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Evidence that the Extracytoplasmic Function Sigma Factor σ^E Is Required for Normal Cell Wall Structure in *Streptomyces coelicolor* A3(2)

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The *sigE* gene of *Streptomyces coelicolor* A3(2) encodes an RNA polymerase sigma factor belonging to the extracytoplasmic function (ECF) subfamily. Constructed *sigE* deletion and disruption mutants were more sensitive than the parent to muramidases such as hen egg white lysozyme and to the CwlA amidase from *Bacillus subtilis*. This correlated with an altered muropeptide profile, as determined by reverse-phase high-performance liquid chromatography analysis of lytic digests of purified peptidoglycan. The *sigE* mutants required high levels of magnesium for normal growth and sporulation, overproducing the antibiotic actinorhodin and forming crenellated colonies in its absence. Together, these data suggest that *sigE* is required for normal cell wall structure. The role of σ^E was further investigated by analyzing the expression of *hrdD*, which is partially *sigE* dependent. The *hrdD* gene, which encodes the σ^{HrdD} subunit of RNA polymerase, is transcribed from two promoters, $hrdDp_1$ and $hrdDp_2$, both similar to promoters recognized by other ECF sigma factors. The activities of $hrdDp_1$ and $hrdDp_2$ were reduced 20- and 3-fold, respectively, in *sigE* mutants, although only $hrdDp_1$ was recognized by $E\sigma^E$ in vitro. Growth on media deficient in magnesium caused the induction of both *hrdD* promoters in a *sigE*-dependent manner.

The σ^E subunit of *Streptomyces coelicolor* RNA polymerase was originally identified by its ability to direct transcription in vitro from $dagAp_2$, one of four promoters of the *dagA* gene, which encodes an extracellular agarase (8, 9). Cloning of the σ^E gene (*sigE*) led to the discovery of a distinct subfamily of sigma factors, named the extracytoplasmic function (ECF) subfamily, which are structurally distinct from other members of the σ^{70} family (39). ECF sigma factors have now been described in a wide range of gram-positive and gram-negative bacteria and, where studied, have been shown to be involved in regulating a variety of functions, typically concerned with the extracytoplasmic environment (44). Well-studied examples include σ^E of *Escherichia coli*, required for transcription of genes involved in the turnover and correct folding of periplasmic proteins (13, 54, 56); σ^{FecI}, which responds to extracellular iron(III) dicitrate and directs the transcription of genes required for its uptake in *E. coli* (2); σ^{CarQ}, required for carotenoid biosynthesis in *Myxococcus xanthus* (22); and σ^{AlgU}, which regulates the production of extracellular alginate in *Pseudomonas aeruginosa* (16, 24).

Members of the ECF subfamily have a number of common features which distinguish them from other members of the σ^{70} family. First, of the four conserved regions in σ^{70}-related sigma factors (38, 39), region 3 and much of region 1 are usually absent, resulting in the typically small size of ECF sigma factors (usually 20 to 30 kDa). Second, promoters regulated by ECF sigma factors are strikingly similar, especially in the −35 region, where a GAAC motif is conserved (39, 44). Third, their activity is often regulated by specific anti-sigma factors encoded by downstream genes. Examples are RseA in the case of *E. coli* σ^E (14, 45) and CarR in the case of σ^{CarQ} (22).

One of the most striking aspects of ECF sigma factors is that relatively few have been identified by traditional genetic means, although very large numbers of ECF sigma factor genes are now being uncovered in a variety of bacteria through genome sequencing. For example, in *Bacillus subtilis* there are seven ECF sigma factor genes (37), none of which was discovered genetically. This seems to imply either that they are functionally redundant or that they control the expression of genes not relevant to normal laboratory culture conditions. Recently, it was shown that there is indeed some redundancy among the ECF sigma factors of *B. subtilis*. A *sigX* mutant of *B. subtilis* has slightly increased sensitivity to heat and oxidative stress but has no other obvious phenotype (30). σ^X contributes to the transcription from at least seven promoters, four of which are also recognized by one or more different ECF sigma factors in vivo (29). The other three promoters are completely dependent on σ^X in vivo, but a second, σ^X-independent promoter also contributes to the expression of the respective genes. Therefore, each of the seven known members of the σ^X regulon is transcribed by more than one RNA polymerase holoenzyme, meaning that disruption of *sigX* will not abolish the expression of any of these genes. This may explain, at least in part, the subtlety of the *sigX* mutant phenotype (29).

To date, the biological role of σ^E in *Streptomyces* has been investigated only in the actinomycin producer *S. antibioticus*. An *S. antibioticus sigE* null mutant was deficient in actinomycin production, although direct targets for σ^E have not been identified (34). Here we investigate the biological role of σ^E in *S. coelicolor*. We show that constructed *sigE* null mutants have an altered cell wall structure, increased sensitivity to cell wall-lytic enzymes, and a distinct peptidoglycan muropeptide profile. We also show that *sigE* mutants require high levels of magnesium

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pIJ2925	Cloning vector based on pUC18 (*bla lacZa*)	33
pMT3000	pIJ2925 with modified polycloning site (*bla lacZa*)	50
pDH5	*E. coli* phagemid cloning vector (*bla tsr lacZa*)	25
pSET151	*E. coli* conjugative plasmid (*bla tsr lacZa oriT*)	5
pSET152	*E. coli*/*Streptomyces* conjugative plasmid containing *attP* and integration functions of ϕC31 [*aac*(*3*)*IV lacZa oriT*]	5
pET11c	T7 expression vector (*bla*)	Novagen
pUZ8002	RK2 derivative with defective *oriT* (*aph*)	62
pIJ2020	The multicopy vector pIJ486 carrying *dagA* on a 5-kb *Bam*HI fragment	7
pIJ2027	The promoter region of *dagA* cloned in pUC18	7
pIJ2036	1.9-kb *Sma*I fragment containing *hrdD* cloned in pIJ2925	7
pIJ5950	2.05-kb *Pvu*II fragment containing *sigE* cloned in pIJ2925	This work
pIJ5951	Δ*sigE* derivative of pIJ5950	This work
pIJ5952	Δ*sigE* cloned in pDH5	This work
pIJ5954	*sigE*::*hyg* derivative of pIJ5950	This work
pIJ5855	*sigE*::*hyg* cloned in pSET151	This work
pIJ2076	0.87-kb *Hin*dIII-*Xho*I fragment of pIJ5950 replaced with two complementary oligonucleotides generating an *Nde*I site at the initiation codon of *sigE*	This work
pIJ2078	*sigE*-containing *Nde*I-*Bgl*II fragment cloned in pET11c	This work

