (TBST) supplemented with 5% non-fat milk for 0.5 h at room temperature, followed by incubation with anti-FLAG

Table 1. Strains used in this study

Strain	Genotype	Source or reference
168	<i>trpC2</i>	Laboratory stock
NCIB3610	Prototrophic, undomesticated parent of 168	Laboratory stock
HB15826	168 <i>amyE::P_{yybS}-lacZ cat</i>	This study
HB15827	168 <i>amyE::P_{gdpPas}-lacZ cat</i>	This study
HB15829	168 <i>flgM::spc amyE::P_{yybS}-lacZ cat</i>	This study
HB15830	168 <i>flgM::spc amyE::P_{gdpPas}-lacZ cat</i>	This study
HB15833	168 <i>sigD::kan amyE::P_{yybS}-lacZ cat</i>	This study
HB15834	168 <i>sigD::kan amyE::P_{gdpPas}-lacZ cat</i>	This study
HB15835	168 <i>sigD::kan flgM::spc amyE::P_{gdpPas}-lacZ cat</i>	This study
HB15885	168 <i>amyE::P_D*-lacZ cat</i>	This study
HB15886	168 <i>sigD::kan amyE::P_D*-lacZ cat</i>	This study
HB15887	168 <i>flgM::spc amyE::P_D*-lacZ cat</i>	This study
HB13056	168 <i>thrC::P_{fabHaF}-fabHa-fabF-FLAG mls</i>	Kingston <i>et al.</i> (2011)
HB15843	168 <i>gdpP::pMUTIN4-gdpP-FLAG mls</i>	This study
HB15845	168 <i>gdpP-FLAG</i>	This study
HB15857	168 <i>gdpP-FLAG thrC::P_{fabHaF}-fabHa-fabF-FLAG mls</i>	This study
HB15858	168 <i>sigD:kan gdpP-FLAG thrC::P_{fabHaF}-fabHa-fabF-FLAG mls</i>	This study
HB15859	168 <i>flgM::spc gdpP-FLAG thrC::P_{fabHaF}-fabHa-fabF-FLAG mls</i>	This study
HB15860	168 <i>sigD:kan flgM::spc gdpP-FLAG thrC::P_{fabHaF}-fabHa-fabF-FLAG mls</i>	This study
HB15836	168 <i>gdpP::pMUTIN4-P_D* mls</i>	This study
HB15837	168 P _D *	This study
HB15901	3610 P _D *	This study
HB15902	3610 <i>flgM::cat</i>	This study
HB15903	3610 <i>flgM::cat P_D*</i>	This study
HB15904	3610 <i>flgM::cat amyE::P_{hyspank}-sigD kan</i>	This study
HB15905	3610 <i>flgM::cat amyE::P_{hyspank}-sigD kan P_D*</i>	This study
HB15906	3610 <i>flgM::cat amyE::P_{hyspank}-sigD kan lytABC::spc lytD::mls lytF::tet</i>	This study
HB15907	3610 <i>flgM::cat amyE::P_{hyspank}-sigD kan lytABC::spc lytD::mls lytF::tet P_D*</i>	This study
HB15908	3610 <i>disA::spc flgM::cat</i>	This study
HB15909	3610 <i>disA::spc flgM::cat P_D*</i>	This study
HB15910	3610 <i>ybbP::tet flgM::spc amy::P_{hag}-cat-lacZ</i>	This study
HB15911	3610 <i>ybbP::tet flgM::spc P_D* amy::P_{hag}-cat-lacZ</i>	This study
HB15912	3610 <i>sigM::kan ybbP::tet flgM::spc amy::P_{hag}-cat-lacZ</i>	This study
HB15913	3610 <i>sigM::kan ybbP::tet flgM::spc P_D* amy::P_{hag}-cat-lacZ</i>	This study
HB15914	3610 <i>disA::spc amyE::P_{spac(hy)}-gdpP cat</i>	This study
HB15915	3610 <i>ybbP::tet amyE::P_{spac(hy)}-gdpP cat</i>	This study
HB15916	3610 <i>yoyj::kan amyE::P_{spac(hy)}-gdpP cat</i>	This study
HB15917	3610 <i>gdpP::mls amyE::P_{spac(hy)}-gdpP cat</i>	This study
HB15918	3610 <i>amy::P_{spac(hy)}-gdpP cat</i>	This study

