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Analysis of the role of *Bacillus subtilis* σ^M in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis

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Summary

The *Bacillus subtilis* extracytoplasmic function (ECF) σ factor σ^M is inducible by, and confers resistance to, several cell envelope acting antibiotics. Here, we demonstrate that σ^M is responsible for intrinsic β-lactam resistance, with σ^X playing a secondary role. Activation of σ^M upregulates several cell wall biosynthetic enzymes including one, PBP1, shown here to be a target for the beta-lactam cefuroxime. However, σ^M still plays a major role in cefuroxime resistance even in cells lacking PBP1. To better define the role of σ^M in β-lactam resistance we characterized suppressor mutations that restore cefuroxime resistance to a *sigM* null mutant. The most frequent suppressors inactivated *gdpP* (*yybT*) which encodes a cyclic-di-AMP phosphodiesterase (PDE). Intriguingly, σ^M is a known activator of *disA* encoding one of three paralogous c-di-AMP cyclases (DAC). Overproduction of the GdpP PDE greatly sensitized cells to β-lactam antibiotics. Conversely, genetic studies indicate that at least one DAC is required for growth with depletion leading to cell lysis. These findings support a model in which c-di-AMP is an essential signal molecule required for cell wall homeostasis. Other suppressors highlight the roles of ECF σ factors in counteracting the deleterious effects of autolysins and reactive oxygen species in β-lactam treated cells.

Keywords

ECF σ factor; β-lactam; cefuroxime; c-di-AMP; *Bacillus subtilis*

Introduction

The bacterial cell envelope is crucial for maintaining cell shape and counteracting turgor pressure and is an important target for many antimicrobial compounds (Walsh, 2003). The cell envelope of *Bacillus subtilis* contains a cytoplasmic membrane surrounded by layers of cross-linked peptidoglycan (PG), membrane-associated lipoteichoic acids (LTA), and wall-associated teichoic acids (WTA) (Foster & Popham, 2002, Scheffers & Pinho, 2005). PG is a polymer of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) glycan chains cross-linked by peptide sidechains. The newly synthesized lipid-linked NAG-NAM units are polymerized to glycan strands by the action of transglycosylase (TG). Concurrent with, or soon after this polymerization, the peptide side chains on the NAM residue are cross-linked by transpeptidase (TP). Both TG and TP are activities of high molecular weight penicillin binding proteins (HMW PBPs), and they are the targets of moenomycin and the β-lactam antibiotics, respectively (Waxman & Strominger, 1983, Macheboeuf *et al*., 2006, Foster & Popham, 2002).

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β-lactam antibiotics are characterized by the presence of a β-lactam ring which mimics the D-Ala-D-Ala dipeptide substrate of HMW PBP and inhibits the transpeptidation reaction by covalent modification of the TG active site (Macheboeuf *et al*., 2006). This inhibition disrupts cell wall biosynthesis, triggers the formation of reactive oxygen species (ROS), and results in cell lysis and death (Kohanski *et al*., 2007, Kohanski *et al*., 2010, Gusarov *et al*., 2009). Synthesis and incorporation of new PG glycan strands into the existing cell wall requires close coordination between the biosynthetic machinery (including HMW-PBPs) and autolytic enzymes that allow the separation of already crosslinked glycan strands. When properly coordinated, the cell grows normally and maintains proper cell shape. Conversely, agents that prevent this coordination by inhibiting TG or TP activities of PBPs, or by activating autolysins, lead to lysis and cell death (Fig. 1). Several models have been advanced to explain how this coordination occurs, but the existence and precise architecture of the proposed biosynthetic holoenzyme is still unclear (Vollmer & Bertsche, 2008, Carballido-Lopez & Formstone, 2007).

There are three major mechanisms that confer high level β-lactam resistance as described for the Gram positive genera *Staphylococcus* and *Streptococcus* and the Gram negative species *Escherichia coli* and *Pseudomonas* spp. These are: (i) expression of β-lactamase(s) that inactivate the antibiotics; (ii) expression of mutated or mosaic PBP alleles that have low affinity for β-lactams; and (iii) the expression of a β-lactam specific efflux pump (Poole, 2004, Wilke *et al*., 2005). *B. subtilis* displays a significant level of intrinsic resistance against a variety of β-lactam antibiotics, but the underlying mechanisms are poorly understood. Although there are three putative β-lactamase genes (*penP*, *ybbE*, and *yblX*) in the genome, no β-lactamase activity is detected in the growing cells or supernatant (Colombo *et al*., 2004). No penicillin-insensitive PBP alleles have been identified nor does an efflux pump-based mechanism appear to be applicable to *B. subtilis* and other Gram positive bacteria. Therefore, the molecular basis of this intrinsic, moderate level β-lactamase resistance is unclear. Recent results suggest that extracytoplasmic function (ECF) σ factors play a role in resistance to β-lactam antibiotics: a triple mutant (strain *sigMWX*) as well as a mutant lacking all 7 ECF σ factors (strain Δ7ECF) is sensitive to β-lactam antibiotics including ampicillin, penicillin G, aztreonam, and cefuroxime (Mascher *et al*., 2007, Luo *et al*., 2010).

B. subtilis harbors 7 ECF σ factors, σ^M, σ^X, σ^W, σ^V, σ^Y, σ^Z and σ^{YlaC}. Of these, the physiological roles of σ^M, σ^W, σ^X, and more recently σ^V, have been well characterized, and their target regulons have been defined (Helmann, 2002, Jervis *et al*., 2007, Eiamphungporn & Helmann, 2008, Guariglia-Oropeza & Helmann, 2011). Both expression and activity of these ECF σ factors are often stimulated by cell wall-active antibiotics. σ^M is strongly induced by vancomycin and moenomycin, and confers resistance to moenomycin (Thackray & Moir, 2003, Mascher *et al*., 2007, Eiamphungporn & Helmann, 2008). Activation of the σ^W regulon contributes to resistance to fosfomycin, sublancin, and a toxic peptide SdpC (Cao *et al*., 2002, Butcher & Helmann, 2006). The σ^X regulon is involved in the resistance to nisin and other cationic antimicrobial peptides (Cao & Helmann, 2002, Cao & Helmann, 2004). Finally, σ^V is induced by and provides resistance to lysozyme (Ho *et al*., 2011, Guariglia-Oropeza & Helmann, 2011).

In this study, we investigated the roles of ECF σ factors in providing intrinsic resistance to β-lactam antibiotics and, in particular, to cefuroxime (CEF). We found that σ^M plays a primary role in β-lactam resistance, with σ^X as a secondary determinant. We identified Tn7 insertions mutations that restored CEF resistance to a *sigM* mutant. Genetic analysis reveals a central role for the recently identified signal molecule cyclic-di-AMP (c-di-AMP), synthesized in part by a σ^M-activated diadenylate cyclase (DAC), in cell wall homeostasis.

In addition, our results highlight the key role of previously defined pathways by which ECF σ factors regulate autolysin activity and resistance to reactive oxygen species.

Results and Discussion

σ^M is the major ECF σ factor involved in the intrinsic resistance to cefuroxime

Previously, we showed that a null mutant lacking all 7 ECF σ factors (strain Δ7ECF) has higher sensitivity to numerous antibiotics (including several β-lactams) compared to the wild type (WT) strain (Luo *et al*., 2010). To clarify the role of ECF σ factors in mediating the intrinsic resistance to β-lactam antibiotics, we here sought to identify both the ECF σ factor(s) and the relevant pathways responsible for resistance using cefuroxime (CEF) as a model ß-lactam.

Isogenic strains carrying single or multiple mutations in genes encoding ECF σ factors were tested for CEF susceptibility using disk diffusion and minimal inhibition concentration (MIC) assays. A *sigM* null mutant showed elevated sensitivity to CEF, whereas other single mutants showed little or no change (Fig. 2). The double *sigM sigX* mutant displayed high sensitivity equivalent to the Δ7ECF strain. A *sigW* mutant showed no effect, although effects on ß-lactam resistance have been seen in other *B. subtilis* strain backgrounds (Lee *et al*., 2012). None of the other four ECF σ factors played a role in CEF resistance, even when a multiple mutant strain was tested (Fig. 2). We conclude that σ^M is the major ECF σ involved in the intrinsic resistance to CEF, with σ^X playing a secondary role apparent in strains lacking σ^M. These results suggest that the major resistance pathway(s) depend exclusively on σ^M for their expression, with one or more additional pathways that can be activated by either σ^M or σ^X (as revealed in the double *sigM sigX* mutant). As described previously, several ECF σ factor promoters can be recognized by more than one ECF σ factor (Huang *et al*., 1998, Qiu & Helmann, 2001, Mascher *et al*., 2007). As we will show later in this study, genes encoding the transcription factors Abh and Spx are recognized by both σ^M and σ^X and are involved in CEF resistance.

