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Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons

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Summary

Bacillus subtilis **encodes seven extracytoplasmic function (ECF) sigma factors. The σ^W regulon includes functions involved in detoxification and protection against antimicrobials, whereas σ^M is essential for growth at high salt concentrations. We now report that antibiotics that inhibit cell wall biosynthesis induce both σ^W and σ^M regulons as monitored using DNA microarrays. Induction of selected σ^W-dependent genes was confirmed using *lacZ* reporter fusions and Northern blot analysis. The ability of vancomycin to induce the σ^W regulon is dependent on both σ^W and the cognate anti-σ, RsiW, but is independent of the transition state regulator AbrB. These results suggest that the membrane-localized RsiW anti-σ^W factor mediates the transcriptional response to cell wall stress. Our findings are consistent with the idea that one function of ECF σ factors is to coordinate antibiosis stress responses and cell envelope homeostasis.**

Introduction

In nature, bacteria frequently secrete substances that inhibit the growth of competing microorganisms, a phenomenon known as antibiosis. Antibiosis was first described in 1877 when Pasteur and Joubert observed that an airborne bacillus could inhibit the growth *of Bacillus anthracis* (Burkholder, 1958). Although similar observations were commonplace in the early days of microbiology, the purification and characterization of antibiotics was not aggressively pursued until the 1940s, fuelled in part by renewed interest in penicillin, discovered 10 years previously by Fleming, and by René Dubos' crystallization of the *Bacillus brevis* antibiotics gramicidin and tyrocidin (Dworkin, 1997). The following decades witnessed a revolution in medicine as antibiotic therapy, together with vaccination, promised to reduce or eliminate many infectious diseases. However, the recent spread of antibiotic resistance, and the emergence of multiply antibiotic-resistant pathogens, has led to widespread alarm and a need to better define the mechanisms of antibiotic resistance and their control (Davies, 1994; Mazel and Davies, 1999). Here, we demonstrate that the Gram-positive bacterium *Bacillus subtilis* harbours an inducible defensive response against antibiotics coordinated by alternative σ factors.

Genome sequencing indicates that *B. subtilis* encodes seven σ factors belonging to the extracytoplasmic function (ECF) subfamily (Kunst *et al*., 1997). Members of this family of regulators typically control cell envelope-related functions such as secretion, synthesis of exopolysaccharides, or ion import or efflux (Missiakas and Raina, 1998; Helmann, 2002). We have investigated the roles of two of these ECF σ factors: σ^W and σ^X. Transcription of *sigW* initiates primarily, if not exclusively, from an autoregulatory promoter that is most active in post-exponential phase cells (Huang *et al*., 1998). We estimate that the σ^W regulon contains a total of $\approx$ 30 operons, most of which were identified by searching the *B. subtilis* genome for candidate promoter elements resembling the *sigW* autoregulatory site (Huang *et al*., 1999; Cao *et al*., 2001; 2002). The nature of the regulon has been defined further by the identification of promoters active *in vitro* using genomic DNA as template and by transcriptional profiling experiments (Wiegert *et al*., 2001; Cao *et al*., 2002).

We hypothesized that a thorough description of the σ^W regulon would provide immediate insight into the function of this σ factor, but that expectation was perhaps overly optimistic. Most of the $\approx$ 60 genes in the σ^W regulon encode proteins of unknown function but, in several cases, functional predictions can be made. The σ^W regulon includes the FosB fosfomycin resistance protein (Cao *et al*., 2001), a penicillin-binding protein (PBP4*, encoded by the *pbpE* gene), a co-transcribed amino acid racemase, an epoxide hydrolase (YfhM), a bromoperoxidase (YdjP), an ABC transporter, two protease IV (signal peptide peptidase) homologues (SppA and YqeZ) and several small peptides that resemble bacteriocin precursors (Huang *et al*., 1998; Cao *et al*., 2002). These observations led us to propose that σ^W may play a role in defending the cell against antimicrobial agents. The best documented example of this role is FosB (Cao *et al*., 2001).

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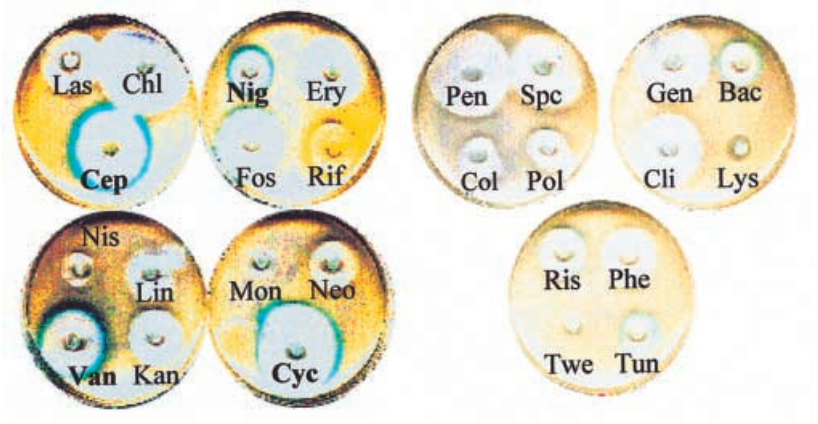

Fig. 1. Induction of *sigW* autoregulatory promoter by antibiotics. Disk diffusion assays were performed using the reporter strain HB0050 [CU1065 with SPβ(P_W-*cat-lacZ*)]. Filter paper disks containing antibiotics were placed on a lawn of *B. subtilis* growing on LB containing Xgal (40 μg per plate). Antibiotics and their respective amounts (mg per disk) were: bacitracin (Bac), 1; cephalosporin C (Cep), 1; chloramphenicol (Chl), 0.5; clindamycin (Cli), 1; colistin (Col), 1; D-cycloserine (Cyc), 1; erythromycin (Ery), 0.1; fosfomycin (Fos), 2; gentamicin sulphate (Gen), 1; kanamycin (Kan), 0.2; lasalocid (Las), 1; lincomycin (Lin), 0.5; lysozyme (Lys), 1; monensin (Mon), 1 nmol; neomycin (Neo), 0.5; nigericin (Nig), 0.1; nisin (Nis), 0.5; penicillin G (Pen), 1; phenol (Phe), 10 μl of saturated solution; polymycin (Pol), 0.1; rifampicin (Rif), 0.2; ristocetin (Ris), 0.05; spectinomycin (Spc), 1; tunicamycin (Tun), 0.05; Tween-20 (Twe), 10 μl of 100% solution; and vancomycin (Van), 1. Plates were incubated overnight at 37°C and kept at room temperature for one additional day for complete colour development.