rabbit antibody (Sigma-Aldrich) in TBST supplemented with 0.5% non-fat milk overnight at room temperature. The membrane was washed three times for 10 min each in TBST, and incubated with alkaline phosphatase-coupled secondary goat anti-rabbit antibody for 1 h (Sigma-Aldrich). After being washed three times for 10 min each in TBST, the membrane was cut into two pieces to separate the GdpP-FLAG- and FabF-FLAG-containing sections, and developed for 10 and 1 min, respectively. The developing reagent contained 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂, 300 µg BCIP (5-bromo-4-chloro-3-indolylphosphate) ml⁻¹ and 300 µg ml⁻¹ Nitro Blue Tetrazolium substrate (Bio-Rad). The relative level of GdpP-FLAG in each strain was normalized to the internal control FabF-FLAG using densitometry analysis with ImageJ (Girish & Vijayalakshmi, 2004).

Disc diffusion and MIC assays. Disc diffusion assays were performed as described previously (Luo *et al.*, 2010), with minor modifications. Mueller–Hinton (MH) broth (Sigma-Aldrich) and a

MOPS-based glucose minimal medium (MM) (Bsat *et al.*, 1996) were used for these assays. The bottom agar was 15 ml MH or MM broth supplemented with 1.5% agar, and the top agar was 4 ml MH or MM broth supplemented with 0.75% agar. Chemical discs were prepared using Whatman filter paper discs (7 mm diameter) and freshly made chemical stocks. For each chemical test, three different amounts were used: aztreonam 6, 30 and 60 µg; cefuroxime 3, 6 and 12 µg; cefixime 5, 10 and 15 µg; and sodium azide 5, 10 and 30 mmol. The zone of growth inhibition was measured after overnight growth at 37 °C. MIC tests were performed as described previously (Luo & Helmann, 2012), except that the media used were MH and MM broths.

Cell lysis assay. Fresh colonies were first grown in LB, MH or MM broth to OD₆₀₀ 0.4, diluted 1:100 in fresh LB, MH or MM, respectively, and inoculated in Bioscreen microtitre plates with a total volume of 200 µl. Growth was measured spectrophotometrically (OD₆₀₀) using a Bioscreen incubator (Growth Curves USA) at 37 °C with vigorous shaking. For cefuroxime-induced lysis, five concentrations of

Table 2. Oligonucleotides used in this study

No.	Oligonucleotide	Sequence (5'–3')*
5586	yybTanti-GSP1	CGATGAGTGAGGTTGAGGCT
5587	yybTanti-GSP2	GGAATACTTCGATCAAGTGCTGA
5588	yybTanti-GSP3	ATCAATATGATTGAAGCAACAGC
5589	yybTanti-GSP4	ACAGCGGAATTGGTGACAGA
5590	yybTanti-GSP5	AGCCGTCACCTCGTCATGGA
5591	yybTanti-GSP6	CGCCTGAAGAAGCAATGGA
5592	yybTanti-GSP7	GTCACCGAAAAGCAGCAATGT
5593	yybTanti-GSP8	AACCCAATGGAGAAAACGAACA
5594	yybTanti-GSP9	CGCAATCCAGCTTGGACTT
5595	yybTanti-GSP10	CTGACGTTAAGCGTCGGTGT
4549	AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
5628	sigD-up-for	CTGATGGAGCTCAGTCAGGT
5629	sigD-up-rev (kan)	CCTATCACCTCAAATGGTTTCGCTGCCTGATCTTCATAATTCAAGGA
5630	sigD-do-for (kan)	CGAGCGCCTACGAGGAATTTGTATCGTCAGATCCATTCAAAGGCATT
5631	sigD-do-rev	GCTTCATAGAAATGACTGACATGT
5632	flgM-up-for	TTCAGCAGAAGGTATGAATATCA
5633	flgM-up-rev(spc)	CGTTACGTTATTAGCGAGCCAGTCGATATGGATTAACGGATTGTGT
5634	flgM-do-for(spc)	CAATAAACCCCTTGCCCTCGCTACGTCATACAAAAGTAGACGCAAATCA
5635	flgM-do-rev	CCGCTGTCTTGTATAACCATCA
5879	flgM-up-rev(cat)	CTTGATAATAAGGGTAACTATTGCCGATATGGATTAACGGATTGTGT
5880	flgM-do-for(cat)	GGGTAAGTACGCTCGCCGGTCCACGTCATACAAAAGTAGACGCAAATCA
5565	PyybS-for (<i>EcoRI</i>)	GAGGAATTCACAAAAGAGGTGAAACACAATG
5566	PyybS-rev (<i>BamHI</i>)	GAGGGATCCCAATCACAGGAACATAAACGA
5598	PyybTanti-for (<i>EcoRI</i>)	GAGGAATTCACAGAGTGACGGCTTATGTGT
5619	PyybTanti-rev2 (<i>BamHI</i>)	GAGGGATCCGTCACCGAAAAGCAGCAATGT
5620	yybT-int-for (<i>EcoRI</i>)	GAGGAATTCATCAAACGGGATGAGCGTCTCT
5621	yybT-PDmut-up-rev	TCGCTGCGCCTATGGAATCCATGTCGGGGAATTTATG
5622	yybT-PDmut-do-for	CATAAATTCGCCGACATGGATTCCATAGGCGCAGCGATCGGGATTTAAAG
5623	yybT-rev (<i>BamHI</i>)	GAAGGATCCCTCATCTCTGTACGCCTCCCT
5684	yybS-int-up-for- (<i>EcoRI</i>)	GAGGAATTCCTTGATATTGTAGAAACTGTAGCGA
5685	yybS-up-rev-(FLAG)	CATCCGCGGTTTATCATCATCATCTTTATAATCCATTTCTATCACTCCCCACCATGT
5246	yybT-for N-flag 1	ATGGATTATAAAGATGATGATGATAAACCGCGGATGCCAAGCTTTTATGAAAAAC
5686	yybT-int-do-rev (<i>BamHI</i>)	GAGGGATCCATCCAATCCTTGTGTACATCA
5298	yybT-for	AGTGATAGAAATGCCAAGCT