for normal growth and sporulation, and we identify a σ^E-dependent promoter that is induced when cultures are grown under conditions of magnesium deficiency.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. coelicolor* A3(2) M600 (11) and its derivatives were cultivated on MM, R2, R2YE (28), MS agar (mannitol plus soy flour) (27), SMMS agar (19), and NMMP liquid media (28), essentially as described previously (28). Unmethylated DNA for introduction into *S. coelicolor* was isolated from *E. coli* ET12567 (*dam dcm hsdS*) (40). Plasmids are described in Table 1.

Overproduction of σ^E. A 2.05-kb *Pvu*II fragment carrying *sigE* was cloned into *Sma*I-cut pIJ2925 (33) such that, in the resulting plasmid, pIJ5950, the *Hin*dIII site in the polylinker was upstream of *sigE*. The 870 bp of DNA between the *Hin*dIII site and a unique *Xho*I site 10 bp downstream of the *sigE* ATG start codon was replaced with two complementary oligonucleotides (5′-AGCTTCCA TATGGGTGAAGTTC-3′ and 5′-TCGAGAACTTCACCCATATGGA-3′) that introduced an *Nde*I site overlapping the ATG start codon and also replaced the second, third, and fourth codons with synonymous codons commonly associated with genes expressed at high levels in *E. coli*. The cassette replacement was verified by sequencing the resulting plasmid (pIJ2076), and *sigE* was excised as a 1.2-kb *Nde*I-*Bgl*II fragment and cloned into the expression vector pET11c (Novagen), which had been cut with *Nde*I and *Bam*HI, to generate pIJ2078.

pIJ2078 was introduced into *E. coli* BL21λDE3(pLysS) (59), and *sigE* expression was induced in exponentially growing cells (optical density at 600 nm, 0.5) by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). σ^E was recovered from inclusion bodies essentially as described previously (48). Inclusion bodies were solubilized with 0.25 (wt/vol) Sarkosyl (*N*-lauroylsarcosine), followed by extensive dialysis to remove Sarkosyl and to allow σ^E to refold. σ^E was further purified by Mono-Q anion-exchange column chromatography.

Approximately 10 μg of purified σ^E was subjected to sequential Edman degradation in order to determine the sequence of the 1st 10 N-terminal residues. The result—GEVLEFEEYV—showed complete agreement with that predicted from the DNA sequence of *sigE* (39) and showed that the N-terminal *N*-formylmethionine had been removed, as it is in *S. coelicolor* (39).

In vitro transcription. Runoff transcription assays were performed with [α-^{32}P]CTP (600 Ci mmol^{-1}; Dupont-NEN) as described by Buttner et al. (8). Typical reaction mixtures contained 1.25 pmol of *E. coli* core RNA polymerase (Epicentre Technologies, Madison, Wis.) and 12.5 pmol of σ^E. Transcription from the *hrdD* promoter region was assayed by using two fragments isolated from pIJ2036: a 490-bp *Hin*dIII-*Nar*I fragment and a 630-bp *Hin*dIII-*Sst*I fragment. Transcripts were analyzed on 6% polyacrylamide-7 M urea gels with heat-denatured ^{32}P-labelled *Hpa*II digests of pBR322 as size standards. *S. coelicolor* RNA polymerase holoenzyme, purified from YEME-grown cultures, was a gift from T. Fujii and E. Takano.

Construction of *sigE* mutants of *S. coelicolor* A3(2). A PCR-based approach was used to generate an internal in-frame deletion in *sigE*. By using pIJ5950 as a template, DNA downstream from *sigE* was amplified with a primer complementary to the C-terminal region of *sigE* which incorporated an *Xho*I site at the 5′ end (MP1; 5′-CGCTCGAGCGTGAGGAGCGG-3′) and a primer complementary to a sequence just downstream from the *Asp*718 site (KB5; 5′-GCCG GTACCCCCGGTCC-3′) (see Fig. 1). The resulting 150-bp product was digested with *Xho*I and *Asp*718, then inserted into *Xho*I-*Asp*718-digested pMT3000 (50) and checked by DNA sequencing. The *Xho*I-*Asp*718 fragment was reisolated from pMT3000 and inserted into pIJ5950, replacing the *sigE*-containing *Xho*I-*Asp*718 fragment. The resulting plasmid, pIJ5951, contains Δ*sigE* flanked by 0.85 kb of DNA upstream and 0.7 kb downstream. Δ*sigE* was isolated as a *Bam*HI-*Bgl*II fragment and inserted into *Bam*HI-digested pDH5 to give pIJ5952. pIJ5952 was passaged through the nonmethylating *E. coli* strain ET12567, then used to transform *S. coelicolor* M600, with selection for thiostrepton resistance (Thio^r). A representative transformant was subcultured on nonselective media for one round of sporulation, and the resulting spores were plated out to allow identification of Thio^s colonies. Thio^s colonies were screened for the presence or absence of the *sigE* gene by Southern hybridization of digested chromosomal DNA. A representative Thio^s isolate in which *sigE* had been deleted was designated J2130.

A disruption mutation was made in *sigE* by inserting a hygromycin resistance cassette (*hyg*) into the internal *Nco*I site. The 1.7-kb *hyg* cassette (63) was blunt ended and cloned into pIJ5950 which had been digested with *Nco*I and filled in, to give pIJ5954. The *sigE*::*hyg* mutant allele was isolated as a *Bgl*II fragment and cloned into pSET151. The resulting plasmid, pIJ5955, was used to transform *S. coelicolor* M600, with selection for hygromycin resistance (Hyg^r). All transformants were also Thio^r, indicating a single crossover event. *sigE*::*hyg* mutants were identified by screening for Hyg^r Thio^s colonies after a round of nonselective growth, and their structures were confirmed by Southern hybridization. A representative *sigE*::*hyg* mutant of M600 was designated J2141.

