Iron-regulatory proteins DmdR1 and DmdR2 of *Streptomyces coelicolor* form two different DNA–protein complexes with iron boxes

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In high G + C Gram-positive bacteria, the control of expression of genes involved in iron metabolism is exerted by a DmdR [divalent (bivalent) metal-dependent regulatory protein] in the presence of Fe^{2+} or other bivalent ions. The *dmdR1* and *dmdR2* genes of *Streptomyces coelicolor* were overexpressed in *Escherichia coli* and the DmdR1 and DmdR2 proteins were purified to homogeneity. Electrophoretic mobility-shift assays showed that both DmdR1 and DmdR2 bind to the 19-nt *tox* and *desA* iron boxes forming two different complexes in each case. Increasing the concentrations of DmdR1 or DmdR2 protein shifted these complexes from their low-molecular-mass form to the high-molecular-mass complexes. Formation of the DNA–protein complexes was prevented by the bivalent metal chelating agent 2,2′-dipyridyl and by antibodies specific against the DmdR proteins. Cross-linking with glutaraldehyde of pure DmdR1 or DmdR2 proteins showed that DmdR1 forms dimers, whereas DmdR2 is capable of forming dimers and probably tetramers. Ten different iron boxes were found in a search for iron boxes in the genome of *S. coelicolor*. Most of them correspond to putative genes involved in siderophore biosynthesis. Since the nucleotide sequence of these ten boxes is identical (or slightly different) with the synthetic DNA fragment containing the *desA* box used in the present study, it is proposed that DmdR1 and DmdR2 bind to the iron boxes upstream of at least ten different genes in *S. coelicolor*.

Key words: divalent metal-dependent regulatory proteins (DmdR1 and DmdR2), genome, iron boxes, iron metabolism, siderophores, *Streptomyces*.

INTRODUCTION

In high G + C Gram-positive bacteria, the control of the expression of genes involved in iron metabolism is exerted by a DmdR [divalent (bivalent) metal-dependent regulatory] protein, previously named DtxR (diphtheria toxin repressor), in the presence of Fe^{2+} or other bivalent ions. This system was first reported in *Corynebacterium diphtheriae*, and controls the expression of the diphtheriae toxin (*tox*) gene [1]. Expression of the *tox* gene, located in the integrated corynephage β DNA, is controlled by the DtxR protein in presence of Fe^{2+}. The *tox* gene promoter region contains a 27 bp palindrome, which overlaps the -10 region of the promoter [2–4]. The minimal consensus sequence for binding of the DtxR protein was confirmed by Tao and Murphy [5] by the CAST (cyclic amplification and selection of targets) technique.

An analysis of the 28 kDa DtxR protein [1] showed that it binds to the *tox* palindromic sequence as a dimer (56 kDa form) [6,7], which was confirmed by cross-linking studies [8]. A bivalent metal (Fe^{2+}, Co^{2+}, Ni^{2+} or Cd^{2+}) is required for binding of the DtxR protein to the palindrome sequence in the DNA [5,6,9].

A gene coding for a 26 kDa DmdR protein homologous with the DtxR repressor of *C. diphtheriae* was cloned from the genome of '*Brevibacterium lactofermentum*' (syn. *Corynebacterium lactofermentum* [10]) [11,12]. Genes encoding similar proteins have been found in *Mycobacterium tuberculosis* [13,14], *Streptomyces pilosus* and *Streptomyces lividans* [15]. The *S. pilosus* protein probably binds to *tox*-like consensus sequence in the promoter of the *desA* gene (encoding lysine decarboxylase), a gene involved in the synthesis of the desferrioxamine B siderophore [16–18].

Recently, we cloned and characterized two different *dmdR* genes (*dmdR1* and *dmdR2*) in the genome of *Streptomyces coelicolor*, which were located in two non-overlapping cosmids. Hybridization studies showed that there are two *dmdR* genes in the species of *Streptomyces* tested, but only one in *Amycolatopsis lactamdurans* and *Streptoverticillium caespitosus* (F. J. Flores and J. F. Martín, unpublished work). Deletion of *dmdR1* resulted in derepression of at least four proteins and repression of five others, as shown by SDS/PAGE and two-dimensional proteomic analysis. In contrast, disruption of *dmdR2* did not affect the protein profile of *S. coelicolor*. The DmdR1 protein was present in *S. coelicolor* at different culture times, as shown by immunodetection analysis, and was absent from the *dmdR1*-disrupted mutants.

Streptomyces species are soil-dwelling micro-organisms that produce a variety of siderophores [19] to solubilize and transport iron. Since *S. coelicolor* appears to have developed an elaborate mechanism to control the expression of iron-regulated genes, it was of great interest to know if both the DmdR regulators have the same affinity to the palindromic sequence of iron-regulated promoters and to study the factors affecting the DNA–protein interaction. Identification of iron boxes and iron-regulated genes in *Streptomyces* is also a subject of great interest.

EXPERIMENTAL

Microbial strains, plasmids and culture conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. *S. coelicolor* cultures were grown in YEME medium [(per litre) 340 g of sucrose/5 g of yeast extract/5 g of peptone/3 g of malt extract/10 g of glucose/1.9 g of $MgCl_2 \cdot 6H_2O$/5 g of glycine] [20]. *Escherichia coli* strains were grown in 2TY or TB media [(per litre) 10 g of tryptone/5 g of yeast extract, pH adjusted to 7.2] [21] following standard procedures.