* Restriction sites are underlined.

cefuroxime (0.5, 1, 2, 4 or 8 $\mu\text{g ml}^{-1}$, final) were added to the cell culture at two growth phases (exponential phase at OD_{600} 0.3–0.4 and transition phase at OD_{600} 0.6–0.7).

Motility and biofilm formation. Swimming and swarming motility tests were performed as described by Kearns & Losick (2005) and Patrick & Kearns (2009), with some modification. Freshly made LB agar was cooled to 55 °C, poured into Petri dishes (25 ml per plate) and dried in a laminar flow hood for 20 min before use. Cells were grown to mid-exponential phase (OD_{600} 0.4) or transition phase (OD_{600} 0.7), harvested and resuspended in phosphate buffer to OD_{600} 10. Ten microlitres of cells was spotted on the swimming plate (LB supplemented with 0.3% agar) or swarming plate (LB supplemented with 0.7% agar and 5 $\mu\text{g tetrazolium violet ml}^{-1}$) and dried in a laminar flow hood for another 10 min. For swimming tests, the plates were incubated at 37 °C, and the swimming zones were measured every 30 min until they reached the edge of the plate (~5 h). For swarming tests, plates were incubated at room temperature (22 °C) overnight. Tetrazolium violet is a redox dye that is imported into the cell and reduced to a purple-coloured formazan. It does not affect cell

growth or motility, but helps to mark the swarming zone. Biofilm formation tests were performed as described by Branda *et al.* (2006) and Romero *et al.* (2010). We tested pellicle formation in Mmsg liquid medium and colony morphology on Mmsg-supplemented 1.5% agar plates.

RESULTS AND DISCUSSION

An antisense transcript is encoded within *gdpP*

The *gdpP* gene (formerly *yybT*) is the second gene in a three-gene operon (*yybS-gdpP-rplI*) (Fig. 1a). Recent transcriptomic surveys using high-density cDNA tiling arrays and RNA-Seq have suggested that there are at least three transcripts emanating from this locus (Irnov *et al.*, 2010; Nicolas *et al.*, 2012; Rasmussen *et al.*, 2009). The major transcript is initiated from a σ^A -dependent promoter located 121 bp upstream of *yybS*. Within the *gdpP* coding