Conjugation. pSET152 and its derivatives were introduced by transformation into ET12567 containing the RK2 derivative pUZ8002 (62). pUZ8002 can supply transfer functions to *oriT*-carrying plasmids, such as pSET152, but is not efficiently transferred itself because of a mutation in its own *oriT*. However, a low level of self-transfer allowed pUZ8002 to be introduced into ET12567. Conjugations between *E. coli* (pUZ8002) and *S. coelicolor* were carried out essentially as described previously (34).

RNA isolation. RNA was isolated from liquid-grown mycelium as described elsewhere (28). However, rather than exhaustive phenol-chloroform extraction and DNase treatment, RNA was purified from contaminating DNA and protein by CsCl gradient centrifugation following an initial phenol-chloroform extraction, as described previously (26). RNA was isolated from solid media by scraping mycelium and spores from cellophane-covered plates and extracting as described above except that, after the addition of phenol-chloroform, the mixture was heated at 65°C for 10 min prior to vortex mixing.

S1 nuclease transcription mapping. The *hrdD* promoter region was mapped by using a probe generated by PCR from pIJ2036 with a 5′-end-labelled oligonucleotide primer internal to *hrdD* (HD1; 5′-TTCAGCGGGTGGTCCGGTGG AC-3′) and the reverse sequencing primer. HD1 (30 pmol) was labelled with [γ-^{32}P]ATP (3,000 Ci mmol^{-1}; Dupont-NEN) (57). The end-labelled PCR product was purified from an agarose gel by using a gel extraction kit (Qiagen). The *dagA* promoter region was mapped by using a 560-bp *Sma*I-*Ava*II fragment isolated from pIJ2027, labelled uniquely at the 5′ end of the *Ava*II site, as described elsewhere (8). The protected DNA fragments were quantified with a phosphorimager (Fujix BAS1000).

Lysozyme sensitivity test. Sensitivity to lysozyme was tested by spotting 5 μl of lysozyme in 10 mM Tris-HCl (pH 8) at various concentrations on confluent lawns of spores on agar (2×10^6 spores per plate). Other cell wall-lytic enzymes tested

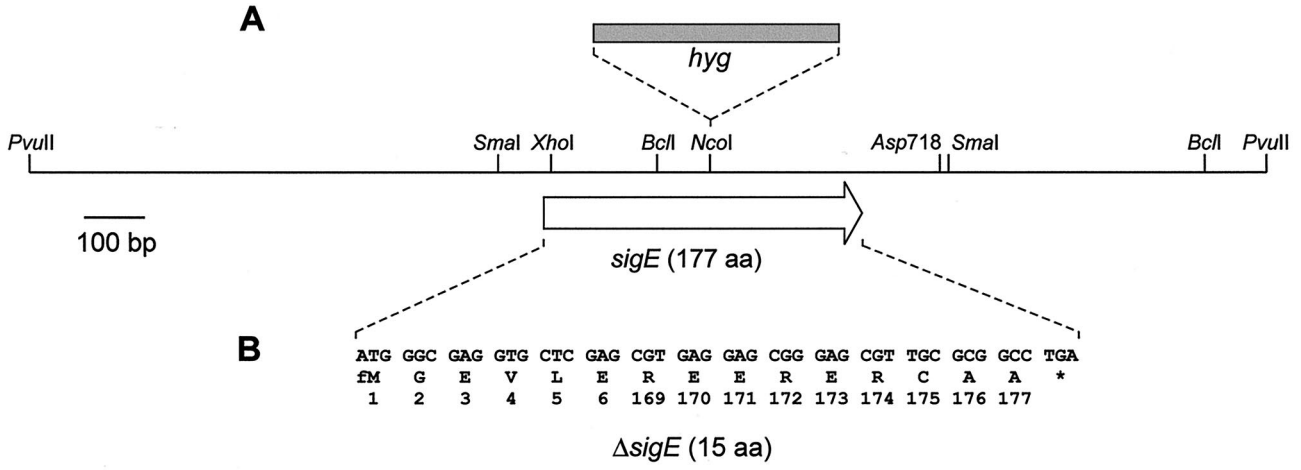

FIG. 1. Restriction map of the *S. coelicolor* 2.05-kb *Pvu*II insert containing *sigE* in pIJ5950. The *sigE* open reading frame is represented by an open arrow; the position of the *hyg* insertion mutation (A) and the extent and sequence of the Δ*sigE* in-frame deletion mutation (B) are shown.

were Cellosyl (a gift from R. Marquardt, Hoechst AG, Frankfurt, Germany), mutanolysin (Sigma), or CwlA amidase (20).

Isolation and analysis of peptidoglycan. Peptidoglycan was prepared by a modification of the method of Atrih et al. (3). Fifty milliliters of mid- to late-exponential cultures in NMMP plus glucose were centrifuged for 2 min at 5,000 × *g*. The mycelium was resuspended in 10 ml of extraction buffer (50 mM Tris-HCl, 2 mM EDTA, 10 mM dithiothreitol [pH 7]), heated in a boiling-water bath for 20 min to inactivate autolysins, and then transferred to ice. The mycelium was disrupted by using a French press, and then 10% sodium dodecyl sulfate was added to a final concentration of 4%, followed by incubation at 37°C for 40 min. Crude walls were collected at room temperature by centrifugation at 11,000 × *g* for 20 min, then washed four times with warm water. The pellet was resuspended in 6 ml of Tris-HCl (pH 7) and treated with pronase (0.5 mg/ml; Sigma) at 60°C for 90 min. Cell walls were collected as before, resuspended in 5 ml of extraction buffer containing 4% sodium dodecyl sulfate, and boiled for 16 min. Purified walls were collected and washed four times with water as above, then resuspended in MilliQ water and stored at −20°C.