In contrast with σ^W, the regulons controlled by the other ECF σ factors in *B. subtilis* are poorly understood. The σ^X regulon includes LytR, a negative regulator of autolysin expression, the *dlt* operon controlling D-alanylation of teichoic acids, the phosphatidylethanolamine biosynthesis operon and *pbpX*, encoding a low-molecular-mass penicillin-binding protein (Huang and Helmann, 1998; M. Cao *et al*., unpublished). Thus, we propose that σ^X acts to modulate cell surface properties by activating the expression of enzymes and pathways affecting the cell envelope. σ^M may also be required for maintaining cell envelope integrity, as mutants are unable to grow in elevated salt concentrations, and swollen, misshapen cells are observed consistent with severe defects in cell wall synthesis or stability (Horsburgh and Moir, 1999).

Additional insights into the roles of ECF σ factors will probably emerge from analysis of conditions that activate the corresponding regulons. In genetic studies, we identified random transposon insertions that activate either the σ^X or the σ^W regulon. The resulting insertions were in multidrug resistance genes, sugar isomerases and antibiotic biosynthesis pathways (Turner and Helmann, 2000). However, as most mutations activate one, but not the other, of these regulons, we surmise that the signals that activate each regulon are largely distinct. Analysis of the alkali shock stimulon led to the unexpected discovery that the σ^W regulon accounts for a large fraction of the genes that are induced most strongly after a sudden shift in pH to 8.9, a growth-limiting alkaline shock (Wiegert *et al*., 2001). However, a *sigW* mutant is unaffected in its ability to survive alkaline shock or to grow at high pH, suggesting that pH homeostasis is not a major function of σ^W.

We now report that antibiotics that inhibit cell wall biosynthesis are strong inducers of both the σ^W and σ^M regulons. The ability of antibiotics to induce the σ^W regulon is dependent on the RsiW anti-σ^W protein, but independent of the transition state regulator, AbrB. These results support a model in which ECF σ factors function to help maintain cell envelope integrity by activating the expression of genes that inactivate or detoxify antibiotics or alter cell surface properties.

Results

Antibiotics that inhibit cell wall biosynthesis induce the sigW *autoregulatory promoter*

To identify stimuli that activate σ^W, we plated *B. subtilis* containing a reporter fusion to the *sigW* autoregulatory promoter (P_W-*cat-lacZ*) on medium containing the indicator dye Xgal and overlaid with filter disks impregnated with various antibiotics and chemicals. This assay allows compounds to be assayed over a wide range of concentrations up to the toxicity limit, as defined by the zone of growth inhibition. As reported previously (Huang *et al*., 1999), many different chemical stimuli (including acids, bases, metal ions and various simple salts) failed to stimulate transcription from P_W. However, the P_W-*cat-lacZ* fusion appeared to be strongly induced by several antibiotics acting on the cell wall, as evidenced by the formation of blue halos (Fig. 1 and Table 1).

Using this qualitative assay, we demonstrated that vancomycin and cephalosporin C are the strongest inducers of P_W, with somewhat less dramatic results observed with D-cycloserine, nigericin and Triton X-100. The weakest inducers were bacitracin, fosfomycin, ristocetin and tunicamycin. Other membrane-active compounds, such as

Table 1. Summary of the inducing activities of various antibiotics[a].

Target of the antibiotics	Pw	P_{ydbS}	P_{yeaA}	P_{fosB}
Cell wall				
Vancomycin (100 mg ml^{-1})	++++	++++	+++	+
Cephalosporin (100 mg ml^{-1})	+++	+++	+++	+
D-cycloserine (100 mg ml^{-1})	++	++	–	–
Fosfomycin (200 mg ml^{-1})	+	+	+	+
Bacitracin (100 mg ml^{-1})	+	+	–	–
Tunicamycin (5 mg ml^{-1})	+	+	+	+
Ristocetin (5 mg ml^{-1})	+	–	–	–
Membrane				
Nigericin (0.01 mg ml^{-1})	++	ND	ND	ND
Triton X-100 (100%)	++	ND	ND	ND

a. The induction ability of various antibiotics was measured by disk diffusion assays, using the reporter strains: HB0050 (CU1065, Pw-*cat-lacZ*), HB7038 (CU1065, P_{ydbS}-*cat-lacZ*), HB7072 (CU1065, P_{yeaA}-*cat-lacZ*), HB0052 (CU1065, P_{fosB}-*cat-lacZ*). A sample of 10 µl of each reagent was applied to the filter disk. Blue halos were observed after overnight incubation: ++++ (dramatic blue) > +++ (strong blue) > ++ (blue) > + (light blue) > –(white after 3 days, no induction).
ND, not done.

monensin, lasalocid, nisin, polymyxin and Tween-20, failed to induce σ^W activity. Nor could antibiotics that interfere with protein synthesis (such as chloramphenicol, kanamycin, lincomycin, spectinomycin and tetracycline) or transcription (such as rifampicin) function as inducers. No induction of the P_W-*cat-lacZ* fusion was observed in a *sigW* mutant background, thereby confirming that the increased expression resulted from an increase in σ^W activity.

Cell wall-active antibiotics induce the σ^W regulon

To determine whether cell wall-active antibiotics induce other members of the σ^W regulon, we tested three *B. subtilis* strains each containing a σ^W-dependent *lacZ* fusion (Table 1). P_{ydbS} displayed a similar pattern of induction to P_W; P_{yeaA} was strongly induced by vancomycin and cephalosporin C, but induction was not observable for D-cycloserine, bacitracin or ristocetin; P_{fosB} was weakly induced by vancomycin and cephalosporin C. These apparent differences in induction ability were probably caused primarily by differences in intrinsic promoter strength. For example, P_{ydbS} is comparable in strength to P_W, and both showed similar patterns of induction with strong blue halos after vancomycin treatment. In contrast, P_{fosB} is a weak promoter and only gave a visible blue halo after treatment with the strongest inducers.

Halo formation is not caused by cell lysis

We hypothesized that the blue halos observed on solid media might be an artifact resulting from the release of β-galactosidase subsequent to cell lysis caused by the cell wall-active antibiotics. To test this idea, we scraped cells from the margin of the inhibition zones (blue areas) and from the edge of the plate (non-induced area) as a control. The collected cells were washed twice to remove cell debris before performing β-galactosidase assays. The results indicated that cells from those zones demonstrating apparent induction do indeed contain higher cell-associated β-galactosidase. Moreover, the results were in qualitative agreement with the visual observations: in this assay, the strongest inducers were Triton X-100 (7.6-fold), cephalosporin (4.8-fold), vancomycin (4.3-fold), ristocetin (3.6-fold) and D-cycloserine (3.0-fold). In contrast, there was no induction in cells isolated proximal to disks containing the non-inducers lasalocid, monensin or kanamycin. Thus, the induction observed on plates results from increased intracellular expression of β-galactosidase, presumably reflecting increased activity of σ^W, and is not an artifact caused by cell lysis.