Abbreviations used: DmdR protein, divalent metal-dependent regulatory protein; DTT, dithiothreitol; DtxR, diphtheria toxin repressor; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase.

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Table 1 Strains and plasmids used in the present study

(a)

Strain	Genotype	Source
E. coli DH5α	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_k^- m_k^+$) *sup44 relA1* λ^- (φ80d*lacZ*ΔM15) Δ(*lacZYA-argF*) U169	Gibco BRL
E. coli BL21	B F$^-$ *dcm ompT hsdS* (rB$^-$ mB$^-$) *gal*	Stratagene
E. coli BL21-CP-RP	B F$^-$ *dcm ompT hsdS* (rB$^-$ mB$^-$) *gal* Tet^r *endA* Hte [*argU proL* Cam^r]	Stratagene
S. coelicolor A3(2)	Wild-type	John Innes Institute (Norwich, U.K.)

(b)

Plasmid	Gene	Source
pGEX-2T	*gst*	Amersham Biosciences
pGEX-dmdR1	*gst-dmdR1*	This study
pGEX-dmdR2	*gst-dmdR2*	This study

Recombinant DNA techniques and DNA sequencing

Plasmid DNA isolation, *E. coli* transformation, DNA sequencing and DNA amplification by PCR were performed by standard methods [21].

Antibodies against the DmdR1 and DmdR2 proteins

Polyclonal antibodies were obtained against the pure DtxR protein from *C. diphtheriae*, and the GST (glutathione S-transferase)-fused DmdR1 and DmdR2 proteins were isolated from SDS/polyacrylamide gel. New Zealand White rabbits were immunized by intradermal injection with the pure proteins, as described by Dunbar and Schwoebel [22]. This immunization process was repeated every 2 weeks for 3 months using incomplete Freund's adjuvant. After the completion of immunization, blood serum was collected by centrifugation, and the IgG fraction was purified by ammonium sulphate precipitation and FPLC using a Protein A–Sepharose column (Amersham Biosciences, Uppsala, Sweden) as described by Harlow and Lane [23].

Immunoaffinity columns

Anti-DtxR rabbit polyclonal antibodies were purified to prepare immunoaffinity columns. Rabbit anti-DtxR antiserum was precipitated with ammonium sulphate and the IgG fraction was purified by FPLC using a Protein A–Superose column (Amersham Biosciences). Immunoaffinity columns were prepared by linking the anti-DtxR IgGs to the Affi-Gel HZ matrix (Affi-Gel Immunoaffinity kit; Bio-Rad Laboratories) according to the manufacturer's instructions.

Purification of DmR1 by immunoaffinity chromatography

Since DmdR1 (but not DmR2) is present at moderate concentrations in cell extracts of *S. coelicolor*, it was purified by immunoaffinity chromatography. Crude extracts of *S. coelicolor* were obtained by cell disruption using a Branson sonicator (Sonifier B12). Cells were sonicated for 10 s, five times with 1.5 min intervals in TE buffer (10 mM Tris/HCl, pH 8.0/1 mM EDTA, pH 8.0) and the disruption was followed by microscopic observation. Cell debris was removed by centrifugation at 18000 ***g*** (for 20 min).

To remove completely the cell debris and the DNA, the extract was centrifuged at 28000 ***g*** (Beckman ultracentrifuge; SW41Ti rotor) for 30 min. The clear supernatant was applied to a column on Affi-Gel matrix with bound anti-DtxR antibodies. The column was washed with 5 vol. of equilibration buffer (100 mM Tris/HCl, pH 7.5) and 10 vol. of washing buffer (1 M NaCl/100 mM Tris/HCl, pH 7.5) to remove non-specific bound proteins. The retained proteins were eluted with elution buffer (50 mM HCl/glycine, pH 2.0) and collected in 1 ml fractions. The fractions were adjusted to pH 7.0 with 40 μl of neutralization buffer (1 M Tris/HCl, pH 9.5) and supplemented with 2 μl of 1 M DTT (dithiothreitol). All the fractions were analysed by SDS/PAGE and revealed by immunodetection with anti-DtxR antibodies.

SDS/PAGE and immunodetection

SDS/PAGE was performed as described by Laemmli [24]. For immunodetection, the proteins resolved by SDS/PAGE were blotted on to PVDF membranes (Immobilon; Millipore). The membrane was incubated with primary anti-DtxR, anti-DmdR1 or anti-DmdR2 antibodies for 2 h, and later with alkaline-phosphatase-conjugated antibodies for an additional 2 h. The immunodetected proteins were revealed with 4-Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-2-indolylphosphate using standard procedures (Roche).

Overexpression in *E. coli* and purification of DmdR1 and DmdR2

Both *dmdR1* and *dmdR2* genes were overexpressed in *E. coli* using the pGEX-2T expression system (Amersham Biosciences). Purification of the GST hybrid proteins in glutathione–Sepharose columns was performed according to the manufacturer's instructions. After elution and separation of the GST by filtration through the GSTrap column, the DmdR1 protein was filtered through a PD-10 column and eluted with 10 mM Tris/HCl (pH 7.5) buffer.