Antibiotic resistance pathways are often transcriptionally activated in the presence of the cognate antibiotic. ECF σ factors typically autoregulate their own expressions and we and others have previously characterized the relevant autoregulatory promoters (Helmann, 2002, Asai *et al*., 2003, Thackray & Moir, 2003). We therefore monitored the effect of CEF on expression from the autoregulatory promoters for *sigM*, *sigW*, and *sigX*. In each case, a 2~3 fold induction was observed (Table 1). In contrast, low (basal) activity and no induction were detected for the other four ECF σ factors (*sigY, sigV, sigZ, ylaC*) (data not shown). This induction profile is consistent with prior results demonstrating that σ^M, σ^X and σ^W are responsive to cell envelope stress and are activated by an overlapping set of inducers (Mascher *et al*., 2007, Eiamphungporn & Helmann, 2008, Hachmann *et al*., 2009, Minnig *et al*., 2003).

CEF targets PBP1, 2a, 2b and 4

The σ^M regulon is known to include several enzymes involved in various aspects of cell wall synthesis including one HMW PBP (PBP1, encoded by *ponA*) (Eiamphungporn & Helmann, 2008). In most cases, σ^M-dependent promoters serve to up-regulate gene expression in response to stress, but are not solely responsible for expression due to the presence of other promoters. In the case of *ponA*, this gene can be transcribed from two promoters: one is σ^M dependent, and the other is σ^A-dependent. We here hypothesized that one mechanism of resistance might be the σ^M-dependent upregulation of PBP1 or other factors involved in assembly or function of cell wall biosynthetic complexes.

To identify the targets of CEF, we performed bocillin-FL competitive labeling assays (Zhao *et al*., 1999, Kawai *et al*., 2009). Five HMW PBPs (PBP1, 2a, 2b, 2c, 4) and one low molecular weight penicillin-binding protein (LMW PBP) (PBP5) were detected by bocillin-FL labeling and CEF competed with bocillin-FL for binding to PBP1, 2b, 2c and 4 (Fig. 3). Since only six PBPs can be detected in this assay, it is possible that other PBPs are also targets for CEF. No differences in either PBP profile or relative affinity for CEF binding were apparent in a comparison of the CEF sensitive *sigMWX* mutant and the WT strain using the bocillin-FL labeling assay (data not shown). This suggests that mutants lacking ECF σ factors are not altered in their CEF susceptibility due to a gross change in the levels of PBPs.

Since PBP1 is a target for CEF, we hypothesized that σ^M-mediated upregulation of PBP1 might contribute to β-lactam resistance. However, deletion of *ponA* did not alter CEF susceptibility (a *ponA* null mutant and WT have an identical zone of inhibition). Thus, upregulation of PBP1 by σ^M does not appear to be a major mechanism of CEF resistance. We next tested whether *B. subtilis* expresses β-lactamase using the chromogenic substrate nitrocefin (Ross *et al*., 2009). No β-lactamase activity could be detected (either prior to or after CEF treatment) in the WT, *sigMWX* or Δ7ECF strains (data not shown). Thus, the role of ECF σ factors in CEF resistance does not appear to be due to alterations in CEF targets or due to degradation by β-lactamases.

Tn7 mutagenesis reveals multiple pathways involved in CEF resistance

To gain insights into the pathways contributing to CEF resistance, we performed Tn7 transposon mutagenesis and selected for mutations that restored CEF resistance to a *sigM* mutant. The Tn7 transposon derivative we used harbors an outward-facing, xylose-inducible promoter which thereby allows recovery of both loss of function (gene disruption) and gain of function (xylose-dependent up-regulation) mutations (Bordi *et al*., 2008). Insertion libraries were generated *in vitro* using WT genomic DNA as a target and then transformed into competent *B. subtilis* cells with selection for both the transposon (spc^R) and CEF resistance. In an initial study, we recovered numerous insertions linked to *sigM*. In these strains, a functional copy of *sigM* had been co-transformed into the recipient cells. Although this result confirms the importance of σ^M in CEF resistance, it was otherwise uninformative. Therefore, all subsequent experiments used a Tn7 mutant library generated in a *sigM* mutant (HB10216) background.

A total of 520 CEF resistant colonies were obtained in 10 separate experiments. DNA sequence analysis identified 25 unique insertions localized to 10 different genes (Table 2). All of the insertions increased CEF resistance in a *sigM* mutant, although none restored resistance to WT levels (Table 2). The most frequently observed insertion occurred in *yybT*, an ortholog of a gene recently renamed *gdpP* (see below). We therefore performed an additional round of selection, transforming the s*igM* Tn7 library into a *sigM gdpP* double mutant strain (HB10257). This selection led to the recovery of insertions in two genes (*lytE* and *clpP*). Both triple mutants (*sigM gdpP lytE*::Tn7 and *sigM gdpP clpP*::Tn7) were at least as CEF resistant as WT (Table 2). These results indicate that *gdpP* likely affects a different resistance pathway than *lytE* and *clpP*. Although our selection plates contained xylose, in no case was CEF resistance dependent on xylose suggesting that in each case we have recovered gene disruption mutations that lead to increased CEF resistance.

Genes identified in this suppressor mutation are involved in a variety of pathways and functions (Table 2). We categorized them into three groups using two criteria: (i) direct or indirect involvement in cell wall metabolism, and (ii) mild or strong effect on CEF resistance. The first group included several insertions that inactivated genes directly involved in cell wall metabolism including *lytE, pbpX, tagA*, and *ymdB*. LytE is a major

autolytic endopeptidase in vegetative cells (Margot *et al*., 1998, Smith *et al*., 2000). LytE interacts with the actin-like protein MreBH along the cylindrical part of cell wall and with FtsZ and PBP2b at the division septum. It is, therefore, closely related to cell wall synthesis (Carballido-Lopez *et al*., 2006). The inactivation of *lytE* presumably increases ß-lactam resistance by delaying cell lysis. PbpX is a LMW PBP that is located at the septum during vegetative growth (Scheffers *et al*., 2004). Its function is unknown, although it was shown previously to be activated by σ^X (Cao & Helmann, 2004). YmdB was recently reported to regulate the expression and/or activity of a transcriptional regulator SlrR, which in turn affects the activity of both σ^D and the regulator of biofilm formation, SinR, and likely indirectly modulates autolysin activity (Diethmaier *et al*., 2011). Finally, TagA is a key enzyme in the synthesis of teichoic acids, a major component of the cell wall (Mauel *et al*., 1991, D'Elia *et al*., 2009). The second and third groups of insertions are not directly linked to cell wall homeostasis. The second group, including *kinD, spo0A, qoxAB* and *ssrA* insertions, had relatively mild effects on CEF resistance. Further studies are needed to define the mechanisms of these effects, but in several cases the mutant strains grew more slowly than WT strain under our experimental conditions and this may contribute to their increased ß-lactam resistance (Table 2).

Here, we focus on the third group of mutations (*gdpP*, *rsiX*, and *clpP*) for further analysis since they resulted in strong CEF resistance and have been linked to σ^M and its regulon members. We recovered 11 independent insertions within the 1980 bp coding sequence of *gdpP* (formerly *yybT*). GdpP is a transmembrane protein containing three functional domains: a heme-binding PAS domain, a degenerate GGDEF domain, and a DHH/DHHA1 phosphodiesterase (PDE) domain (Rao *et al*., 2010, Rao *et al*., 2011). The *S. aureus* ortholog has recently been renamed GdpP to indicate that it is a GGDEF domain protein containing phosphodiesterase (Corrigan *et al*., 2011) and we therefore adopt this same designation for *B. subtilis*. RsiX is the anti-σ factor cognate for σ^X. We hypothesized that the *rsiX*::Tn7 insertion increased ß-lactam resistance by upregulation of σ^X. Tn7 insertions in *clpP* led to the highest level of CEF resistance observed in this study (Table 2). ClpP is a component of the Clp protease. In *B. subtilis*, the ClpP proteolytic core can pair with any of the three Clp ATPases (ClpX, ClpC and ClpE) and form a large hetero-oligomeric Clp protease. Clp protease recognizes and degrades a wide range of proteins, including non-native proteins and stress response regulators, and it is therefore involved in multiple cell development and stress response pathways (Frees *et al*., 2007). Here, we present evidence that these three insertion mutations affect three inter-related pathways for CEF resistance (Fig. 1).

The role of σ^X in CEF resistance is in part through regulation of *abh* and *spx*

We hypothesized that the Tn7 insertion in *rsiX* restored CEF resistance by up-regulation of σ^X which, as noted above, plays a secondary role in CEF resistance that becomes important in the absence of σ^M (Fig. 1). As predicted, epistasis experiments indicated that σ^X is downstream of RsiX: a *sigM sigX rsiX* strain was as sensitive to CEF as the *sigM sigX* strain (Fig. 4A).

