A response-regulator-like activator of antibiotic synthesis from Streptomyces coelicolor A3(2) with an amino-terminal domain that lacks a phosphorylation pocket

Ellen P. Guthrie,† Christine S. Flaxman, Janet White, David A. Hodgson, Mervyn J. Bibb and Keith F. Chater

Author for correspondence: Keith F. Chater. Tel: +44 1603 452571. Fax: +44 1603 456844.

e-mail: chater@bbsrc.ac.uk

1 John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK
2 Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

In Streptomyces coelicolor A3(2), bldA mutants that lack the tRNA for the rare leucine codon UUA fail to make the red undecylprodigiosin antibiotic complex. To find out why, red-pigmented while bald (Pwb) derivatives of a bldA mutant were isolated. Using a cloning strategy that allowed for (and demonstrated) dominance of the mutations, they were localized to the red gene cluster. By using insert-mediated integration of a φC31 phage-based vector, one of the Pwb mutations was more precisely located between red structural genes to a segment of approximately 1 kb about 4 kb from the known pathway-specific regulatory gene redD. The segment contained most of an ORF (redZ) encoding a protein (RedZ) with end-to-end similarity to response regulators of diverse function from a variety of bacteria. Remarkably, in RedZ hydrophobic residues replace nearly all of the charged residues that usually make up the phosphorylation pocket present in typical response regulators, including the aspartic acid residue that is normally phosphorylated by a cognate sensory protein kinase. A single TTA codon in redZ provided a potential explanation for the bldA-dependence of undecylprodigiosin synthesis. This codon was unchanged in three Pwb mutants, but further analysis of one of the mutants revealed a potential up-promoter mutation. It seems possible that a combination of low-level natural translation of the UUA codon by a charged non-cognate tRNA, coupled with increased transcription of redZ in the Pwb mutant, allows the accumulation of a threshold level of the RedD protein.

Keywords: Streptomyces coelicolor A3(2), undecylprodigiosin, bldA, RedZ, RedD

INTRODUCTION

Antibiotics and other secondary metabolites are among the most important products of bioindustry. It is therefore a matter of pressing interest to understand how production levels are regulated so that well-informed approaches can be used for strain improvement and fermentation development (Chater, 1990; Baltz & Hosted, 1996). In streptomycetes, which produce many commercially important antibiotics, the regulation of secondary metabolism is also of fundamental interest, because it is responsive to both developmental and environmental signals (Chater & Bibb, 1997).

For studies of this subject Streptomyces coelicolor A3(2) provides an excellent model system because of its well-developed genetics and its ability to produce at least four quite different antibiotics. Among at least 21 loci known to have pleiotropic effects on production of most or all of these compounds (Champness & Chater, 1994; Bibb, 1996; Chater & Bibb, 1997; Chakraburtty et al., 1996; Martínez-Costa et al., 1996) are several bld genes whose action is also important for normal morphological differentiation (Champness & Chater, 1994). Most or all of these pleiotropically acting genes seem likely to influence the activity of pathway-specific regulatory genes (Bibb, 1996; Chater & Bibb, 1997).
In *S. coelicolor* A3(2) all antibiotic production (as well as normal aerial mycelium development) is prevented on media by mutations in *bldA* (Merrick, 1976), which encodes the only tRNA that can efficiently translate the leucine codon UUA (Lawlor et al., 1987; Leskiw et al., 1991a). This codon is rarely used in *Streptomyces* spp., which contain more than 70 mol% GC in their DNA (Wright & Bibb, 1992). Indeed, the unimpaired growth and viability of *bldA* mutants shows that the TTA codon is not present in any essential gene and most of its known occurrences are in pathway-specific genes, often regulatory, that are involved in antibiotic production. This has given rise to the idea that *bldA* exerts a regulatory role (Leskiw et al., 1991b), though searches for evidence of such a function have given differing results under different conditions (Leskiw et al., 1993; Gramajo et al., 1993).

At least some of the genes encoding enzymes for biosynthesis of three of the *S. coelicolor* antibiotics are not transcribed in *bldA* mutants of *S. coelicolor* (Guthrie & Chater, 1990; Bruton et al., 1991; A. Wietzorrekk & K. F. Chater, unpublished). In the case of actinorhodin biosynthesis the explanation is simple: *actII-ORF4*, encoding the pathway-specific activator of actinorhodin biosynthesis, contains a TTA codon and substitution of the donor DNA with Sau3AI to generate fragments of about 1.5 kb from promoter derivatives produced by *bldA* independent production of blue-pigmented actinorhodin (Fernández-Morenco et al., 1991).