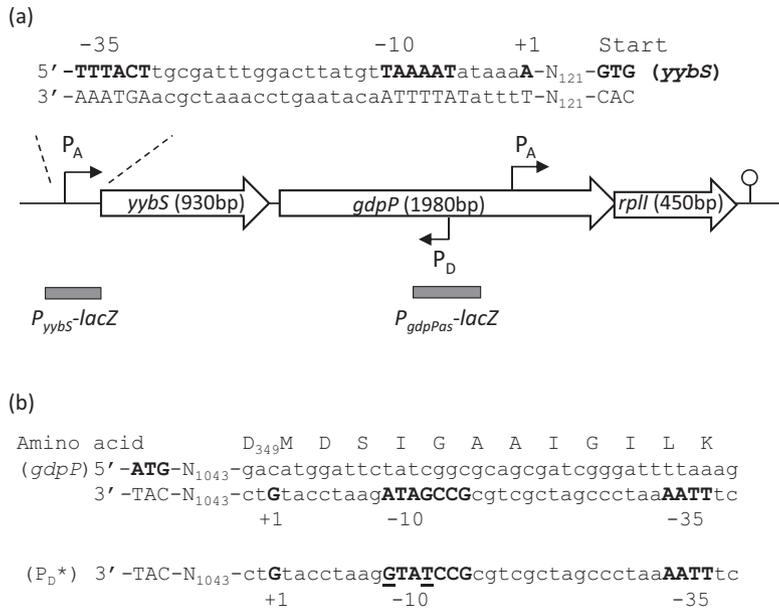


Fig. 1. A σ^D -dependent promoter in *gdpP*. (a) Schematic map of the *yybS-gdpP-rplI* operon. Promoter sites are indicated by bent arrows with a subscript to indicate the relevant holoenzyme. The -35, -10 and +1 elements of P_{yybS} and the start codon of *yybS* are in bold type. The balloon indicates a transcriptional terminator. The DNA regions included in the $P_{yybS-lacZ}$ and $P_{gdpPas-lacZ}$ fusions are also illustrated. (b) Promoter sequence of $gdpP_{as}$. The sense *gdpP* start codon and the -35, -10 and +1 elements of the antisense P_{gdpPas} are shown in bold type. P_D^* mutations are underlined.

region, there is a putative σ^A -dependent promoter located upstream of the essential gene *rplI* (Nicolas *et al.*, 2012). The third transcript is a putative antisense RNA encoded within *gdpP*. As this antisense transcript could potentially affect the expression of GdpP we investigated its expression and function.

The antisense *gdpP* (*gdpP_{as}*) was first annotated as transcript shd124 by Rasmussen *et al.* (2009), and more recently as S1559 by Nicolas *et al.* (2012). However, assignment of the start site and length for this antisense RNA differed in those two studies. While Rasmussen and co-workers suggested a 1429 nt transcript starting from genome nucleotide position 4164574 (GenBank accession no. AL009126.3), Nicolas and co-workers assigned its start site to position 4164565 and predicted its length as 667 nt. To verify expression of this antisense transcript and define its transcriptional start site, we performed 5'-RACE with total RNA isolated from exponential phase cells grown in LB broth. Five pairs of primers were used to map any antisense transcripts initiated within a region of 800–1980 bp downstream of the *gdpP* start codon. One transcript, designated *gdpP_{as}*, was identified and its start site was mapped to 1048 bp downstream of the start codon, corresponding to the genome nucleotide location of 4164576. The start site of *gdpP_{as}* is proximal to a candidate σ^D promoter (Fig. 1) that is positionally conserved within the genomes of closely related bacilli (Fig. 2).

We next tried to determine the size and abundance of the sense *gdpP* transcript and antisense *gdpP_{as}* transcript using Northern blotting and radioactively labelled oligonucleotide probes. However, we were unable to detect signals corresponding to either transcript (data not shown). It is possible that the expression levels of these transcripts are below the detection threshold, or perhaps more likely that

the transcripts are unstable due to the formation of an RNA–RNA duplex. By examining the tiling data in detail, we noticed the transcript signals fading away after about 600 nt (Nicolas *et al.*, 2012; Rasmussen *et al.*, 2009). No terminators were found in the vicinity of this region, nor was an extended RNA transcript detected in RNA prepared from a *rho* mutant as monitored using tiling arrays (Nicolas *et al.*, 2012). The termination mechanism for the antisense transcript therefore remains unknown. For simplicity reasons, here we adopt the annotation of 667 nt as suggested by Nicolas *et al.* (2012), which is a conservative estimate.

