MbtH-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in *Streptomyces coelicolor* M145

Sylvie Lautru,^{1†} Daniel Oves-Costales,¹ Jean-Luc Pernodet² and Gregory L. Challis¹

Correspondence

Gregory L. Challis
G.L.Challis@warwick.ac.uk

¹Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK

²Institut de Génétique et Microbiologie, UMR CNRS 8621, Université Paris-Sud 11, 91405 Orsay Cedex, France

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MbtH-like proteins are a family of small proteins encoded by genes found in many, but not all, non-ribosomal peptide synthetase-encoding gene clusters that direct the biosynthesis of peptide antibiotics and siderophores. Studies published to date have not elucidated the function of MbtH-like proteins, nor have they clarified whether they are required for metabolite biosynthesis. Here it is shown that only one of two genes (*cdaX* or *cchK*) encoding MbtH-like proteins in *Streptomyces coelicolor* is required for biosynthesis of the peptide siderophore coelichelin and the calcium-dependent peptide antibiotic (CDA). The *cdaX* and *cchK* genes can functionally complement each other *in trans*, suggesting that CdaX and CchK can cross-talk with the coelichelin and CDA biosynthetic pathways, respectively. Transcriptional analyses of wild-type *S. coelicolor* and a double *cchK/cdaX* replacement mutant indicate that CchK and CdaX may not be involved in transcriptional regulation of coelichelin and CDA biosynthetic gene clusters.

INTRODUCTION

One consequence of large-scale bacterial genome sequencing is that a growing number of orphan genes encoding proteins of unknown function are appearing in the databases. Among such 'hypothetical proteins' are members of the MbtH-like family that typically contain around 70 aa and are encoded within gene clusters directing the non-ribosomal biosynthesis of peptide antibiotics and siderophores. The family is named after the protein encoded by the *mbtH* gene within the gene cluster directing the biosynthesis of the peptide siderophore mycobactin in *Mycobacterium tuberculosis* (Cole *et al.*, 1998; Quadri *et al.*, 1998). However, by no means all gene clusters encoding non-ribosomal peptide synthetase (NRPS) biosynthetic systems contain a gene encoding an MbtH-like protein and some MbtH-like proteins do not appear to be associated with NRPS gene clusters (e.g. NFA5500 from *Nocardia farcinica*). The MbtH-like family is rapidly expanding and searches of protein sequence databases indicate that about

130 members have been identified thus far, all in bacteria. Interestingly, no MbtH-like proteins have been identified in fungal NRPS gene clusters.

In a few cases, it has been shown that genes encoding MbtH homologues are cotranscribed with other genes encoding biosynthetic proteins. Thus, in the vicibactin gene cluster, *vbsG* (an *mbtH*-like gene) is cotranscribed with *vbsS* (an NRPS gene) (Carter *et al.*, 2002). The *ybdZ* (*mbtH*-like) gene in the *Escherichia coli* enterochelin biosynthetic gene cluster is cotranscribed with the *fes* and *entF* genes encoding enterochelin esterase and an NRPS, respectively (Pettis & McIntosh, 1987), and *mbtH* from the peptidoglycolipid biosynthetic gene cluster of *Mycobacterium smegmatis* is cotranscribed with two NRPS-encoding genes (Sondén *et al.*, 2005).

Two studies have examined whether a gene encoding an MbtH-like protein that is clustered with an NRPS-encoding gene is required for metabolite biosynthesis *in vivo*. In the first study, interruption of the *vbsG* gene within the vicibactin biosynthetic gene cluster by transposon insertion abrogated vicibactin production (Carter *et al.*, 2002). *vbsG* is the first gene of an operon containing the *vbsS* gene encoding the vicibactin NRPS. Therefore, this observation could stem from a polar effect on *vbsS* expression. However, insertions of *Tnlac* transposons are not normally polar on downstream genes. In a more recent study, Wohlleben and

[†]Present address: Institut de Génétique et Microbiologie, UMR CNRS 8621, Université Paris-Sud 11, 91405 Orsay Cedex, France.

Abbreviations: CDA, calcium-dependent peptide antibiotic; NRPS, non-ribosomal peptide synthetase.