Further purification of DmdR2 by MonoS HR cation exchange

It was difficult to separate the DmdR2 protein from the GST using the GSTrap column. Therefore advantage was taken of the high pI value (8.1) deduced from the amino acid sequence, to purify the DmdR2 protein further by filtration through a MonoS HR 5/5 cation-exchange column. The DmdR2 protein was eluted with 300 mM NaCl in 20 mM sodium phosphate buffer (pH 7.0). Both the DmdR1 and DmdR2 proteins obtained by the GST-fusion procedure contained two extra amino acids (Gly-Ser) at their N-terminal end.

DNA–protein interaction assays

The binding of the DmdR1 and DmdR2 proteins to the synthetic 50-mer oligonucleotides, containing the iron box, was performed in a 20 μl reaction volume by the method of Tao and Murphy [5]. The 50-mer probes were labelled by 3′-end filling with [^{32}P]dCTP. Formation of DNA–protein complexes was monitored by EMSAs (electrophoretic mobility-shift assays) using SDS/PAGE (7% gel).

Cross-linking of protein monomers with glutaraldehyde

Protein was cross-linked with glutaraldehyde using pure preparations of DmdR1 in PBS buffer (obtained from the GSTrap column) and DmdR2 (obtained from the cation-exchange column) in 20 mM phosphate buffer (pH 7.0) with 300 mM NaCl. The solution of each protein (approx. 19 μg of protein/ml) was supplemented with 20 mM KCl, 5 mM $MgCl_2$, 0.125 mM $MnCl_2$ and 2 mM DTT (final concentrations) and incubated for 45 min at

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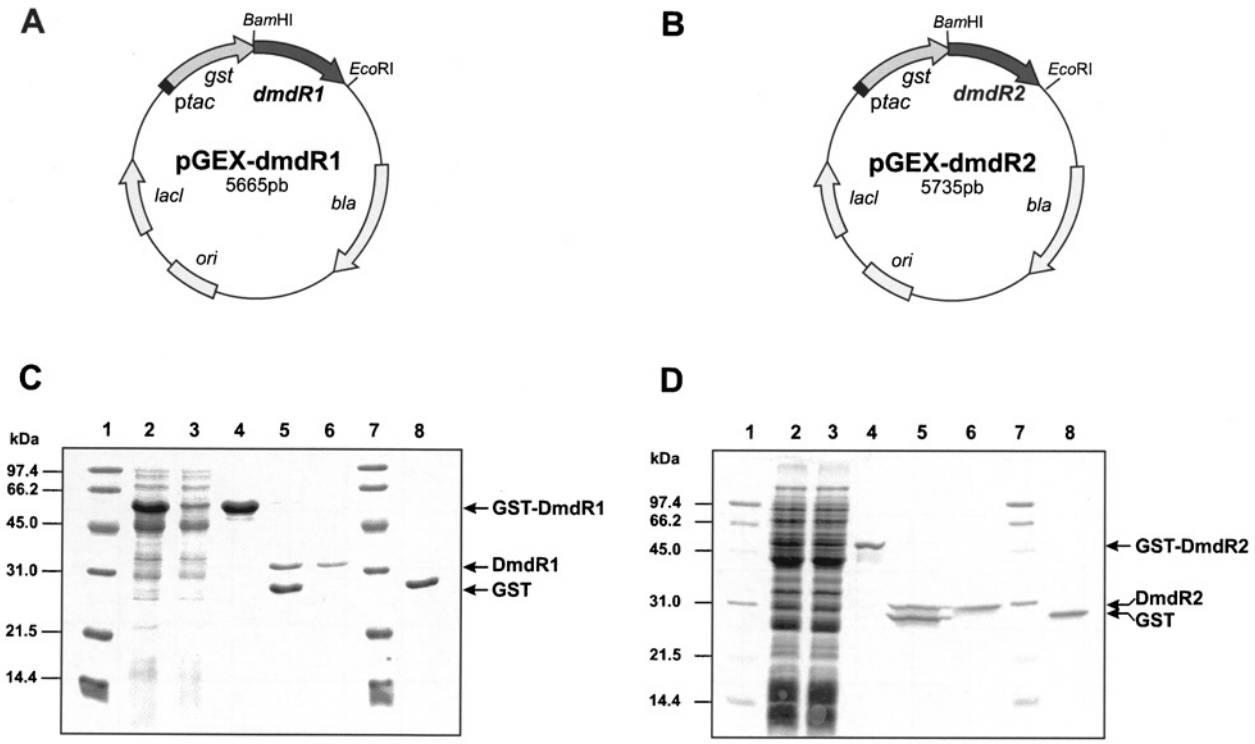

Figure 1 Overexpression of the *dmdR1* and *dmdR2* genes of *S. coelicolor* and purification of the DmdR1 and DmdR2 proteins