Antibiotics induce transcription of the sigW–rsiW *operon*

To complement the *lacZ* reporter studies, we measured directly the amount of *sigW* mRNA from cells treated with various antibiotics. We extracted total RNA from cell cultures after 10 min treatment with various antibiotics and performed Northern blot analysis. A single band about 1.2 kb in size appeared in each lane corresponding to the *sigWrsiW* operon (Fig. 2). Strong induction was observed when cells were treated with vancomycin or Triton X-100, and more modest induction was observed with either bacitracin or tunicamycin. In this assay, induction was not observed with nisin, polymyxin, cephalosporin C or D-cycloserine, even though the last two were strong inducers in the disk diffusion assays. Presumably, the concentrations of these two antibiotics were not appropriate to trigger gene expression in these cultures, whereas a gradient was produced on plates allowing a greater chance of observing the transcriptional induction.

The global transcriptional response to vancomycin

We used DNA microarrays to characterize the global transcriptional response to vancomycin, one of the strongest inducers of σ^W-dependent promoters. We isolated RNA from early logarithmic phase cells ($OD_{600} \approx 0.3$) after 3 and 10 min of treatment with or without 2 µg ml^{-1} vancomycin (10× minimum inhibitory concentration, MIC). The results clearly demonstrated that the σ^W regulon comprises a significant portion of the vancomycin stimulon (Table 2): after 3 min of treatment with vancomycin, 19 σ^W-dependent genes were induced about two- to fivefold, representing 32% of the σ^W regulon (Cao *et al.*, 2002). After 10 min of vancomycin treatment, about 80% of the σ^W regulon (41

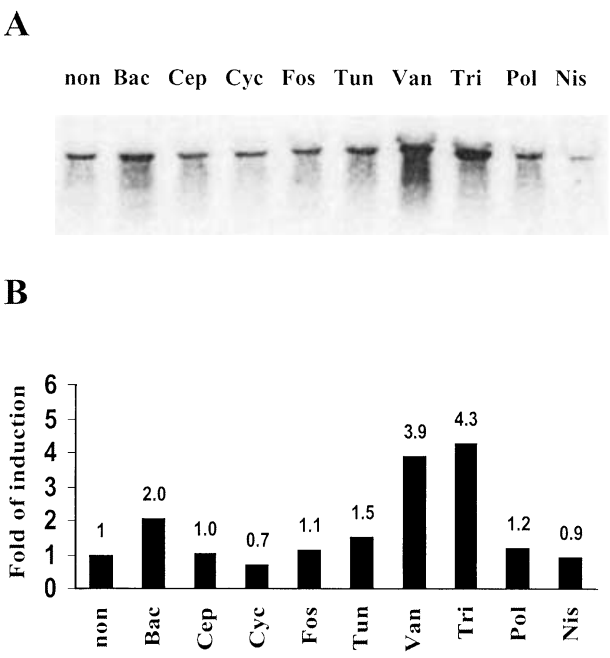

Fig. 2. Northern blot analysis of the expression of *sigWrsiW* in response to various antibiotics.
A. Total RNA (10 μg) from each sample was loaded on the formaldehyde agarose gel. The mRNA of *sigWrsiW* was detected using a labelled PCR fragment from the *sigW* coding region.
B. To quantify the amount of mRNA signal in each sample, IMAGEQUANT data analysis software was used. The data shown are representative results from one of two experiments. Antibiotics and their final concentration used for induction (μg ml^{-1} except as noted) were: no induction (non); bacitracin (Bac), 200; cephalosporin C (Cep), 5; D-cycloserine (Cyc), 200; fosfomycin (Fos), 500; tunicamycin (Tun), 10; vancomycin (Van), 1; Triton (Tri), 1%; polymyxin (Pol), 10; and nisin (Nis), 50.

genes) was induced at least twofold in the vancomycin-treated cells. The most strongly induced genes included *yuaF* (fivefold after 3 min and 49-fold after 10 min), *fosB* (encoding the fosfomycin resistance protein, 21-fold after 10 min), *yeaA* (19-fold), *pbpE* (encoding a penicillin-binding protein, 18-fold) and *yjoB* (similar to cell division protein FtsH homologue, 14-fold). However, *sigW* itself was not strongly induced.

Although the microarray results indicate that the σ^W regulon was strongly induced after 10 min of treatment with vancomycin, another ECF sigma factor, σ^M, and several candidate σ^M-controlled genes (A. Moir, personal communication) were also strongly induced, and this response was readily apparent even after only 3 min of vancomycin treatment (Table 2). These genes include *sigM* and its downstream genes *yhdL* and *yhdM* (encoding the anti-σ^M factors; Horsburgh and Moir, 1999), the DNA repair protein homologues *radA* (originally *sms*) and *radC* (originally *ysxA*), *ydaH* and *ypbG*.

Several genes controlled by multiple ECF sigma factors were also induced by vancomycin: the *divIC* gene, encoding a cell division initiation protein, is controlled by both σ^X and σ^W (Huang *et al*., 1998), whereas *ywoA*, encoding a bacitracin permease (M. Cao and J. D. Helmann, unpublished results), is controlled by both σ^X and σ^M. Interestingly, two other ECF sigma factors, σ^V and σ^Y, were also induced by vancomycin after 3 and 10 min of treatment. Thus, antibiotic treatment affects the expression of as many as four or more regulons controlled by ECF sigma factors.

Table 2. Genes induced by vancomycin that are controlled by ECF sigma factor(s).

Gene[a]	3 min	10 min	Regulator(s)
fosB	2.7 ± 0.5	20.6 ± 2.9	σ^W
*pbpE**	–	17.7 ± 4.2	σ^W
*pspA**	2.8 ± 0.3	12.1 ± 1.5	σ^W
rsiW[b]	–	4.9 ± 1.8	σ^W
*xpaC**	3.2 ± 0.6	8.3 ± 0.4	σ^W
*ybfO**	2.1 ± 0.3	4.2 ± 0.1	σ^W
*yceC**	2.5 ± 0.3	9.6 ± 0.8	σ^W (also by σ^A)
*ydbS**	–	8.3 ± 0.5	σ^W
*yeaA**	3.9 ± 1.0	19.4 ± 1.1	σ^W
*yfhL**	–	9.1 ± 1.8	σ^W
yjoB	2.3 ± 0.2	14.0 ± 6.7	σ^W
*yknW**	–	4.8 ± 0.1	σ^W
yoaF	2.9 ± 0.4	7.2 ± 0.7	σ^W
yoaG	–	4.5 ± 0.1	σ^W
yobJ	–	8.2 ± 2.0	σ^W
yozO	2.4 ± 0.3	4.4 ± 0.4	σ^W
*yqeZ**	–	5.7 ± 0.2	σ^W
ysdB	–	2.6 ± 0.9	σ^W
*ytel**	–	5.7 ± 0.3	σ^W
ythQ[c]	–	6.7 ± 0.8	σ^W
*yuaF**	5.0 ± 1.0	49.1 ± 6.3	σ^W
yvlC[d]	–	8.6 ± 5.7	σ^W
ywrE	2.6 ± 0.2	6.1 ± 1.7	σ^W
*yxjI**	2.7 ± 0.2	16.0 ± 2.3	σ^W
*sigM**	5.8 ± 1.0	6.2 ± 0.9	σ^M
*radA**	4.8 ± 0.4	11.2 ± 1.1	σ^M
radC	4.0 ± 0.4	3.6 ± 0.1	σ^M
ydaH	4.7 ± 0.2	5.9 ± 0.7	σ^M
*ypbG**	3.8 ± 0.0	9.0 ± 0.6	σ^M
divIC	2.2 ± 0.3	3.1 ± 0.1	σ^X/σ^W
*yjbC**	3.6 ± 0.3	9.0 ± 0.5	$\sigma^X/\sigma^W/\sigma^M$ (also by σ^B)
*yrhH**	6.3 ± 0.3	14.9 ± 1.2	σ^X/σ^W
ywnJ	–	2.0 ± 0.8	σ^X/σ^W
ywoA	3.4 ± 0.6	5.0 ± 0.5	σ^X/σ^M
yqjL	9.3 ± 0.7	14.1 ± 1.7	$\sigma^X/\sigma^W/\sigma^M$
*sigV**	2.6 ± 0.2	3.3 ± 0.5	σ^V
*sigY**	2.1 ± 0.6	5.0 ± 1.1	σ^Y