A table of oligonucleotide primers used in this study is available as supplementary data with the online version of this paper.

coworkers showed that an in-frame deletion of *orf1* of the balhimycin biosynthetic gene cluster (encoding a homologue of MbtH) does not abolish balhimycin production in *Amycolatopsis balhimycina* (Stegmann *et al.*, 2006). This seems to indicate that Orf1 is not required for balhimycin biosynthesis, although the authors do not exclude the possibility of complementation by the other *mbtH* homologues (encoding AmyBal2 and AmyBal3) present in the genome of *A. balhimycina*.

Streptomyces coelicolor possesses two homologues of MbtH. The first one, CchK, is encoded by a gene within the *cch* cluster that directs coelichelin biosynthesis (Fig. 1a). Coelichelin is a peptide siderophore assembled by an unusual NRPS system encoded by *cchH* and *cchJ* (Lautru *et al.*, 2005). The second *S. coelicolor* MbtH homologue is CdaX, encoded by a gene within the calcium-dependent antibiotic (CDA) biosynthetic gene cluster (Fig. 1b) that directs NRPS-mediated assembly of an anionic lipopeptide (Hojati *et al.*, 2002).

Here, we investigate the involvement and role of CchK and CdaX in coelichelin and CDA biosynthesis, respectively.

METHODS

Strains, plasmids and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Micrococcus luteus* strains were grown in LB medium supplemented as necessary with appropriate antibiotics. Genetic manipulations of *S. coelicolor* and spore stock preparations were carried out on SFM medium (Kieser *et al.*, 2000). Oxoid nutrient agar was used for CDA bioassays and HPLC analyses of coelichelin production were carried out on culture supernatants of *S. coelicolor* strains grown for 4 days at 30 °C in the dark in an iron-deficient liquid medium (Müller & Raymond, 1984).

Preparation and manipulation of DNA. DNA extraction and manipulations, *E. coli* transformations, *Streptomyces* protoplast transformations and *E. coli*/*S. coelicolor* conjugations were performed according to standard procedures (Sambrook & Russell, 2001; Kieser *et al.*, 2000). The Expand high-fidelity DNA polymerase kit (Roche) was used to amplify *cchK* and *cdaX* from cosmids SCF34 and SCE8, respectively, and the *oriT-aac(3)IV* cassette from pIJ773. PCR reactions for verification of the replacement or deletion of genes in *S. coelicolor* were carried out using *Taq* polymerase from Fermentas. DNA fragments were purified from solution or agarose gels using

the Qiagen Qiaquick gel extraction kit. *S. coelicolor* chromosomal DNA for mutant analyses was obtained using the FastDNA Spin kit for soil from Q-biogene.

Disruptions of *cchK* and *cdaX*. PCR-based REDIRECT technology was used to construct the *cchK* and *cdaX* mutants (Gust *et al.*, 2004). In the SCF34 and SCE8 cosmids from the *S. coelicolor* ordered genomic library, the *cchK* and *cdaX* genes, respectively, were replaced with the *oriT-aac(3)IV* cassette from pIJ773. The oligonucleotides used for the replacements were as follows (bases identical to regions flanking *cchK* and *cdaX*, respectively, are underlined): for *cchK*, 5'-TTAGGCGAGCCTAACCTAATCCACTGGGAGGTAC-CGGGTATTCGGGGATCCGTCGACC-3' (forward primer) and 5'-AGTTGGGAGTTCACGGGCGACGCTTGACGGGGCTCGGCC-TGTAGGCTGGAGCTGCTTC-3' (reverse primer); and for *cdaX*, 5'-GAGTCTCCAGCCGACGCTCCCGAAGGAATGCGACGTGATTCCGGGGATCCGTCGACC-3' (forward primer) and 5'-CCCC-CTGCCGGGGACGTACGGGCCGTCGTGGTCCGGTCAATGTAGGCTGGAGCTGCTTC-3' (reverse primer).