Cell walls were treated with aqueous hydrofluoric acid to remove the accessory polymers, then digested with Cellosyl and reduced with sodium borohydride (at a final concentration of 2 mg/ml) as previously described (3). The muropeptide profile was investigated by applying Cellosyl-digested peptidoglycan to a TSK SWXL2000 gel filtration column (7.8 mm by 30 cm; Anachem, Luton, United Kingdom). The muropeptides were eluted with 40 mM sodium phosphate (pH 6.5) at a flow rate of 0.3 ml min^{-1}, and the eluted compounds were detected by the absorbance at 202 nm (A_{202}). Muropeptide separations carried out at a higher temperature (45°C versus the standard 40°C) or over a longer gradient (180 min versus the standard 160 min) resulted in similar profiles. Amino acid analysis was performed by the Pico Tag method (3). The cross-linking index was determined as described elsewhere (42).

RESULTS

***sigE* mutants are sensitive to cell wall-hydrolytic enzymes.** To investigate the biological role of *sigE* in *S. coelicolor*, two mutant alleles were constructed in vitro: one with an in-frame internal deletion mutation and one with a hygromycin resistance gene (*hyg*) insertion mutation (Fig. 1). These mutant alleles were used to replace the wild-type allele in M600, a plasmid-free derivative of the wild-type strain, creating J2130 (Δ*sigE*) and J2141 (*sigE*::*hyg*). The former mutation should be nonpolar on potential downstream genes, whereas the *hyg* insertion mutation might affect the expression of any genes cotranscribed with *sigE*.

Compared to M600, the *sigE* mutants grew well and sporulated with equal vigor on MM, R2, R2YE, and SMMS media. They produced normal levels of the pigmented antibiotics actinorhodin and undecylprodigiosin and of the calcium-dependent lipopeptide antibiotic CDA. The *sigE* mutants were also unaffected in their resistance to heat, their ability to produce siderophores, and their resistance to oxidizing agents—phenotypes controlled by ECF sigma factors in other bacteria (44).

However, the *sigE* mutants were up to 50 times more sensitive to egg white lysozyme (Fig. 2), an enzyme which hydrolyzes the β1-4 linkage between adjacent *N*-acetylmuramic acid and *N*-acetylglucosamine units in the glycan backbone of peptidoglycan. In our standard assay, lysozyme was spotted onto freshly plated confluent lawns of spores and zones of clearing were noted after 48 h. Under these conditions, lysozyme sensitivity probably arises soon after germination because *Streptomyces* spores are lysozyme resistant, whereas germlings are especially sensitive (55). Indeed, identical results were ob-

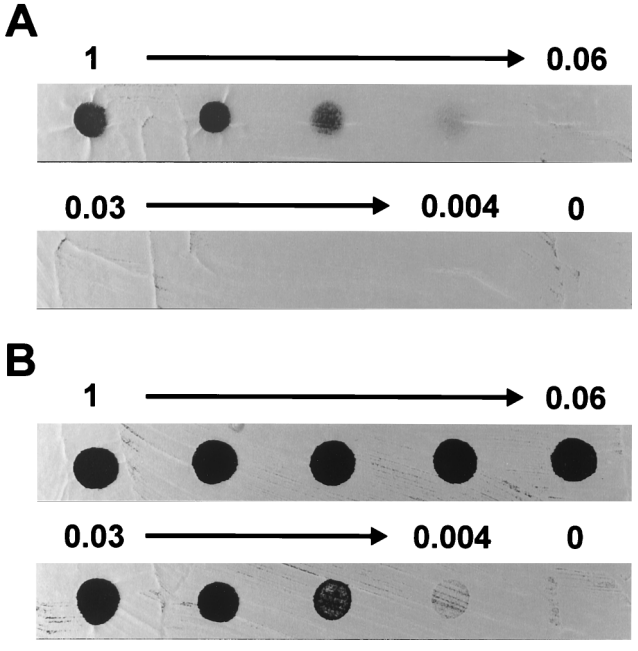

FIG. 2. Lysozyme sensitivity of M600 (*sigE*$^+$) (A) compared to that of J2130 (Δ*sigE*) (B). Spores of each strain were plated on Difco nutrient agar to give confluent lawns and then a twofold dilution series of egg white lysozyme (from 1 mg ml^{-1}, as indicated) was spotted onto the plates immediately after plating. Zones of clearing, seen here as dark circles, were photographed after 2 days of incubation at 30°C.

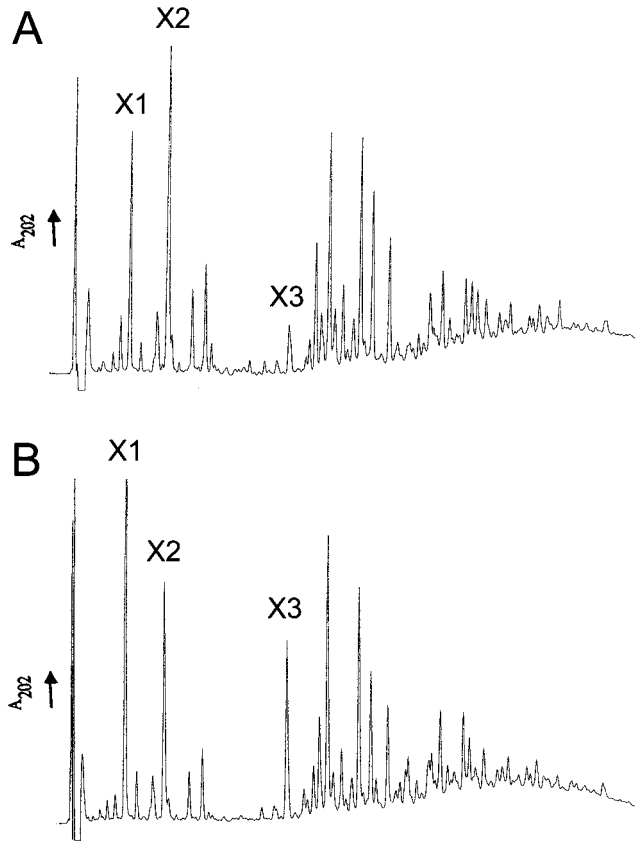

FIG. 3. Analysis of peptidoglycan composition by RP-HPLC. Samples of peptidoglycan isolated from M600 (*sigE*$^+$) (A) or J2130 (Δ*sigE*) (B) were digested with Cellosyl and subjected to HPLC analysis, and the A_{202} values of the eluates were monitored. Muropeptides that show particularly different abundances in the two strains are marked (X1 through X3).