a. Only the first gene of an operon is listed. Those genes whose downstream genes were also induced by vancomycin are labelled with an '*'. '–' indicates that the gene was not induced (<twofold). Evidence for regulation of the indicated genes by σ^W (Huang *et al*., 1999; Cao *et al*., 2002), σ^X (Huang and Helmann, 1998; M. Cao and J. D. Helmann, unpublished results), σ^M (A. Moir, personal communication) sB (Antelmann *et al*., 2000), and σ^V and σ^Y (M. Cao and J. D. Helmann, unpublished results).
b. The upstream gene of *rsiW*, *sigW*, was only induced in one array experiment. It is not listed.
c. Induction of the upstream gene *ythP* was not observed in this experiment.
d. Induction of the upstream genes *yvlA* and *yvlB* was not observed in this experiment.

Table 3. Genes induced by vancomycin that are not known to be controlled by ECF sigma factors.

Gene[a]	Fold induction (±SD) 3 min	10 min	Functions
I. Cell wall-related genes			
cwlD	7.0 ± 1.9	2.4 ± 0.4	N-acetylmuramoyl-L-alanine amidase
maf	4.0 ± 0.4	3.6 ± 0.5	Septum formation
rodA	3.0 ± 0.5	2.4 ± 0.3	Maintenance of the rod shape and extension of the lateral walls of the cell
murG	2.3 ± 0.3	3.2 ± 1.0	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide)pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
murB	2.4 ± 0	3.2 ± 0.1	UDP-N-acetylenolpyruvoylglucosamine reductase
divIB	2.4 ± 0.1	2.1 ± 0.2	Cell division initiation protein
ylxW	2.2 ± 0.1	2.2 ± 0	Unknown
recU	2.0 ± 0.2	–[b]	PBP-related factor A
ponA	2.0 ± 0.1	–	Penicillin-binding proteins 1A and 1B
yrrR	2.2 ± 0.2	2.5 ± 0.1	Similar to penicillin-binding protein
II. Genes that were induced (≥ fivefold) after 3 min			
yoeB	13.0 ± 2.2	18.6 ± 3.7	Unknown
yuiF	12.5 ± 0.8	–	Unknown
yjeA	10.6 ± 0.9	3.2 ± 0.2	Unknown
yozB	7.4 ± 1.3	–	Unknown
ytrB	5.9 ± 0.1	2.2 ± 0.1	ABC transporter (ATP-binding protein)
ytrC	5.8 ± 0.8	2.5 ± 0.1	Similar to cytochrome *c* oxidase subunit
ytrD	4.1 ± 0.3	2.3 ± 0.5	Similar to cytochrome *c* oxidase subunit
ytrE	3.3 ± 0.1	3.0 ± 1.1	ABC transporter (ATP-binding protein)
III. Genes that were induced (≥10-fold) after 10 min			
yhaA	–	29.2 ± 3.2	Similar to aminoacylase
ywfH	–	20.5 ± 2.0	Similar to 3-oxoacyl-acyl-carrier protein reductase
yhaS	2.1 ± 0.6	13.5 ± 1.4	Unknown
yhaT	–	11.2 ± 0.5	Unknown
rapK	–	11.6 ± 0.8	Response regulator aspartate phosphatase (sporulation)
yabD	2.1 ± 0.0	10.0 ± 3.5	Unknown
yvqI[c]	4.3 ± 0.3	117 ± 6.1	Unknown
yvqH	3.3 ± 0.3	–	Unknown
yvqG	–	16.9 ± 0.7	Unknown
yvqF	–	11.3 ± 0.4	Unknown
clpE	–	21.0 ± 2.3	ATP-dependent Clp protease-like
ctsR	–	10.9 ± 0.6	Transcriptional repressor of class III stress genes
mcsA	–	12.0 ± 1.6	Modulator of CtsR repression
mcsB	–	14.3 ± 0.5	Modulator of CtsR repression
htrA	3.0 ± 0.4	10.1 ± 1.3	Serine protease Do (heat shock protein)

a. Downstream genes in operons are indented.
b. Minus sign indicates that the ratio was less than twofold or the signal was missing.
c. The *yvqIH* genes were also found to be strongly induced in response to alkali stress (Wiegert *et al*., 2001).

In summary, approximately half the genes in the vancomycin stimulon are known to be controlled by ECF sigma factors. Forty-seven genes (44% of the total) that were induced more than twofold by 3 min of treatment are controlled by ECF sigma factors. After 10 min of induction, the overall transcriptional response is much stronger. Using fivefold induction as a cut-off, 57 of the 129 induced genes (44%) are regulated by ECF sigma factors.

Vancomycin also induced many genes not known to be regulated by ECF sigma factors (Table 3). For the purposes of discussion, we can arbitrarily divide these genes into four groups. The first includes several cell wall-related genes: *cwlD* (encoding a cell wall hydrolase; Sekiguchi *et al*., 1995), the *murG* operon (MurG and MurB are key enzymes in peptidoglycan biosynthesis; Henriques *et al*., 1992; Miyao *et al*., 1992), *rodA* (maintenance of the rod shape and essential for growth and viability; Henriques *et al*., 1998) and *ponA* (encoding penicillin-binding proteins 1A and 1B; Popham and Setlow, 1995; Murray *et al*., 1998; Pedersen *et al*., 1999). Many of these genes are induced only modestly after vancomycin treatment, but this appears to be a rapid transcriptional response. A second group includes eight additional genes that were strongly induced (>fivefold) after 3 min of vancomycin treatment. Although four of these genes encode proteins of unknown functions, the remaining four belong to the *ytrABCDEF* operon thought to play a role in import of acetoin during stationary phase (Yoshida *et al*., 2000). The six genes of the *ytrA* operon are co-transcribed from a putative σ^A-dependent promoter (Yoshida *et al*., 2000). However, we did not observe induction on the first gene.