The mutagenized cosmids (SCF34-*cchK*::*aac* and SCE8-*cdaX*::*aac*) were introduced into *S. coelicolor* M145 by conjugation from *E. coli* ET12567 containing pUZ8002, selecting for apramycin resistance. The primary transconjugants were screened for kanamycin sensitivity resulting in the double cross-over mutants W7 and W9. The desired gene replacements were confirmed in these mutants by PCR and sequencing using the following oligonucleotides: for *cchK*, 5'-CACGGCAGTTGGGAGTTCAC-3' (forward primer) and 5'-CCGGAAGACTAAGCTCATCG-3' (reverse primer); and for *cdaX*, 5'-GGCGGGATGCGCTTTAAGTG-3' (forward primer) and 5'-GGAAGGAAGACGGTCTCAG-3' (reverse primer).

To construct the *cchK* and *cdaX* double mutant, an in-frame *cchK* deletion mutant was first created. The SCF34-*cchK*::*aac* cosmid was introduced into *E. coli* BT340 to excise the disruption cassette, resulting in SCF34- Δ *cchK*. To introduce this cosmid into *S. coelicolor* M145 by conjugation, it was re-engineered to replace the *neo* gene in the superCos1 vector backbone with the *oriT-aac(3)IV* cassette, as described by Barona-Gómez *et al.* (2004), yielding SCF34- Δ *cchK*-*aac*. After conjugal transfer of this plasmid from *E. coli* ET12567/pUZ8002 to *S. coelicolor* M145, single cross-over recombination events were first selected for with apramycin. After a round of non-selective growth, potential double cross-over recombinants were identified by their sensitivity to apramycin. PCR using the same oligonucleotides as for W7 was used to identify a double cross-over mutant W8 from this pool of mutants.

cdaX in *S. coelicolor* W8 was disrupted using the same procedure for construction of *S. coelicolor* W9, resulting in the double *cchK*⁻ and *cdaX*⁻ mutant W10.

Fig. 1. Schematic representation of coelichelin (a) and CDA (b) biosynthetic gene clusters. The *mbtH*-like genes, inactivated in this study, are in bold. The names of the genes investigated by transcriptional analysis are shown.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Properties*	Source/reference
Strain		
<i>E. coli</i> DH5 α	General cloning host	Promega
<i>E. coli</i> BW25113/pIJ790	Strain used for λ RED mediated recombination	Gust <i>et al.</i> (2004)
<i>E. coli</i> ET12567/pUZ8002	Strain used for <i>E. coli</i> / <i>S. coelicolor</i> conjugation	Gust <i>et al.</i> (2004)
<i>E. coli</i> BT340	Strain used for FLP-mediated excision of disruption cassette	Datsenko & Wanner (2000)
<i>M. luteus</i>	Strain used for CDA bioassays	DSM 1790
<i>S. coelicolor</i> M145	Wild-type strain	John Innes Centre
<i>S. coelicolor</i> W7	M145 strain with <i>cchK</i> replaced with the <i>oriT-aac(3)IV</i> cassette from pIJ773	This study
<i>S. coelicolor</i> W8	M145 strain with in-frame deletion of <i>cchK</i>	This study
<i>S. coelicolor</i> W9	M145 strain with <i>cdaX</i> replaced with the <i>oriT-aac(3)IV</i> cassette from pIJ773	This study
<i>S. coelicolor</i> W10	M145 strain with in-frame deletion of <i>cchK</i> and <i>cdaX</i> replaced with the <i>oriT-aac(3)IV</i> cassette from pIJ773	This study
<i>S. coelicolor</i> W10/pSL75	W10 strain harbouring the plasmid pSL75	This study
<i>S. coelicolor</i> W10/pSL76	W10 strain harbouring the plasmid pSL76	This study
Plasmids		
pGEM-T Easy	Cloning vector for PCR products; Ap ^R	Promega
pUWL201	<i>E. coli</i> / <i>Streptomyces</i> shuttle vector, contains the <i>ermE</i> * promoter for expression of cloned genes in <i>Streptomyces</i> ; Ap ^R Ts ^R	Doumith <i>et al.</i> (2000)
pIJ773	Source of the apramycin cassette, containing the <i>aac(3)IV</i> gene and <i>oriT</i> flanked by FRT sites; Ap ^R Am ^R	Gust <i>et al.</i> (2004)
SCF34	Cosmid containing the <i>cch</i> cluster and used for the deletion of <i>cchK</i> ; Ap ^R Km ^R	PBL Biomedical Laboratories
SCE8	Cosmid containing part of the <i>cda</i> cluster and used for the deletion of <i>cdaX</i> ; Ap ^R Km ^R	PBL Biomedical Laboratories
SCF34- <i>cchK</i> :: <i>aac</i>	F34 cosmid with <i>cchK</i> replaced with the <i>oriT-aac(3)IV</i> cassette from pIJ773; Ap ^R Km ^R Am ^R	This study
SCF34- Δ <i>cchK</i>	F34 cosmid with in-frame deletion of <i>cchK</i> ; Ap ^R Km ^R	This study
SCF34- Δ <i>cchK</i> - <i>aac</i>	F34 cosmid with in-frame deletion of <i>cchK</i> and replacement of <i>neo</i> gene by the pIJ773 <i>oriT-aac(3)IV</i> cassette; Ap ^R Am ^R	This study
SCE8- <i>cdaX</i> :: <i>aac</i>	E8 cosmid with <i>cdaX</i> replaced with the <i>oriT-aac(3)IV</i> cassette from pIJ773; Ap ^R Km ^R Am ^R	This study
pSL73	<i>cchK</i> cloned into pGEM-T Easy	This study
pSL74	<i>cdaX</i> cloned into pGEM-T Easy	This study
pSL75	<i>cchK</i> cloned into pUWL201	This study
pSL76	<i>cdaX</i> cloned into pUWL201	This study

