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A single amino acid substitution in region 1.2 of the principal $\sigma$ 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 and essential signalling molecule (SCB1), that can elicit precocious Act and Red production. Clones encoding the principal butyrolactone, SCB1, that can elicit precocious Act and Red production. 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 Act-deficient mutants of *Streptomyces griseus* (A-factor is a $\gamma$-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., 1993). Their promoters (actII-ORF4p and redDp) are recognized efficiently in vitro by an RNA polymerase (RNAP) holoenzyme containing $\sigma^{HrdD}$ (Fuji et al., 1996), a non-essential $\sigma$ factor that is a close homologue of $\sigma^{HrdB}$, the principal and essential $\sigma$ factor in *S. coelicolor* (Buttner et al., 1990). Regions 2.4 and 4.2 of these two $\sigma$ factors, which are responsible for recognition of the $-10$ and $-35$ regions, respectively, of cognate promoters, are almost identical, and the two $\sigma$ 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 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.

Introduction
Streptomycetes produce approximately 70% of all known microbial antibiotics, including with 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 Act-deficient mutants of *Streptomyces griseus* (A-factor is a $\gamma$-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., 1993). Their promoters (actII-ORF4p and redDp) are recognized efficiently in vitro by an RNA polymerase (RNAP) holoenzyme containing $\sigma^{HrdD}$ (Fuji et al., 1996), a non-essential $\sigma$ factor that is a close homologue of $\sigma^{HrdB}$, the principal and essential $\sigma$ factor in *S. coelicolor* (Buttner et al., 1990). Regions 2.4 and 4.2 of these two $\sigma$ factors, which are responsible for recognition of the $-10$ and $-35$ regions, respectively, of cognate promoters, are almost identical, and the two $\sigma$ 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...
one other σ factor in _S. coelicolor_ that can recognize actII-ORF4p and _redDp_ in _in vivo_. As a protein with the same electrophoretic mobility as σ hrdB characterized 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 pJ698 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 HindIII 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 cosmids 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.

**Plasmid**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>whiH</th>
<th>hrdB</th>
<th>gyrB</th>
<th>Red production in BH5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIJ4320</td>
<td>+</td>
<td>+</td>
<td>2000</td>
<td>+</td>
</tr>
<tr>
<td>pIJ4321</td>
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<td>pIJ4323</td>
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<td>+</td>
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<tr>
<td>pIJ4330</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>

**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 μg 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).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation time</th>
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<tr>
<td></td>
<td>27 h</td>
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<tr>
<td>A. Red (μg mg⁻¹ dry weight)</td>
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</tr>
<tr>
<td>A700[pHJL401]</td>
<td>0.01</td>
</tr>
<tr>
<td>BH5[pHJL401]</td>
<td>0.00</td>
</tr>
<tr>
<td>BH5[pIJ4330]</td>
<td>0.02</td>
</tr>
<tr>
<td>B. Act (μg mg⁻¹ dry weight)</td>
<td></td>
</tr>
<tr>
<td>A700[pHJL401]</td>
<td>1.41</td>
</tr>
<tr>
<td>BH5[pHJL401]</td>
<td>0.00</td>
</tr>
<tr>
<td>BH5[pIJ4330]</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Strains were grown in SMM, and antibiotic production was measured at three different times.
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 HrdB. G243 lies at the C-terminal end of region 1.2 of HrdB (Fig. 2) and is highly conserved among essential and non-essential factors from phylogenetically diverse bacteria (Lonetto et al., 1992).

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

pIJ4330 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.

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
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 $\phi$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 rmD 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 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 rmD, one of the six rRNA gene sets of S. coelicolor, was assessed using the same RNA samples. rmD is transcribed from four promoters (P1 to P4; Baylis and Bibb, 1988), at least one of which, P2, is recognized by $\sigma^{\text{hrdB}}$ in vitro (Kang et al., 1997). The characteristic pattern of rmD 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.
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 $\sigma^{HrdB}$

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 $\sigma^{HrdB}$. A protein of 66 kDa, corresponding in size to $\sigma^{HrdB}$, 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 $\sigma^{HrdB}$. Extended exposure of the Western blot revealed several putative $\sigma^{HrdB}$ degradation products that were present at much higher levels (five- to 10-fold) in M760, suggesting that $\sigma^{HrdB}$ (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 $\sigma$ factor, $\sigma^{HrdB}$, at the end of conserved region 1.2. This G is conserved in a large number of $\sigma$ 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 $\sigma^{HrdB}$, which is likely to be extremely similar to $\sigma^{HrdB}$ in its promoter specificity (Tanaka et al., 1991), in vitro transcription of redDp was also directed by a protein corresponding in size to $\sigma^{HrdB}$ (Fujii et al., 1996). As none of the close homologues of $\sigma^{Hrb}$ ($\sigma^{HrdD}$, $\sigma^{HrdA}$ and $\sigma^{HrdC}$) 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. 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.](image1)