The pathway for synthesis of another pigmented antibiotic of *S. coelicolor*, underprodigiosin (a tripyrrole compound produced as the major component of a mixture of red-pigmented prodigiones here termed Red; Tsao et al., 1985), is positively controlled by the *actII-ORF4 homologue redD* (Narva & Feitelson, 1990; Takano et al., 1992), which is located at one end of the red gene cluster that encodes the enzymes for Red production (Malpartida et al., 1990; Hopwood et al., 1995). Although transcription of red pathway genes in most conditions appears to depend on both *redD* and *bldA*, the nature of this dual requirement is obscure because *redD* contains no TTA codons (Narva & Feitelson, 1990). Moreover, Red is produced at approximately wild-type levels by *bldA* mutants on certain media, apparently in response to low phosphate levels (Guthrie & Chater, 1990; White & Bibb, 1997). Here we describe the use of a genetic approach to investigate further the reason for the *bldA* dependence of Red gene expression. The analysis of mutations that bypass this dependence has led to the identification of *redZ*, a gene in the red cluster that encodes an unusual homologue of a well-known family of transcriptional activators. *redZ* contains a TTA codon and is therefore expected to depend on *bldA* for translation of its mRNA.

**METHODS**

**Strains, media and microbiological methods.** *S. coelicolor* A3(2) derivatives used were: M145 (prototrophic SCP1 SCP2; and M124 (proA1 argA1 cysD18 SCP1 SCP2) (Hopwood et al., 1985); J1501 (hisA1 uraA1 strA1 pgl SCP1 SCP2; Chater et al., 1982); J1700, a *bldA39* derivative of J1501 (Pirer & Chater, 1985); GE6 and EG9, red-pigmented (Pwb, "pigmented while bald") derivatives of J1700 isolated after UV irradiation (containing the Pwb-6 and Pwb-9 mutations, respectively); GE16, a Pwb-16 derivative isolated after treatment of J1700 with NTG; and EG311, EG111 and EG441, Pwb-6, Pwb-9- and Pwb-16-containing *mthB2 strA1 bldA39* NF recombinants, respectively, from crosses of EG6, EG9 and EG16 with J668 (*mthB2 cysD18 agaA7 bldA39 NF SCP2*) (Merrick, 1976). Propagation of *c31* derivatives was on *S. lividans* 66. The rich agar medium R2YE and the minimal agar medium MM, and methods for subculturing strains, and making, transforming and regenerating protoplasts were as described in Hopwood et al. (1985); SMM was described by Takano et al., 1992; SY contained (w/v) 1.5% soluble starch, 0.1% Difco yeast extract, 0.1% K2HPO4, 0.1% MgSO4, 7H2O, 0.3% NaCl, 1.5% Difco agar, pH 7.4. Standard conditions were used for the transformation and growth of *Escherichia coli* strains ED7876 (the host for routine subcloning using pBR vectors) and TG1 (used for subcloning using pUC and other lacZ a-containing vectors) (Sambrook et al., 1989).

**Plasmid and bacteriophage vectors, cloning procedures and DNA techniques.** The *Streptomyces* vector for shotgun cloning of DNA from Pwb mutants was pIJE68 (Kieser & Melton, 1988), a low-copy-number SCP2*-derived vector containing the selectable *srs* gene encoding thiotrepton resistance. pIJE68 propagated in *S. lividans* was prepared for ligation by digestion with *BglII* and treatment with calf intestine alkaline phosphatase. In separate experiments it was then ligated with DNA from EG111, EG311 and EG441 after partial digestion of the donor DNA with Sau3AI to generate fragments of about 7–10 kb (fractionated on a sucrose gradient as described in Hopwood et al., 1985). The ligated DNA was used to transform protoplasts of *S. coelicolor* M124 to thiotrepton resistance as described in Hopwood et al. (1985). After sporulation (about 6 d) the colonies were replica-plated to R2YE plates spread with the *bldA* mutant J1700. After about 6 d these plates were replica-plated to MM containing histidine, uracil, streptomycin and thiotrepton to select for J1700 transconjugants containing pIJE68 (potentially with DNA inserts) and to counter-select the M124-based donor. After a further 6 d these were replicated to MM with histidine or uracil or both supplements to distinguish between pigmented colonies able to grow on singly supplemented plates that must have arisen from chromosomal recombination between M124 and J1700, and those probably arising from plasmid transfer (retaining both auxotrophic requirements). Standard procedures (Hopwood et al., 1985) were used to isolate and analyse plasmid DNA from selected strains. The presence of HindIII sites flanking the *BglII* cloning sites of pIJE68 and the rarity of such sites in *E. coli* host (Dam+ or Dcm+ or EcoK+) is introduced directly into *S. coelicolor* A3(2) (Kieser & Hopwood, 1991). The wild-type red DNA-containing plasmids pCLL1 (vector pBR327) (Covarrubias & Bolivar, 1982) for physical analysis and further subcloning. Where DNA was to be re-introduced into *S. coelicolor* derivatives, it was subcloned either into pIJE68 or pC31 KC861 (Bruton et al., 1991) with *S. lividans* as an intermediate host to bypass the restriction barrier observed when DNA from a methylating *E. coli* host (Dam+ or Dcm+ or EcoK+) is introduced directly into *S. coelicolor* A3(2) (Kieser & Hopwood, 1991). The wild-type red DNA-containing plasmids pCLL1 (vector pBR327, insert 16-kb EcoRI fragment including redB, redE and redF; Feitelson et al., 1983; Malpartida et al., 1990; DNA supplied by J. Feitelson) and pIJ2341 (vector pBR329, insert 13-kb PstI fragment including redD; Malpartida et al., 1990) were used as probes in Southern blotting (Hopwood et al., 1985).
The low-copy-number plasmid plJ2351, containing the right ca 50% of the red cluster in the vector plJ922 (Malpartida et al., 1990), was used to evaluate the effects of extra copies of redD and redZ on Red production by J1700.