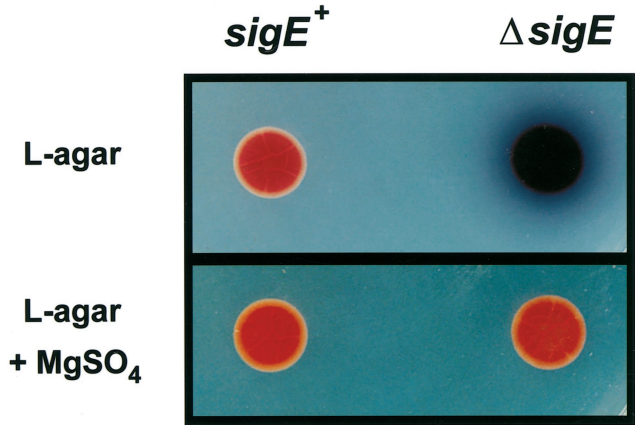

FIG. 4. The Δ*sigE* mutant, J2130, overproduces the blue-pigmented antibiotic actinorhodin on medium deficient in Mg^{2+}. Ten microliters of a spore suspension (10^8 spores ml^{-1}) was spotted on L agar alone or on L agar plus 1.6 mM $MgSO_4$. Plates were photographed after 3 days of incubation at 30°C.

tained by spotting lysozyme on newly germinated spores. Vegetative mycelia from *sigE* mutants were also more sensitive to lysozyme than that from the wild type, as judged by spotting lysozyme on 12-h-old confluent plates. To rule out the possibility that lysozyme sensitivity was caused by polar effects on possible downstream genes, *sigE* was reintroduced into the mutants by using the vector pSET152, which integrates site-specifically at the phage ϕC31 *attB* site (5). A derivative of pSET152 carrying a 2.05-kb *Pvu*II fragment containing *sigE* restored lysozyme resistance to J2130 and J2141, whereas pSET152 carrying the Δ*sigE* mutation in the same fragment did not (data not shown).

In other organisms, resistance to lysozyme has been attributed to O acetylation of peptidoglycan at the C-6 hydroxyl moiety of muramyl residues (18). However, the *sigE* mutants were also much more sensitive than their parent to Cellosyl and mutanolysin, muramidases which are insensitive to this type of O acetylation, suggesting that the C-6 position is unaltered in acetylation.

To see if the *sigE* mutants were also susceptible to cell wall-lytic enzymes that cut other linkages in the peptidoglycan, we tested their sensitivity to the CwlA amidase from *B. subtilis* (20), which cleaves the peptide side chain from the glycan backbone. By the plate assay, the *sigE* mutants were found to be at least 50-fold more sensitive than the wild type to the CwlA amidase (data not shown). Sensitivity to more than one type of cell wall-hydrolytic enzyme suggested that the mutants have an altered cell wall structure, which allowed hydrolytic enzymes increased access to the peptidoglycan.

Muropeptide analysis of *sigE* mutant cell walls indicates an altered composition. In an attempt to detect possible changes in cell wall structure, the muropeptide profiles of a *sigE* mutant and its congenic parent were determined. Cell walls were isolated from exponentially growing cultures and subjected to enzymatic hydrolysis followed by reverse-phase high-performance liquid chromatography (RP-HPLC). The muropeptide profiles obtained after RP-HPLC for M600 (*sigE*$^+$) and J2130 (Δ*sigE*) are shown in Fig. 3A and B, respectively. For each strain, the profiles were reproducible both in peak retention time and in the relative amounts of different muropeptides for three independent cultures. The two strains presented an identical complement of muropeptides but showed differences in the abundances of certain muropeptides. For example, muropeptides X1 and X3, most likely a monomer and a dimer, respectively, are present in larger amounts in J2130, while muropeptide X2 is less abundant in J2130. Amino acid analysis revealed, in addition to glucosamine and muramic acid, comparable ratios of glutamic acid, glycine, alanine, and diaminopimelic acid in both strains. These amino acids have been identified previously in the peptidoglycan of streptomycetes (58). One possible explanation for the altered ratio of muropeptides would be a difference in the cross-linking of the peptidoglycan. To determine whether the *sigE* mutant was affected in peptidoglycan cross-linking and in the distribution of oligomers, muropeptides digested with Cellosyl were separated by gel permeation HPLC. The cross-linking index, as determined by the method of Martin and Gmeiner (42), was 47 for M600 and 46.4 for J2130, indicating no significant difference between the two strains.

***sigE* mutants conditionally overproduce actinorhodin on media deficient in Mg^{2+}.** Although the *sigE* mutants appeared identical to the parent strain on most solid media, they overproduced the blue-pigmented antibiotic actinorhodin on certain complex media such as L agar (Fig. 4) and MS agar (data not shown). On these media the colonies sporulated very poorly and also had a crenellated appearance, which was not caused by actinorhodin overproduction, because it remained in a constructed *sigE act* double mutant (data not shown). Again, the overproduction of actinorhodin and the crenellation could be complemented in *trans* by integrating a functional copy of

dagAp2 GTTCCGGAACTTTTTGCACGCACGCGAGCTCTCGAATTTT
phsAp TTCAGGGAACGCGCGAGGGGCGCCGGGCGTCTCATGGGGCAA
hrdDp1 CCGTGGCAACCCTCAGGCGGTACGGGCCGTCTTCAGGGT
hrdDp2 GGTTGGGAATTCTGTCCGGATTCCAGTCGTTGTTTCCAT
-35 -10

FIG. 5. The three classes of promoter described in this paper. $dagAp_2$ and *phsAp* are recognized by $E\sigma^E$ in vitro, but their activities are unaffected in *sigE* null mutants; $hrdDp_1$ is recognized by $E\sigma^E$ in vitro and is highly *sigE* dependent in vivo; $hrdDp_2$ is not recognized by $E\sigma^E$ in vitro but is partially *sigE* dependent in vivo. The putative −35 and −10 regions are underlined, and the conserved sequences are shown in boldface. The transcription start points are italicized and boldfaced.

sigE into the chromosome at the ϕC31 *attB* site by using pSET152.

