

A single amino acid substitution in region 1.2 of the principal σ factor of *Streptomyces coelicolor* A3(2) results in pleiotropic loss of antibiotic production

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Summary

Antibiotic production in streptomycetes generally occurs in a growth phase-dependent and developmentally co-ordinated manner, and is subject to pathway-specific and pleiotropic control. *Streptomyces coelicolor* A3(2) produces at least four chemically distinct antibiotics, including actinorhodin (Act) and undecylprodigiosin (Red). *afsB* mutants of *S. coelicolor* are deficient in the production of both compounds and in the synthesis of a diffusible γ -butyrolactone, SCB1, that can elicit precocious Act and Red production. Clones encoding the principal and essential σ factor (σ^{HrdB}) of *S. coelicolor* restored Act and Red production in the *afsB* mutant BH5. A highly conserved glycine (G) at position 243 of σ^{HrdB} was shown to be replaced by aspartate (D) in BH5. Replacement of G243 by D in the *afsB*⁺ strain M145 reproduced the *afsB* phenotype. The antibiotic deficiency correlated with reduced transcription of *actII-ORF4* and *redD*, pathway-specific regulatory genes for Act and Red production respectively. Exogenous addition of SCB1 to the G-243D mutants failed to restore Act and Red synthesis, indicating that loss of antibiotic production was not a result of the deficiency in SCB1 synthesis. The G-243D substitution, which lies in the highly conserved 1.2 region of undefined function, had no effect on growth rate or morphological differentiation, and appears specifically to affect antibiotic production.

Introduction

Streptomycetes produce approximately 70% of all known microbial antibiotics, including many with important applications in human medicine and agriculture. Antibiotic production in streptomycetes generally occurs in a growth

phase-dependent manner, coinciding with the onset of aerial mycelium formation in agar-grown cultures and with stationary phase in liquid-grown cultures (Chater and Bibb, 1997). The gene clusters that encode antibiotic biosynthetic pathways are generally regulated by pathway-specific transcriptional activators, which are in turn controlled by pleiotropic regulatory genes, some of which are also required for morphological differentiation (Champness and Chater, 1994). Expression of both the pleiotropic and the pathway-specific regulatory genes is determined by a variety of physiological and environmental factors that include growth rate, small diffusible signalling molecules, imbalances in metabolism and various physiological stresses (Bibb, 1996). However, little is understood of the underlying regulatory mechanisms.

The genetically well-studied *Streptomyces coelicolor* A3(2) produces at least four chemically diverse antibiotics, and a variety of mutants are pleiotropically deficient in their synthesis (Bibb, 1996). Among the less well characterized are *afsB* mutants, which are deficient in actinorhodin (Act) and undecylprodigiosin (Red) production and in the synthesis of a diffusible signalling molecule that can cross-feed A factor-deficient mutants of *Streptomyces griseus* (A-factor is a γ -butyrolactone required for streptomycin production and aerial mycelium formation in *S. griseus*; Hara *et al.*, 1983). Transcription of *actII-ORF4*, the pathway-specific regulatory gene for Act synthesis, was abolished in the *afsB* mutant BH5 (Horinouchi *et al.*, 1989). *actII-ORF4* and its homologue *redD*, a pathway-specific regulatory gene for Red production, play pivotal roles in determining the onset of Act and Red biosynthesis, and their levels of transcription increase markedly upon entry into stationary phase (Takano *et al.*, 1992; Gramajo *et al.*, 1993). Their promoters (*actII-ORF4p* and *redDp*) are recognized efficiently *in vitro* by an RNA polymerase (RNAP) holoenzyme containing σ^{HrdD} (Fujii *et al.*, 1996), a non-essential σ factor that is a close homologue of σ^{HrdB} , the principal and essential σ factor in *S. coelicolor* (Buttner *et al.*, 1990). Regions 2.4 and 4.2 of these two σ factors, which are responsible for recognition of the -10 and -35 regions, respectively, of cognate promoters, are almost identical, and the two σ factors are likely to recognize similar promoter sequences. As disruption of *hrdD* had no effect on Act and Red production (Buttner *et al.*, 1990), there must be at least

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one other σ factor in *S. coelicolor* that can recognize *actII-ORF4p* and *redDp* *in vivo*. As a protein with the same electrophoretic mobility as σ^{HrdB} conferred on core RNAP the ability to transcribe from *redDp* *in vitro*, it was possible that this σ factor was σ^{HrdB} (Fujii *et al.*, 1996).

The results presented here show that, in the *afsB* mutant BH5, σ^{HrdB} contains a G-243D substitution. This mutation, in the poorly understood region 1.2 of σ factors, appears to affect antibiotic production by reducing the level of transcription of *actII-ORF4* and *redD* without any other apparent consequences.