A third group includes 15 genes that were strongly induced (>10-fold) after 10 min but not strongly induced at the earlier time point. These include some of the class III stress genes: *clpE* (Derre *et al*., 1999), the *ctsR* operon (Kruger and Hecker, 1998) and *htrA* (Pallen and Wren, 1997). Finally, we note that 33 members of the large σ^B (general stress) regulon were induced from two- to 10-fold (average 3.6-fold) after 10 min (data not shown; see supple-mentary material at http://www.micro.cornell.edu/faculty.JHelmann.html). As this response was not observed at the 3 min time point, and the σ^B general stress response can be activated very rapidly in response to other stresses (e.g. a maximal response at 3 min in our previous study of the heat shock stimulon; Helmann *et al*., 2001), we speculate that activation of the σ^B regulon is a secondary response to antibiotic treatment.

RsiW, but not AbrB, is required for sensing cell wall stress

To determine whether the RsiW anti-σ^W factor is involved in sensing cell wall stress, we repeated the vancomycin induction experiment with an *rsiW* knock-out strain, HB0010. None of the genes controlled directly by σ^W could be induced in the *rsiW* mutant (see supplementary material at http://www.micro.cornell.edu/faculty.JHelmann.html). Similarly, an *rsiW* mutant strain containing the Pw-*cat*-*lacZ* fusion does not form the striking blue halos indicative of antibiotic induction when grown on solid medium. Instead, the whole plate becomes light blue, indicative of the elevated expression from the reporter constructs in the absence of RsiW.

Recently, the σ^W regulon was shown to be part of the AbrB regulon (Qian *et al*., 2002). AbrB represses transcription of *sigW* itself and also directly regulates selected σ^W-dependent target genes including *pbpE*, *yvlA*, *yxzE* and *yknW* (Strauch, 1995; Qian *et al*., 2002). As a result of this regulation, the σ^W regulon is repressed in a *spo0A* mutant strain in which AbrB expression is elevated. To determine whether AbrB activity might respond to antibiotic stress, we repeated the induction experiments using both P_W and P_{ydbS} reporter fusions in an *abrB* mutant background. The formation of the characteristic blue halos in response to vancomycin, cephalosporin, D-cycloserine, tunicamycin and Triton X-100 demonstrates that AbrB is not an essential part of the antibiotic stress-sensing pathway.

ECF σ factors affect antibiotic resistance

As vancomycin and other cell wall-active antibiotics can strongly induce the expression of *sigW* and a large number of the σ^W regulon genes, we tested the *sigW* mutant for sensitivity to various antibiotics. As expected from previous studies, the *sigW* mutant is very sensitive to fosfomycin, an early-stage inhibitor of peptidoglycan biosynthesis (Cao *et al*., 2001). However, the *sigW* mutant was not significantly affected in sensitivity to vancomycin, cephalosporin, D-cycloserine, ristocetin and tunicamycin. In contrast, the *sigM* mutant is slightly sensitive to vancomycin (MIC of 100 ng ml^{-1} for *sigM* compared with 200 ng ml^{-1} for the wild-type strain) and to D-cycloserine (MIC of 100 μg ml^{-1} for *sigM* versus 150 μg ml^{-1} for the wild type). The *sigWsigM* double mutant has the same MICs as the *sigM* mutant. Strains carrying disruption mutations in the *sigV* and *sigY* genes were not noticeably affected in antibiotic resistance.

Discussion

The *B. subtilis* σ^W regulon, including *sigW* itself, was induced by several antibiotics, most of which interfere with cell wall biosynthesis. Fosfomycin and D-cycloserine are inhibitors that block early steps in cell wall biosynthesis, whereas bacitracin, cephalosporin, ristocetin, tunicamycin and vancomycin are late-stage inhibitors (Bugg and Walsh, 1993). The σ^W regulon was also activated by some compounds thought to act primarily on the cell membrane (nigericin and Triton X-100) but not others (monensin, lasalocid and Tween-20). Transcriptional profiling studies demonstrate that a significant portion of the vancomycin stimulon is accounted for by genes known to be controlled by σ^W and another ECF σ factor, σ^M.

There is ample precedence for the induction of genes in response to antibiotics. In the best understood systems, the inducer is the antibiotic itself: well-characterized examples include the regulation of the tetracycline efflux pump by the TetR repressor (Orth *et al*., 2000) and the activation of genes encoding multidrug efflux pumps by the BmrR and QacR proteins (Heldwein and Brennan, 2001; Schumacher *et al*., 2001). In other cases, the inducer is generated as a direct consequence of antibiotic action. For example, inducible beta-lactamases are found in both Gram-positive and Gram-negative bacteria (Philippon *et al*., 1998). In Gram-negative bacteria, such as *Citrobacter freundii*, induction requires the AmpR activator, which senses directly the relative levels of cytosolic peptidoglycan precursor (UDP-MurNAC-pentapeptide) and recycling products (anhMurNac-tripeptide) (Jacobs *et al*., 1997).

In many other cases, the mechanisms of antibiotic induction are poorly defined. For example, treatment of *Staphylococcus aureus* with cell wall synthesis inhibitors leads to the upregulation of several proteins, including PBP2, Drp35 and methionine sulphoxide reductase, but the regulatory pathways are not known (Murakami *et al*., 1999; Singh *et al*., 2001a,b). In *Mycobacterium tuberculosis*, the alternative σ factor σ^F has been implicated in the

response to various antibiotics (including those targeted to cell wall synthesis, RNA polymerase and translation; Michele *et al*., 1999). However, this response is not specific to antibiotics: *sigF* is also induced by entry into stationary phase, cold shock and oxidative stress, consistent with the observation that this gene is an orthologue of the *B. subtilis* general stress response regulator σ^B. Interestingly, resistance to teicoplanin, an antibiotic with a mechanism of action very similar to vancomycin, is correlated with increased σ^B activity (Bischoff and Berger-Bachi, 2001).

There is considerable interest in defining antibiotic-inducible reporter systems as a tool for identifying novel antimicrobial compounds. For such applications, systems that respond to a wide range of inhibitors are advantageous. Towards this end, several studies have taken advantage of the VanR–VanS two component system that regulates inducible vancomycin resistance in some vancomycin-resistant enterococci. The ability of both glycopeptide and non-glycopeptide antibiotics to activate the VanS sensor kinase argues that this regulator probably senses structural properties of the cell wall, rather than a specific molecule. Most studies have found that this system is selective for late-stage inhibitors of peptidoglycan synthesis (Allen and Hobbs, 1995; Baptista *et al*., 1996; Lai and Kirsch, 1996; Grissom-Arnold *et al*., 1997; Mani *et al*., 1998) but, under some conditions, early-stage inhibitors may also be active (Ulijasz *et al*., 1996). We suggest that promoters regulated by σ^W or σ^M may provide an alternative screening system for antimicrobials with a selectivity distinct from that offered by the VanRS system.