Since the effect of *sigX* on CEF resistance is greatly enhanced in a *sigM* mutant background (Figs. 2 and 4A), we hypothesized that the relevant genes involved in CEF resistance can be activated by either σ^M or σ^X. The regulons of σ^M and σ^X have been characterized, and several target promoters have been defined that are activated by both ECF σ factors (Eiamphungporn & Helmann, 2008, Cao & Helmann, 2004). We chose six such target operons (*abh*, *spx*, *dltABCDE*, *lytR*, *yceCDEF*, and *bcrC*) for further analysis. In a WT background, only the *spx* null mutant showed increased CEF susceptibility. When introduced into the *sigM* null mutant, the *abh* and *spx* mutations both increased CEF sensitivity (Fig. 4B). The *abh* and *spx* CEF sensitive phenotypes in both *sigM* and *sigMX* background can be complemented using IPTG-inducible *abh* or *spx* alleles, respectively

(Figs. S1 and S2). These results suggest that *spx* and *abh* can account for at least part of the role of σ^X in CEF resistance. We also defined the MIC of single and multiple mutant strains of *sigM*, *abh*, and *spx*. Although their differences in CEF susceptibility are readily detected in the disk diffusion assay (Fig. 4A and B), mutant strains of *sigMX*, *sigM abh*, *sigM abh spx* have the same MIC of 0.03 μg/ml when measured in liquid medium (Table S3). We therefore focus here on the differences observed on solid medium.

Abh is a paralog of AbrB and together these two transition state regulators regulate biofilm formation, autolysin activity, and antibiotic production and resistance (Strauch *et al*., 2007, Murray *et al*., 2009, Luo & Helmann, 2009, Murray & Stanley-Wall, 2010). The transcription of *abh* is dependent on σ^X and σ^M, with σ^X being the major regulator (Huang & Helmann, 1998, Luo & Helmann, 2009). Recently, an *abh* mutant was shown to be sensitive to β-lactam antibiotics ampicillin, carbenicillin, and cephalexin (Murray & Stanley-Wall, 2010). Resistance to ampicillin was restored by inducing the expression of the transcriptional regulator *slrR*, or by inactivating genesencoding major autolysins (*lytC* and *lytF* encoding amidase and DL-endopeptidase, respectively) (Murray & Stanley-Wall, 2010). These results support a model (Fig. 1) in which Abh indirectly activates the expression of SlrR (Murray *et al*., 2009). SlrR forms a heteromeric complex with SinR which represses both the *lytABC* and *lytF* operons (Chai *et al*., 2010b). Thus, σ^X and σ^M play partially redundant roles in ß-lactam resistance by activating Abh, which in turn activates SlrR to enable repression of autolytic enzymes.

Accumulation of Spx can increase CEF resistance

Next, we investigated the genetic basis for increased CEF resistance in the *clpP* mutant strains. Several of the reported phenotypes of *clpP* mutants have been linked to increased accumulation of Spx (Nakano *et al*., 2001, Nakano *et al*., 2003), a global regulator of oxidative stress responses (Zuber, 2009). There are at least four promoters that control expression of Spx, including one activated by σ^M and σ^X (Eiamphungporn & Helmann, 2008). Previously, we determined that *spx* was transcriptionally activated ~3-fold by vancomycin in a σ^M-dependent manner (Eiamphungporn & Helmann, 2008) and a similar induction was also reported by Jervis *et al*. (2007) using *lacZ*-fusions. Other cell wall antibiotics also induce the Spx regulon including amoxicillin (Hutter *et al*., 2004, Eiamphungporn & Helmann, 2008) and enduracidin (Rukmana *et al*., 2009).

β-lactam antibiotics trigger the production of ROS (Kohanski *et al*., 2007, Gusarov *et al*., 2009), and Spx is known to protect against oxidative stress (Nakano *et al*., 2003, Choi *et al*., 2006, Pamp *et al*., 2006, You *et al*., 2008). We therefore hypothesized that the upregulation of Spx by σ^M might provide a pathway by which ECF σ factors contribute to antibiotic resistance (Fig. 1). Indeed, in *S. aureus* mutation of the adaptor protein YjbH was recently found to lead to a modest increase in β-lactam resistance which may be due to stabilization of Spx (Gohring *et al*., 2011).

We used a genetic approach to explore the role of ClpP and Spx in ß-lactam resistance. As noted above (Table 2), a *clpP*::Tn7 mutation greatly increased CEF resistance in the *sigM gdpP* mutant strain (HB10264). The *clpP* null mutation also increased CEF resistance in WT and null mutant strains of *sigM* and both *sigM* and *sigX* (Fig. 4C). Spx is a ClpXP substrate (Nakano *et al*., 2002). The *spx* mutation masked the effect of *clpP* in the WT, *sigM*, and *sigM sigX* strain backgrounds (Fig. 4C). These epistasis results imply that *spx* is downstream of *clpP* in the CEF resistance pathway and is the major ClpP substrate that plays a role in ß-lactam resistance. Thus, we predict that the major impact of the *clpP* mutation is to enhance accumulation of Spx in the cell. To test this idea, an IPTG inducible copy of *spx* or spx^{DD} (a Clp protease insensitive variant; Nakano *et al*., 2003) was introduced in the *sigM* and *sigM sigX* mutant strains. An increase in CEF resistance was observed when either *spx* or spx^{DD}

was induced (although the effect was much more dramatic with the protease-insensitive allele), suggesting that the accumulation of Spx can increase resistance to CEF in *B. subtilis* (Fig. S2). In addition, we performed disk diffusion assays with strains lacking either *clpX* or *clpC* (Fig. S3). Deletion of *clpX* can strongly increase CEF resistance in both strain backgrounds of WT and *sigM* mutant, while deletion of *clpC* only showed minor effect. This result is consistent with the major role of ClpP in CEF resistance being the ClpXP-dependent degradation of Spx.

We also note that the effect of the *clpP* mutation may not be limited to enhancing accumulation of Spx, since mutation of *clpP* also led to a small increase in CEF resistance in an *spx* mutant background. This effect was most notable in strains mutant for *sigM* or *sigM* and *sigX* (Fig. 4C). A small increased in CEF resistance was also found with a *clpC* mutant (Fig. S3). Therefore, we suggest that there are other ClpP protease substrates that also contribute, albeit modestly, to CEF resistance. One candidate is SlrR which, as noted above, has been implicated in the down-regulation of autolysins and is subjected to degradation by ClpCP (Chai *et al*., 2010a) (Fig. 1). A second candidate and a ClpCP-degraded substrate is MurAA. MurAA is a UDP-N-acetylglucosamine 1-carboxyvinyltransferase, which catalyzes the first committed step in PG biosynthesis (Kock *et al*., 2004).

c-di-AMP as an emerging second messenger found in Bacteria

The most frequent insertions recovered in our selection (Table 2) were in *gdpP* and inactivate a PDE known to degrade c-di-AMP, an emerging second messenger found in Bacteria and likely in Archaea (Romling, 2008). c-di-AMP was discovered as a metabolite bound in the crystal structure of DisA which catalyzes its synthesis from ATP (Witte *et al*., 2008). DisA was initially characterized as a DNA integrity scanning protein that signals the integrity of the DNA and thereby enables sporulation to proceed (Bejerano-Sagie *et al*., 2006). This led to a model in which the DisA diadenylate cyclase (DAC; DUF147 domain) signals chromosome integrity: DAC activity can be strongly inhibited by binding of DisA to branched chain nucleic acid structures that might form as recombination intermediates.

DisA is the only confirmed c-di-AMP cyclase (DAC) in *B. subtilis* (Witte *et al*., 2008, Oppenheimer-Shaanan *et al*., 2011). However, *B. subtilis* encodes two additional candidate DAC proteins (containing DUF147 domains): YbbP and YojJ (Romling, 2008). The DisA DAC domain is linked to a helix-hairpin-helix non-specific DNA-binding domain which allows DAC activity to be regulated by DNA integrity. In contrast, YbbP is predicted to be membrane-localized and YojJ cytosolic, but little is known of how their activities might be regulated. Of relevance to the present study, transcription of *disA* is regulated by both σ^A and σ^M (Eiamphungporn & Helmann, 2008).

The level of c-di-AMP in the cell is controlled by both its rate of synthesis by DAC and its degradation by a c-di-AMP specific phosphodiesterase (PDE) (Fig. 1). *B. subtilis* GdpP (formerly YybT) is a c-di-AMP PDE *in vitro (*Rao *et al*., 2010, Rao *et al*., 2011) and *in vivo* (Oppenheimer-Shaanan *et al*., 2011). In vegetatively growing *B. subtilis*, 1.7 μM c-di-AMP was measured which increased, in a DisA-dependent manner, to near 5 μM early during sporulation. A *gdpP* deletion strain of *B. subtilis* was shown to have a >4-fold increase in c-di-AMP levels in early sporulating cells (Oppenheimer-Shaanan *et al*., 2011). Similarly, a ~15 fold increase was observed with a *gdpP* mutation in *S. aureus* (from 2.8 μM to 42.9 μM). In *S. aureus*, elevated levels of c-di-AMP suppress the growth defects associated with an inability to synthesize LTA and alter both autolysin expression and the level of PG crosslinking (Corrigan *et al*., 2011).