*Abbreviations: Ap^R, ampicillin resistance; Am^R, apramycin resistance; Km^R, kanamycin resistance; Ts^R, thiostrepton resistance.

Complementation of W10. The two genes *cchK* and *cdaX* were cloned into the *E. coli*/*Streptomyces* shuttle vector pUWL201 under the control of the *ermE** promoter. To ensure good translation initiation, a 17 bp long sequence corresponding to the one immediately upstream of the initiation codon in the expression vector pIJ6021 (Takano *et al.*, 1995) was introduced into the forward primers. The genes were amplified by PCR using the following oligonucleotides: for *cchK*, 5'-GGGGGAAGCTTGAGAAGGGAGCGGACATATGAGCACCAACCCCTTC-3' (forward primer, *Hind*III site underlined) and 5'-TTTTACTAGTTCAGGCGTCCGCGGTC-CG-3' (reverse primer, *Spe*I site underlined); and for *cdaX*, 5'-GGGGGAAGCTTGAGAAGGGAGCGGACATATGACCAATCCG-TTCGAAGA-3' (forward primer, *Hind*III site underlined) and 5'-GGGTACTAGTTCAGTTGCCGGTGCTCAT-3' (reverse primer, *Spe*I site underlined).

PCR products were first cloned into the pGEM-T Easy vector, yielding pSL73 and pSL74. After sequencing of the inserts, the *Hind*III-*Spe*I fragments from these plasmids were subcloned into *Hind*III/*Spe*I

digested pUWL201 vector (resulting in plasmids pSL75 and pSL76). The plasmids were introduced separately into *S. coelicolor* W10 by protoplast transformation.

HPLC analyses. A 1 M FeCl₃ solution (20 μ l) was added to 50 ml culture supernatants of *S. coelicolor* strains M145, W7, W8, W9, W10, W10/pSL75 and W10/pSL76 grown in iron-deficient medium, to form the ferricoelichelin complex. Supernatants were concentrated using a rotary evaporator and the residues were redissolved in the minimum amount of water. Samples were filtered through a Vivaspin 0.5 ml concentrator (10 000 Da molecular mass cut-off) and analysed on a Supelco Discovery HSF5 column (150 $\times$ 4.6 mm, 5 μ m, column temperature 20 $^{\circ}$ C) using an Agilent 1100 HPLC instrument equipped with a binary pump. Isocratic elution was carried out at 1 ml min⁻¹ with 1:9 10 mM ammonium carbonate (pH 7.0)/MeOH. The ferricoelichelin complex was detected by monitoring A₄₃₅ (Lautru *et al.*, 2005). Coelichelin production was evaluated in wild-type *S. coelicolor* and the W7 mutant by quantification of the peak area and expressed as mAU (g wet cells)⁻¹.