(**A**, **B**) Constructions with the pGEX plasmids for overexpression of *dmdR1* and *dmdR2*. *ori*, origin of replication; *gst*, GST gene. Only the relevant restriction sites are shown. (**C**) SDS/PAGE (13 % gel) of different fractions during purification of DmdR1: lanes 1 and 7, molecular-mass standards; lane 2, crude extract of *E. coli* DH5α [pGEX-*dmdR1*] after induction for 2 h; lane 3, non-retained proteins in the glutathione–Sepharose 4B column; lane 4, proteins retained and eluted from the glutathione–Sepharose 4B column; lane 5, proteins obtained after thrombin digestion of the protein eluted from the glutathione–Sepharose 4B column (note the formation of only DmdR1 and GST; arrows); lane 6, protein non-retained in the GSTrap column (pure DmdR1); lane 8, eluted protein retained in the GSTrap column (pure GST). (**D**) Purification of DmdR2. Lanes 1 and 7, molecular-mass standards; lane 2, crude extract of *E. coli* BL21-CP-RP [pGEX-*dmdR2*]; lane 3, non-retained protein in the glutathione–Sepharose 4B column; lane 4, proteins retained and eluted from the glutathione–Sepharose 4B column; lane 5, proteins obtained after thrombin digestion of the protein eluted from the glutathione–Sepharose 4B column (note the formation of only DmdR2 and GST; arrows); lane 6, eluted protein from the MonoS HR 5/5 cation-exchange column (pure DmdR2); lane 8, non-retained protein in MonoS HR 5/5 cation exchange.

30 °C to facilitate dimerization. Glutaraldehyde was then added to a final concentration of 0.2 % and allowed to cross-react with the protein for 1 min. The reaction was stopped after 1 min by addition of 1 vol. of loading buffer [1 ml of 2 M Tris/HCl (pH 6.8), 3.2 ml of glycerol, 0.8 ml of 0.1 % Bromophenol Blue, 0.64 ml of 2.5 M DTT] and heating for 3 min at 90 °C. The proteins were loaded on to SDS/polyacrylamide gels and, after electrophoresis, were visualized by Western blotting with anti-DmdR1 or anti-DmdR2 antibodies.

Modelling of the DmdR1 and DmdR2 proteins by homology

On the basis of homology of the DmdR1 and DmdR2 proteins with the known structure of *Mycobacterium tuberculosis* IdeR (DmdR) [25,26] DmdR1 and DmdR2 were modelled with the SWISS-MODEL [27–29] (http://www.expasy.ch/swissmod/SWISS-MODEL.htm).

RESULTS

Purification of DmdR1 and DmdR2 to near-homogeneity

Overexpression of each separate *dmdR* gene was made in *E. coli* using plasmid constructions pGEXdmdR1 and pGEXdmdR2 on the basis of the pGEX-2T expression system (Figure 1). Expression in *E. coli* DH5α or BL21 of the DmdR1 protein was good, but formation of the DmdR2 protein was poor even after optimization of the different isopropyl β-D-thiogalactoside-induction temperatures. This problem was solved in *E. coli* BL21-CP-RP, a strain with high levels of the tRNAs for the rare CCC, AGA and AGC codons.

Both proteins DmdR1 and DmdR2 were purified by the GST-fusion procedure using plasmid constructions pGEXdmdR1 and pGEXdmdR2 on the basis of the pGEX-2T expression system (Figure 1) as described in the Experimental section. The fusion proteins after expression in *E. coli* were purified by affinity chromatography through glutathione–Sepharose 4B and digested with thrombin. DmdR2 was further purified by an FPLC ion-exchange column using a MonoS HR 5/5 column. Electrophoretically pure forms of DmdR1 and DmdR2 were obtained (Figures 1C and 1D).

The DmdR1 and DmdR2 proteins bind to the *tox* and *des*A iron boxes forming different complexes

DNA fragments (iron boxes) were synthesized using the known nucleotide sequences of the iron-regulated promoters *tox* (of the diphtheriae toxin) of *C. diphtheriae* (50 nt) and *des*A of the desferrioxamine B biosynthesis (lysine decarboxylase gene) of *S. pilosus* (50 nt) (see the Experimental section). Binding results showed that the DmdR1 protein formed two complexes with high electrophoretic mobility (Figure 2) with the *des*A iron box, and

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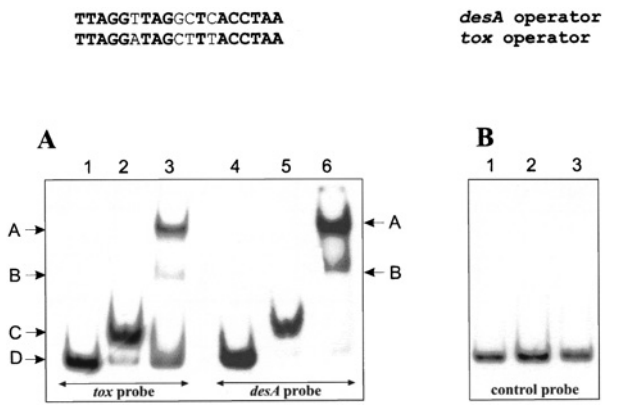

Figure 2 DNA–protein complexes formed by binding of DmdR1 and DmdR2 to the *tox* and *desA* iron boxes

(**A**) EMSA showing the interaction of the *tox* probe (lanes 1–3) or *desA* probe (lanes 4–6) with 40 ng of pure DmdR1 (lanes 2 and 5) and 8 ng of pure DmdR2 (lanes 3 and 6) proteins. Lanes 1 and 4, control *tox* and *desA* probes respectively without DmdR proteins. Note the formation of complexes C and D with the DmdR1 protein, and complexes A and B of larger molecular mass with the DmdR2 protein. The nucleotide sequence of the *desA* and *tox* iron boxes is shown in the upper part of the Figure. The nucleotides in common in both sequences are highlighted in boldface letters. (**B**) Lack of binding (negative control) of DmdR1 and DmdR2 to a 34 nt DNA fragment of the *S. clavuligerus ccaR* gene that is known to bind a different regulatory protein. Lane 1, DNA fragment without protein; lane 2, with DmdR1 (40 ng); lane 3, with DmdR2 (16 ng).