Results

Isolation of a DNA fragment that restores antibiotic production to the *afsB* mutant BH5

A previously constructed genomic library of *S. coelicolor* M145 DNA made using the low-copy-number plasmid vector pIJ698 in strain J1501 was introduced into the *afsB* mutant BH5 by conjugation according to the method of Ryding *et al.* (1998). Of approximately 2400 BH5 exconjugants, 44 produced Act and/or Red on SMMS agar. Plasmid DNA from 30 antibiotic-producing exconjugants was used to transform BH5. Although none of the preparations conferred a wild-type phenotype, two plasmids, pIJ4310 and pIJ4311, restored Red production, and a third, pIJ4312, restored Act production. Restriction analysis indicated that pIJ4310 and pIJ4311 contained essentially the same insert. The 10 kb insert of pIJ4310 and the 11 kb insert of pIJ4312 were cloned as *Hind*III fragments in the *Escherichia coli*-*Streptomyces* shuttle vector pHJL401 (approximately 10 copies per chromosome in *Streptomyces*; Larson and Hershberger, 1986), yielding pIJ4320 and pIJ4313 respectively. Partial complementation of the *afsB* phenotype of BH5 by each of the cloned fragments was again observed. Southern analysis of pulsed field gel electrophoresis (PFGE) gels of *Asel*-digested *S. coelicolor* M145 DNA (kindly provided by H. M. Kieser) localized the pIJ4313 insert (the 'Act' clone) to *Asel* fragment A and the pIJ4320 insert to *Asel* fragment B (Kieser *et al.*, 1992). Only the location of the pIJ4320 insert was consistent with the position of *afsB* on the combined genetic and physical map of the *S. coelicolor* chromosome (data not shown; Hara *et al.*, 1983). The pIJ4320 insert was subsequently localized to the unique region of cosmid 5B8 in the ordered *S. coelicolor* cosmid library (Redenbach *et al.*, 1996). Earlier studies had shown that the 4.1 kb insert of pIJ6201 that contained the sporulation gene *whiH* and the 5' region of the principal σ factor gene, *hrdB*, also mapped to the unique region of 5B8 (Ryding *et al.*, 1998). Southern hybridization revealed that most, if not all, of this 4.1 kb fragment was contained in pIJ4320.

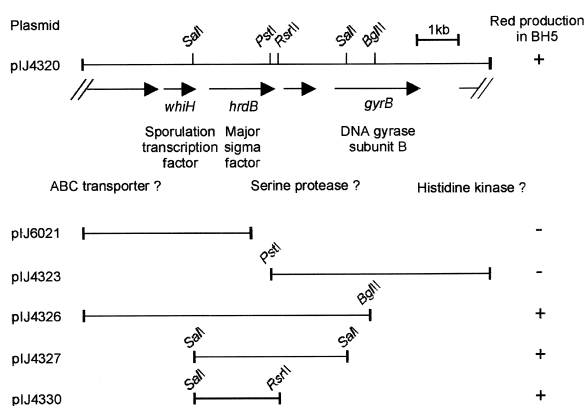


Fig. 1. Localization of the region of pIJ4320 that restored Red production in BH5. The location of the different ORFs in pIJ4320 and their respective functions are shown (EMBL AL022374). To assay Red production, the BH5 transformants were grown on SMMS plates containing $5 \mu\text{g ml}^{-1}$ thiostrepton, and pigment production was visualized after 4 days at 30°C .

hrdB restores the production of Act and Red in BH5

A set of overlapping clones of the pIJ4320 insert was made in pHJL401 and used, with pIJ6201, to transform BH5. pIJ4330 possessed the shortest fragment able to restore Red production on SMMS agar (Fig. 1). Moreover, the same plasmid restored both Red and Act production to BH5 grown in liquid SMM, and to levels that were similar to those observed in the parental strain A700 containing the vector pHJL401 (Table 1). pIJ4330 contained little more than the coding sequence and promoter region of *hrdB*. Thus, the mutant phenotype of BH5 can be partially or completely restored by *hrdB*, the gene encoding the principal and essential σ factor of *S. coelicolor*.

The *afsB* mutant BH5 carries a point mutation in *hrdB*

To determine whether *afsB* was a mutant allele of *hrdB*, the *hrdB* genes of BH5 and A700 were cloned and

Table 1. Production of (A) Red and (B) Act by *S. coelicolor* A700(pHJL401), BH5(pHJL401) and BH5(pIJ4330).

Strain	Incubation time		
	27 h	48 h	66 h
A. Red ($\mu\text{g mg}^{-1}$ dry weight).			
A700[pHJL401]	0.01	0.11	0.15
BH5[pHJL401]	0.00	0.00	0.01
BH5[pIJ4330]	0.02	0.15	0.23
B. Act ($\mu\text{g mg}^{-1}$ dry weight)			
A700[pHJL401]	1.41	28.00	82.50
BH5[pHJL401]	0.00	0.00	13.00
BH5[pIJ4330]	0.24	30.00	66.50

Strains were grown in SMM, and antibiotic production was measured at three different times.