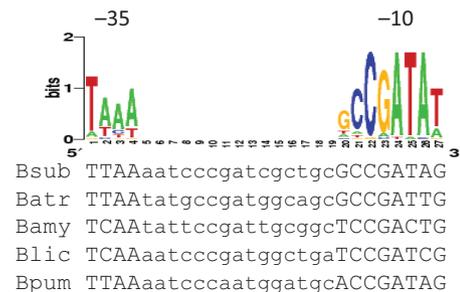


Fig. 2. Alignment of P_{gdpPas} elements from various bacilli species. The *gdpP* sequence of *B. subtilis* 168 (Bsub) was aligned with the corresponding sequences from *Bacillus atrophaeus* 1942 (Batr), *Bacillus amyloliquefaciens* FZB42 (Bam), *Bacillus licheniformis* ATCC 14580 (Blic) and *Bacillus pumilus* SAFR-032 (Bpum) using CLUSTALW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The WebLogo of the σ^D promoter consensus from *B. subtilis* is shown above the alignment (Sierra *et al.*, 2008). The -35 and -10 elements are indicated.

The $gdpP_{as}$ transcript is dependent on σ^D

σ^D is an alternative σ factor that mainly regulates the expression of autolysins and genes involved in flagella biosynthesis and chemotaxis. The activity of σ^D is mediated primarily by the anti- σ factor FlgM, which sequesters σ^D and inhibits its activity. An *flgM* mutant displays increased *sigD* activity and hence increased expression of genes within the σ^D regulon (Caramori *et al.*, 1996; Fredrick & Helmann, 1996). To determine whether the predicted $gdpP_{as}$ promoter is active, we constructed an ectopic promoter-*lacZ* reporter (*amyE::P_{gdpPas}-lacZ*) and introduced it into a WT strain (strain 168) and into isogenic strains possessing *sigD* and/or *flgM* mutations. This promoter fusion includes sequences from -230 to +60 bp relative to the transcription start site. The sense *yybS* promoter-*lacZ* fusion was also constructed and used as a control (Fig. 1a). Expression of *P_{gdpPas}-lacZ* was completely eliminated in a *sigD* mutant or a *sigD flgM* double mutant, but was highly induced in an *flgM* mutant (Fig. 3). In contrast, transcription of *P_{yybS}-lacZ* was not affected by mutations in either *sigD* or *flgM*. This result suggests that the antisense promoter is active and that it is σ^D -dependent.

To further verify that the $gdpP_{as}$ promoter activity is due to σ^D , we constructed a *P_D*-lacZ* fusion in which the -10 element GCCGACA sequence was changed to GCCTATG (mutated nucleotides underlined). These two nucleotide mutations alter two of the most conserved residues in the σ^D promoter consensus, yet maintain the sense GdpP amino acid sequence (Figs 1b and 2). As expected, *P_D*-lacZ* expressed no β -galactosidase in the WT strain or the *sigD/flgM* mutant backgrounds (Fig. 3).

$gdpP_{as}$ transcription reduces the level of GdpP protein in the cell

As we could not detect any ORFs within the $gdpP_{as}$ transcript, and as $gdpP_{as}$ shares perfect complementarity

with the sense *gdpP* mRNA, we hypothesized that $gdpP_{as}$ functions as a *cis*-acting regulatory RNA. Base-pairing between a *cis*-acting RNA and its target mRNA typically alters target RNA stability and/or modulates sense translation (Georg & Hess, 2011), both of which alter expression levels of the sense-encoded protein. To measure the expression level of GdpP and the effects of $gdpP_{as}$ transcription, we constructed an at-locus *gdpP* allele encoding an N-terminal FLAG-tag for immunodetection. This tagged allele of *gdpP* was introduced into WT and isogenic mutants of *sigD*, *flgM* and *sigD flgM*. Direct attempts to monitor GdpP-FLAG accumulation by Western blotting using crude cell lysates and anti-FLAG antibodies were unsuccessful, probably due to the low abundance of GdpP in all strain backgrounds. We therefore utilized immunoprecipitation to enrich GdpP-FLAG and then used Western blotting to quantify the recovered protein. As an internal control we used strains also expressing a FabF-FLAG protein (Kingston *et al.*, 2011). Using this strategy, we determined that GdpP-FLAG accumulation increased in a *sigD* or a *sigD flgM* mutant, but decreased in an *flgM* mutant (relative to levels in the WT strain background) (Fig. 4). As σ^D activity is known to be quite heterogeneous in growing cell populations (Kearns & Losick, 2005), perhaps the most biologically relevant comparison of GdpP-FLAG accumulation is between the *flgM* mutant (wherein $gdpP_{as}$ is highly expressed) and the *sigD* mutant (wherein $gdpP_{as}$ is not expressed). We estimate an overall change in GdpP-FLAG accumulation of about 2.5- to threefold when comparing the *sigD* and *flgM* mutant strains. Thus, the $gdpP_{as}$ transcript can serve as a negative regulator of GdpP expression.