In *B. subtilis*, the synthesis and degradation of c-di-AMP is correlated with ß-lactam resistance

GdpP is a transmembrane protein with three functional domains: a heme-binding PAS domain, a degenerate GGDEF domain, and a DHH/DHHA1 PDE domain (Rao *et al*., 2010, Rao *et al*., 2011). In accordance with the emerging model of c-di-AMP as a signal molecule, we hypothesized that it was the loss of GdpP PDE activity that conferred CEF resistance. We therefore complemented the *sigM gdpP* strain with an IPTG-inducible GdpP, a truncated GdpP lacking the DHH/DHHA1 domain ($GdpP_{1-303}$), or a mutated GdpP ($GdpP_{D420A}$) carrying a single amino acid substitution which abolishes PDE activity (Rao *et al*., 2010). Induction of WT GdpP conferred an extreme CEF sensitivity (Fig. 5). In contrast, neither of the mutant GdpP proteins increased sensitivity to CEF (Fig. 5), suggesting that it is the PDE activity that affects CEF sensitivity.

One consequence of antibiotic stress is the activation of σ^M which leads to elevated expression of the DisA DAC. We therefore hypothesized that a *sigM* null mutant might have decreased c-di-AMP levels that could be compensated by mutation of GdpP, the c-di-AMP degrading PDE. Indeed, a *disA* deletion mutant displayed a small but reproducible increase in sensitivity to CEF, with an MIC of 3 μg/ml compared to 4 μg/ml for WT (Table S3). This is consistent with the recent report that DisA accounts for perhaps 50% of the c-di-AMP present in cells as monitored early in sporulation (Oppenheimer-Shaanan *et al*., 2011). As expected, the induction of GdpP in the *disA* mutant led to a large increase in CEF susceptibility (Fig. 6), consistent with the notion that even *disA* cells contain substantial c-di-AMP that contributes to CEF resistance. This suggests that *B. subtilis* contains at least one additional DAC, presumably encoded by either or both the DAC-domain containing proteins YbbP and YojJ.

c-di-AMP is essential for cell growth

To gain insights into the relative contributions of *disA*, *ybbP*, and *yojJ* to c-di-AMP synthesis we mutated each of these loci individually and in combination. Deletion of *ybbP* resulted in the highest CEF sensitivity (as seen in the uninduced sample in Fig. 6, and MIC of 1 μg/ml, Table S3). Deletion of *yojJ*, however, had no effect. Induction of GdpP increased CEF sensitivity in all three DAC mutant backgrounds (Fig. 6). We conclude that YbbP is the major DAC contributing to intrinsic β-lactam resistance in growing cells, and that both synthesis and degradation of c-di-AMP affects CEF resistance. This result is consistent with the recent suggestion that DisA functions primarily in early sporulation, with a comparatively minor contribution in (unstressed) vegetative phase cells (Oppenheimer-Shaanan *et al*., 2011). It is interesting to note that YbbP and GdpP are both membrane-localized, although the signals that might control their synthesis and activity are unknown.

The expression of YbbP is poorly characterized, but it is noteworthy that it is encoded immediately downstream of the *sigW-rsiW* operon and it may be, in part, activated by σ^W. However, σ^W has no effect in CEF resistance in our *B. subtilis* WT strain background (Fig. 2). We therefore asked whether σ^M or σ^X have a role in regulating *ybbP*. Multiple null mutants of *sigM*, *sigX*, and *ybbP* were constructed and tested for their susceptibilities to CEF. The mutation in *ybbP* is clearly additive to both *sigM* and *sigX* mutations (Fig. S4). In addition, the transcriptional start site of *ybbP* was mapped to 72 bp upstream of its start codon using 5′RACE. A σ^A promoter is present upstream of the assigned start site (TTCACTtgctaaatcgaaatgtggTATAATgggctcG; upper case letters indicate the −35, −10, +1 regions, respectively). Together, these results suggest that *ybbP* is not part of the σ^M or σ^X regulatory pathways.

We next sought to construct double and triple null mutants of *disA*, *ybbP*, and *yojJ*. A *disA ybbP* double mutant strain could not be obtained, suggesting that this combination of mutations is lethal, whereas double mutants of *disA yojJ* and *ybbP yojJ* were viable. We conclude that c-di-AMP is essential for viability and that the basal level of expression of either DisA or YbbP is sufficient to support growth. An essential role for DAC proteins has also been suggested in *Listeria monocytogenes* since it was impossible to disrupt the single DAC encoding gene in this organism (Woodward *et al*., 2010). Similarly, DAC genes were identified in screens for essential genes in *Mycoplasma* spp., *Streptococcus pneumoniae*, and *S. aureus* (Chaudhuri *et al*., 2009, Glass *et al*., 2006, French *et al*., 2008, Song *et al*., 2005).

To determine whether all three DAC proteins (DisA,YbbP, YojJ) are active and could support growth, we integrated an IPTG-inducible copy of each gene into a *ybbP* null mutant and then attempted to introduce a *disA* null mutation by chromosomal transformation. Indeed, a *disA ybbP* double mutant could be obtained when any one of the three genes (*disA, ybbP, or yoj*) was induced (Fig. 7A). This strategy also allowed construction of IPTG-dependent *disA ybbP yojJ* triple mutant strains in which growth could be supported by any one of three DAC-encoding genes. We note that the Pspac(hy) promoter used in this work is slightly leaky and, as a result, the *disA ybbP* $P_{spac(hy)}$-*disA* strain was able to grow even in the absence of IPTG. However, the *disA ybbP* $P_{spac(hy)}$-*yojJ* strain grew slowly and the *disA ybbP* $P_{spac(hy)}$-*ybbP* was unable to grow unless at least 50 μM IPTG was present (data not shown). These results suggest that all three of these putative DAC proteins are biologically active and able to support growth when expressed.

The essential role of c-di-AMP is linked to PG homeostasis

Since a reduced level of c-di-AMP is linked to high CEF sensitivity, we tested whether c-di-AMP is involved in cell wall homeostasis. Depletion of c-di-AMP in strain *disA ybbP* $P_{spac(hy)}$-*ybbP* by growth in the absence of inducer IPTG led to cell lysis as monitored both by following optical density (Fig. 7B) and by light microscopy (Fig. S5). The lysis phenotype can be suppressed either by the presence of IPTG (inducing the expression of *ybbP*), or by supplementation of the growth medium with SMM (sucrose, $MgSO_4$ and maleic acid), sucrose, or $MgSO_4$. SMM has been used previously to stabilize protoplasts and support the growth of cell wall-free L-form cells (Chang & Cohen, 1979, Leaver *et al*., 2009). Similarly, sucrose likely functions as an osmotic protectant, and Mg^{2+} has been shown to restore growth and WT morphology of many PG defective mutants including single mutants of *ponA, rodA, mreB, mreC, mreD, mbl* and a double mutant of *pbpAH* (Murray *et al*., 1998, Formstone & Errington, 2005, Leaver & Errington, 2005, Kawai *et al*., 2009, Schirner & Errington, 2009, Kawai *et al*., 2011). This is reminiscent of recent results from Corrigan *et al*. (2011) who showed that osmotic protectants support the growth of a LTA deficient mutant of *S. aureus* and that this requirement can be bypassed by a *gdpP* mutation. The *S. aureus gdpP* mutant displayed an increase in both c-di-AMP and PG cross-linking. Collectively, these results suggest that c-di-AMP plays an essential role in PG homeostasis (Fig. 1).

σ^M and c-di-AMP are involved in resistance to other cell wall antibiotics

We next tested whether c-di-AMP is involved in resistance to other antibiotics. Induction of GdpP in strain *sigM gdpP* $P_{spac(hy)}$-*gdpP* leads to high sensitivity to aztreonam, cefixime, and moenomycin in addition to CEF as monitored using disk diffusion assays (Fig. 8). Cefixime is a third generation cephalosporin, aztreonam is a monobactam, and moenomycin is a glycolipid. As β-lactams, cefixime and aztreonam target PBP transpeptidases. Moenomycin, on the other hand, targets the TG activity of HMW-PBPs (Lovering *et al*., 2007). Although aztreonam is generally found to have poor activity against Gram positive bacteria (Georgopapadakou *et al*., 1982, Guay & Koskoletos, 1985) we observed using

bocillin-FL labeling that aztreonam can derivatize PBP 1, 2c, and 4 in *B. subtilis* (Fig. 3). As also noted for CEF, mutation of *sigM* converts *B. subtilis* from an aztreonam non-susceptible to a susceptible strain, and this susceptibility is modulated by *gdpP* (Fig. 8). Thus, the function of c-di-AMP is not limited to CEF resistance, as would be expected if it functions to support balanced cell wall synthesis.