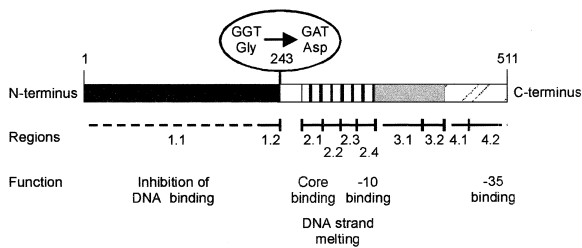


Fig. 2. Location of the single amino acid substitution in σ^{HrdB} of *S. coelicolor* BH5. Conserved regions (Lonetto *et al.*, 1992) and their proposed functions are shown. Dotted lines indicate poorly conserved regions. Note that G243 in σ^{HrdB} corresponds to G174 in Lonetto *et al.* (1992).

sequenced from 100 bp upstream of the *hrdB* transcription start point to 80 bp downstream of the stop codon, a 1887 bp region that included the likely terminator of *hrdB* transcription. A single base change (guanine to adenine) was observed in the *afsB* mutant BH5 that results in the replacement of a glycine (G) with an aspartate (D) at residue 243 of σ^{HrdB} . G243 lies at the C-terminal end of region 1.2 of σ^{HrdB} (Fig. 2) and is highly conserved among essential and non-essential σ factors from phylogenetically diverse bacteria (Lonetto *et al.*, 1992).

Allele exchange experiments confirm that afsB is a mutant allele of hrdB

pJ4330 did not fully restore the wild-type phenotype to BH5 on agar medium; moreover, replacement of the *hrdB*-G243D allele by the wild-type gene only partially restored Act and Red production (data not shown). As BH5 was obtained by chemical mutagenesis of A700 (Hara *et al.*, 1983), it was conceivable that it contained additional mutations impairing antibiotic production and contributing to the *afsB* phenotype. To assess unambiguously the effect of the G-243D substitution on antibiotic production, the *hrdB* allele of strain M145 was replaced by the mutant allele of BH5, yielding M760. M760 produced no Act or Red even after 5 days of incubation on nitrogen-limited SMMS agar (Fig. 3), in contrast to M145, which produced both in 3 days. On phosphate-limited R2 plates, Red and Act production was delayed by 2 days in M760. On rich R5 medium, there was little difference between the two strains. Thus, the G-243D substitution does indeed impair Act and Red production, but the phenotype is medium dependent. Bioassays of M145 and M760 failed to reveal any effect of the mutation on the production of the calcium-dependent antibiotic (CDA) made by *S. coelicolor*, consistent with an earlier comparison of BH5 with an *afsB*⁺ strain (Adamidis and Champness, 1992). There was no apparent difference between M145 and M760 in their ability to produce aerial hyphae and spores, or in the rate and extent of growth in liquid minimal medium.

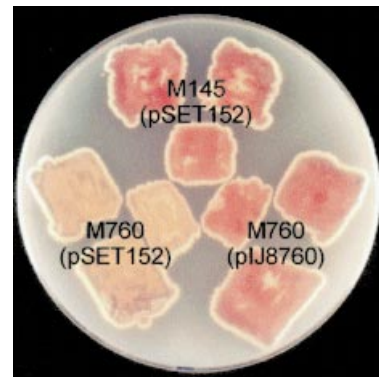


Fig. 3. Antibiotic production by *S. coelicolor* M145(*hrdB*⁺) and M760(*hrdB*-G-243D) and complementation of M760 by *hrdB* (pIJ8760). Three independent transformants of each strain were grown on SMMS for 5 days at 30°C. The plates were photographed from below to show Act and Red production. On SMMS at this pH, Act remains in the mycelium as a purple pigment. pIJ8760 is a derivative of pSET152 containing *hrdB*.

The afsB mutation impairs the production of a diffusible signalling compound

BH5 is deficient in the production of a diffusible signalling molecule that can restore antibiotic production and sporulation to A-factor-deficient mutants of *S. griseus* (Hara *et al.*, 1983). Recently, the γ -butyrolactone SCB1 was isolated from *S. coelicolor* M145 and shown to induce precocious production of both Act and Red in M145 (Takano *et al.*, 2000). SCB1 is one of at least four stimulatory factors, all believed to be γ -butyrolactones, made by *S. coelicolor* (Takano *et al.*, 2000). Supernatants of exponential, transition and stationary phase SMM-grown

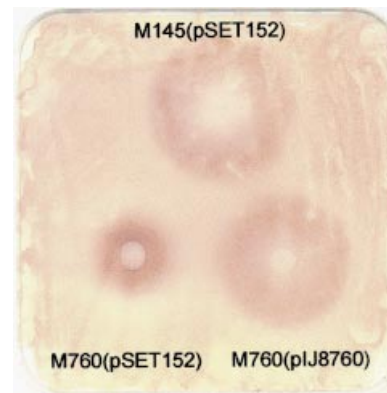


Fig. 4. Bioassay for the production by M145(pSET152), M760(pSET152) and M760(pIJ8760) of diffusible factors capable of inducing antibiotic production in *S. coelicolor*. Supernatants from transition phase cultures used for the S1 protection analysis were extracted with ethyl acetate, and samples were added to a lawn of the indicator strain M145 plated on SMMS. The absence of induction immediately around the point of application reflects inhibition of induction by a high concentration of γ -butyrolactones (Takano *et al.*, 2000).