CDA bioassay. Production of CDA was detected using the bioassay adapted from Kieser *et al.* (2000). Patches of strains to be tested were grown on Oxoid nutrient agar (ONA) containing 200 µM of the iron chelator 2,2'-dipyridyl. In addition, *S. coelicolor* W9 was grown on ONA with 100 µM FeCl₃ (without 2,2'-dipyridyl). After 2 days incubation at 30 °C, each plate (20 ml) was overlaid with 2.5 ml soft nutrient agar containing the indicator strain *Micrococcus luteus*, FeCl₃ to a final concentration of 300 µM and Ca(NO₃)₂ to a final concentration of 15 mM. In the overlay of the control plate, Ca(NO₃)₂ was omitted. Inhibition of growth of *M. luteus* was observed after overnight incubation at 37 °C.

Transcriptional analysis. The *S. coelicolor* M145 and W10 strains were grown on cellophane sheets placed on plates of ONA medium containing 200 µM 2,2'-dipyridyl. After 48 h growth at 30 °C, the mycelium was collected and RNA was extracted using the method described by Oh & So (2003). The purified RNA samples were treated with DNase (Ambion) followed by extraction twice with phenol and once with chloroform. Oligonucleotides were designed to amplify gene fragments of about 400 bp (see Table S1 available as supplementary data with the online version of this paper) from the genes *sco3210*, *sco3216*, *sco3219*, *cdaR*, *absA2*, *cdaPS1*, *cdaPS2* and *fabH4* for the *cda* gene cluster, and *cchA*, *cchB*, *cchG*, *cchH* and *cchJ* for the *cch* gene cluster. They were first tested by carrying out PCR using wild-type *S. coelicolor* M145 genomic DNA and then used in PCRs with DNase-treated RNA samples from *S. coelicolor* M145 and W10 to ensure that no DNA was left in the preparations. The RT-PCR reactions were carried out using the One step RT-PCR kit (Qiagen) on 1 µg RNA using the following conditions: 50 °C for 30 min, 95 °C for 15 min, then 25 cycles of 95 °C for 45 s, 54 °C for 45 s and 72 °C for 40 s, and finally 10 min at 72 °C. RT-PCR products were analysed on a 1 % agarose gel by electrophoresis.

RESULTS

Sequence analysis of the MbtH-like protein family

MbtH homologues are encoded by genes of a mean size of around 216 bp. In a few cases, however, *mbtH*-like genes appear to be fused with other biosynthetic genes. Thus, an *mbtH*-like gene is fused to the 5' end of an NRPS-encoding gene in the nikkomycin biosynthetic gene cluster (NikP1,

accession no. CAC11137) (Lauer *et al.*, 2000), a cytochrome P450-encoding gene in the lyngbyatoxin biosynthetic gene cluster (LtxB, AAT12284) (Edwards & Gerwick, 2004) and a biotin carboxylase-encoding gene (Plu1218, CAE13512) in *Photorhabdus luminescens*. In the bleomycin gene cluster, *orf13*, encoding an MbtH homologue, has a C-terminal extension of about 100 aa showing no similarities to known proteins (Du *et al.*, 2000).

A sequence alignment of selected MbtH-like proteins is shown in Fig. 2. Sequence alignment of all members of this protein family reveals an overall high degree of sequence conservation among the various MbtH homologues. In particular, CchK and CdaX share 74 % amino acid identity and 83 % similarity. The two tryptophan residues W36 and W56 (CchK numbering), separated by 19–27 amino acid residues, are completely conserved in all members of the family. Removing the sequences of the MbtH homologues that are fused to other proteins (especially Plu1218, but also NikP1) allows two new universally conserved residues, W26 and S24, to be identified. This suggests that MbtH homologues fused to other proteins may no longer be functional. Among other well conserved residues are G35 (conserved in all homologues except VbsG from *Rhizobium etli* CFN 42; conserved in VbsG from *Rhizobium leguminosarum*), N18 (conserved in all but three homologues) and P61 (conserved in all but four homologues). Finally, MbtH homologues seem to possess two regions rich in residues with acidic side chains (Fig. 2).