Lack of $gdpP_{as}$ transcription does not lead to obvious cell lysis or motility phenotypes

We next investigated the possible biological effects of $gdpP_{as}$ transcription by monitoring functions known or postulated to be related to GdpP function. Previously, we

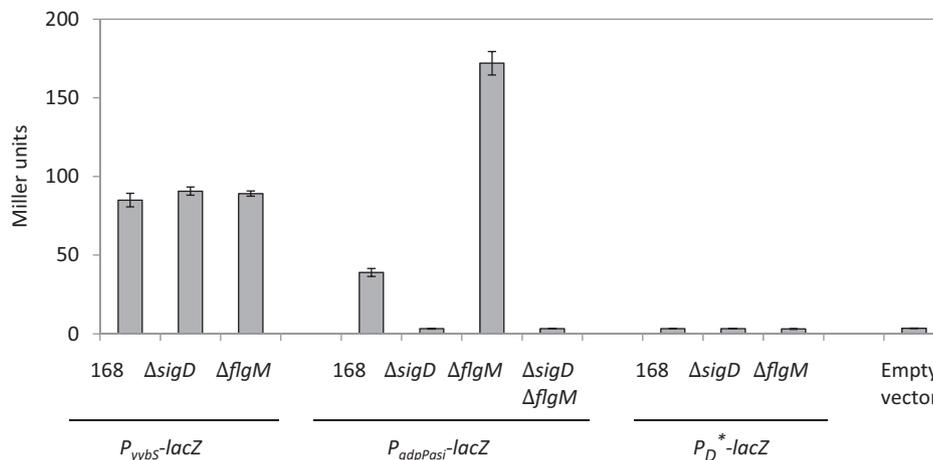


Fig. 3. β -Galactosidase activity of *P_{yybS}-lacZ*, *P_{gdpPas}-lacZ* and *P_D*-lacZ* in different strain backgrounds in LB overnight cultures. This experiment was performed in biological triplicate and repeated three times. Error bars, SEM.

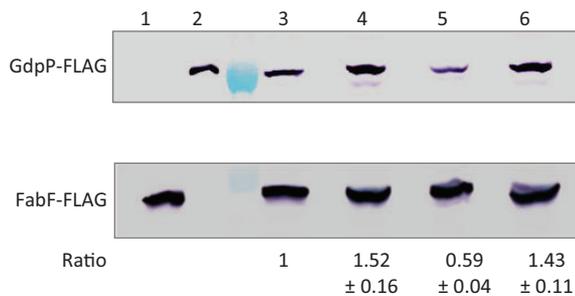


Fig. 4. Detection of GdpP-FLAG and FabF-FLAG by immunoprecipitation followed by SDS-PAGE and Western blotting with anti-FLAG antibodies. The sections of the membrane containing GdpP-FLAG and FabF-FLAG were separated after antibody hybridization, and developed separately using a chromogenic assay. Strains used were: lane 1, *fabF-FLAG* (HB13056); 2, *gdpP-FLAG* (HB15845); 3, *gdpP-FLAG fabF-FLAG* (HB15857); 4, *sigD gdpP-FLAG fabF-FLAG* (HB15858); 5, *flgM gdpP-FLAG fabF-FLAG* (HB15859); 6, *sigD flgM gdpP-FLAG fabF-FLAG* (HB15860). The lane between lanes 2 and 3 is a protein ladder, where the 75 and 37 kDa markers are visible at the sections of GdpP-FLAG and FabF-FLAG proteins, respectively. This experiment was repeated three times, and one representative experiment is shown. The intensities of GdpP-FLAG bands were normalized with the internal protein control FabF-FLAG. The numbers below each band represent the fold change (mean \pm SEM) of normalized GdpP-FLAG relative to strain HB15857 (lane 3).