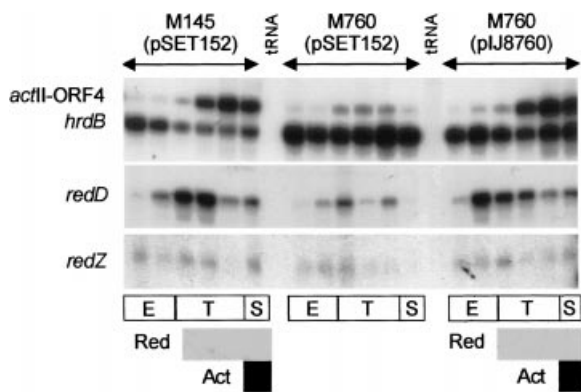


Fig. 5. Transcription of *actII-ORF4*, *redD*, *redZ* and *hrdB* in SMM-grown cultures of *S. coelicolor* M145(pSET152), M760(pSET152) and M760(pIJ8760). RNA was isolated during exponential (E), transition (T) and stationary (S) phases and subjected to S1 nuclease protection analysis. tRNA, yeast tRNA control. The grey and black boxes denote the production of Red and Act, respectively, by M145(pSET152) and M760(pIJ8760).

cultures of M145 and M760 were examined for the presence of SCB1. Although high-performance liquid chromatography (HPLC) analysis detected SCB1 in transition and stationary phase cultures of M145, it was barely detectable in M760. Furthermore, ethyl acetate extracts of transition phase culture supernatants of M145 induced markedly precocious Act and Red production in M145, whereas extracts from M760 showed only slight stimulation (Fig. 4). The ability of BH5 and its parent A700 to produce stimulatory activity was also examined by bioassay. Activity was readily detected in the A700 extract, but none was observed in that from BH5 even when concentrated 100-fold (data not shown).

The wild-type hrdB allele fully complements the afsB phenotype of M760

A derivative of pSET152 (Bierman *et al.*, 1992), pIJ8760, with a 2.1 kb *SalI*–*RsrII* fragment containing the promoter and coding region of *hrdB*, which integrates at the chromosomal Φ C31 *attB* site, fully restored Act and Red production in M760 (Fig. 3). The complemented mutant also synthesized SCB1 at the wild-type level, as indicated by both HPLC and bioassay (Fig. 4).

The afsB mutation reduces transcription of actII-ORF4 and redD, but does not affect redZ or rrnD transcription

In SMM-grown cultures, M145 started to produce Red at the beginning of transition phase and Act during stationary phase; M760 did not produce either antibiotic. S1 nuclease protection assays with RNA isolated from these cultures showed that, compared with M145, there was a marked reduction in M760 in the transition phase-associated

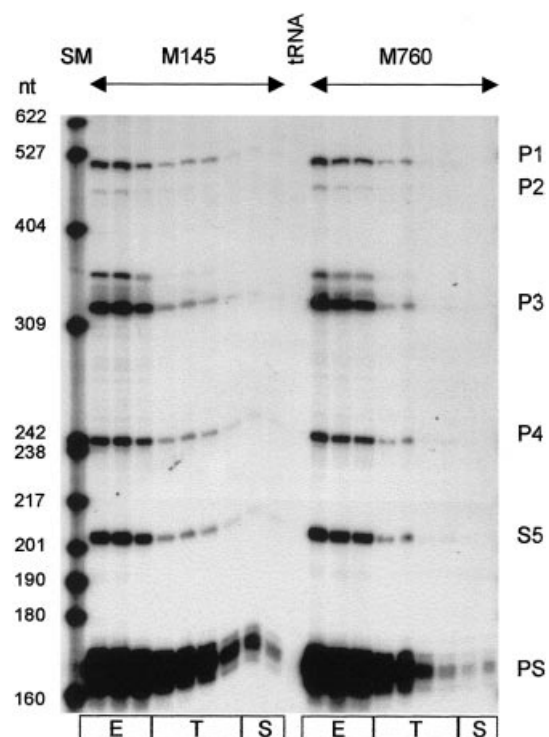


Fig. 6. Transcription of *rrnD* in SMM-grown cultures of *S. coelicolor* M145(*hrdB*⁺) and M760(*hrdB*-G-243D). RNA was isolated during exponential (E), transition (T) and stationary (S) phases and subjected to S1 nuclease protection analysis. Total RNA (1 μ g) was used for each reaction. P1, P2, P3 and P4 reflect transcripts originating at the four *rrnD* promoters, PS represents a processing site, and S5 is of unknown origin. tRNA, yeast tRNA control. SM, ³²P end-labelled, *Hind*III-digested pBR322. nt, nucleotides.

elevation of transcription of the pathway-specific regulatory genes *actII-ORF4* and *redD*, which usually precedes antibiotic production (Takano *et al.*, 1992; Gramajo *et al.*, 1993) (Fig. 5). When M760 was complemented with pIJ8760, the levels of *actII-ORF4* and *redD* transcription were fully restored, and Act and Red production occurred as in M145 (Fig. 5). Transcription of *redD* *in vivo* is absolutely dependent on a second pathway-specific regulatory gene, *redZ* (White and Bibb, 1997), but the G-243D substitution had no effect on *redZ* transcription (Fig. 5; although the *redZ* signal is weak, repeated experiments failed to reveal any significant effect of the *afsB* mutation on *redZ* transcription). Transcription of *rrnD*, one of the six rRNA gene sets of *S. coelicolor*, was assessed using the same RNA samples. *rrnD* is transcribed from four promoters (P1 to P4; Baylis and Bibb, 1988), at least one of which, P2, is recognized by σ^{HrdB} *in vitro* (Kang *et al.*, 1997). The characteristic pattern of *rrnD* transcription was observed in both strains (Fig. 6), i.e. strong expression during exponential phase, with a sharp decrease during transition phase. Despite repeated attempts, no significant difference could be detected