The media on which the SigE phenotype was apparent (L agar and MS agar) lack added Mg^{2+}, in contrast to the media on which the phenotype was not manifested (SMMS, R2YE, R2, and MM). When $MgSO_4$ (1.6 mM) was included in the L-agar plates, the Δ*sigE* mutant J2130 no longer overproduced actinorhodin (Fig. 4) and the colony surface was no longer crenellated (data not shown). Also, when the concentration of $MgSO_4$ in SMMS agar was reduced from the usual 5 mM to less than 1 mM, the *sigE* mutants produced actinorhodin earlier and in greater amounts than the parental wild type (data not shown). The suppression effect was caused by Mg^{2+} and not by the counterion, since it was observed with either $MgSO_4$ or $MgCl_2$. The addition of Ca^{2+} could also suppress the overproduction of actinorhodin and colony crenellation in the *sigE* mutants, although to a slightly lesser extent.

However, Mg^{2+} could not suppress the lysozyme sensitivity phenotype of *sigE* mutants. The presence of 5 mM Mg^{2+} in SMMS agar increased the resistance to lysozyme of both the *sigE* mutants and the parent, M600, but the *sigE* mutants remained substantially more sensitive to lysozyme than M600 in the presence of high (5 mM) or low (50 μM) concentrations of Mg^{2+}.

The $hrdDp_1$ promoter is σ^E dependent. To facilitate in vitro analysis of σ^E, the protein was overproduced in *E. coli* and purified to homogeneity. A *sigE* overexpression plasmid, pIJ2078, based on the T7 expression vector pET11c, was constructed as described in Materials and Methods. Active σ^E was recovered from inclusion bodies by using a minor modification of the method of Nguyen et al. (48). This involved the purification of the inclusion bodies, their solubilization with 0.25% (wt/vol) Sarkosyl, extensive dialysis to remove the detergent, and further purification by Mono-Q anion-exchange column chromatography.

Previous work showed that the σ^E holoenzyme ($E\sigma^E$) purified from *S. coelicolor* can direct transcription from the $dagAp_2$ promoter in vitro (9). An alignment of streptomycete promoters compiled by Bourn and Babb (6) revealed that the $hrdDp_1$ promoter is identical to $dagAp_2$ at 5 of 6 and 4 of 6 bases in the putative −35 and −10 regions, respectively (Fig. 5). $hrdDp_1$ is one of two promoters that drive expression of the gene encoding σ^{HrdD}, one of three *S. coelicolor* sigma factors that are very closely related in amino acid sequence and promoter specificity to σ^{HrdB}, the principal, essential sigma factor of this species. However, the function of σ^{HrdD} is unknown; *hrdD* null mutants are apparently unaffected in growth, morphological development, and antibiotic production (10). To see if σ^E could direct transcription from $hrdDp_1$ in vitro, recombinant σ^E was added to core RNA polymerase and used to transcribe $hrdDp_1$-containing templates. The two templates used, *Hin*dIII-*Nar*I and *Hin*dIII-*Sst*I, would be expected to lead to the formation of runoff products of 316 and 456 nucleotides (nt), respectively. Products of the expected sizes were obtained in a σ^E-dependent manner (Fig. 6). In contrast, no transcription from $hrdDp_2$ was detected. In vitro transcription using total RNA polymerase isolated from YEME-grown cultures of *S. coelicolor* M145 produced a low level of $hrdDp_1$ transcripts but abundant transcription from $hrdDp_2$ (Fig. 6).

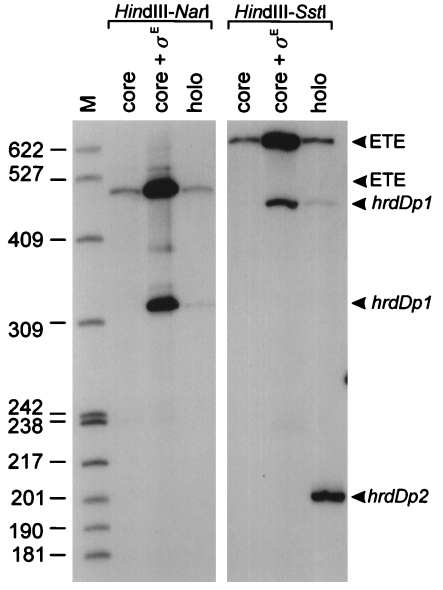

FIG. 6. In vitro transcription of *hrdD*. Core RNA polymerase (core), core RNA polymerase plus recombinant σ^E (core + σ^E), or total RNA polymerase isolated from *S. coelicolor* M145 (holo) was used in in vitro transcription reactions. Transcription from $hrdDp_1$ would be expected to lead to the formation of a runoff product of 316 or 456 nt with either a *Hin*dIII-*Nar*I or a *Hin*dIII-*Sst*I fragment, respectively, used as a template. Transcription from $hrdDp_2$ would be expected to lead to the formation of a runoff product of 204 nt with the *Hin*dIII-*Sst*I template. ETE, end-to-end transcription. The mobilities of the size markers (M) are indicated on the left.

To see if $E\sigma^E$ also transcribed $hrdDp_1$ in vivo, S1 nuclease mapping of *hrdD* was performed with RNA isolated from M600 and the *sigE* mutants J2130 and J2141. For this experiment, RNA was isolated from surface-grown MS agar cultures because growth on this medium gave a clear mutant phenotype, suggesting that σ^E was active in the wild-type strain under these conditions. RNA was isolated from 36-h cultures; by this time aerial mycelium was present and a few spores could be seen in both M600 and the *sigE* mutants. Representative results (Fig. 7A) showed that the level of transcription from $hrdDp_1$ was severely reduced (~20-fold) in J2130 and J2141 compared to that in M600. However, weak promoter activity could still be detected in RNA samples from both *sigE* mutants. Taken together with the in vitro data, these data show that $hrdDp_1$ is an in vivo target for $E\sigma^E$ but that another form of RNA polymerase holoenzyme also contributes to transcription from $hrdDp_1$. Interestingly, the level of $hrdDp_2$ activity was also significantly lower (approximately threefold) in RNA isolated from the *sigE* mutants, suggesting that $hrdDp_2$ also depends partially on *sigE*. There is considerable similarity between $hrdDp_1$ and $hrdDp_2$, with 4 of 6 nt in both the proposed −35 and −10 promoter recognition sequences being identical (Fig. 5), but since $E\sigma^E$ cannot direct transcription from $hrdDp_2$ in vitro, it is not clear if $E\sigma^E$ recognizes $hrdDp_2$ in vivo.