DNA sequencing. Single-stranded DNA templates were sequenced by the chain-termination technique (Sanger et al., 1977) using defined restriction fragments cloned in M13 mp18/19 (Yanisch-Perron et al., 1985) and in some cases specially designed oligonucleotide primers. The sequence was determined on both strands and analysed using the program FRAME (Bibb et al., 1984). Database searches used the BLAST (Altschul et al., 1990) and FASTA (Devereux et al., 1984) programs.

S1 nuclease protection assays. A 1·2 kb BamHI–EcoRI fragment (sites 1–4 in Fig. 3 of White & Bibb, 1997), uniquely labelled at the 5' end of the EcoRI site located in the redZ coding region using [γ-32P]ATP and T4 polynucleotide kinase (Sambrook et al., 1989), was used to map the redZ transcriptional start site. About 0·02 pmol (approx. 108 Cerenkov counts min–1) of labelled probe was hybridized to 60 μg RNA in sodium TCA buffer (Murray, 1986) at 45 °C overnight after denaturation at 65 °C for 15 min. All subsequent steps were as described by Janssen et al. (1989). The RNA-protected fragment was subjected to electrophoresis alongside a sequence ladder generated using a 19 nt primer whose 5' end corresponded to the 5' end of the labelled EcoRI site used for S1 nuclease mapping.

Phylogenetic analysis. The program CLUSTAL W (Thompson et al., 1994) was used to align the sequences. Distances were calculated using PROTDIST, which employs maximum likelihood estimates based on the Dayhoff PAM matrix. The tree was constructed using FITCH, which employs the Fitch–Margoliash method, and displayed with TREEVIEW (Page, 1996). PROTDIST and FITCH were from the PHYLIP package, version 3.5 (Felsenstein, 1989).

RESULTS

Identification of mutants showing the Pwb (pigmented while bald) phenotype

To investigate the role of bldA in controlling antibiotic production, we sought mutants of the normally unpigmented bldA39 mutant J1700 that produced blue (γ-actinorhodin; Bystrykh et al., 1996) or red (undecylprodigiosin) antibiotic pigments on R2YE medium. Unexpectedly, deep red colonies were very frequent. These nearly all turned out also to be sensitive to chloramphenicol (Cm*). A high frequency of Cm* mutants with deep red mycelium has been noted previously in morphologically wild-type S. coelicolor (Sermonti et al., 1977) and is associated with DNA rearrangements (Altenbuchner & Cullum, 1984; Dyson & Schrempf, 1987). Although an explanation of the red pigmentation of such mutants would be of interest, their complex nature led us to avoid them in this analysis. We therefore screened again for mutants showing the Pwb phenotype, this time incorporating chloramphenicol (10 μg ml–1) into the medium. This caused a dramatic drop in the frequency of Pwb mutants, necessitating the use of mutagenesis. Even after mutagenesis we could find no blue (γ-actinorhodin-producing) colonies, but several showing moderate red coloration were obtained among the ca 1800 colonies screened after UV treatment and ca 2100 colonies after NTG treatment. We selected EG6 (Pwb-6) and EG9 (Pwb-9) (isolated following UV irradiation) and EG16 (Pwb-16) (isolated after NTG treatment) for further study (Fig. 1a). (In this paper we found it convenient to use the Pwb designation equally to describe phenotype, mutants and mutations; we have avoided its use in the italicized form, pwb, as a genetic designation, preferring the term redZ for the gene eventually identified through the Pwb mutants.) The mutants all showed perceptible red pigmentation on SY medium but EG6 differed from EG9 and EG16 in failing to develop pigment on MM with either mannitol or glucose as carbon source. Attempts to establish genetic map locations for the three mutations in crosses with a differently marked bldA mutant (J668; Merrick, 1976) did not give clear-cut results: although the phenotypic difference between the Pwb mutants and the J1700 parent was reproducible, intermediate or low levels of pigmentation were obtained among some recombinants, which could therefore not be scored satisfactorily. In the course of these crosses, some recombinants showing an unambiguous Pwb phenotype were retained, including the three strains EG311 (Pwb-6), EG111 (Pwb-9) and EG441 (Pwb-16) used as sources of DNA for cloning.