The molecular basis for the induction of the σ^W and σ^M regulons by cell wall-active antibiotics is not yet clear. Our genetic studies indicate that the σ^W response is mediated by RsiW, a predicted membrane protein that functions as an anti-σ factor. The wide diversity of antibiotics that activate σ^W makes it likely that the signal is either a chemical or a physical consequence of defects in the cell envelope rather than a direct interaction of RsiW with the antibiotic. Indeed, the σ^W regulon is strongly activated by a sudden shift to high pH (alkaline shock) (Wiegert *et al*., 2001). This activation probably reflects the fact that cell wall biosynthetic enzymes are critical for growth but are located outside the protective confines of the cell membrane. Although both the σ^W gene and several *sigW* target operons are repressed by the global transcriptional repressor AbrB (Qian *et al*., 2002), this protein is not involved in the antibiotic-sensing pathway.

As a soil bacterium, *B. subtilis* co-exists with many microorganisms in a complex and often competitive microenvironment. Many microorganisms, including the Bacilli, secrete substances that inhibit the growth of their competitors, a phenomenon known as antibiosis. In this environment, the cell must develop defensive mechanisms. Indeed, many of the antibiotic resistance genes currently gaining notoriety for their ability to spread rapidly in clinical settings presumably arose in response to the selective pressures imposed by soil microorganisms. Our results demonstrate that σ^W, and perhaps σ^M, control antibiosis regulons that respond to these chemical attacks. Indeed, these two regulons account for a large fraction of the genes induced by vancomycin treatment, which includes several known and presumptive antibiotic resistance determinants. These two regulons may also play a role in cell wall homeostasis during normal growth or growth phase transitions. An analogous function has been proposed for the *Streptomyces coelicolor* σ^E regulon, which is also found to be strongly induced by vancomycin and other cell wall-active antibiotics (Hong *et al*., 2002).

Experimental procedures

Bacterial strains

All *B. subtilis* strains are derivatives of CU1065 (W168 *trp*C2 *attSP*β). The strains HB0020 (*sigW*::*erm*), HB0010 (*rsiW*::*kan*), HB0050 (CU1065 containing P_w-*cat-lacZ* reporter fusion), HB0021 (HB0020 containing P_W-*cat-lacZ* reporter fusion), HB7038 (CU1065 with P_{ydbS}-*cat-lacZ*), HB7072 (CU1065 with P_{yeaA}-*cat-lacZ*) and HB0052 (CU1065 with P_{fosB}-*cat-lacZ*) have been described previously (Huang *et al*., 1998; 1999; Cao *et al*., 2001). All reporter fusions are carried on an integrated SPβ prophage as described previously (Slack *et al*., 1993).

Primers #331(5′-CCCAAGCTTGGGTATATTCCATTGTG CCA) and #332 (5′-CCGGAATTCCTCAAATGCAGTCATTT CCT) were used to amplify the *sigM* gene from *B. subtilis* strain CU1065 chromosomal DNA. The polymerase chain reaction (PCR) fragment (about 790 bp; including the coding region of *sigM* as well as proximate 210 bp upstream sequence and 75 bp downstream sequence) was digested with *Hin*dIII and *Eco*RI and ligated into pUC18 to generate plasmid pMC63. pMC63 was digested with *Sac*I and *Cla*I and ligated with a Kan^R cassette (from pJM114; digested with *Sac*I and *Cla*I) to generate pMC67. Thus, an internal fragment (about 320 bp) of *sigM* was replaced by the Kan^R cassette. pMC67 was linearized with *Sca*I and transformed into *B. subtilis* strain CU1065, selecting for Kan^R, to generate strain HB0031 (*sigM*::*kan*). The Kan cassette is oriented in the opposite direction to the *sigM* gene. Chromosomal DNA from HB0020 (*sigW*::*erm*) was transformed into strain HB0031 to generate strain HB0096 (*sigWsigM* double mutant).

Demonstration of antibiotic induction on solid medium

Bacillus subtilis strains were grown in LB medium at 37°C overnight, diluted 1:100 into fresh LB medium and grown at 37°C with shaking. At mid-log phase, 20 μl of culture was mixed with 2 ml of 0.7% soft LB medium agar (containing 40 μg of Xgal per plate) and poured onto the bottom agar. After cooling, filter paper disks (6 mm diameter) carrying antibiotics

as indicated were placed on the top of the agar, and the plates were incubated at 37°C overnight. After 12–24 h incubation, the plates were scored for the appearance of a blue ring at or near the edge of the zone of growth inhibition produced by the diffusion of antibiotics from the filter paper disks (disk diffusion experiments).

Measurement of induction by β-galactosidase assays

Bacillus subtilis cells were scraped directly from the blue halos of the fresh plates (from disk diffusion experiments) and washed twice with working buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 400 nM dithiothreitol (DTT)] to remove cell debris and released enzymes. The pellets were resuspended in 0.8 ml of working buffer and assayed for β-galactosidase activity as described with normalization to cell density (Miller, 1972). As a control, cells were recovered from the edge of the plates, where there was no demonstrable effect from the antibiotics. To quantify induction of the σ^W regulon in liquid medium, cells were grown in Luria broth (LB), 2× YT, minimal medium (MM), DSM + 2% glucose or the very rich medium 4× SG. Antibiotics were added at 5× MIC after the cultures had reached early, mid or late exponential or early stationary phase, and cells were harvested after 5, 10, 30 min or 1 h of induction. Despite trying a variety of conditions, we could not reproducibly measure the strong transcriptional response expected from the observations made on solid medium.

Total RNA preparation for Northern blot

Total RNA was prepared from 3 ml of *B. subtilis* cell culture with and without antibiotic treatment. Antibiotics were added to the cultures at an OD_{600} of 0.8 (late log phase), and samples were collected 10 min after induction by centrifugation at 5000 r.p.m. for 2 min at 4°C. The cell pellets were resuspended in 50 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mg ml^{-1} lysozyme) and incubated at 37°C for 10–15 min. The RNAwiz (total RNA purification reagent; Ambion) reagent and RNeasy mini kit (Qiagen) were used as two different methods to extract total RNA from the cell lysates. Recovered RNA was dissolved in 50 μl of 0.1% diethyl pyrocarbonate (DEPC)-treated water and stored at −80°C.