Neither coelichelin nor CDA production is abolished in *cchK* or *cdaX* single disruption mutants

To examine the requirement of CchK for coelichelin biosynthesis, we replaced *cchK* on the chromosome of *S. coelicolor* M145 with an *oriT-aac(3)IV* cassette derived from pIJ773 to create *S. coelicolor* W7. The *cchK* gene is at one extremity of the *cch* cluster and does not form part of an

Fig. 2. Sample alignment of MbtH-like protein sequences. Numbering is according to the CchK sequence. Conserved residues are in red. Residues coloured pink are residues or families of related residues conserved at 85 %. Families of related residues are as follows: (I, L, V), (S, T), (W, F, Y), (D, E), (Q, N) and (H, K, R). The two regions rich in acidic residues are highlighted in blue.

operon with the flanking genes which are in the opposite orientation (Fig. 1a). Thus any polar effects on the expression of other *cch* genes in this mutant are excluded. HPLC analysis of coelichelin production by the mutant W7 showed that coelichelin is still produced (Fig. 3b), although a clear reduction of coelichelin titre is observed (about 2.5-fold from a mean of five measurements) compared to coelichelin production by the wild-type strain M145.

Fig. 3. HPLC analysis of coelichelin production by wild-type *S. coelicolor* and mutants M145 (a), W7 (b), W9 (c), W10 (d), W10/pSL75 (e) and W10/pSL76 (f).

Similarly, *cdaX* was inactivated in *S. coelicolor* M145 by replacement with the same *oriT-aac(3)IV* cassette to generate *S. coelicolor* W9. As with the W7 mutant, the organization of the genes around *cdaX* excludes the possibility of any polar effects in this mutant on the expression of other genes in the *cda* cluster. Production of CDA by the W9 strain was examined using a bioassay based on growth inhibition of the indicator strain, *M. luteus*, in the presence of calcium. Contrary to what was observed for the W7 mutant regarding coelichelin production, CDA production was abolished in the W9 mutant under these growth conditions (Table 2), suggesting the involvement of CdaX in CDA biosynthesis.

Results from the two sets of experiments appeared to be contradictory. However, because of the high number of identical amino acid residues in CdaX and CchK (74 %), it was hypothesized that CchK and CdaX can complement each other and that cross-talk can occur between the *cch* and *cda* biosynthetic pathways. Coelichelin is a siderophore and is therefore only biosynthesized under iron-deficient conditions. CDA, on the other hand, is an antibiotic whose production is temporally regulated and begins at the onset of aerial hyphae formation, or in liquid cultures at the transition between the exponential and stationary phases. The ONA medium used for CDA bioassay is a rich medium in which iron is abundant and expression of the *cch* gene cluster is therefore likely to be repressed. To create conditions of intracellular iron deficiency and thus elicit expression of the *cch* cluster, 2,2'-dipyridyl, a ferrous iron chelator, was added to ONA medium to a final concentration of 200 μ M. CDA production is restored in W9 under these conditions, demonstrating that cross-talk occurs between the two pathways (Table 2).

***cchK* or *cdaX* is required for coelichelin and CDA biosynthesis**

The W7 (*cchK*[−]) mutant still produced coelichelin and the W9 (*cdaX*[−]) mutant still produced CDA under appropriate

Table 2. CDA production by wild-type *S. coelicolor* and mutants with and without addition of 2,2'-dipyridyl (dipy), calcium nitrate (Ca²⁺) or ferric chloride (Fe³⁺)

<i>S. coelicolor</i> strain	dipy	Ca ²⁺	Fe ³⁺	CDA production*
M145	+	+	−	+
M145	+	−	−	−
W9	−	+	+	−
W9	+	+	−	+
W10	+	+	−	−
W10/pSL76	+	+	−	+