Cloning of Pwb DNA suggests a location for Pwb mutations in the red cluster

Cloning of the Pwb mutations was expected to provide access to DNA involved in red gene regulation. Because we did not know whether the Pwb mutations would be dominant or recessive, we used a cloning strategy that allowed the comparatively easy recognition of the desired clones in either case. The approach was to make a library of DNA from each Pwb mutant in the low-copy-number transmissible Streptomyces plasmid vector plJ698 and then to transfer the library from its primary host (M124) into the unpigmented bldA mutant J1700 using plate mating. The transconjugant colonies were scored for the Pwb phenotype on the presumption that they would be uniformly red if the Pwb mutations were dominant and specked with red if the mutations were recessive (because of the occasional production of Pwb/Pwb homogenotes by gene conversion). In fact, a few uniformly red transconjugant patches were obtained for each mutant (Pwb-6, 4/4800 colonies; Pwb-9, 8/1760 colonies; Pwb-16, 2/1200 colonies), suggesting that the Pwb mutations were dominant. For each mutant, plasmid DNA was isolated from one of the pigmented J1700 transconjugant colonies and designated plJ2530 (Pwb-6), plJ2520 (Pwb-9) and plJ2540 (Pwb-16). When each DNA sample was used to transform J1700, nearly all transformants were pigmented, verifying that the Pwb phenotype was associated with the plasmids and reinforcing the suggestion that the mutations were dominant.

The inserts from the three apparently Pwb-containing plasmids were subcloned as HindIII fragments into pBR327 in E. coli, yielding plJ2531 (Pwb-6), plJ2521 (Pwb-9) and plJ2541 (Pwb-16), and analysed by BamHI

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digestion. Several fragments appeared to be common to all three inserts and were similar in mobility to fragments present near the right-hand end of the red cluster. Proof that the plasmid clones indeed contained red DNA came from Southern blotting using the red-containing plasmid pIJ2341 (Malpartida et al., 1990) as probe. All three clones contained a common region of the red cluster (Fig. 2) that included the pathway-specific regulatory gene redD (Narva & Feitelson, 1990; Malpartida et al., 1990). It was possible that the Pwb phenotype caused by the clones might have been attributable to an additional copy of redD (or other red genes) rather than to cloned Pwb DNA. We therefore introduced pIJ2351, a pIJ922 derivative containing a segment of wild-type red DNA that spanned the inserts present in the three clones, into J1700 (pIJ2351 is based on the low-copy-number SCP2* replicon also present in the pIJ698 vector used for Pwb cloning). Slight pigmentation resulted, but it was much weaker than was seen with the Pwb clones (Fig. 1b), suggesting that the cloned DNA did indeed contain the Pwb mutations.

**Use of insert-directed integration of φC31 vectors to localize the Pwb-9 mutation**

Partial Sau3AI digests (fragment sizes ca 4 kb) of the Pwb-9-containing insert of pIJ2520 were used to provide quasi-random fragments for insertion into the BamHI
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Fig. 2. Restriction map of the part of the red cluster containing the Pwb mutations. The lower part shows details of subcloning of Pwb-9-containing DNA into \( \phi C31 \) KC861 after Sau3A1 digest of the plJ2520 insert. Solid lines indicate the approximate lengths of the inserts, vertical lines indicate restriction sites known to be present in each insert and dotted lines indicate the extent of uncertainty about the precise positions of the insert ends. The effects of the inserts on Red production in J1700, J1501 and EG9 are shown to the left of the map. Inserts giving a Red\(^+\) phenotype in J1700 presumably carry the Pwb-9 mutation, while those giving a Red\(^-\) phenotype on both J1501 and EG9 are likely to reflect disruption of a red transcription unit. The chromosomal segments present in plJ2520, plJ2530 and plJ2540, and the extents and directions of the coding regions of redZ (this paper) and redD (Narva & Feitelson, 1990) are shown above the map. The chromosomal insert in plJ2351 includes the entire mapped region and additional flanking sequences (Malpartida et al., 1990). The shading indicates the region that is common to all of the inserts that confer the Pwb phenotype on J1700 (with the exception of isolate 19) and that presumably contains the Pwb-9 mutation. The extent of the nucleotide sequence shown in Fig. 3 is indicated by the triangles and the BamHI fragment previously used to define redX (Guthrie & Chater, 1990) is marked by circles. B, BamHI; E, EcoRI; G, BgIII; N, NcoI; P, PvuII; S, SphI; X, XhoI.

site of KC861, a Streptomyces vector based on an attP-site-deleted derivative of the temperate phage \( \phi C31 \) (Bruton et al., 1991). Phages containing inserts were able to lysogenize S. coelicolor strains by single crossover integration at the homologous chromosomal region. If the insert contained a functional, dominant Pwb mutation, then at least some lysogens of the \( \text{bldA} \) mutant J1700 should have shown the Pwb phenotype (unless the insert also disrupted a red transcription unit needed for pigmentation).