Expression of *hrdD* is induced in cultures deficient in Mg^{2+} in a σ^E-dependent manner. The actinorhodin overproduction and altered colony morphology of the *sigE* mutants could be

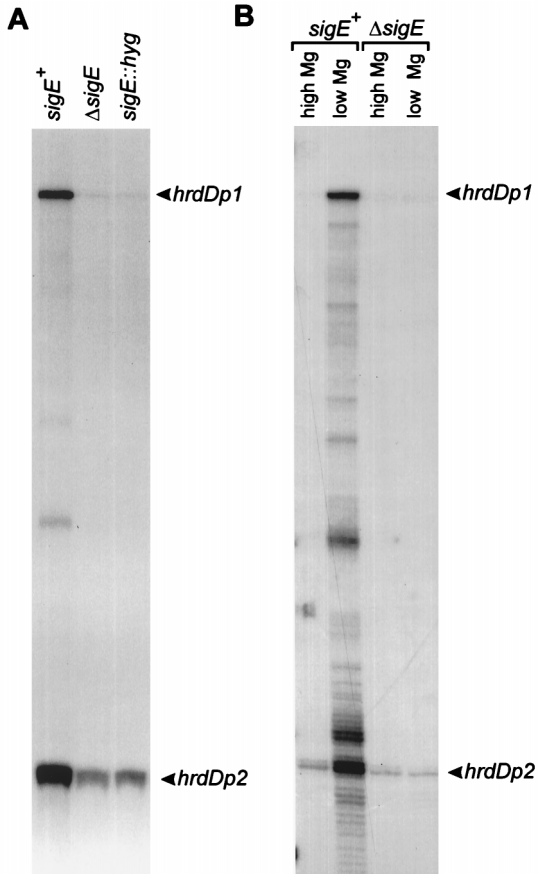

FIG. 7. (A) S1 mapping of the *hrdD* promoter region using RNA isolated from *S. coelicolor* M600 (*sigE*$^+$), J2130 (Δ*sigE*), and J2141 (*sigE*::*hyg*) grown on MS agar. (B) S1 mapping of the *hrdD* promoter region using RNA isolated from *S. coelicolor* M600 (*sigE*$^+$) and J2130 (Δ*sigE*) grown in NMMP liquid medium with 2 mM (high Mg) or 50 μM (low Mg) $MgCl_2$. The uniquely 5′-end-labelled probe was prepared as described in Materials and Methods. Protected fragments corresponding to initiation at $hrdDp_1$ and $hrdDp_2$ are indicated.

suppressed by the addition of Mg^{2+} to the medium. To see if σ^E-directed expression of *hrdD* varied in response to changing Mg^{2+} concentrations, RNA was isolated from NMMP liquid cultures containing high (2 mM) or low (50 μM) concentrations of Mg^{2+}, and transcription was assessed by S1 nuclease mapping. In the *sigE*$^+$ strain, M600, the levels of $hrdDp_1$ and $hrdDp_2$ transcripts increased approximately 12- and 4-fold, respectively, in Mg^{2+}-deficient medium (Fig. 7B), whereas in J2130 (Δ*sigE*) both promoters were transcribed at low basal levels and were insensitive to Mg^{2+} concentrations.

σ^E is not essential for $dagAp_2$ transcription in vivo. σ^E was originally identified by its ability to direct transcription from the $dagAp_2$ promoter in vitro (9). To see if $dagAp_2$ required σ^E for activity in vivo, transcript levels were investigated by S1 nuclease mapping. There was no difference between J2130 (Δ*sigE*) and its parent, M600, in the level of the $dagAp_2$ transcript, indicating that σ^E does not contribute significantly to $dagAp_2$ transcription in vivo, at least under the growth conditions used here (data not shown). To see if σ^E was required when the copy number of $dagAp_2$ was increased, S1 nuclease mapping was performed on RNA isolated from J2130 or M600 carrying the *dagA* gene on a multicopy plasmid (pIJ2020). Again, there was no difference in the level of the $dagAp_2$ transcript between the two strains (data not shown).

DISCUSSION

The evidence presented here suggests that *sigE* is required for normal cell wall structure in *S. coelicolor*. *sigE* null mutants are particularly sensitive to cell wall-lytic enzymes, including muramidases and amidases, and their peptidoglycan has an altered muropeptide composition. Although the Δ*sigE* mutant showed alterations in the ratio of certain muropeptides, a wild-type complement of muropeptides appeared to be maintained. One possible explanation for this is a change in the activity of autolysins, enzymes that hydrolyze peptidoglycan and play important roles in cell wall growth, cell separation, and differentiation (60). However, because the activities of autolysins can be affected by changes in the cell wall itself (see below), it would be difficult to identify the root cause of the altered muropeptide composition.

Sensitivity to cell wall-lytic enzymes often correlates with increased sensitivity to autolysins and can be attributed to a range of different changes in the cell wall, including O acetylation of the peptidoglycan (18) and modification of the accessory polymers (see, e.g., references 43 and 61). A change in the degree of O acetylation of the C-6 hydroxyl moiety of muramyl residues is unlikely to be the cause of increased sensitivity to lysozyme because *sigE* mutants are also more sensitive to the muramidases Cellosyl and mutanolysin, enzymes which are unaffected by this type of acetylation. In addition, *sigE* mutants are also more sensitive to the CwlA amidase, which cuts peptidoglycan at a different position (20). Preliminary investigations into possible changes in the accessory polymers have not revealed any differences in the teichoic acid content and composition of *sigE* mutants (53). The future identification of genes under the control of σ^E should help define its precise role in cell wall structure. Helmann and colleagues have used consensus-based computer searches of the complete *B. subtilis* genome sequence to identify promoters, and hence genes, under the control of the ECF sigma factors σ^X and σ^W (29, 32). The ongoing *S. coelicolor* genome sequencing project (www.sanger.ac.uk/Projects/S_coelicolor/) should permit the same approach to be used in identifying candidate members of the σ^E regulon.