also with the *C. diphtheriae tox* box (Figure 2, arrows C and D). On the other hand, the protein DmdR2 formed a complex with very low mobility (i.e. high molecular mass) with both the *des*A and the *tox* iron boxes (Figure 2). The protein DmdR2 showed a very high affinity for the *desA* iron box, producing a complete mobility shift of the *desA*–DNA fragment even at very low protein concentration (4 ng). The DmdR2 protein also formed two distinct complexes of high molecular mass with the *tox* iron box (Figure 2, lanes 3 and 6, arrows A and B). A control was made using a 34 nt DNA fragment corresponding to the upstream region of the *S. clavuligerus ccaR* gene that binds a different protein. No gel mobility shift was observed for this DNA fragment with the DmdR1 or DmdR2 proteins (Figure 2B).

Metal requirement and specificity of the binding of DmdR1

Interaction of the DmdR1 protein with the *tox* (Figure 3, left panel) and the *desA* (Figure 3, right panel) iron boxes, as shown by EMSAs, was diluted with unlabelled probe (cf. lanes 2, 3 and 4 or 9, 10 and 11), and it was prevented by complexing the iron with the chelating agent 0.1 mM 2,2′-dipyridyl (lanes 6 and 13). Inactivation of the DmdR1 protein by boiling prevented the mobility shift (lanes 5 and 12). An interesting finding was the observation that the formation of the DmdR1 complex with both *desA* and *tox* iron boxes was prevented by addition of anti-DtxR antibodies (lanes 7 and 14), but not by the addition of non-specific anti-IgG rabbit antibodies (lanes 3 and 10). These results provided evidence showing that the interaction of the DmdR1 protein with the *tox* and *desA* iron boxes is highly specific.

The interaction of the DmdR1 protein with the *tox* and *desA* iron boxes requires Fe^{2+} (or another bivalent metal), as shown by the lack of gel shift of the DNA fragments when 2,2′-dipyridyl is used as bivalent metal chelating agent (Figure 3, lanes 6 and 13).

Metal requirement and specificity of the binding of DmdR2

Similar results were found when the pure DmdR2 protein was used in the EMSAs instead of DmdR1. As shown in Figure 4,

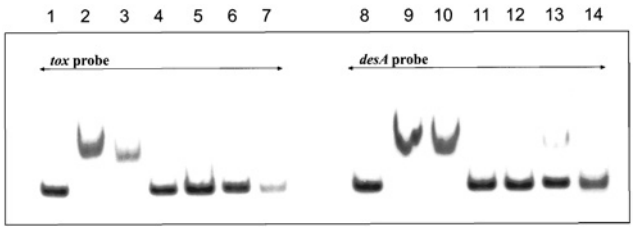

Figure 3 Metal requirement and specificity of the binding of DmdR1

Specificity of interaction and metal requirement of the DmdR1 protein (20 ng) with the *tox* probe (lanes 1–7) and the *desA* probe (lanes 8–14), as shown by EMSA. The DNA-binding reaction mixtures contain 20 mM $MnCl_2$ (except in lanes 3 and 10). Lanes 1 and 8, free probes without protein; lanes 2 and 9, probe with DmdR1 protein; lanes 3 and 10, probes with DmdR1 protein in the absence of metal ($MnCl_2$) and with control anti-IgG rabbit antibodies; lanes 4 and 11, competition with excess unlabelled probe: labelled probe, DmdR1 protein and excess unlabelled probe; lanes 5 and 12, probe with denatured DmdR1 protein (100 °C, 10 min); lanes 6 and 13, probe and DmdR1 protein with addition of the metal chelating agent 2,2′-dipyridyl (100 mM). Note the lack of binding when the metal ion is complexed. Lanes 7 and 14, probe and DmdR1 protein with specific anti-DmdR1 antibodies. Note that specific antibodies prevent formation of the complex.

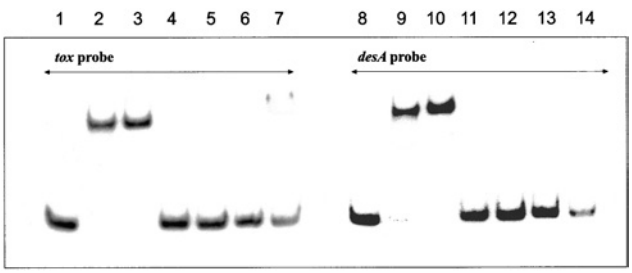

Figure 4 Metal requirement and specificity of the binding of DmdR2

Specificity of interaction and metal requirement of the DmdR2 protein (4 ng) with the *tox* (lanes 1–7) and *desA* probes (lanes 8–14). The DNA-binding reaction mixtures contain 20 mM $MnCl_2$ (except in lanes 3 and 10). Lanes 1 and 8, control probes without protein in the reaction mixture; lanes 2 and 9, probe with DmdR2 protein; lanes 3 and 10, probe with DmdR2 protein in the absence of metal ($MnCl_2$) and with anti-IgG antibodies. Lanes 4 and 11, probe with DmdR2 and excess unlabelled probe (competition effect); lanes 5 and 12, probe with denatured DmdR2 protein (100 °C, 10 min); lanes 6 and 13, probe with DmdR2 and the metal chelating agent 2,2′-dipyridyl. Note the lack of shift in the absence of metal ions; lanes 7 and 14, probe with DmdR2 and specific anti-DmdR2 antibodies. Note that specific antibodies prevent formation of the complex.