*CDA production was analysed as follows. Patches of *Streptomyces* strains were grown for 2 days on ONA medium, then overlaid with *Micrococcus luteus* and Ca(NO₃)₂, or just *Micrococcus luteus* (negative control).

conditions. The results reported above suggested that this was probably due to complementation of the *cchK* mutation by *cdaX* and vice versa. Consequently, we undertook the construction of a *cchK/cdaX* double knockout mutant. A *cchK* in-frame deletion mutant was constructed first, and *cdaX* was then replaced in this mutant with the *oriT-aac(3)IV* cassette used for construction of the W9 mutant to create *S. coelicolor* W10. HPLC analysis of this double mutant showed that coelichelin production was completely abolished (Fig. 3d). CDA bioassays carried out on ONA medium containing 200 µM 2,2'-dipyridyl indicated that W10 did not produce any CDA either (Table 2).

In trans* complementation of *S. coelicolor* W10 with *cchK* and *cdaX

To confirm that the abolition of coelichelin and CDA production in the W10 mutant was due to the disruption of *cchK* and *cdaX*, respectively, both genes were separately reintroduced into this strain. The *cchK* and *cdaX* genes were cloned into pUWL201, an *E. coli*/*Streptomyces* shuttle vector containing a *Streptomyces* origin of replication under the control of the strong constitutive *ermE** promoter. To ensure good translation, an artificial ribosome-binding site was introduced into the forward PCR primer 7 bp upstream of the start codon of each gene. Production of coelichelin was restored in *S. coelicolor* W10/pSL75 (complementation with *cchK*, Fig. 3e) and production of CDA was restored in *S. coelicolor* W10/pSL76 (complementation with *cdaX*, Table 2) confirming that CchK and CdaX are involved in coelichelin and CDA biosynthesis, respectively. In addition, W10/pSL75 produced CDA and W10/pSL76 produced coelichelin, consistent with the hypothesis that CchK and CdaX can functionally complement each other.

Inactivation of *cchK* and *cdaX* does not abolish transcription of the *cda* or *cch* gene clusters

To examine whether CdaX and CchK are involved in the transcriptional regulation of the CDA and coelichelin biosynthetic gene clusters, RNA from the wild-type M145 strain and the W10 double mutant was extracted after 48 h growth on ONA medium containing 200 µM 2,2'-dipyridyl (to relieve ferrous iron-promoted repression of the *cch* cluster). Purified RNA was used to examine transcription of a number of genes from the *cda* (*sco3210*, *sco3216*, *sco3219*,

cdaR, *absA2*, *cdaPS1*, *cdaPS2* and *fabH4*) and *cch* (*cchA*, *cchB*, *cchG*, *cchH* and *cchJ*) gene clusters using RT-PCR. These genes, which are indicated in Fig. 1 and mostly belong to different transcriptional units, were chosen to span the entire clusters and include the NRPS-encoding genes. Fig. 4 shows that all the genes examined in the two clusters are transcribed in both strains, indicating that inactivation of *cckK* and *cdaX* has no major effect on the transcription of the *cda* and *cch* genes.

DISCUSSION

Conflicting conclusions have been reported in previous studies of the requirement for MbtH-like proteins for non-ribosomal peptide biosynthesis. While a gene within the vicibactin biosynthetic gene cluster encoding an MbtH homologue is required for vicibactin production in *Rhizobium leguminosarum* (Carter *et al.*, 2002), an analogous gene in the bahlmimycin biosynthetic gene cluster has been reported not to be required for bahlmimycin production in *Amycolatopsis bahlmimycinia* (Stegmann *et al.*, 2006). A possible explanation for the latter result is that other MbtH homologues encoded by genes outside the bahlmimycin gene cluster in *A. bahlmimycinia* are able to functionally replace the protein encoded by the gene within the bahlmimycin cluster (Stegmann *et al.*, 2006). Our results provide strong support for this hypothesis by demonstrating that CchK can function in place of CdaX and vice versa in the biosynthesis of CDA and coelichelin in *S. coelicolor*. We suggest that such MbtH-like protein-mediated cross-talk between non-ribosomal peptide biosynthetic pathways is likely to be a general phenomenon in bacteria containing multiple NRPS gene clusters. It is not immediately obvious what selective pressure maintains two genes encoding functionally interchangeable proteins in the genome of *S. coelicolor*. The *cda* cluster directs biosynthesis of an antibiotic and its expression is activated during the transition from exponential to stationary phase in *S. coelicolor*. On the other hand, expression of the *cch* cluster is activated by intracellular ferrous iron deficiency and results in production of the siderophore coelichelin which sequesters extracellular ferric iron and transports it into the cell (Barona-Gómez *et al.*, 2006). Once iron homeostasis has been re-established by this iron uptake mechanism, the expression of this cluster should be repressed. Thus, the preservation of two genes encoding MbtH-like proteins in *S.*