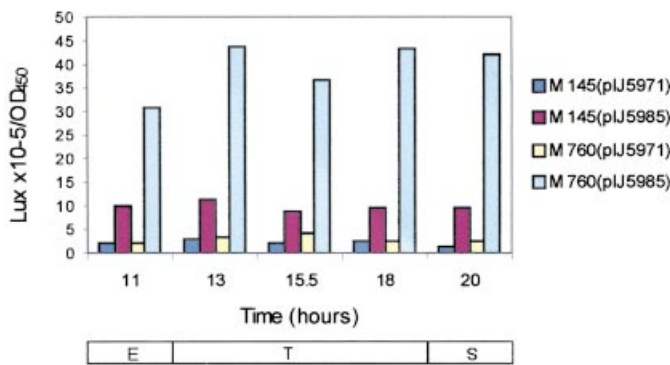


Fig. 7. $hrdBp$ activity in *S. coelicolor* M145($hrdB^+$) and M760($hrdB$ -G-243D). Transcription from $hrdBp$ was monitored by following $hrdBp$ -dependent luciferase activity in SMM-grown cultures of *S. coelicolor* M145(pIJ5985) and M760(pIJ5985) during exponential (E), transition (T) and stationary (S) phases. M145 and M760 containing the vector pIJ5971 were used as controls.

between M760 and M145 for any of the *rrnD* promoters, including P2, consistent with the absence of any detectable difference in growth rate between the two strains on a range of media.

The G-243D substitution enhances transcription of hrdB but not the level of σ^{HrdB}

S1 nuclease protection analysis of $hrdB$ transcription was carried out using the same RNA stocks used to assess *actII-ORF4* and *redD* expression (Fig. 5). The level of the $hrdB$ transcript was markedly increased in M760 compared with M145, regardless of growth phase. Complementation with wild-type $hrdB$ resulted in intermediate transcript levels [Fig. 5, M760(pIJ8760)]. To distinguish between increased transcription of $hrdB$ and increased mRNA stability, $hrdB$ promoter ($hrdBp$) activity was assessed in M145 and M760 using the luciferase-based reporter plasmid pIJ5971 (M. S. B. Paget, personal communication). pIJ5985, a derivative of pIJ5971 carrying *luxAB* under the control of $hrdBp$, was introduced into both strains. Luciferase activity was three- to sixfold higher in M145(pIJ5985) than in M145(pIJ5971) (Fig. 7), reflecting transcription from $hrdBp$, and three- to 4.5-fold higher in M760(pIJ5985) than in M145(pIJ5985), indicating elevated levels of $hrdB$ transcription in the $hrdB$ mutant. The intermediate level of promoter activity observed in M760(pIJ8760) (Fig. 5) presumably reflects the presence of two copies of $hrdB$ (one wild-type and one mutant allele). Proteins extracted from the same M145 and M760 cultures that had been used to isolate RNA were subjected to Western analysis using antibody raised against *S. coelicolor* σ^{HrdB} . A protein of 66 kDa, corresponding in size to σ^{HrdB} , was detected with equal intensity in both extracts, irrespective of growth phase (Fig. 8). Thus, elevated transcription of $hrdB$ -G-243D does not result in overproduction of σ^{HrdB} . Extended exposure of the Western blot revealed several putative σ^{HrdB} degradation products that were present at much higher levels (five- to 10-fold) in M760, suggesting that

σ^{HrdB} (G-243D) may be less stable than the wild-type protein.

Discussion

The *afsB* mutant BH5 is deficient in antibiotic production and in the synthesis of a diffusible signalling molecule. Here, we show that this mutant phenotype is attributable to a single nucleotide change, resulting in a G to D substitution at amino acid position 243 of the principal and essential σ factor, σ^{HrdB} , at the end of conserved region 1.2. This G is conserved in a large number of σ factors of different classes from phylogenetically diverse bacteria (Lonetto *et al.*, 1992). The mutation has no other apparent phenotypic consequences.

Although both *actII-ORF4p* and *redDp* are recognized efficiently *in vitro* by σ^{HrdD} , which is likely to be extremely similar to σ^{HrdB} in its promoter specificity (Tanaka *et al.*, 1991), *in vitro* transcription of *redDp* was also directed by a protein corresponding in size to σ^{HrdB} (Fujii *et al.*, 1996). As none of the close homologues of σ^{HrdB} (σ^{HrdD} , σ^{HrdA} and σ^{HrdC}) is required *in vivo* for antibiotic production (Buttner and Lewis, 1992), it is likely that both *actII-ORF4p* and *redDp* are recognized by RNAP containing

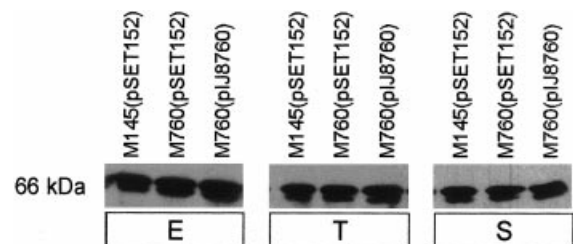


Fig. 8. Western analysis of σ^{HrdB} levels in *S. coelicolor* M145(pSET152), M760(pSET152) and M760(pIJ8760). Proteins were extracted from the same exponential (E), transition (T) and stationary (S) phase cultures used for the S1 nuclease protection analyses (Fig. 5). Because there was no significant difference between the time courses, only one point from each growth phase is shown. The presence of pSET152 did not affect expression of σ^{HrdB} .