the specificity of DmR2 for both the *desA* and *tox* iron boxes is similar to that of DmdR1 (cf. Figures 3 and 4). The interaction of DmdR2 with the iron boxes of both genes also requires Fe^{2+} or Mn^{2+} and was prevented by 2,2′-dipyridyl and neutralized with anti-DtxR antibodies.

Increasing the amount of DmdR1 or DmdR2 resulted in the formation of two complexes of different masses with the *tox* iron box

DmdR1 and DmdR2 showed a high affinity for the *tox* and *desA* iron boxes. To study the type of complex formed, increasing concentrations of either DmdR1 (1.25, 2.5, 5, 10, 20 and 40 ng) or DmdR2 protein (0.125, 0.25, 0.5, 1, 2, 4 and 8 ng) were added to a constant amount of labelled probe. The lower amount of either DmdR1 or DmdR2 was limiting for complex formation, and the low-molecular-mass complex started to accumulate at increasing protein concentration in the reaction mixture (Figure 5A, lanes 3 and 10). The DmdR2 protein was active at lower concentrations, and formation of DNA–protein complexes were already observed at DmdR2 concentration of 0.5 ng (Figure 5B, lane 3).

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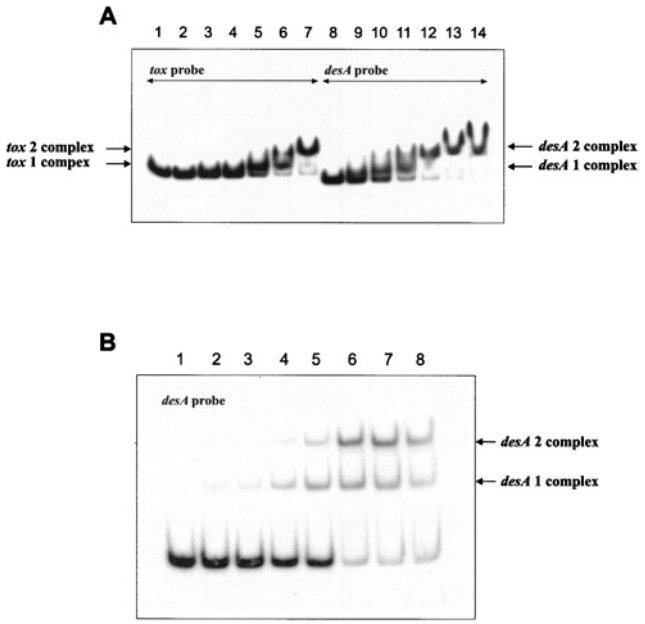

Figure 5 DNA–protein complexes formed by increasing amounts of DmdR1 (A) or DmdR2 protein (B)

(**A**) Complexes of DmdR1 with the *tox* probe (lanes 1–7) or the *desA* probe (lanes 8–14). Lanes 1 and 8, control probes without DmdR1 protein. Lanes 2–7 and 9–14, probe with increasing amounts of DmdR1 protein (2.5, 5, 10, 20, 40 and 80 nM respectively). (**B**) Complexes of DmdR2 with the *desA* probe. Lane 1, control probe without DmdR2. Lanes 2–8, probe with increasing DmdR2 concentration (0.5, 1, 2, 4, 8, 16 and 32 nM respectively). Note the formation of the *tox1* and *tox2* and the *desA1* and *desA2* complexes (arrows) with increasing concentration of the DmdR1 protein (**A**) or the DmdR2 protein (**B**).

The high-molecular-mass complex accumulated at higher DmdR2 concentrations (2, 4 and 8 ng). Essentially, all *desA* DNA probe in the assay was retarded when high concentrations of DmdR2 were used, whereas this does not occur at low concentration of this regulatory protein. These results prove that the DmdR2 protein forms two distinct complexes with the *desA* promoter, which most probably correspond to the binding of the dimer and/or tetramer (see below) forms respectively of this protein. The basic unit for binding of the DtxR repressor is the dimer.

Cross-linking with glutaraldehyde shows that DmdR2 forms tetramers

DtxR is known to form dimers by interaction of two monomers through the dimerization domain of the protein. The initial EMSA results suggested that DmdR2, but not DmdR1, is capable of forming a high-molecular-mass complex in the DNA–protein interaction. To ascertain this, glutaraldehyde-mediated cross-linking of pure DmdR1 and DmdR2 proteins in solution was performed in the presence or absence of Mg^{2+} and Mn^{2+} ions. Formation of the complexes was detected by immunoblotting with antibodies prepared against the pure DmdR1 or DmdR2 protein. Results (Figure 6) showed that DmdR1 forms a dimer (molecular mass of approx. 65 kDa) when cross-linked with glutaraldehyde either in presence or absence of bivalent ions, indicating that DmdR1 is capable of forming dimers in solution. Similarly, dimer formation was observed when pure DmdR2 was cross-linked with glutaraldehyde. In addition, DmdR2 formed tetramers (molecular mass of approx. 130 kDa), as shown by immunodetection with anti-DmdR2 antibodies. This ability of DmdR2 to form dimers and tetramers explains the formation of a high-molecular-mass complex with the *tox* or *desA* DNA boxes.