Probe preparation and Northern blot analysis

Primers #143 (5′-ggggtaccatggaaatgatgattaaaaaa-3′) and #144 (5′-cgggatccttaaagatcccttaattg-3′) were used to amplify the coding region of *sigW* from the chromosomal DNA of *B. subtilis* CU1065. The resulting PCR fragment (582 bp) was purified using a Qiagen PCR purification kit and digested with *Hin*dIII. There are two *Hin*dIII restriction sites inside the fragment. Thus, *Hin*dIII cut the fragment into three pieces: 160 bp, 254 bp and 168 bp. The digestion mixture was purified again by Qiagen PCR kit and labelled by the 3′ fill-in method using Klenow fragment (3′→5′ exo^-) (New England BioLabs) and [α-^{32}P]-dATP (NEN, 3000 Ci $mmol^{-1}$, 10 mCi μl^{-1}). Unincorporated [α-^{32}P]-dATPs were removed by NucAway spin column (Ambion).

The NorthernMax formaldehyde-based system (Ambion) was used to perform the Northern analysis. Total RNA (10 μg) was denatured and loaded on 1% formaldehyde agarose gel. After electrophoresis, RNA was transferred to Zeta-Probe blotting membrane (Bio-Rad) using a downward transfer apparatus. The damp membrane was exposed to ultraviolet light (λ = 302 nm) for 1 min before hybridization to cross-link the RNA. The blot was prehybridized at 42°C for 1 h, and then labelled probe (preheated at 95°C for 10 min) was added to the hybridization tube. Hybridization was performed at 42°C overnight. On the second day, the blot was washed twice with low-stringency buffer (2× SSC) at room temperature followed by two washes with high-stringency buffer (0.1× SSC) at 42°C. The blot was wrapped in plastic wrap and exposed to a Phosphor screen (Molecular Dynamics). To quantify the amount of *sigWrsiW* mRNA in each sample, a Storm imaging system (Storm 840, Molecular Dynamics) was used together with IMAGEQUANT data analysis software.

Microarray analysis

Total RNA was prepared from *B. subtilis* strain CU1065 grown aerobically in LB medium with (van) and without (control) the addition of vancomycin. The cell culture was grown to an OD of 0.3 and split into two flasks with equal volume. Vancomycin was added to one flask to a final concentration of 2 μg ml^{-1} (10× MIC), and cells were harvested 3 or 10 min after treatment. The protocol for RNA isolation, cDNA synthesis, slide hybridization and labelling with random hexamers was described previously (Ye *et al*., 2000). Each RNA preparation was used to make both Cy3- and Cy5-labelled cDNA, and all competitive hybridizations were done twice, once with each cDNA preparation, to control for any differences in labelling between the two fluorophores. As all PCR products are spotted twice on each slide, all signal intensities and calculated ratios are the average of four values. Two microarray experiments (van versus control) were performed for each time point with RNAs prepared from two independent batches of cell cultures. For data analysis, signal intensities were detected and quantified using ARRAYVISION software (Molecular Dynamics) and assembled into EXCEL spreadsheets. Mean values and standard deviations were calculated using EXCEL. Genes with standard deviation greater than the mean value were ignored. Complete data sets are available as supplementary material at http://www.micro.cornell.edu/faculty.JHelmann.html.

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References

Allen, N.E., and Hobbs, J.N., Jr (1995) Induction of vancomycin resistance in *Enterococcus faecium* by non-glycopeptide antibiotics. *FEMS Microbiol Lett* **132:** 107–114.

Antelmann, H., Scharf, C., and Hecker, M. (2000) Phosphate

starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. *J Bacteriol* **182:** 4478–4490.

Baptista, M., Depardieu, F., Courvalin, P., and Arthur, M. (1996) Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. *Antimicrob Agents Chemother* **40:** 2291–2295.

Bischoff, M., and Berger-Bachi, B. (2001) Teicoplanin stress-selected mutations increasing sigma (B) activity in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **45:** 1714–1720.

Bugg, T.D.H., and Walsh, C.T. (1993) Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. *Nature Prod Rep* **9:** 199–215.

Burkholder, P.R. (1958) *Trends in Antibiotic Research. The Pasteur Fermentation Centennial: 1857–1957.* New York: Pfizer, pp. 41–57.

Cao, M., Bernat, B.A., Wang, Z., Armstrong, R.N., and Helmann, J.D. (2001) FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma (W), an extracytoplasmic-function sigma factor in *Bacillus subtilis*. *J Bacteriol* **183:** 2380–2383.

Cao, M., Kobel, P.A., Morshedi, M.M., Wu, M.F.W., Paddon, C., and Helmann, J.D. (2002) Defining the *Bacillus subtilis* sigma-W regulon: a comparative analysis of promoter consensus search, run-off transcription/macroarray analysis (ROMA), and transcriptional profiling approaches. *J Mol Biol* **316:** 443–457.

Davies, J. (1994) Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264:** 375–382.

Derre, I., Rapoport, G., Devine, K., Rose, M., and Msadek, T. (1999) ClpE, a novel type of HSP100 ATPase, is part of the CtsR heat shock regulon of *Bacillus subtilis*. *Mol Microbiol* **32:** 581–593.

Dworkin, M. (1997) Multiculturalism versus the single microbe. In *Bacteria as Multicellular Organisms*. Shapiro, J.A., and Dworkin, M. (eds). New York: Oxford University Press, pp. 3–13.

Grissom-Arnold, J., Alborn, W.E., Jr, Nicas, T.I., and Jaskunas, S.R. (1997) Induction of VanA vancomycin resistance genes in *Enterococcus faecalis*: use of a promoter fusion to evaluate glycopeptide and nonglycopeptide induction signals. *Microb Drug Resist* **3:** 53–64.

Heldwein, E.E., and Brennan, R.G. (2001) Crystal structure of the transcription activator BmrR bound to DNA and a drug. *Nature* **409:** 378–382.

Helmann, J.D. (2002) The extracytoplasmic function (ECF) sigma factors. *Adv Microb Phys* **46:** 47–110.

Helmann, J., Wu, M.F.W., Kobel, P.A., Gamo, F.-J., Wilson, M., Morshedi, M.M., *et al.* (2001) Global transcriptional response of *Bacillus subtilis* to heat shock. *J Bacteriol* **183:** 7318–7328.

Henriques, A.O., de Lencastre, H., and Piggot, P.J. (1992) A *Bacillus subtilis* morphogene cluster that includes *spoVE* is homologous to the *mra* region of *Escherichia coli*. *Biochimie* **74:** 735–748.

Henriques, A.O., Glaser, P., Piggot, P.J., and Moran, C.P., Jr (1998) Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. *Mol Microbiol* **28:** 235–247.

Hong, H.J., Paget, M.S.B., and Buttner, M.J. (2002) A signal transduction system in *Streptomyces coelicolor* that activates expression of a putative cell wall glycan operon in response to vancomycin and other cell wall-specific antibiotics. *Mol Microbiol* **44:** 1199–1211.

Horsburgh, M.J., and Moir, A. (1999) Sigma M, an ECF RNA polymerase sigma factor of *Bacillus subtilis* 168, is essential for growth and survival in high concentrations of salt. *Mol Microbiol* **32:** 41–50.