Of 26 insert-containing phages tested, 11 caused pigmentation, further indicating that the cloned Pwb DNA was dominant. DNA isolated from a representative of each of the 26 types of lysogen was analysed by Southern blotting after EcoRI, BamHI, XhoI, NcoI and SphI digestion with plJ2341 as probe. DNA of the 26 phages was also subjected to restriction analysis with EcoRI, BamHI and PvuII. The analysis was somewhat complicated by the use of Sau3A1 as the enzyme for subcloning into KC861, but clear-cut results were obtained for 16 of the 26 phages (Fig. 2). The data confirmed the expected integration of the prophages into the red region and gave information on the extent, localization and orientation of the inserts. The inserts giving rise to the Pwb phenotype overlapped, containing a common segment of approximately 1 kb located about 4 kb to the left of redD. This segment was also present in one of the phages (isolate 19) that did not give rise to the Pwb phenotype. One explanation for this disparity could be recombination of the insert with the homologous region of the host chromosome to restore the wild-type sequence, which might have taken place during replication of the \( \phi C31 \) derivative in S. lividans (which contains a red cluster; Malpartida et al., 1990) during phage propagation.

To test whether any of the 26 insert-containing phages might contain fragments internal to red transcription units, they were also introduced into J1501, the \( \text{bld}^+ \) (hence Red-producing) parent of J1700, and into the Pwb mutant EG9 (Fig. 2). Of the 16 well-defined phages, isolates 9, 12 and 25 caused a loss of red pigmentation in J1501 and EG9, suggesting that they, and no other phages, contained inserts internal to red transcription units. Of these, numbers 9 and 12 contained inserts from the left end of the region, and number 25 an insert from the region between the 'common Pwb' region and redD. The insert in number 25 overlaps with a BamHI fragment shown previously – on the same criterion – to be internal to a biosynthetic transcription unit termed redX (Guthrie & Chater, 1990). Thus, the Pwb-9
Fig. 3. Nucleotide sequence of the redZ region. The sequence is oriented to correspond to Fig. 2 and extends between the marked BglII and EcoRI sites shown in that figure. The deduced amino acid sequence of RedZ is shown in single letter code above the nucleotide sequence. The position in the EcoRI site marked by a solid arrowhead and the transcription start point is marked by a solid hexamer for recognition by RNA polymerase holoenzyme are underlined, as is the TTA (leucine) codon at positions 675-677. The putative helix-turn-helix motif of RedZ is overlined. An inverted repeat that may play a role in phosphorylation are boxed. The putative helix-turn-helix motif of RedZ is overlined. An inverted repeat that may play a role in termination of both the redZ and redY transcripts is shown by converging arrows above the nucleotide sequence (positions 923-945). Usually, such DNA contains about 72 mol% GC and only 70% of codons end with G or C, whereas the central ca 40% of this gene contains about 60 mol% GC and the third base in at least 90% of codons is a G or C, whereas the central ca 40% of this gene contains about 60 mol% GC and only 70% of codons end with G or C, with several very rare codons being used. Most notably, the 'low GC' region ends at a TTA codon, providing a potential target for the action of bldA and therefore a possible explanation for the Red phenotype of bldA mutants. This, and the absence of any other complete ORF from the Pwb region, suggested that the gene, termed redZ, was responsible for the Pwb phenotype.

A gene in the Pwb region contains a TTA codon, potentially a target for the action of bldA