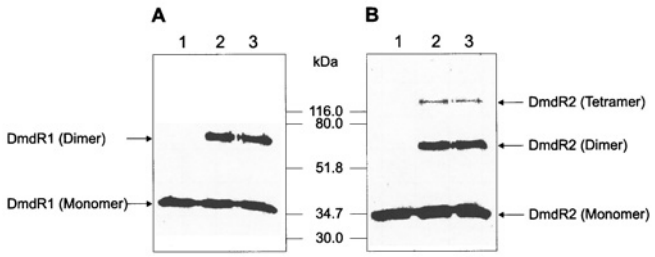

Figure 6 Cross-linking with glutaraldehyde shows that DmdR2 forms tetramers

Cross-linking with glutaraldehyde of DmdR1 (**A**) or DmdR2 (**B**). Lane 1, DmdR1 (or DmdR2) without glutaraldehyde. Lane 2, DmdR1 (or DmdR2) without $MnCl_2$ or $MgCl_2$ cross-linked with glutaraldehyde. Lane 3, DmdR1 (or DmdR2) with $MnCl_2$ and $MgCl_2$ cross-linked with glutaraldehyde. Note that DmdR1 forms dimers, whereas the DmdR2 protein is able to form dimer and tetramer forms. The proteins were revealed with anti-DmdR1 antibodies (**A**) or anti-DmdR2 antibodies (**B**).

Iron boxes in the *S. coelicolor* genome

The nucleotide sequence of iron boxes of the *desA* and *tox* promoters have 14 nt in common, including the inverted repeat. Availability of the *S. coelicolor* genome sequence [30] prompted us to search for genes containing iron boxes in *S. coelicolor*.

A search of the *S. coelicolor* genome revealed the presence of ten putative genes containing well-conserved iron boxes (Figure 7). Many of the proteins encoded by these genes are poorly characterized. They include: (i) CD1, located in the 5′ region of a pyridoxal-phosphate-dependent decarboxylase gene similar to the lysine decarboxylase encoded by the *desA* gene; (ii) CD2, corresponding to an ATP-binding-cassette transporter; (iii) CD3, CD4, CD5 and CD6, located upstream of several *cch* genes (*cchJ*, *cchF*, *cchE* and *cchA*), corresponding to the colichelin siderophore cluster [31]; (iv) CD8 located in the 5′ region of a second iron-siderophore ATP-binding-cassette transporter; (v) CD10, corresponding to an integral membrane protein; and (vi) CD7 and CD9, upstream of two hypothetical 25.8 and 33.0 kDa proteins.

The finding of CD1 allowed us to identify a gene cluster containing a pyridoxal-phosphate-dependent putative lysine decarboxylase (*S. coelicolor* open reading frame SC02782), which appears to be involved in the biosynthesis of the desferrioxamines G1 and E siderophores [19] (F. J. Flores and J. F. Martín, unpublished work).

Comparison of the consensus iron box deduced from the 10 CD sequences showed a sequence ttaggttAGGCTcaCCTaa, essentially identical with that of the *desA* box. The t in the second position and the a in position 18 is conserved in only 70 % of the CD sequences.

DISCUSSION

Soil actinomycetes are able to extract iron usually from iron salts in the soil. They probably contain a DmdR protein similar to DtxR to regulate iron metabolism. This hypothesis is supported by the observation that in *S. coelicolor* and other *Streptomyces* species, iron starvation leads to a change in the pattern of several proteins in extracts of these bacteria (F. J. Flores and J. F. Martín, unpublished work).

A few examples of proteins homologous with the DtxR repressor of *C. diphtheriae* have been reported in different Gram-positive bacteria, including members of the genera *Corynebacterium*, e.g. *C. lactofermentum* [11,12], *Mycobacterium* [13], *Streptomyces* [15], and *Rhodococcus*, e.g. *R. fascians* [4] and

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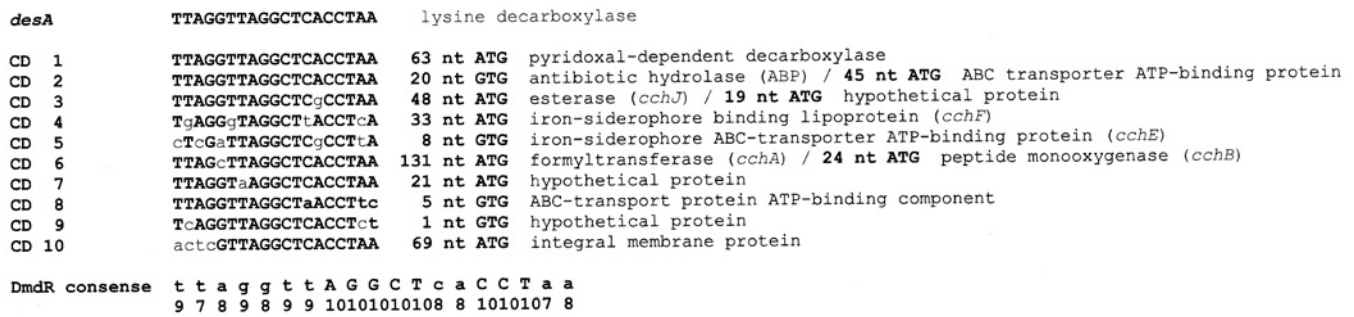