![Fig. 8. Western analysis of $\sigma^{HrdB}$ 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 $\sigma^{HrdB}$.](image2)
α^{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 α^{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 α^{HrdB} protein. This, together with the apparent decreased stability of the mutant protein, suggests that α^{HrdB} may negatively regulate its own synthesis. In Bacillus subtilis, spo0H encodes the early sporulation-specific α factor, α^{H} (Dubnau et al., 1988). Analysis of two temperature-sensitive alleles of spoOH, spoOH1 and spoOH5, which show a sporulation-deficient phenotype at 43°C revealed rapid degradation of the α^{H} protein in both mutants (Ohashi et al., 1999). spoOH5 contains a single nucleotide replacement that results in a G-30E substitution in region 1.2 of α^{H}; this position corresponds precisely to G243 in α^{HrdB}. In both spoOH5 and afsB mutants, the small neutral G in the wild-type sequence is replaced by a large acidic residue (E for α^{H} and D for α^{HrdB}). As suggested for spoOH5, the G-243D substitution in α^{HrdB} could decrease the affinity of the α factor for core RNAP (spoOH mutations are suppressed by an amino acid substitution in the β-subunit), potentially resulting in enhanced proteolysis of the free α 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 α factor have such a specific effect on antibiotic production? α 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 α^{70} of E. coli, which extends from the C-terminal part of region 1.2 (including G126, which corresponds to G243 in α^{HrdB}) to the N-terminal portion of region 2.4, has been determined (Malhotra et al., 1996). α^{70} possesses a large non-conserved segment between regions 1.2 and 2.1 that is absent from most other principal α factors, including α^{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 α^{70} (corresponding to G243 in α^{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 α^{70} and α^{30} of E. coli (Lesley and Burgess, 1989; Lesley et al., 1991), as well as a point mutation in region 2.1 of α^{E} of Bacillus subtilis (Shuler et al., 1995), all reduce binding of α 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 α with core RNAP. In Pseudomonas fluorescens, overexpression of the principal α factor enhances production of the antibiotics pyoluteorin and 2,4-diacytethylphloroglucinol 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 α factor, might reflect limited availability of this α 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 α^{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 α^{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 α^{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 (Chakraburty 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 (Chakraburty 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 σ⁷₀-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 σ²⁰₄ 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 (Florian and Bibb, 1996); MM with mannitol, R2 and R5 (Hopwood et al., 1985); or in 50 ml of SMM (Takano et al., 1991) 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 DH5α (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 bios assay (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 (Florian and Bibb, 1996).

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**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 electrophoresis after agarose gel electrophoresis. The fragments were cloned in SalI-digested and dephosphorylated pJ2925 (Janssen and Bibb, 1993), and the ligation mixture was used to transform *E. coli* DH5α. Colony hybridization, using the 4.1 kb BglII hrdB insert of pJ2934 (Buttner et al., 1990) as probe, identified two hrdB-containing clones (pJ4341 and pJ4344) from the BH5 partial library and one (pJ4343) from the A700 partial library. The BH5 and A700 hrdB alleles were sequenced by the dye-deoxy 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 pJ4341 from BH5 was cloned in pDH5, a plasmid unable to replicate in *Streptomyces* (Hilleman et al., 1991), yielding pJ4351, which was used to transform M145 to thioestreptone resistance. Transformants were presumed to contain pJ4351 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 thioestrepton-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 as described by Florian and Bibb (1996), except that the 294 nucleotide actII-ORF4 probe was made with the unlabelled primer 5'-ATAGGAGATCGCTTGTGACCGGCA-3' and yielded a 228 nucleotide protected product. The 259 nucleotide redD probe, which yields a 155 nucleotide protected fragment, was generated using pJ4132 (White and Bibb, 1997) as template and 5'-CAGGATGACGCGTTCGAGACCA-3' and 5'-TCAC-GACAAAGATCTTCTTGAAGT-3' as labelled and unlabelled primers respectively. For hrdB, the 5' end of the labelled primer 5'-GCCATGACAGACGAGACTCGGCGGCA-3' was located 217 nucleotides downstream of the hrdB transcription start site, and the 5' end of the unlabelled primer 5'-CGGCCGCAAGG-TACCCTTGTAGTGA-3' 126 nucleotides upstream; the PCR template was pJ2034 (Buttner et al., 1990). For rmd, pJ2820 (Baylis and Bibb, 1988) was the PCR template, with the labelled and unlabelled primers 5'-GTATCCGG-TAATCGGTTTGGA-3' and 5'-GGGCCGCGTACCATCGGCGG-3' respectively. Hybridizations were carried out using NaTCA buffer (Murray, 1986; Janssen et al., 1989). S1 nuclease

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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 plJ5985, a derivative of the integrative luxAB-based reporter plasmid plJ5971 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 (Beattie, M.). 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 (Complete™, 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-α30 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.

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