The region common to all the Pwb-9-containing phages consisted largely of an EcoRI fragment of approximately 800 bp. Accordingly, this fragment was subcloned from wild-type DNA present in pl2341 into M13 mp18 and sequenced. The sequence contained two converging protein-coding sequences, both truncated at their 5' ends. Further sequencing of the adjacent BglII-EcoRI fragment that contained most of the sequence common to the Pwb-containing phages revealed the 5' end of the longer of the two incomplete coding sequences found initially. The whole sequence is shown in Fig. 3. The central part of the coding sequence deviates somewhat from typical protein-coding DNA of streptomycetes. Usually, such DNA contains about 72 mol% GC and the third base in at least 90% of codons is a G or C, whereas the central ca 40% of this gene contains about 60 mol% GC and only 70% of codons end with G or C, with several very rare codons being used. Most notably, the deduced amino acid sequence of RedZ was compared with known proteins using the FASTA (Devereux et al., 1984) and BLAST programs (Altschul et al., 1990). RedZ
Fig. 5. Alignment of RedZ with the most closely related response regulators found in the databases. The positions of the highly conserved amino acids that appear to play an essential role in phosphorylation in this family of proteins, but that are absent from RedZ, are marked by triangles. The secondary structural features of the corresponding regions of CheY (Volz, 1995) discussed in the text are shown above the N-terminal domain. The putative helix-turn-helix motif (residues 163-182) is underlined. The sequences were aligned and displayed using the PILEUP and PRETTYBOX programs, respectively, of the UWGGG package (Devereux et al., 1984). The origin of amino acid sequences and their database accession numbers (SWISS-PROT unless stated otherwise) are: Dnrn-Strpe, Bacillus megaterium (P11454); Ramr-Strco, B. subtilis (P13800); Uvry-Ecoli, E. coli (P07027); Degu-Bacsu, Bacillus subtilis (P11470); Absa2-Strco, B. subtilis (P13800); Yfik-Bacsu, B. subtilis (EMBL D78508); Yxjl-Bacsu, B. subtilis (EMBL Y07902). Black boxes indicate positions in the alignment where the same amino acid is found in at least 8 of the 14 sequences; grey boxes indicate amino acids that are similar to those marked in black.

is related to a large family of regulatory proteins, many of which are response regulators partnered by sensory histidine protein kinases in so-called two-component systems (Fig. 5; Hackenbeck & Stock, 1996). The similarities extend through both of the characteristic domains of response regulators: the N-terminal domain that typically interacts with the sensor kinase, leading to phosphorylation of the response regulator; and the C-
terminal domain that contains an α-helix-turn-α-helix motif responsible for binding to specific DNA sequences in or near promoters, leading to gene activation or repression. In its N-terminal domain, RedZ shows moderate similarity to the cluster 3 sub-group of receiver modules of response regulators (Pao & Saier, 1995), the most similar being UhpA (27% identity), NarP (27%) and UvrY (28%) of E. coli, TrpO (25%) of Pseudomonas aeruginosa and BvgA (28%) of Bordetella pertussis (all percentage identities are for a region of overlap of 109 residues; Fig. 5). Cluster 3 receiver modules are generally, but not always, associated with DNA-binding domains classified as family 3 by Pao & Saier (1995). RedZ conforms to this tendency; its C-terminal putative DNA-binding region shows considerable similarity to family 3 DNA-binding domains, including DnrN (46%), AbsA2 (42%) and RamR (42%) from streptomycetes, DegU (44%) and GerE (45%) from Bacillus subtilis and MalT (37%) from E. coli (all percentage identities are for regions of overlap of 60 residues). In a detailed phylogenetic analysis (Fig. 6; Thompson et al., 1994; Felsenstein, 1989) the putative DNA-binding domains of RedZ, DnrN, AbsA2 and RamR, all from streptomycetes, formed a discrete subcluster. At least one family 3 response regulator from a Streptomyces sp. (BrpA) was well separated from this subcluster, showing that the clustering is not a trivial consequence of the high GC content of Streptomyces genes (Fig. 6). The receiver-like domain of RedZ did not appear to be particularly closely related, in evolutionary terms, to any other cluster 3 receiver module (data not shown).

Surprisingly, RedZ lacks several of the highly conserved residues that appear to be essential for phosphorylation of the receiver modules of response regulators. The invariant aspartate at position ca 55, which is the residue phosphorylated by the cognate histidine protein kinases, is replaced by valine; and the highly conserved lysine at position ca 100, which is generally required for efficient phosphotransfer, and which would usually be hydrogen-bonded to the unphosphorylated aspartate at position ca 55, is replaced by an alanine. Moreover, one of the pair of aspartates conserved at positions ca 11 and 12 is absent from RedZ. These two aspartates would usually be embedded in close apposition to the conserved aspartate/lysine pair, playing important roles in phosphorylation. Thus, the structure of this part of RedZ may be maintained by hydrophobic interactions, in contrast to the hydrogen bonding and Mg^2+ contacts that determine the structure of the phosphorylation pocket in typical response regulators (Volz, 1995).