A model for the role of ECF σ factors in ß-lactam resistance

The genetic analyses presented herein lead to an integrated model in which the ECF σ factors σ^M and σ^X contribute to ß-lactam resistance by the antibiotic-inducible activation of regulatory proteins that affect three distinct pathways (Fig. 1). *B. subtilis* PG is a dynamic structure, which is continuously synthesized, modified, and hydrolyzed. It is notable that σ^M-activated promoters have been previously mapped preceding several genes involved in PG synthesis (including mreB, bcrC, divIB, divIC, ddl, murB, murF, rodA, pbpX, and *ponA*), one of the four paralogous LTA synthases (*yfnI*), and cell wall modification enzymes (*dltABCDE*) (Eiamphungporn & Helmann, 2008). Thus, σ^M appears to function to positively regulate cell wall assembly and structure in response to antibiotic stress. β-lactam antibiotics inhibit the TP activity of PBPs and thereby inhibit glycan strand cross-linking. This inhibition disrupts the balance between PG synthesis and hydrolysis and endogenous autolysins trigger cell lysis. In addition, ß-lactams trigger ROS formation and cell death. Both autolysin-dependent and independent mechanisms contribute to the bactericidal effect (Dubee *et al*., 2011, Kohanski *et al*., 2007).

ECF σ factors counteract the effects of ß-lactams by activating at least three distinct pathways (Fig.1). First, σ^M contributes to the expression of one of three c-di-AMP synthases (DisA). The cellular level of c-di-AMP is regulated by both DAC synthases (DisA, YbbP and YojJ) and the cognate PDE (GdpP). At least one DAC is required for cell growth, indicating an essential role of c-di-AMP. The cell lysis phenotype of our DAC depletion strain together with the recent report from Corrigan *et al*. (2011) suggest a positive link between c-di-AMP and PG cross-linking. However, the role of c-di-AMP may be not limited to cross-linking, since c-di-AMP also modulates susceptibility to moenomycin, which targets the TG domain of PBP and thereby inhibits the polymerization of the PG glycan strands.

Second, ECF σ factors affect the expression and regulation of autolysins. Both σ^M and σ^X activate the transcription of *abh*, whose product indirectly activates the expression of SlrR, which directly represses expression of LytC and LytF (Luo & Helmann, 2009, Murray & Stanley-Wall, 2010, Chai *et al*., 2010b). Another autolytic endopeptidase (LytE) was identified by Tn7 mutagenesis as a contributor to ß-lactam susceptibility. These findings support the notion that preventing autolysis can increase ß-lactam resistance.

Third, our analysis of the ß-lactam resistance phenotype of a *clpP* null mutant identified Spx, a regulator of pathways that protect the cell against ROS (Zuber, 2009), as a contributor to ß-lactam resistance. The *clpP* mutant strain may also have elevated levels of SlrR, a known inhibitor of autolysin expression (Chai *et al*., 2010a). Although the model we have developed here (Fig. 1) is already quite complex, it certainly underestimates the true complexity of the adaptive responses mediated by ECF σ factors and other regulators that conspire to protect cells against antibiotics and other chemical insults.

Experimental Procedures

Bacterial strains and growth conditions

B. subtilis strains used are derivatives of strain168 (*trpC2*) and are shown in Table 3. *Escherichia coli* strain DH5α was used for standard cloning procedures. Bacteria were

grown in Luria-Bertani (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) broth at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg/ml ampicillin for *E. coli*, and 1 μg/ml erythromycin plus 25 μg/ml of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg/ml chloramphenicol, 100 μg/ml spectinomycin (Spc), 5 μg/ml tetracycline and 10 μg/ml kanamycin for *B. subtilis*. OD_{600} readings were taken on a Spectronic 21 spectrophotometer.

Strain Constructions

Gene deletions were generated by replacing the coding region with an antibiotic resistance cassette using long flanking homology PCR (LFH-PCR) followed by DNA transformation as previously described (Mascher *et al*., 2003). Chromosomal DNA transformations were performed as described previously (Harwood & Cutting, 1990).

The IPTG inducible constructs were generated using vector pPL82 (Quisel *et al*., 2001). PCR products were amplified from *B. subtilis* 168 chromosomal DNA, digested with endonucleases, and cloned into pPL82. pPL82 contains a chloramphenicol resistance cassette, a multiple cloning site downstream of the $P_{spac(hy)}$ promoter, and the *lacI* gene between the two arms of the *amyE* gene. Primer pairs used for PCR amplification are 5249/5250 for *disA*, 5252/5253 for *ybbP*, 5255/5256 for *yojJ*, 5244/5245 for *gdpP*, and 5244/5258 for $gdpP_{1\text{-}303}$. All oligonucleotide sequences are listed in SI Table S1. The sequences of the inserts were verified by DNA sequencing (Cornell DNA sequencing facility). pPL82-$gdpP_{D420A}$ was generated using overlap joining PCR with pPL82-*gdpP* as DNA template. Primer pairs 5244/5293, and 5294/5245 were first used to amplify the up and down fragments of *gdp*, respectively. The $gdpP_{D420A}$ mutation was generated using primers 5293 and 5294. A joining PCR was then performed with the up and down fragments as template and primer pairs 5244/5245. The PCR product was cloned into pPL82 as above, and the insert was verified by DNA sequencing. Plasmids were linearized by ScaI and used to transform *B. subtilis*, where they integrated into the *amyE* locus.

Antibiotic susceptibility tests

Susceptibility tests for antibiotics were conducted using disk diffusion assay and minimal inhibitory concentration (MIC) test. Mueller Hinton (MH, Sigma-Aldrich) medium was used for both assays. Disk diffusion assays were performed as previously described (Luo *et al*., 2010). The bottom agar is 15 ml MH broth supplemented with1.5% agar, and the top agar is 4 ml MH broth supplemented with 0.75% agar. We used BBL™ Sensi-Disc™ Susceptibility Test Discs (BD; cefixime 5 μg, cefoxitin 30 μg, ceftriaxone 30 μg, ceftazidime 30 μg, cefoperazone 75 μg, amoxicillin 30 μg, ampicillin 10 μg, piperacillin 100 μg, oxacillin 1 μg, piperacillin 100 μg, imipenem 10 μg, meropenem 10 μg, and Isoniazid 1μg) and also prepared disks using Whatman filter paper disks (7 mm in diameter) and freshly made stocks of antibiotics (aztreonam 30 μg, cefuroxime 6 μg , penicillin G 10 U, nalidixic acid 30 μg, novobiocin 250 μg, vancomycin 30 μg, polymycin B 250 μg, and moenomycin 50 μg). The zone of growth inhibition was measured after overnight growth at 37°C. For MIC test, fresh single colonies were first grown in MH broth to an OD_{600} of 0.4, and diluted 1:100 in MH broth, and 200 μl of the diluted culture was dispensed in Bioscreen 100-well microtiter plate. Growth was measured spectrophotometrically (OD_{600}) using a Bioscreen incubator (Growth Curves USA, Piscataway, NJ) at 37°C with vigorous shaking. The absorbance was recorded every 30 minutes for 24 hours. Inhibition was defined as a final OD_{600}<0.2 at the 12 hour time point (after 12 h, suppressor mutants started to grow up). All antibiotics susceptibility tests were performed with biological triplicates and repeated at least twice.

Bocillin-FL competitive labeling assay

The bocillin-FL labeling assay was performed as previously described (Zhao *et al*., 1999, Kawai *et al*., 2009) with modifications. Overnight cultures of *B. subtilis* cells in LB were diluted 1:100 into 5 ml fresh LB broth, and incubated at 37°C with vigorous shaking. When cell cultures reached mid-log phase (OD_{600} 0.4), the cultures were treated with either 0.05 μg/ml (final conc.) of bocillin-FL, or with additional challenge of 0.00625μg/ml (final conc.) of CEF, or an additional 5 μg/ml aztreonam (final conc.) for 10 min. The cells were pelleted by centrifugation and kept at −20°C overnight. The pellet was thawed on ice and resuspended in 50 μl 0.85% NaCl. The cell resuspension was boiled for 5 min with SDS loading buffer, and proteins were separated by 4~12% SDS-PAGE. To visualize the labeled PBPs, the gels were scanned with a Molecular Dynamics Typhoon PhosporImager (excitation at 488 nm and emission at 530 nm), and the images were analyzed using ImageQuant TL (Amersham Biosciences).

Tn7 mutagenesis

The Tn7 mutagenesis libraries were generated with chromosomal DNA using *in vitro* transposition as described (Bordi *et al*., 2008). The library DNA was transformed into WT *B. subtilis* or a *sigM* mutant strain (HB10216), and the resulting transposants were grown in the presence of 100 μg/ml spectinomycin (Spc) with and without xylose (final concentration of 1%). Chromosomal DNA was prepared from these cultures using phenol-chloroform extraction (Sambrook & Russell, 2001) and considered an amplified Tn7 library. The amplified Tn7 library DNA was transformed into the *sigM* mutant strains (HB10016 or HB10216), and cells were plated on LB agar supplemented with 100 μg/ml Spc, 1% xylose and 2 μg/ml CEF (32 x MIC of the *sigM* strain). Resulting transformants were streaked onto the same selection plate twice. In order to confirm that the increased CEF resistance was due to the presence of the transposon, we performed linkage tests by transforming the chromosomal DNA of the Tn7 mutants into the *sigM* mutant again and selecting with 100 μg/ml Spc. The resulting transformants (20 colonies for each strain) were then streaked on LB agar supplemented either with 100 μg/ml Spc or with 100 μg/ml Spc plus 2 μg/ml CEF. The transformants that can grow on both plates were counted as linked mutants, and strains with 100% linkage were subjected to Tn7 insertion position mapping using arbitrary PCR as previously described (Bordi *et al*., 2008). The dependence on xylose was tested by streaking cells on LB agar supplemented with 2 μg/ml CEF or with 2 μg/ml CEF plus 1% xylose. Tn7 mutagenesis with strain *sigM gdpP* (HB10257) was performed as described above, except that 4 μg/ml of CEF (MIC of the WT strain, and 64 x MIC of the *sigM* strain) was used for selection.