showed that c-di-AMP is involved in PG homeostasis (Luo & Helmann, 2012). A *gdpP* mutant (exhibiting increased c-di-AMP levels) is more resistant to PG-targeting antibiotics such as the β -lactam drugs and, conversely, overexpression of *gdpP* confers β -lactam sensitivity. As demonstrated above, transcription of *gdpP_{as}* is dependent on σ^D , and σ^D is known to regulate the expression of the major vegetative autolysins (LytC, LytD and LytF) which degrade PG (Blackman *et al.*, 1998; Helmann *et al.*, 1988; Lazarevic *et al.*, 1992; Margot *et al.*, 1994, 1999; Serizawa *et al.*, 2004). We therefore hypothesized that σ^D downregulates GdpP expression (thereby leading to elevated c-di-AMP levels) as a mechanism to upregulate PG biosynthesis concomitant with the upregulation of autolysins under σ^D control. To test this idea, we mutated the *gdpP_{as}* promoter and generated an at-locus marker-less P_D^* mutant, which eliminates σ^D -dependent *gdpP_{as}* transcription without affecting the expression of other σ^D regulon genes (Figs 1b and 3). We predicted that cells carrying P_D^* would be unable to downregulate GdpP synthesis when σ^D is activated, and that this might lead to an imbalance between PG biosynthetic and autolytic functions. In an attempt to detect such an imbalance we treated both a WT strain (P_D^{wt} , strain 168) and the strain carrying the mutant promoter (P_D^* , HB15837) with the cell autolysis inducers sodium azide and three β -lactam antibiotics (cefuroxime, aztreonam and cefixime) and monitored cell lysis rates by OD₆₀₀ and c.f.u. counts. We also examined chemical

susceptibility by disc diffusion and MIC assays. However, the response differences between P_D^{wt} and P_D^* strains were either not significant or not reproducible with large standard errors (data not shown). We reasoned that this is probably due to the temporary and heterogeneous nature of σ^D activity in the our laboratory strain (strain 168) background.

The heterogeneity of σ^D is due to multiple factors, including the magnitude of processive transcription through the long *fla/che* (*sigD*) operon, expression of the anti- σ factor FlgM, and transcriptional regulators SwrA, SwrB, DegU and SlrA/SinR/SlrR (Amati *et al.*, 2004; Calvio *et al.*, 2008; Cozy & Kearns, 2010; Cozy *et al.*, 2012; Fredrick & Helmann, 1996; Hsueh *et al.*, 2011; Kearns & Losick, 2005; Tsukahara & Ogura, 2008). As a result, a growing *B. subtilis* population contains both σ^D active (ON) and inactive (OFF) cells. The fraction of σ^D ON cells is influenced by growth phase, nutrient levels and the parental strain background: exponential phase, the presence of amino acids in the growth medium and domestic strains (e.g. strain 168) are associated with low σ^D activity, whereas transition phase, the absence of amino acids and the undomesticated strain (strain NCIB 3610) appear to have a relatively high fraction of σ^D ON cells. There is no known specific growth condition that can universally induce σ^D activity in a population. To test the effect of the P_D^* mutation in a population with relatively high levels of σ^D activity, we conducted the same experiments using strain NCIB 3610 in a variety of different growth media (LB, MH and minimal medium) and growth phases (exponential, transition, early stationary and late stationary phases). However, no significant differences were observed between the P_D^{wt} and P_D^* strains, at least with respect to cell autolysis and β -lactam susceptibility. It has been reported that a full σ^D ON population can be achieved by simultaneously mutating *flgM* and overexpressing *sigD* (Cozy & Kearns, 2010). In this case, however, the highly expressed autolysins in this strain background caused rapid cell lysis and may have masked the effect of *gdpP_{as}*, as no phenotypes were attributable to the P_D^* mutation (data not shown). To separate the effects of loss of *gdpP_{as}* transcription from autolysin activity, we mutated all three major autolysins, *lytC*, *lytD* and *lytF*, in this σ^D ON strain background (*flgM P_{spank}-sigD*). Again, we did not observe differences between P_D^* and P_D^{wt} strains in terms of cell lysis and β -lactam susceptibility. It is important to note that this autolysin-defective strain grows mainly as chains of un-separated cells, which may obscure the analysis of cell lysis using OD₆₀₀ as an indicator. We conclude that the detection of a *gdpP_{as}* phenotype may require other factors besides high cellular σ^D activity.