Fig. 6. Phylogenetic tree of the presumptive DNA-binding domains found in class 3 response regulators (Pao & Saier, 1995), including RedZ and other streptomycete homologues. The amino acid sequences analysed correspond to positions 146-205 of RedZ shown in Fig. 5. The DNA-binding domain of AfsQ1 (AfsQ1-Strco, SWISS-PROT Q04942), a response regulator from S. coelicolor that belongs to class 2, not class 3, response regulators is given as an outgroup. Branch length is proportional to phylogenetic distance. The scale bar represents the number of substitutions per site during the evolutionary period represented by that length of branch. The origin of amino acid sequences not shown in Fig. 5 and their database accession numbers are: MalT-Escco, E. coli (P06993); AlkS-Pseol, Pseudomonas oleovorans (P17051); AgmR-Pseae, P. aeruginosa (P29369); LuxR-Vibfi, Vibrio fischerii (P12746); SdiAEscco, E. coli (P07026); NarLEscco, E. coli (P10957); ComABacsu, B. subtilis (P14204); BrpAStrhy, S. hygroscopicus (Q01108); FimLEscco, E. coli (P21502); RcsB-Escco, E. coli (P14374); RcsA_Kleae, Klebsiella aerogenes (P05338); NodW-Braja, Bradyrhizobium japonicum (P15940); FixJ-Rhime, Br. meliloti (P10958); FixJ-Rhime, Br. japonicum (P23221).
A Pwb mutant has a base change in the —35 region of the redZ promoter

Using pIJ2520, pIJ2530 and pIJ2540 as sources of DNA, the Pwb mutant copies of the 796 bp EcoRI fragment containing most of redZ were sequenced. No base changes were detected and the TTA codon present in the wild-type sequence was retained in each of the DNAs derived from the mutants. Analysis of the sequence upstream of this from Pwb-6 DNA revealed a base change in the —35 region of the redZ promoter (sequences upstream of the coding regions of redZ in Pwb-9 and Pwb-16 DNA have not yet been determined). This change caused a closer resemblance to the likely consensus —35 sequence for promoters presumed to be recognized by the major vegetative form of RNA polymerase holoenzyme:

- TCTCCA Pwb+
- TTTCCA Pwb-6
- TTGACA —35 consensus

To explain how this change might give rise to the Pwb phenotype, we postulate that it may improve interaction of the redZ promoter with RNA polymerase to give higher levels of the redZ transcript. Assuming a low degree of natural mis-reading of the UUA codon by charged non-cognate tRNAs, increased redZ mRNA levels would lead to proportionately greater production of RedZ protein, presumably to levels sufficient to activate RedZ target genes. Indeed, the presence of multiple (ca 10 copies) of redZ in a bldA mutant restores Red production, consistent with the notion of a low level of natural mis-translation (White & Bibb, 1997). Similar observations were also made with the TTA-containing gene actII-ORF4, which regulates actinorhodin production (Passantino et al., 1991; Fernández-Moreno et al., 1991). Quantitative analysis of redZ transcription in all three Pwb mutants is currently in progress.

DISCUSSION

The nature of the RedZ protein

Through the isolation of suppressor mutations causing pigment production in a bldA mutant, we discovered redZ, a gene encoding a response regulator-like protein, within the red biosynthetic cluster. Where sufficiently studied, all known end-to-end homologues of RedZ are subject to phosphorylation by a cognate histidine protein kinase, an interaction involving an N-terminal ‘receiver module’ domain of the protein. A few proteins consisting only of detached receiver domains are also known and the detailed structure of one of these, CheY, has provided the basis for much of the current modelling of receiver domains in more complex proteins. Here we use the CheY co-ordinates to compare residues conserved in the receiver domains of response regulators with those in equivalent positions in RedZ.

Volz (1995) presented a table of the structural roles of conserved residues of receiver modules. Most of these residues are of similar character in RedZ (Fig. 5), such that it should retain the overall ($\beta$/$\alpha$)5 structure of receiver domains. The most striking changes are those affecting the part of the protein that, in response regulators, is involved in phosphorylation; the normal hydrogen-bonding and Mg2+ binding that characterizes this pocket (Volz, 1995) would be replaced by hydrophobic interactions, preventing all possibility of conventional phosphorylation. However, the N-terminal domain of RedZ should retain the ability to take part in the general kinds of protein–protein interactions common to all receiver modules attached to DNA-binding domains. Typically these would include association with sensor kinases, intramolecular interaction with the DNA-binding domain, perhaps homo- (or hetero-) oligomerization and interaction with the transcription initiation complex at target promoters. We speculate that RedZ exhibits an unusual variation on the theme of receiver modules in which the normal phosphorylating pocket, made up of elements from different parts of the primary sequence, has become entirely reconstructed to fulfil a different biochemical function. For example, it could provide a binding site for a small co-regulator molecule that contains a hydrophobic feature, as in the case of LuxR which has an unrelated, N-acyl homoserine lactone-binding N-terminal domain coupled to its RedZ-like C-terminal DNA-binding domain (Pao & Saier, 1995). Alternative hypotheses, including the possibility that the N-terminal domain has no regulatory influence on the activity of RedZ, cannot be excluded at this stage.