σ^{HrdB} . The G-243D substitution also resulted in decreased and delayed transcription of *scbA* and *scbR* (data not shown), genes likely to be involved in the synthesis and regulation of SCB1, respectively (E. Takano, unpublished data), presumably explaining the low level of SCB1 production in the G-234D mutants. Comparison of the -35 and -10 regions of the *actII-ORF4*, *redD*, *scbA* and *scbR* promoters reveals sequences that are similar to the consensus sequence for σ^{HrdB} -dependent promoters (Brown *et al.*, 1992). Although the deficiency in Act and Red production could simply have reflected reduced SCB1 synthesis, exogenous addition of SCB1 to BH5 and M760 failed to restore antibiotic production, indicating that the effect of the G-243D substitution was not mediated solely through *scbA* and *scbR* expression.

The G-243D substitution results in elevated levels of the *hrdB* transcript, but not of σ^{HrdB} protein. This, together with the apparent decreased stability of the mutant protein, suggests that σ^{HrdB} may negatively regulate its own synthesis. In *Bacillus subtilis*, *spo0H* encodes the early sporulation-specific σ factor, σ^{H} (Dubnau *et al.*, 1988). Analysis of two temperature-sensitive alleles of *spo0H*, *spo0H1* and *spo0H5*, which show a sporulation-deficient phenotype at 43°C revealed rapid degradation of the σ^{H} protein in both mutants (Ohashi *et al.*, 1999). *spo0H5* contains a single nucleotide replacement that results in a G-30E substitution in region 1.2 of σ^{H} ; this position corresponds precisely to G243 in σ^{HrdB} . In both *spo0H5* and *afsB* mutants, the small neutral G in the wild-type sequence is replaced by a large acidic residue (E for σ^{H} and D for σ^{HrdB}). As suggested for *spo0H5*, the G-243D substitution in σ^{HrdB} could decrease the affinity of the σ factor for core RNAP (*spo0H* mutations are suppressed by an amino acid substitution in the β -subunit), potentially resulting in enhanced proteolysis of the free σ factor. Elevated transcription of *hrdB* in the G-243D mutants would then compensate for the increase in protein turnover.

Why should a mutation in such a highly conserved region of a principal σ factor have such a specific effect on antibiotic production? σ factors possess a number of conserved regions, and functions have been ascribed to several (Fig. 2; Lonetto *et al.*, 1992). For example, regions 2.4 and 4.2 interact directly with the -10 and -35 regions of cognate promoters. In contrast, the function of region 1 is relatively poorly understood. Region 1.1 appears to inhibit DNA binding by region 4 (Dombroski *et al.*, 1992; 1993). Deletion analysis revealed that region 1.1 is also required for efficient isomerization of a closed promoter complex to an open complex and for the transition from the open complex to a ternary initiated complex (Wilson and Dombroski, 1997). Further deletion of region 1.2 resulted in arrest of initiation at the earliest closed complex, suggesting that region 1.2 is required for

open complex formation (Wilson and Dombroski, 1997). The 2.6 Å crystal structure of a segment of σ^{70} of *E. coli*, which extends from the C-terminal part of region 1.2 (including G126, which corresponds to G243 in σ^{HrdB}) to the N-terminal portion of region 2.4, has been determined (Malhotra *et al.*, 1996). σ^{70} possesses a large non-conserved segment between regions 1.2 and 2.1 that is absent from most other principal σ factors, including σ^{HrdB} . Nevertheless, regions 1.2–2.4 are likely to be similarly disposed regardless of the presence or absence of the non-conserved region (Malhotra *et al.*, 1996). The C-terminal G126 of region 1.2 of *E. coli* σ^{70} (corresponding to G243 in σ^{HrdB}) lies close to the N-terminus of region 2.1 (the regions both form parts of antiparallel helices). Deletion of region 2.1 in σ^{70} and σ^{32} of *E. coli* (Lesley and Burgess, 1989; Lesley *et al.*, 1991), as well as a point mutation in region 2.1 of σ^{E} of *Bacillus subtilis* (Shuler *et al.*, 1995), all reduce binding of σ factor to core RNAP. Conceivably, an amino acid substitution at the end of region 1.2 that influences the positioning of region 2.1 could affect the interaction of σ with core RNAP. In *Pseudomonas fluorescens*, overexpression of the principal σ factor enhances production of the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol in the wild-type strain (Schnider *et al.*, 1995), whereas in *S. coelicolor*, the presence of *hrdB* on a multicopy plasmid results in precocious overproduction of Red (M. J. Buttner, personal communication). In each case, the antibiotics are normally made in stationary phase, and the elevated levels of production, apparently associated in both species with increased synthesis of the principal σ factor, might reflect limited availability of this σ factor once growth has ceased. Thus, a reduction in core binding mediated by the G-243D mutation might account for the loss of Act and Red production in *S. coelicolor*, as well for the decrease in SCB1 synthesis.