We next considered the possibility that modulation of GdpP levels by *gdpP_{as}* is most important under conditions where c-di-AMP synthesis is reduced. Therefore, we also introduced *disA*, *sigM* (encoding a *disA* activator) and *ybbP* mutations into NCIB 3610 strains carrying *flgM* and *flgM P_D^{*}* mutations. These three mutations are known to reduce

c-di-AMP levels and result in increased sensitivity to cefuroxime (Luo & Helmann, 2012). However, we could not detect any differences between P_D^* and P_D^{wt} alleles in these strains in terms of either cell lysis rates or β -lactam susceptibility. Despite our various efforts, we were not able to associate $gdpP_{as}$ with an observable phenotype. Nevertheless, the presence of a conserved, σ^D -dependent $gdpP_{as}$ among closely related *Bacillus* species (Fig. 2) suggests that it likely plays a (still elusive) biological role.

Besides autolysis and β -lactam susceptibility, we also tested whether c-di-AMP affects cell motility for two reasons: (1) σ^D directs expression of genes involved in flagella biosynthesis and chemotaxis, and is therefore essential for cell motility; and (2) c-di-GMP, a related yet distinct secondary messenger molecule in bacteria, is known to regulate the transition between a motile and a sessile lifestyle, where cells with high c-di-GMP are non-motile and form robust biofilms (Hengge, 2009). c-di-GMP has not been detected in *B. subtilis*. However, a $gdpP$ mutant (with increased c-di-AMP levels) in *Staph. aureus* has been reported to form about threefold more biofilm than the WT, which is reminiscent of the effect of c-di-GMP (Corrigan *et al.*, 2011). To test cell motility and biofilm formation, strains harbouring mutations in the c-di-AMP cyclase genes (*disA*, *ybbP* and *yojJ*) or the hydrolase gene $gdpP$ were constructed. An IPTG-inducible $gdpP$ construct (*amyE::P_{spac(hy)}-gdpP*) was introduced into these strains to reduce the level of c-di-AMP. We did not observe significant changes under these genetic conditions when compared with the WT strain NCIB 3610, suggesting that c-di-AMP may not be involved in cell motility (swimming and swarming) or biofilm formation, at least under our test conditions.

Concluding remarks

c-di-AMP is an essential second messenger in *B. subtilis*. Here, we show that the c-di-AMP hydrolase GdpP is subject to post-transcriptional regulation via an antisense RNA, $gdpP_{as}$, which thereby represents a novel means of regulating c-di-AMP levels in the cell. Antisense RNAs are a widespread regulatory mechanism and include both *trans*- and *cis*-acting RNAs. In both cases, the regulatory RNA typically acts by annealing to its target and affecting either translation or mRNA stability (Thomason & Storz, 2010). Recent genome-wide studies have revealed over 100 potential regulatory RNA transcripts in *B. subtilis* (Irnov *et al.*, 2010; Nicolas *et al.*, 2012; Rasmussen *et al.*, 2009). Only a few antisense RNA molecules have been studied in detail, including three *cis*-acting (*ratA*, SR4 and antisense of *yabE*) and two *trans*-acting (*fsrA* and *bsrF*) sRNAs (Eiamphungporn & Helmann, 2009; Gaballa *et al.*, 2008; Jahn *et al.*, 2012; Preis *et al.*, 2009; Silvaggi *et al.*, 2005). Antisense RNA typically base-pairs with its target RNA, forming an RNA–RNA duplex, which alters the half-life of the target RNA, and therefore influences protein product accumulation. Transcription of $gdpP_{as}$ is initiated in the middle of the $gdpP$ gene and is dependent on

σ^D . As demonstrated here, this *cis*-acting regulatory RNA can modulate the cellular levels of GdpP, although the biological role of this regulation remains elusive. Further studies are needed to better understand the regulatory network that links σ^D , the $gdpP_{as}$ regulatory transcript, $gdpP$ expression and c-di-AMP levels with effects on PG homeostasis.

ACKNOWLEDGEMENTS

We thank Dr Win Chai (Harvard University) for strain NCIB 3610, and Dr Daniel Kearns (Indiana University) for strains DS793 (3610 *amyE::P_{hag}-lacZ cat*), DS2447 (NCIB 3610 *lytABC::spc lytD::mly lytF::tet amyE::P_{hag}-GFP cat*) and DS3988 (NCIB 3610 Δ *flgM amyE::P_{hyspank}-sigD kan thrC::P_{sigD}-lacZ mls*). This work was supported by NIH grant GM-047446 to J. D. H.

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Edited by: J. Stülke