The C-terminal domain of RedZ shows up to 46% identity with the sub-group of DNA-binding domains of response regulators known as family 3 (Pao & Saier, 1995). Within this sub-group, RedZ and several other streptomycete proteins involved in antibiotic production or morphological differentiation cluster together by themselves (Fig. 6). This clustering suggests a comparatively recent origin of the genes for these response regulators from a common ancestral gene that may have controlled a much simpler range of stationary phase options.

The significance of RedZ in the regulation of undecylprodigiosin biosynthesis

The regulatory roles played by RedZ-like proteins, the presence of a TTA codon in the redZ coding region and the discovery of a base change in the redZ promoter of one of the mutants all provide strong circumstantial evidence that redZ is the target for bldA in the red cluster. We have shown elsewhere that redZ is directly or indirectly required for transcription of red biosynthetic genes (White & Bibb, 1997). Its transcripts were present during exponential growth and increased in amount during transition and stationary phase, while transcription of redD, the previously identified pathway-specific regulatory gene, was confined to the two latter stages of growth. Moreover, transcription of redD was highly dependent on redZ, whereas mutation of redD had no effect on redZ transcription, suggesting that
targets of signal input that might influence the system. The Fig. 7. The involvement of they might respond are ppGpp (Chakraburtty et from Takano, personal communication) and phosphate levels, putative co-regulator is proposed to interact with the although the restoration of Red production under conditions of other genes (see Introduction). Among the signals to which RedZ is a transcriptional activator of RedZ is a transcriptional activator of positively acting pathway-specific regulatory gene con.

The finding that Red synthesis involves more than one regulatory gene in the red cluster may reflect the complexity of the converging biochemical pathway for Red biosynthesis (Coco et al., 1991). Such complex pathways may involve a modular genetic origin with different parts of the pathway having been brought into a common genetic background by lateral transfer of gene sets that had evolved separately. If so, each part of the pathway is likely to have co-evolved with its own pathway-specific regulatory system. Although transcription of redD depends, directly or indirectly, on redZ (White & Bibb, 1997), it will be interesting to determine whether RedZ and RedD independently activate different red genes or, at the other extreme, whether they are simply steps in a linear regulatory cascade. The latter seems to be the case in the closest parallel to the redZ/redD situation: the S. peucetius genes for daunorubicin production are transcriptionally dependent on DnrI, a RedD homologue and the dnrI gene is itself transcriptionally dependent on dnrN, which encodes a response regulator homologous to RedZ (Stutzman-Engwall et al., 1992; Madduri & Hutchinson, 1995; Otten et al., 1995; Figs 5 and 6). Although DnrN retains the aspartyl group which would be phosphorylated by interaction with a cognate sensor kinase, as well as most of the other conserved features of the active site, there is no nearby gene specifying an obvious potential protein kinase and a mutation of the potentially phosphorylated aspartyl residue had only a small effect on daunorubicin production (Otten et al., 1995). Thus, DnrN and RedZ may both fulfil their activating roles in an unphosphorylated state.

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Baltz, R. H. & Hosted, T. J. (1996). Molecular genetic methods for gene has been discovered. This gene (actII-ORF4) encodes a RedD-like protein. The additional component in the red gene cluster, RedZ, seems to offer opportuni
ties for further levels of regulatory signal input (Fig. 7) at the levels of redZ transcription [is it repressed by AbsA2 (Brian et al., 1996) and is its transcription affected by other pleiotropic regulatory genes?], translation (which is predicted to be bldA-dependent) and RedZ activity (which might be modulated post-translationally).

RedZ is a transcriptional activator of redD. Transcrip
tion of redZ appeared to be unaffected in a bldA mutant, but redD mRNA was undetectable, consistent with translational dependence of redZ on bldA and transcriptional dependence of redD on redZ.

Fig. 7. The involvement of redZ in Red biosynthesis provides multiple opportunities for the input of diverse signals. Bold arrows trace the pathway-specific regulatory system leading from redZ via redD to the expression of red structural genes (White & Bibb, 1997; Narva & Feitelson, 1990). Dashed arrows from shaded ovals indicate the major possible sources and targets of signal input that might influence the system. The putative co-regulator is proposed to interact with the hydrophobic pocket of RedZ that replaces the phosphorylation pocket of typical response regulators. The pleiotropic regulators referred to include various abs, aba, afs, bld and other genes (see introduction). Among the signals to which they might respond are ppGpp (Chakraburtty et al., 1996; Chakraburtty & Bibb, 1997), extracellular autoregulators (E. Takano, personal communication) and phosphate levels, although the restoration of Red production under conditions of phosphate-limited growth (Guthrie & Chater, 1990) may reflect elevated levels of mistranslation rather than a specific regulatory role (White & Bibb, 1997). We also speculate that the activity of RedD may itself be susceptible to modulation by some such signal input.

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