Alternatively, the G-243D substitution may cause a conformational change in σ^{HrdB} that perturbs its interaction with a regulatory protein. The latter could act negatively, perhaps in an analogous fashion to Rsd of *E. coli*, which has an inhibitory effect on σ^{70} -dependent transcription (Jishage and Ishihama, 1998; 1999), or positively as a transcriptional activator (e.g. upon exposure to DNA-methylating agents, Ada of *E. coli* activates transcription of specific genes by contacting σ^{70} ; Landini and Busby, 1999). However, none of the *S. coelicolor* mutants that are pleiotropically deficient in antibiotic production through a mutation in a potential transcriptional regulatory gene have a phenotype similar to that of the G-243D mutants. Alternatively, the mutation may perturb interaction with a small molecule effector, such as guanosine 3',5'-bis(pyrophosphate) (ppGpp). Under conditions of nitrogen limitation, the *S. coelicolor* *relA* mutant M570, which is deficient in ppGpp synthesis,

fails to produce Act and Red, but still makes CDA at the wild-type level (Chakraborty and Bibb, 1997), a phenotype that is strikingly similar to that of M760. Loss of Act and Red in M570 reflects diminished transcription of *actII-ORF4* and *redD* respectively (Chakraborty and Bibb, 1997). Moreover, induction of ppGpp synthesis in exponentially growing cultures correlates with enhanced transcription of both genes (Takano and Bibb, 1994). Although the mechanism whereby ppGpp exerts a positive influence on transcription is not understood, ppGpp binds to the β -subunit of *E. coli* RNAP (Chatterji *et al.*, 1998), and mutations that confer resistance to the growth-inhibitory effects of high levels of ppGpp synthesis in *E. coli* are found in genes encoding the β -, β' - and σ^{70} -subunits of RNAP (Hernandez and Cashel, 1995). Thus, it is conceivable that the G-243D substitution prevents the formation of an effective interaction between core RNAP containing ppGpp and σ^{HrdB} that is required for the activation of transcription of *actII-ORF4* and *redD*.

In conclusion, we have identified a single amino acid substitution at a highly conserved region of the principal and essential σ factor of *S. coelicolor* that diminishes the production of two antibiotics and a diffusible signalling molecule without any other apparent phenotypic consequences. Further analysis of this mutation may provide new insights into the role of this relatively uncharacterized region in σ factor function.

Experimental procedures

Bacterial manipulations

S. coelicolor A3(2) strains used were M145 (SCP1⁻, SCP2⁻; Hopwood *et al.*, 1985) and its *afsB* derivative M760 (*hrdB*-G-243D), A700 (*argA1*, *proA1*, *cysD18*; Hopwood *et al.*, 1985) and its *afsB* derivative BH5 (Hara *et al.*, 1983) and J1501 (*hisA1*, *uraA1*, *strA1*, *pgl*, SCP1⁻, SCP2⁻; Hopwood *et al.*, 1985). The strains were grown on various agar media: SMMS (Floriano and Bibb, 1996); MM with mannitol, R2 and R5 (Hopwood *et al.*, 1985); or in 50 ml of SMM (Takano *et al.*, 1992) or YEME (Hopwood *et al.*, 1985) liquid media. For transformation of *S. coelicolor* (Hopwood *et al.*, 1985), unmethylated DNA was isolated from *E. coli* ET12567 (MacNeil *et al.*, 1992). *E. coli* K-12 strain DH5 α (Sambrook *et al.*, 1989) was used for routine subcloning. Conjugation between *E. coli* and *S. coelicolor* was as described by Paget *et al.* (1999) and Flett *et al.* (1997).

Antibiotic and γ -butyrolactone production assays

Act and Red production were assayed in SMM-grown cultures (Strauch *et al.*, 1991). γ -Butyrolactone production was analysed by HPLC and by bioassay (Takano *et al.*, 2000). CDA assays were carried out on Oxoid nutrient agar (Hopwood *et al.*, 1985) or SMMS plates using *B. subtilis* as indicator (Floriano and Bibb, 1996).

Cloning and sequencing of the *hrdB* alleles of BH5 and A700

hrdB lies on a 4.1 kb *SalI* fragment (Buttner *et al.*, 1990). DNA from BH5 and A700 was digested with *SalI*, and fragments in the size range 4.0–4.4 kb were recovered by electroelution after agarose gel electrophoresis. The fragments were cloned in *SalI*-digested and dephosphorylated pIJ2925 (Janssen and Bibb, 1993), and the ligation mixture was used to transform *E. coli* DH5 α . Colony hybridization, using the 4.1 kb *BglII* *hrdB* insert of pIJ2034 (Buttner *et al.*, 1990) as probe, identified two *hrdB*-containing clones (pIJ4341 and pIJ4344) from the BH5 partial library and one (pIJ4343) from the A700 partial library. The BH5 and A700 *hrdB* alleles were sequenced by the dideoxy chain termination method (TaqTrack kit, Promega) using synthetic primers.