Fig. 4. Transcriptional analyses of *cda* and *cch* genes in *S. coelicolor* M145 and W10. Lanes: 1, *cchA*; 2, *cchB*; 3, *cchG*; 4, *cchH*; 5, *cchJ*; 6, *hrdB*; 7, *sco3210*; 8, *sco3216*; 9, *cdaR*; 10, *absA2*; 11, *cdaPS1*; 12, *cdaPS2*; 13, *fabH4*; 14, *sco3219*.

coelicolor probably stems from the differential regulation of the *cda* and *cch* clusters. If the *cchK* gene was absent from the genome, coelichelin-mediated iron acquisition could only occur during stationary phase. Similarly, if the *cdaX* gene was absent from the genome, CDA production could only occur in the stationary phase under conditions of iron deficiency. Our data speak to the importance of coelichelin for ferric iron acquisition during vegetative growth of *S. coelicolor* (Barona-Gómez *et al.*, 2006).

Although our study demonstrates the requirement for CchK and CdaX for the biosynthesis of coelichelin and CDA *in vivo* in *S. coelicolor*, the role of these proteins in biosynthesis remains unclear. Another family of small proteins in *Streptomyces* species (the Wbl family) is involved in regulation, probably acting as transcriptional activators (Soliveri *et al.*, 2000). Moreover, a small domain, the Myb-like DNA-binding domain, which contains three conserved tryptophans, is found in the Myb family of eukaryotic proteins, a family of transcriptional regulators. Thus, a role for MbtH homologues in transcriptional regulation, either direct or as a 'helper' of another regulatory protein, as recently identified for *tylU* in tylosin biosynthesis regulation (Bate *et al.*, 2006), could be envisaged. However, our results show that neither CchK nor CdaX is required for transcription of either the *cda* or the *cch* gene clusters, suggesting that these proteins do not play a significant role in transcriptional regulation. A role in post-transcriptional regulation, however, cannot be ruled out. The *E. coli* enterochelin biosynthetic gene cluster contains a gene (*ybdZ*) encoding an MbtH-like protein. However, enterochelin biosynthesis *in vitro* can be reconstituted from EntB, EntE and EntF, suggesting that YbdZ may not play a significant role in this catalytic process (Gehring *et al.*, 1998). It would therefore be interesting to examine whether YbdZ is required for enterochelin production in *E. coli*. Other possible roles for MbtH-like proteins include participation in export of the metabolites and the mediation of protein-protein interactions that are important for metabolite assembly *in vivo*. Small domains with conserved tryptophan residues in other proteins have been shown to mediate such protein-protein interactions: for example, the W2 domain (two conserved tryptophans separated by 22–30 aa), found at the C-terminal extremity of some eukaryotic initiation factors and the WW domain (two conserved tryptophans separated by 20–23 aa) that binds to proline-rich regions of some proteins.

While the precise function of MbtH-like proteins remains unclear, it seems likely that they play an important role in the production of many non-ribosomal peptide metabolites in bacteria. Future studies aimed at examining the role of these proteins in post-transcriptional regulation, metabolite export and in mediating protein-protein interactions between components of NRPS biosynthetic systems should help to elucidate their function. Structural studies of these proteins may also provide valuable insight into their mode of action.

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