β-galactosidase activity test

Strains harboring ECF σ promoter-*lacZ* fusions were grown overnight in LB broth containing appropriate antibiotics and diluted 1:100 into 5 ml LB medium. The culture was grown at 37°C with vigorous shaking to OD_{600}~0.4 (mid-log growth phase), and then split into two aliquots. One was challenged with 8 μg/ml of CEF and the other was untreated. The cultures were returned to 37°C, and samples were collected after 30 min. β-galactosidase assays were performed as described by Miller (26), and each strain was tested in biological triplicates and repeated three times. Data were reported as the mean and SE.

5′-RACE

The transcriptional start site of *ybbP* was determined using 5′ rapid amplification of cDNA ends (5′-RACE). Five micrograms of total RNA from a mid-log-phase LB culture was reversed transcribed to cDNA using TaqMan reverse transcription reagents (Roche) and oligo *ybbP*-rev-GSP3 (5584) as primer. The 3′ end of cDNA was tailed with poly-dCTP

using terminal deoxynucleotidyl transferase (New England Biolabs). The tailed cDNAs were then amplified by PCR with primers AAP (3314) and *ybbP*-rev-GSP4 (5585). The PCR products were subjected to DNA sequencing (Cornell DNA sequencing facility).

Growth rate test

Fresh single colonies were first grown in MH broth to OD_{600} of 0.4, and diluted 1:100 in MH broth, and inoculated in Bioscreen microtiter plates with a total inoculum of 200 μl. Growth was measured spectrophotometrically (OD_{600}) using a Bioscreen incubator (Growth Curves USA, Piscataway, NJ) at 37°C with vigorous shaking. The specific growth rate of each strain was calculated from the exponential growth phase. Each test was performed with biological triplicates and repeated twice.

Depeletion of c-di-AMP and microscopic imaging

Strain HB10359 was grown in MH broth supplemented with 1mM IPTG to mid-exponential phase, and collected by centrifugation. The cells were washed twice with MH medium, and resuspended to OD_{600} of 0.2 in fresh MH broth, or MH broth supplemented with 1mM IPTG, SMM (20 mM $MgCl_2$, 10% sucrose, 20 mM maleic acid, pH 7.0), 10% sucrose, or 10mM $MgSO_4$. 200ul of each cell resuspension was added a Bioscreen microtiter plate, and incubated at 37°C with vigorous shaking. For phase contrast and fluorescence microscopy, 1μg/ml (final concentration) of cell membrane stain FM 4-64 (Invitrogen) was added to the cell culture, and incubated at 37 °C for 30min with shaking. 5 μl of cells were then mounted on microscope slide coated with a thin film of 1% agarose as previously described in (Glaser *et al*., 1997). Microscopy was performed using an Olympus BX61 epifluorescence microscope. Images were acquired using Cooke SensiCam and Slidebook software (Intelligent Imaging Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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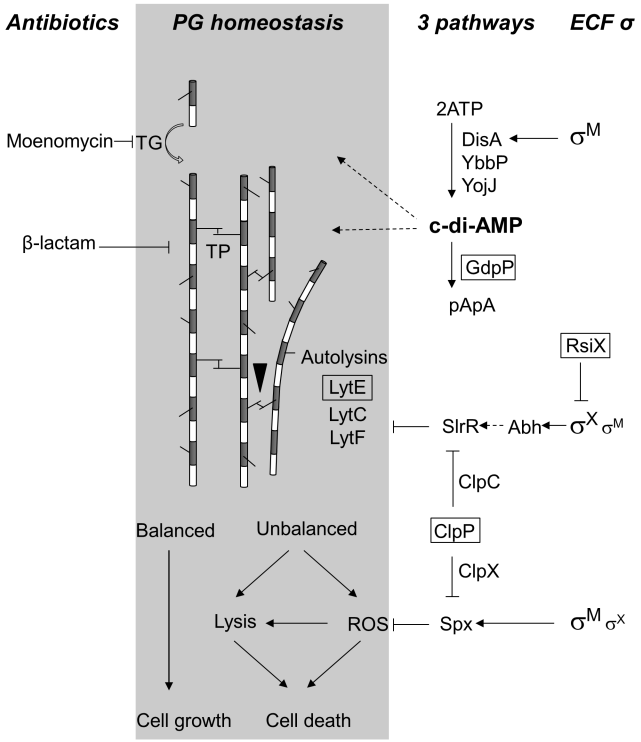
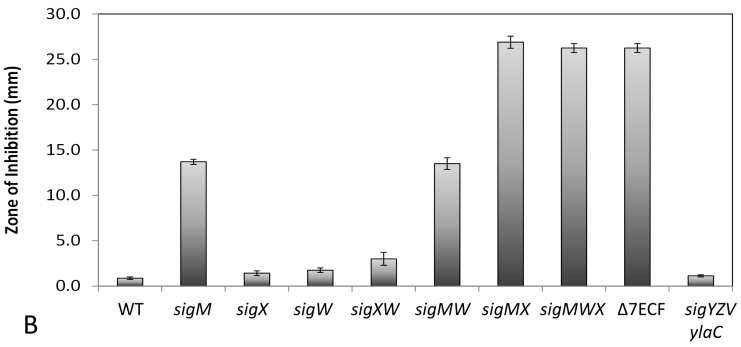


Fig. 1.

Model of peptidoglycan (PG) homeostasis and the contributions of σ^M and σ^X to cell wall antibiotic resistance. The alternating grey and white bars represent N-acetylmuramic acid and N-acetylglucosamine, respectively, which comprise the glycan chains. Peptide crosslinks between strands are introduced by transpeptidation (TP) and are broken by autolytic endopeptidases (black triangle). Moenomycin targets the transglycosylation (TG) step in glycan chain elongation while ß-lactams inhibit TP-mediated crosslinking. The results reported herein, combined with previous results (see text), indicate that σ^M and σ^X contribute to antibiotic resistance by three distinct pathways as shown on the right. Genes identified by Tn7 mutagenesis are boxed. ROS, reactive oxygen species; straight arrow, direct positive regulation; dashed arrow, indirect positive regulation; ---| negative regulation.

A



B

MIC (μg/ml)	4	0.06	2	4	0.5	0.06	0.03	0.03	0.015	4

Fig. 2.

σ^M is the major ECF σ involved in the intrinsic resistance to CEF and σ^X plays a secondary role. **A**. The susceptibility of each strain was tested using disk diffusion assay with 6 μg CEF. The zone of inhibition is expressed as the total diameter of the clearance zone minus the diameter of filter paper disk (7mm). The means and SE from at least 3 biological replicates are reported. **B**. MIC values are shown under the bar graph.

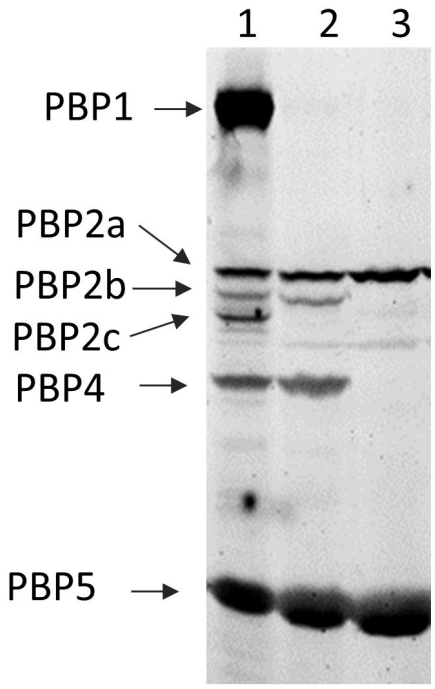


Fig. 3.
CEF binds to PBP 1, 2b, 2c, and 4. PBPs in vegetatively growing cells were labeled with bocillin-FL (lane 1). The binding of bocillin-FL to PBPs was subjected to competitive inhibition by the addition of aztreonam (Lane 2) or CEF (Lane 3). Proteins were separated by 4~12% gradient SDS-PAGE, and visualized by using a Typhoon Fluorimager.