Interestingly, *B. subtilis* σ^X is involved in transcribing a number of genes concerned with cell wall structure, including *lytR*, a regulator of autolysin expression, and *csbB*, which encodes a putative membrane-bound glycosyl transferase, possibly involved in peptidoglycan biosynthesis (29). It is therefore conceivable that σ^E and σ^X play related roles in *S. coelicolor* and *B. subtilis*, respectively.

sigE mutants required Mg^{2+} or Ca^{2+} for normal growth and sporulation. Divalent cations can stabilize cell walls (17, 36, 41, 47) and can protect cells from autolysis as well as from exogenous lytic enzymes (36, 61). Thus, it seems likely that Mg^{2+} stabilizes the defect in the cell walls of *sigE* mutants, thereby suppressing the phenotype. However, even in the presence of Mg^{2+}, the defect in the cell walls of the *sigE* mutants remains because they are still more sensitive to cell wall-lytic enzymes than the parental wild-type strain. It is not clear why the *sigE* mutants overproduce the antibiotic actinorhodin on medium with low levels of Mg^{2+}. Antibiotic production can be induced by a number of different stresses (4), and it is possible that the overproduction of actinorhodin seen in *sigE* mutants is an indirect consequence of stresses induced by the cell wall defect when it is not stabilized by Mg^{2+}.

σ^{HrdD} is one of three *S. coelicolor* sigma factors that are very closely related in amino acid sequence and promoter specificity to σ^{HrdB}, the principal, essential sigma factor of this species. However, the function of σ^{HrdD} is unknown; *hrdD* null mutants

are apparently unaffected in growth, morphological development, and antibiotic production (10). Expression of *hrdD* was found to be partially dependent on *sigE*, but since *hrdD* mutants do not resemble *sigE* mutants, the *sigE* mutant phenotype is not manifested through *hrdD*. Expression of *hrdD* increased in cultures grown under conditions of Mg^{2+} deficiency in a *sigE*-dependent manner. However, it is not clear whether the cells were responding directly to Mg^{2+} deficiency or to possible changes in the cell wall resulting from this deficiency. Magnesium has been shown to affect the expression of cell wall proteins in *Bacillus brevis* 47 (1) and to affect lipopolysaccharide composition in *Salmonella typhimurium* via the PhoP-PhoQ virulence regulatory system (23). However, only in the case of the PhoP-PhoQ system has Mg^{2+} been shown to act directly as an extracellular signal (21).

In *sigE* mutants, transcription from $hrdDp_1$ is severely reduced but not abolished, showing that another RNA polymerase holoenzyme also contributes to $hrdDp_1$ transcription in vivo. Given that $hrdDp_1$ is clearly similar to cognate promoters of other ECF sigma factors, it is likely that this holoenzyme contains another ECF sigma factor. In addition to σ^E, there are at least another five ECF sigma factors encoded by the *S. coelicolor* genome (46, 49, 51). Considering that there are 10 ECF sigma factors in another actinomycete, *Mycobacterium tuberculosis* (not including σ^F, which was classified as an ECF sigma factor by Cole et al. [12] but is more similar to the sporulation subfamily [15]), we suspect that many more ECF sigma factors will be discovered in *S. coelicolor* by genome sequencing. It will be interesting to establish the extent to which different ECF sigma factors have overlapping promoter specificity and thus overlapping function. Indeed, there is ample evidence, from this work and from work with *B. subtilis* (29, 31), that more than one ECF sigma factor can recognize certain promoters.

Although transcription from both $hrdDp_1$ and $hrdDp_2$ was reduced in the *sigE* mutants, only $hrdDp_1$ was recognized by $E\sigma^E$ in vitro. This could mean that the dependence of $hrdDp_2$ on σ^E is indirect or that transcription from $hrdDp_2$ in vivo requires an accessory factor absent from the in vitro reactions, or that the in vitro reaction conditions do not reflect the in vivo conditions in some other way. It is feasible that σ^E can direct transcription from $hrdDp_2$ in vivo, because the promoter is very similar to $hrdDp_1$ in both the -35 and -10 recognition sequences (Fig. 5). The residual activity of $hrdDp_2$ in a *sigE* null mutant is due, at least in part, to a newly identified ECF sigma factor, σ^R (51). σ^R directs transcription from $hrdDp_2$ in vitro (35), and $hrdDp_2$ depends partially on σ^R in vivo (52). The construction of a *sigE sigR* double mutant will be needed to establish if yet other holoenzymes are involved in the transcription of $hrdDp_2$ in vivo.

Although σ^E was originally identified by its ability to direct transcription from $dagAp_2$ in vitro, σ^E did not contribute to transcription from $dagAp_2$ in vivo, at least under the conditions used here. Similarly, in a related paper we showed that while $E\sigma^E$ could direct transcription of the *phsA* gene of *S. antibioticus* in vitro, *sigE* was not required for normal levels of *phsA* transcription in vivo (34). Thus, three classes of promoter have been described in this paper: $dagAp_2$ and *phsAp* are recognized by $E\sigma^E$ in vitro, but their activities are unaffected in *sigE* null mutants; $hrdDp_1$ is recognized by $E\sigma^E$ in vitro and is highly *sigE* dependent in vivo; $hrdDp_2$ is not recognized by $E\sigma^E$ in vitro but is partially *sigE* dependent in vivo. Although all the promoters are quite similar in both the -35 and -10 recognition regions, there are some clear differences (Fig. 5). Major challenges for the future will be to establish which nucleotides in these promoter regions determine sigma specificity and to establish the extent to which promoter dependence is affected by the growth conditions used.

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