Huang, X., and Helmann, J.D. (1998) Identification of target promoters for the *Bacillus subtilis* σ^X factor using a consensus-directed search. *J Mol Biol* **279:** 165–173.

Huang, X., Fredrick, K.L., and Helmann, J.D. (1998) Promoter recognition by *Bacillus subtilis* σ^W: autoregulation and partial overlap with the σ^X regulon. *J Bacteriol* **180:** 3765–3770.

Huang, X., Gaballa, A., Cao, M., and Helmann, J.D. (1999) Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor, σ^W. *Mol Microbiol* **31:** 361–371.

Jacobs, C., Frere, J.M., and Normark, S. (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell* **88:** 823–832.

Kruger, E., and Hecker, M. (1998) The first gene of the *Bacillus subtilis clpC* operon, *ctsR*, encodes a negative regulator of its own operon and other class III heat shock genes. *J Bacteriol* **180:** 6681–6688.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., *et al.* (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390:** 249–256.

Lai, M.H., and Kirsch, D.R. (1996) Induction signals for vancomycin resistance encoded by the *vanA* gene cluster in *Enterococcus faecium*. *Antimicrob Agents Chemother* **40:** 1645–1648.

Mani, N., Sancheti, P., Jiang, Z.D., McNaney, C., DeCenzo, M., Knight, B., *et al.* (1998) Screening systems for detecting inhibitors of cell wall transglycosylation in *Enterococcus*. Cell wall transglycosylation inhibitors in *Enterococcus*. *J Antibiot (Tokyo)* **51:** 471–479.

Mazel, D., and Davies, J. (1999) Antibiotic resistance in microbes. *Cell Mol Life Sci* **56:** 742–754.

Michele, T.M., Ko, C., and Bishai, W.R. (1999) Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis sigF* gene: implications for chemotherapy against mycobacterial persistors. *Antimicrob Agents Chemother* **43:** 218–225.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 352–355.

Missiakas, D., and Raina, S. (1998) The extracytoplasmic function sigma factors: role and regulation. *Mol Microbiol* **28:** 1059–1066.

Miyao, A., Yoshimura, A., Sato, T., Yamamoto, T., Theeragool, G., and Kobayashi, Y. (1992) Sequence of the *Bacillus subtilis* homolog of the *Escherichia coli* cell-division gene *murG*. *Gene* **118:** 147–148.

Murakami, H., Matsumaru, H., Kanamori, M., Hayashi, H., and Ohta, T. (1999) Cell wall-affecting antibiotics induce expression of a novel gene, *drp35*. *Staphylo-coccus aureus*. *Biochem Biophys Res Commun* **264:** 348–351.

Murray, T., Popham, D.L., and Setlow, P. (1998) *Bacillus*

subtilis cells lacking penicillin-binding protein 1 require increased levels of divalent cations for growth. *J Bacteriol* **180:** 4555–4563.

Orth, P., Schnappinger, D., Hillen, W., Saenger, W., and Hinrichs, W. (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nature Struct Biol* **7:** 215–219.

Pallen, M.J., and Wren, B.W. (1997) The HtrA family of serine proteases. *Mol Microbiol* **26:** 209–221.

Pedersen, L.B., Angert, E.R., and Setlow, P. (1999) Septal localization of penicillin-binding protein 1 in *Bacillus subtilis*. *J Bacteriol* **181:** 3201–3211.

Philippon, A., Dusart, J., Joris, B., and Frere, J.M. (1998) The diversity, structure and regulation of beta-lactamases. *Cell Mol Life Sci* **54:** 341–346.

Popham, D.L., and Setlow, P. (1995) Cloning, nucleotide sequence, and mutagenesis of the *Bacillus subtilis ponA* operon, which codes for penicillin-binding protein (PBP) 1 and a PBP-related factor. *J Bacteriol* **177:** 326–335.

Qian, Q., Lee, C.Y., Helmann, J.D., and Strauch, M.A. (2002) AbrB is a regulator of the σ^W regulon in *Bacillus subtilis. FEMS Microbiol Lett* (in press).

Schumacher, M.A., Miller, M.C., Grkovic, S., Brown, M.H., Skurray, R.A., and Brennan, R.G. (2001) Structural mechanisms of QacR induction and multidrug recognition. *Science* **294:** 2158–2163.

Sekiguchi, J., Akeo, K., Yamamoto, H., Khasanov, F.K., Alonso, J.C., and Kuroda, A. (1995) Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis*. *J Bacteriol* **177:** 5582–5589.

Singh, V.K., Jayaswal, R.K., and Wilkinson, B.J. (2001a) Cell wall-active antibiotic induced proteins of *Staphylococcus aureus* identified using a proteomic approach. *FEMS Microbiol Lett* **199:** 79–84.

Singh, V.K., Moskovitz, J., Wilkinson, B.J., and Jayaswal, R.K. (2001b) Molecular characterization of a chromosomal locus in *Staphylococcus aureus* that contributes to oxidative defence and is highly induced by the cell-wall-active antibiotic oxacillin. *Microbiology* **147:** 3037–3045.

Slack, F.J., Mueller, J.P., and Sonenshein, A.L. (1993) Mutations that relieve nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *J Bacteriol* **175:** 4605–4614.

Strauch, M.A. (1995) Delineation of AbrB-binding sites on the *Bacillus subtilis spo0H*, *kinB*, *ftsAZ*, and *pbpE* promoters and use of a derived homology to identify a previously unsuspected binding site in the *bsuB1* methylase promoter. *J Bacteriol* **177:** 6999–7002.

Turner, M.S., and Helmann, J.D. (2000) Mutations in multidrug efflux homologs, sugar isomerases, and antimicrobial biosynthesis genes differentially elevate activity of the sigma (X) and sigma (W) factors in *Bacillus subtilis*. *J Bacteriol* **182:** 5202–5210.

Ulijasz, A.T., Grenader, A., and Weisblum, B. (1996) A vancomycin-inducible *lacZ* reporter system in *Bacillus subtilis*: induction by antibiotics that inhibit cell wall synthesis and by lysozyme. *J Bacteriol* **178:** 6305–6309.

Wiegert, T., Homuth, G., Versteeg, S., and Schumann, W. (2001) Alkaline shock induces the *Bacillus subtilis* σ^W regulon. *Mol Microbiol* **41:** 59–71.

Ye, R.W., Tao, W., Bedzyk, L., Young, T., Chen, M., and Li, L. (2000) Global gene expression profile of *Bacillus subtilis* growth under anaerobic conditions. *J Bacteriol* **182:** 4458–4465.

Yoshida, K.I., Fujita, Y., and Ehrlich, S.D. (2000) An operon for a putative ATP-binding cassette transport system involved in acetoin utilization of *Bacillus subtilis*. *J Bacteriol* **182:** 5454–5461.