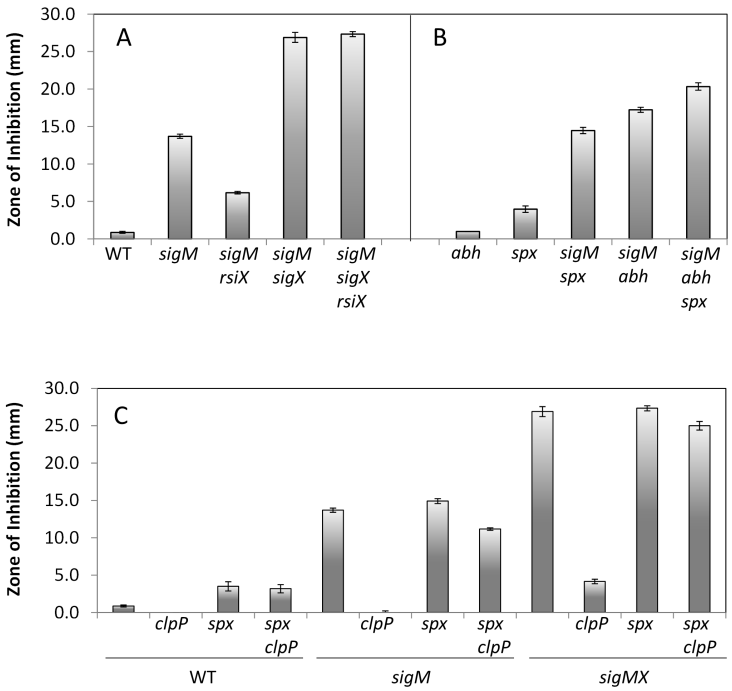


Fig. 4.

The role of *rsiX* and *clpP* mutations in CEF resistance. **A**. Increased CEF resistance due to an *rsiX* null mutation depends on σ^X. **B**. *abh* and *spx* mutations are additive to *sigM* with respect to CEF sensitivity. **C**. Increased CEF resistance due to a *clpP* null mutation depends on Spx in all three strain backgrounds. For ease of comparison, some strains are shown in multiple panels. The susceptibility of each strain was tested using disk diffusion assays with 6 μg CEF. The means and SE at least from 3 biological replicates and 2 independent experiments are reported.

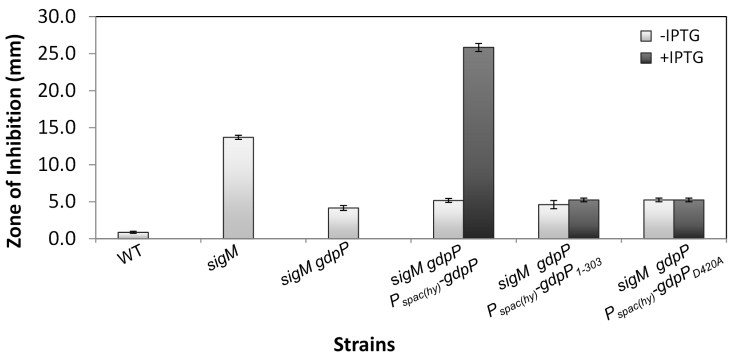


Fig.5.

The DHH/DHHA1 domain of GdpP is required to restore CEF sensitivity to the resistant strain *sigM gdpP*. Disk diffusion tests were performed with 6 μg CEF. The means and SE based on 3 biological replicates and 2 independent experiments are shown. 1 mM IPTG was added where indicated.

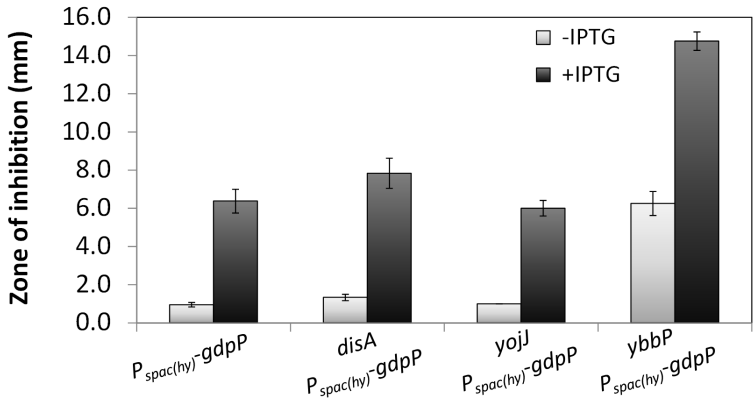


Fig.6.

Induction of the GdpP PDE increases CEF sensitivity in WT and cells individual DAC enzymes. Disk diffusion tests were performed with 6 μg CEF. The means and SE based on 3 biological replicates and 2 independent experiments are reported. 1 mM IPTG was added where indicated.

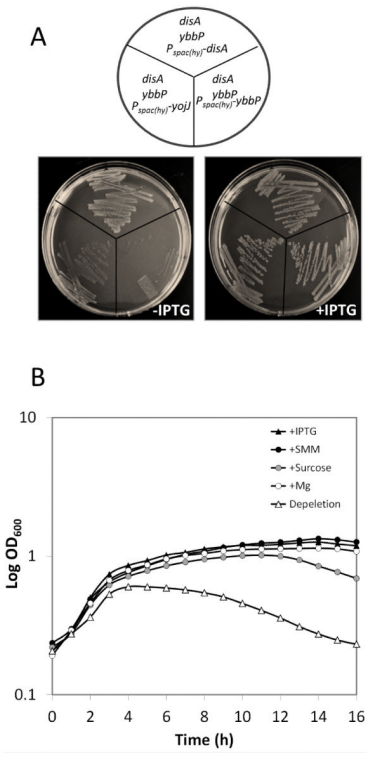


Fig.7.

disA and *ybbP* are synthetically lethal. **A**. Strains of *disA ybbP* harboring IPTG inducible *disA*, *ybbP* or *yojJ* were grown on MH agar plates supplemented with or without 1 mM IPTG. **B.** Depletion of *ybbP* in strain *disA ybbP* $P_{spac(hy)}$-*ybbP* results in cell lysis. Cells were grown in presence of 1 mM IPTG to mid-log phase, washed, resuspended in fresh MH medium alone or with additional 1 mM IPTG, SMM, 10% sucrose or 10 mM Mg, and returned to 37°C incubation with vigorous shaking. Growth was measured by OD_{600} using a Bioscreen incubator. Ten biological replicates were tested, and showed similar growth pattern. Growth curves from one representative experiment are shown.

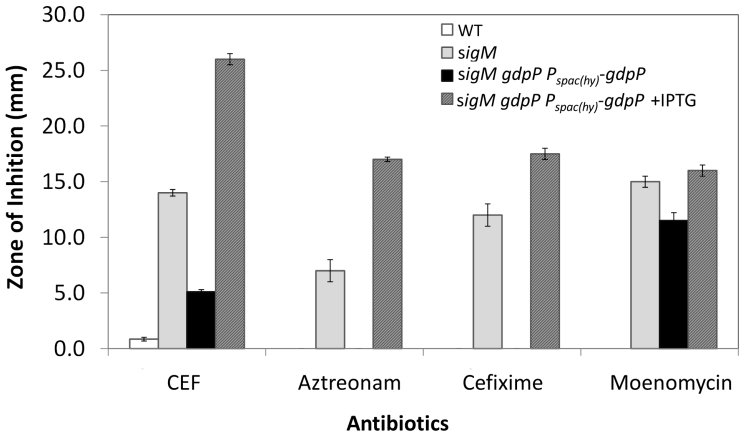


Fig. 8.

c-di-AMP is involved in intrinsic resistance to other cell wall antibiotics. Disk diffusion tests were performed with CEF (6 μg), aztreonam (30 μg), cefixime (5 μg) and moenomycin (50 μg). The means and SE from biological triplicates are shown. Note that no zone of inhibition could be detected with aztreonam or cefixime in WT and the uninduced s*igM gdpP* $P_{spac(hy)}$-*gdpP* strain.

Table 1

ECF σ promoter activities induced after treatment with 8 μg/ml CEF for 30 min

Activities (Miller Units) were measured using β-galactosidase assays and the means and SE are reported.

Reporter fusion	Untreated	CEF treated
P_{sigM}-lacZ	3.7±0.5	10.1±0.5
P_{sigX}-lacZ	38.6±1.3	99.7±3.5
P_{sigW}-lacZ	35.6±1.9	71.3±2.3

Table 2

Tn7 insertions that can restore CEF resistance in a *sigM* or a *sigM gdpP* mutant.

Tn7 Mutants	Unique insertions	Gene annotation	Resistance to CEF[a]	Growth rate relative to WT (%)[b].
Insertions in a sigM background				
gdpP::Tn7	11	phosphodiesterase	++	98
rsiX::Tn7	1	anti-sigma X	++	100
lytE::Tn7	1	autolysin	++	98
pbpX::Tn7	1	penicillin-binding endopeptidase X	++	95
tagA::Tn7	1	wall teichoic acid biosynthesis	++	81 *
ymdB::Tn7	1	Regulate expression of SlrR	+	68 *
kinD::Tn7	1	negative regulator of Spo0A~P	+	95
spo0A::Tn7	2	initiation of sporulation	+	102
qoxAB:Tn7	3	cytochrome aa3-600 quinol oxidase	+	52 *
ssrA::Tn7	1	transfer-messenger RNA (tmRNA)	+	79 *
Insertions in a sigM gdpP background				
lytE::Tn7	2	autolysin	+++	96
clpP::Tn7	2	ATP-dependent Clp protease proteolytic subunit	++++	81 *

[a]The resistance to CEF was tested using disk diffusion assay with biological triplicates, and repeated twice. The zone of inhibition (mean ± SE) was used for the score. The resistance level of wt is defined as "+++", and Δ*sigM* is "-".