Replacement of wild-type *hrdB* by the BH5 *hrdB* allele in *S. coelicolor* M145

The 4.1 kb *SalI* insert of pIJ4341 from BH5 was cloned in pDH5, a plasmid unable to replicate in *Streptomyces* (Hillemann *et al.*, 1991), yielding pIJ4351, which was used to transform M145 to thiostrepton resistance. Transformants were presumed to contain pIJ4351 integrated at *hrdB*. To obtain derivatives that had lost the plasmid for allele replacement, two transformants were grown non-selectively for one round of sporulation on R5 followed by two rounds in liquid YEME medium. The cultures were spread on MM mannitol to give about 2500 colonies. Two independent thiostrepton-sensitive clones were identified by replica plating (one from each of the original clones). Allele exchange in both clones was confirmed using the polymerase chain reaction (PCR) and sequencing. Southern analysis failed to reveal any rearrangements in the *hrdB* region of both isolates. One of the strains was designated M760.

RNA isolation and S1 nuclease protection analysis

RNA from SMM-grown mycelium (Strauch *et al.*, 1991) was subjected to S1 nuclease protection assays using PCR-generated probes for *redD* and *actII-ORF4* prepared as described by Floriano and Bibb (1996), except that the 294 nucleotide *actII-ORF4* probe was made with the unlabelled primer 5'-ATAGGAGATCGCTTGTGACGGCA-3' and yielded a 228 nucleotide protected product. The 259 nucleotide *redZ* probe, which yields a 155 nucleotide protected fragment, was generated using pIJ4132 (White and Bibb, 1997) as template and 5'-CAGGATGACGCGGTCCGACACA-3' and 5'-TCAC-GACAAGATCTTCTTGAGGT-3' as labelled and unlabelled primers respectively. For *hrdB*, the 5' end of the labelled primer 5'-GCCATGACAGAGACGGACTCGGCG-3' was located 217 nucleotides downstream of the *hrdB* transcription start site, and the 5' end of the unlabelled primer 5'-CGGCCGCAAGG-TACGCGTTGATGA-3' 126 nucleotides upstream; the PCR template was pIJ2034 (Buttner *et al.*, 1990). For *rrnD*, pIJ2820 (Baylis and Bibb, 1988) was the PCR template, with the labelled and unlabelled primers 5'-GTATACCGG-TAATCGGGTGA-3' and 5'-TGGGCCCGCATCACCATCGG-3' respectively. Hybridizations were carried out using NaTCA buffer (Murray, 1986; Janssen *et al.*, 1989). S1 nuclease

protection experiments were performed with 25 fmol of each probe and with 30 or 40 μg of total RNA, with the exception of *rrnD*, where 1 μg was used (the same amount of total RNA was used within a set of comparable hybridizations). Experiments were carried out at least twice using RNA isolated from independently grown cultures.

Luciferase activity tests

hrdB promoter (*hrdBp*) activity was assessed using pIJ5985, a derivative of the integrative *luxAB*-based reporter plasmid pIJ5971 containing a 0.5 kb *hrdBp* fragment (M. S. B. Paget, personal communication). Plasmids were introduced into *S. coelicolor* by conjugation from *E. coli* ET12567 (pUZ8002) (Paget *et al.*, 1999). Plasmid-containing strains were grown in 50 ml of SMM, and culture samples were harvested at different times of growth. Luciferase activity was determined by adding 100 μl of 1% *n*-decylaldehyde (Sigma; in 9% ethanol) to 100 μl of culture and measuring light production over 30 s using a Lumat LB9501 detection system (Beathood). The value for each sample corresponds to the average of three measurements; light production was standardized to OD₄₅₀.

Immunoblotting

Proteins were extracted from 5–10 ml of the SMM-grown cultures used for RNA analysis. Mycelium was harvested by centrifugation, frozen quickly in liquid nitrogen and kept at -80°C until extraction, when it was resuspended in extraction buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT)] containing protease inhibitors (CompleteTM; Boehringer Mannheim) and sonicated. After centrifugation at 13 000 r.p.m. and 4°C for 45 min, 1 μg of soluble proteins from each sample was separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for immunodetection using the ECL Western blotting analysis system (Amersham Pharmacia Biotech). Anti- σ^{HrdB} antibody, kindly provided by J.-H. Roe, was used as primary antibody (dilution 1:10000) with peroxidase-labelled anti-mouse antibody (Amersham, dilution 1:2000) as the second antibody.

Acknowledgements

We thank Sueharu Horinouchi (University of Tokyo) for strain BH5, Jung-Hye Roe (National University of Seoul) for σ^{HrdB} antibody, Mark Paget for the luciferase constructions, Jamie Ryding for the genomic library, and Helen Kieser for the PFGE gels. Mark Buttner, Keith Chater, David Hopwood and Tobias Kieser are thanked for their comments on the manuscript. This work was supported by grants to M.J.B. from the Biotechnology and Biological Sciences Research Council (MOL04653 to B.A. and A.W., and CAD04400 to E.T.).

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