Figure 7 Iron boxes in the *S. coelicolor* genome

Iron box sequences (CD1–CD10) found in the genome of *S. coelicolor* by searching with the *desA* box of *S. pilosus* (upper line). The distance in nucleotides of the iron boxes to the ATG-translation start codon is indicated. The consensus iron box sequence and the percentage of conservation is indicated in the lower line. Capital letters indicate full conservation of the nucleotides [10, conservation in the ten CD sequences (100 %); 9, 90 % conservation; 8, 80 % conservation; 7, 70 % conservation]. The designation of the genes and the putative proteins encoded by them are indicated on the right-hand side (see text for further details).

R. equi [32], and *Bacillus subtilis* [33]. A protein, SirR (staphylococcal iron regulator repressor) showing a lower identity (29 % identical residues) has been reported in *Staphylococcus epidermis* [34]. A related protein named ScaR has been described in *Streptococcus mutans* [35,36] and *Streptococcus gordonii* [35]. All these proteins require Fe^{2+} (with the exception of ScaR) or other bivalent metals, including Cd^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+}, and, therefore, belong to a common family of bivalent-metal-dependent repressors that include at least two subfamilies: (i) the DtxR-like repressors of corynebacteria, *Rhodococcus*, *Mycobacterium* and filamentous actinomycetes; and (ii) the Gram-positive cocci (*Staphylococcus* and *Streptococcus*) subfamily. The ScaR protein requires Mn^{2+}, Ni^{2+} or Zn^{2+} (but not Fe^{2+}). A second DtxR-like protein, MntR, has been described recently in *C. diphtheriae*, which works by a Mn^{2+}-dependent mechanism [37]

The *S. coelicolor* DmdR1 and DmdR2 proteins belong to the DtxR-like subfamily. As reported in this study, both the DmdR1 and DmdR2 proteins recognize and bind the *tox* box of *C. diphtheriae* and the *desA* box of *S. pilosus*. The DmdR2 protein showed a higher affinity (approx. 2-fold) for these sequences when compared with DmdR1. As shown in Figure 4, binding of DmdR1 or DmdR2 was prevented by the addition of the iron chelator 2,2′-dipyridyl. The binding of DmdR1 and DmdR2 was avoided by boiling the protein or by neutralizing the DmdR proteins with anti-DtxR- or anti-DmdR1- or anti-DmdR2-specific antibodies, but was not completely prevented by the absence of the metal ion in the binding mixture, suggesting that a sufficient amount of Fe^{2+} (or bivalent metal) is bound to the protein in the *E. coli* extracts. Previously reported purified protein (e.g. IdeR) [14] was obtained by the nitrilotriacetate-based Ni^{2+}-affinity chromatography, and this technique may favour the purification of metal-free protein due to the nitrilotriacetate metal-complexing activity, whereas, in our experiments, purification was performed by glutathione affinity of the GST–DmdR hybrid protein. However, the protein-bound metal was chelated by 2,2-dipyridyl, thereby preventing the reaction. Since 2,2′-dipyridyl is particularly active in chelating iron when compared with Mn^{2+}, our results suggest that both MdmR1 and DmdR2 require Fe^{2+}, although they may also work with other bivalent ions.

An important observation is the formation of two DNA–protein complexes with DmdR1 or DmdR2. These results indicate that the DmdR1 or DmdR2 protein binds to the DNA as two basic units (each functional unit being a dimer); therefore the low-molecular-mass complex disappears when more protein is added to the binding reaction and is converted into the high-molecular-mass complex. This is in agreement with the results of crystallization studies of DNA-bound DtxR and IdeR proteins of *C. diphtheriae* and *M. tuberculosis* [25,38,39], which show binding of two protein dimers to the iron-box sequence.

Although the molecular masses of both DmdR1 and DmdR2 proteins are similar, the mass of the DNA–protein complex formed is very different (cf. lanes 2 and 3 or 5 and 6 in Figure 2). Our initial results suggested that DmdR2 is capable of forming tetramer molecules. This hypothesis was confirmed by glutaraldehyde cross-linking experiments in the absence of DNA. Immunodetection of the cross-linked protein with antibodies against DmdR1 or DmdR2 revealed that DmdR2, but not DmdR1, is able to form tetramers. This is probably due to the presence (in addition to the dimerization domain that occurs in both DmdR1 and DmdR2) of eight additional residues in a proline- and alanine-rich region in DmdR2. This highly hydrophobic amino acid stretch is exposed in the proposed structure for DmdR2 based on the known structure of IdeR (results not shown) and may explain the formation of tetramers by an hydrophobic interaction between the two dimers.

Several putative iron boxes were found upstream of ten different open reading frames in the genome of *S. coelicolor*. This allowed us to define a consensus iron box for *Streptomyces* species (TTAGGTTAGGCTCACCTAA; Figure 7).

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