[b]The *sigM* strain has the same growth rate as WT (100%). Strains with noticeably reduced growth rates are labeled with *.

Table 3

Strains used in this study

Strain[1]	Genotype	Reference / construction[2]
168	*trpC2*	lab strain
CU1065	*trpC2 attSPβ*	lab strain
PS832	Prototrophic revertant of strain 168	lab strain
BSU2007	168 *sigMWXYZV ylaC* (Δ7ECF)	(Asai *et al*., 2008)
HB0031	CU1065 *sigM::kan*	(Cao *et al*., 2002)
HB10216	168 *sigM::kan*	chrDNA of HB0031 -->168
HB10016	168 *sigM::tet*	(Luo & Helmann, 2009)
HB10103	168 *sigX::kan*	(Luo & Helmann, 2009)
HB10102	168 *sigW::mls*	(Luo & Helmann, 2009)
HB10114	168 *sigX::kan, sigW::mls*	(Luo & Helmann, 2009)
HB10117	168 *sigM::tet, sigW::mls*	(Luo & Helmann, 2009)
HB10113	168 *sigM::tet sigX::kan*	(Luo & Helmann, 2009)
HB7007	CU1065 *sigX::spc*	(Huang *et al*., 1997)
HB15815	168 *sigM::kan sigX::spc*	chrDNA of HB7007 --> HB10216
HB10107	168 *sigM::tet, sigX::kan sigW::mls*	(Luo & Helmann, 2009)
HB10236	168 *sigZ::kan sigV::cat sigY::mls ylaC::spc*	(Luo *et al*., 2010)
HB5421	CU1065 *amyE::*P_{sigX}*-lacZ cat*	Lab strain
HB5422	CU1065 *amyE::*P_{sigW}*-lacZ cat*	Lab strain
HB5423	CU1065 *amyE::*P_{sigM}*-lacZ cat*	Lab strain
HB10183	168 *amyE::*P_{sigM}*-lacZ cat*	chrDNA of HB5423 --> 168
HB10184	168 *amyE::*P_{sigX}*-lacZ cat*	chrDNA of HB5421 --> 168
HB10185	168 *amyE::*P_{sigW}*-lacZ cat*	chrDNA of HB5422 --> 168
PS2062	PS832 *ponA::spc*	(Popham & Setlow, 1995)
HB10386	168 *ponA::spc*	chrDNA of PS2062 --> 168
HB0047	CU1065 *rsiX::spc*	lab strain
HB10118	168 *rsiX::spc*	chrDNA of HB0047 --> 168
HB10379	168 *sigM::tet rsiX::spc*	chrDNA of HB10118 -->
HB10016	HB10536 CU1065 *sigX rsiX::kan*	LFH -->CU1065
HB10378	168 *sigM::tet sigX rsiX::kan*	chrDNA of HB10536 -->HB10016
HB10131	168 *abh::spc*	(Luo & Helmann, 2009)
HB4728	CU1065 *spx::spc*	lab strain
HB10328	168 *spx::spc*	chrDNA of HB4728 --> 168
HB10348	168 *spx::mls*	LFH -->168
HB10329	168 *sigM::kan spx::spc*	chrDNA of HB4728 --> HB10216
HB15808	168 *sigM::kan abh::spc*	chrDNA of HB10131 --> HB10216
HB15811	168 *sigM::kan abh::spc spx::mls*	chrDNA of HB10348--> HB15808
HB10316	168 *clpP::tet*	LFH-->168
HB10332	168 *spx::spc clpP::tet*	chrDNA of HB10316 --> HB10328
HB10320	168 *sigM::kan clpP::tet*	chrDNA of HB10316 --> HB10216

Strain[1]	Genotype	Reference / construction[2]
HB15814	168 *sigM::kan spx::spc clpP::tet*	chrDNA of HB10316 --> HB10329
HB15816	168 *sigM::kan sigX::spc clpP::tet*	chrDNA of HB10316 --> HB15815
HB15823	168 *sigM::kan sigX::spc spx::mls*	chrDNA of HB10348 --> HB15815
HB15824	168 *sigM::kan sigX::spc spx::mls clpP::tet*	chrDNA of HB10316 --> HB15823
HB10278	168 *amyE:: $P_{spac(hy)}$- gdpP cat*	pPL82-*gdpP* -->168
HB10287	168 *amyE::$P_{spac(hy)}$- gdp_{1-303} cat*	pPL82-*$gdpP_{1-303}$* -->168
HB10309	168 *amyE::$P_{spac(hy)}$- $gdpP_{D420A}$ cat*	pPL82- *$gdpP_{D420A}$* -->168
HB10352	168 *gdpP::mls*	LFH -->168
HB10257	168 *sigM::kan gdpP::mls*	chrDNA of HB10352 --> HB10216
HB10295	168 *sigM::kan gdpP::mls amyE::$P_{spac(hy)}$- gdpP cat*	chrDNA HB10278--> HB10257
HB10298	168 *sigM::kan gdpP::mls amyE::$P_{spac(hy)}$- $gdpP_{1-303}$cat*	chrDNA HB10287 --> HB10257
HB10310	168 *sigM::kan gdpP::mls amyE::$P_{spac(hy)}$- $gdpP_{D420A}$ cat*	chrDNA HB10309 --> HB10257
HB10353	168 *disA::spc*	LFH -->168
HB10334	168 *ybbP::tet*	LFH -->168
HB10335	168 *yojJ::kan*	LFH -->168
HB10365	168 *disA::spc amyE:: $P_{spac(hy)}$- gdpP cat*	chrDNA of HB10278 --> HB10353
HB10366	168 *ybbP::tet amyE:: $P_{spac(hy)}$- gdpP cat*	chrDNA of HB10278 --> HB10334
HB10367	168 *yojJ::kan amyE:: $P_{spac(hy)}$- gdpP cat*	chrDNA of HB10278 --> HB10335
HB10354	168 *disA::spc yojJ::kan*	chrDNA of HB10353 -->HB10335
HB10356	168 *ybbP::tet yojJ::kan*	chrDNA of HB10334 -->HB10335
HB10281	168 *amyE::$P_{spac(hy)}$-disA cat*	pPL82-disA -->168
HB10283	168 *amyE::$P_{spac(hy)}$-ybbP cat*	pPL82-ybbP -->168
HB10285	168 *amyE::$P_{spac(hy)}$-yojJ cat*	pPL82-yojJ -->168
HB10357	168 *disA::spc amyE:: $P_{spac(hy)}$-disA cat*	chrDNA of HB10353 --> HB10281
HB10358	168 *ybbP::tet amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10334 --> HB10283
HB10374	168 *ybbP::tet amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10334 --> HB10285
HB10359	168 *disA::spc ybbP::tet amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10353 --> HB10358
HB10360	168 *disA::spc ybbP::tet amyE:: $P_{spac(hy)}$-disA cat*	chrDNA of HB10334 --> HB10357
HB10375	168 *disA::spc ybbP::tet amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10353 --> HB10374
HB15802	168 *ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10358 --> HB10356
HB15803	168 *ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10374 --> HB10356
HB15801	168 *disA::spc ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$--disA cat*	chrDNA of HB10354 --> HB10360
HB15806	168 *disA::spc ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10353 --> HB15802
HB15807	168 *disA::spc ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10353 --> HB15803
HB10209	168 *sigM::tet spo0A::Tn7*	WT Tn7 library --> HB10016
HB10210	168 *sigM::tet tagA::Tn7*	WT Tn7 library --> HB10016
HB10253	168 *sigM::kan gdpP::Tn7 sigM::kan*	Tn7 library --> HB10216

Strain[1]	Genotype	Reference / construction[2]
HB10247	168 *sigM::kanrsiX::Tn7 sigM::kan*	Tn7 library --> HB10216
HB10248	168 *sigM::kan lytE::Tn7 sigM::kan*	Tn7 library --> HB10216
HB10246	168 *sigM::kan pbpX::Tn7 sigM::kan*	Tn7 library --> HB10216
HB10273	168 *sigM::kan ymdB::Tn7 sigM::kan*	Tn7 library --> HB10216
HB10249	168 *sigM::kan kinD::Tn7 sigM::kan*	Tn7 library --> HB10216
HB10245	168 *sigM::kan qoxAB:Tn7 sigM::kan*	Tn7 library --> HB10216
HB10274	168 *sigM::kan ssrA::Tn7 sigM::kan*	Tn7 library --> HB10216
HB10263	168 *sigM::kan gdpP::mls lytE::Tn7 sigM::kan*	Tn7 library --> HB10257
HB10264	168 *sigM::kan gdpP::mls clpP::Tn7 sigM::kan*	Tn7 library --> HB10257

[1] Some genes have multiple Tn7 insertion positions. Only one representative strain number for each gene is listed here.

[2] The donor DNA and recipient strain of transformation are indicated before and after the arrows, respectively.