Identification and functional analysis of enzymes required for precorrin-2 dehydrogenation and metal ion insertion in the biosynthesis of sirohaem and cobalamin in *Bacillus megaterium*

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In *Bacillus megaterium*, the *hemAXBCDL* genes were isolated and were found to be highly similar to the genes from *Bacillus subtilis* that are required for the conversion of glutamyl-tRNA into uroporphyrinogen III. Overproduction and purification of HemC (porphobilinogen deaminase) and -D (uroporphyrinogen III synthase) allowed these enzymes to be used for the *in vitro* synthesis of uroporphyrinogen III from porphobilinogen. A second smaller cluster of three genes (termed *sirABC*) was also isolated and found to encode the enzymes that catalyse the transformation of uroporphyrinogen III into sirohaem on the basis of their ability to complement a defined *Escherichia coli* (*cysG*) mutant. The functions of SirC and -B were investigated by direct enzyme assay, where SirC was found to act as a precorrin-2 dehydrogenase, generating sirohydrochlorin, and SirB was found to act as a ferrochelatase responsible for the final step in sirohaem synthesis. CbiX, a protein found encoded within the main *B. megaterium* cobalamin biosynthetic operon, shares a high degree of similarity with SirB and acts as the cobaltochelatase associated with cobalamin biosynthesis by inserting cobalt into sirohydrochlorin. CbiX contains an unusual histidine-rich region in the C-terminal portion of the protein, which was not found to be essential in the chelation process. Sequence alignments suggest that SirB and CbiX share a similar active site to the cobaltochelatase, CbiK, from *Salmonella enterica*.

Key words: CbiX, SirA, SirB, SirC, sirohydrochlorin.

INTRODUCTION

All modified tetrapyrroles, including haem, chlorophyll, cobalamin (vitamin B_{12}), sirohaem, coenzyme F_{430} as well as the open chain bilins, are synthesized along a branched biosynthetic pathway [1]. One of the ways in which flux along a particular branch of the pathway is regulated is by the insertion of a specific metal ion at the branching-point intermediate. For example, insertion of magnesium into protoporphyrin IX determines that the molecule is destined for chlorophyll (or bacteriochlorophyll) synthesis, whilst insertion of ferrous iron forms haem [2]. Similarly, in bacteria, the formation of sirohydrochlorin represents another branch point, where the presence of cobalt destines the intermediate for cobalamin synthesis whereas insertion of ferrous iron generates sirohaem (Scheme 1) [3]. In this study we examine the enzymes that generate sirohydrochlorin from uroporphyrinogen III within *Bacillus megaterium* and identify the chelatases associated with sirohaem and cobalamin biosynthesis.

The reasons for studying tetrapyrrole biosynthesis in *B. megaterium* are multiple, including its large size, ease of genetic manipulation, its inherent neat genetic organisation and its previous record as a commercial source of vitamin B_{12} [4]. *Bacillus subtilis* is better characterized among the Bacilli, but it does not house the metabolic ability to synthesize vitamin B_{12} *de novo*. Indeed we hope to be able to use *B. megaterium* as a model organism for understanding not only how cobalamin is synthesized, but also how this synthesis is controlled and regulated in relation to the biosynthesis of sirohaem and haem. Such studies could lead to the generation of improved *B. megaterium* strains with enhanced vitamin B_{12} production.

Sirohaem, the prosthetic group of sulphite and nitrite reductases, is synthesized from uroporphyrinogen III in three steps (Scheme 1). Firstly uroporphyrinogen III is transformed into precorrin-2 by two *S*-adenosyl-L-methionine (SAM)-dependent methylations, at the base of the acetate side chains at positions 2 and 7, by an enzyme called uroporphyrinogen III methyltransferase. The second step results in an overall oxidation of the macrocycle in a reaction catalysed by precorrin-2 dehydrogenase, which requires NAD^+ as a cofactor. Finally, sirohaem is synthesized by insertion of ferrous iron into sirohydrochlorin in a reaction catalysed by sirohydrochlorin ferrochelatase. In *Saccharomyces cerevisiae* the transformation of uroporphyrinogen III into sirohaem is catalysed by two enzymes, Met1p and Met8p, the former responsible for the generation of precorrin-2 (methyltransferase activity) whilst the latter is a bifunctional enzyme that converts precorrin-2 into sirohaem (dehydrogenase and chelatase activities) ([5]; Scheme 2). In bacteria such as *E. coli*, gene equivalents of *MET1* and *MET8* would appear to have fused to encode a single trifunctional enzyme called CysG [6] (Scheme 2). This 457-amino-acid protein can be envisaged as containing two major functional domains; the C-terminal domain contains the methyltransferase activity and is referred to as $CysG^A$ (amino acids 203–457), whilst the N-terminus of the protein houses the dehydrogenase/chelatase activities and is termed $CysG^B$ (amino acids 1–202) [7]. The comparable enzyme activities of Met1p and $CysG^A$ are reflected in the sequence

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani (broth); PBG, porphobilinogen; SAM, *S*-adenosyl-L-methionine.

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Sequence data for *B. megaterium hemAXBCDL* have been deposited with the accession number AJ508220.

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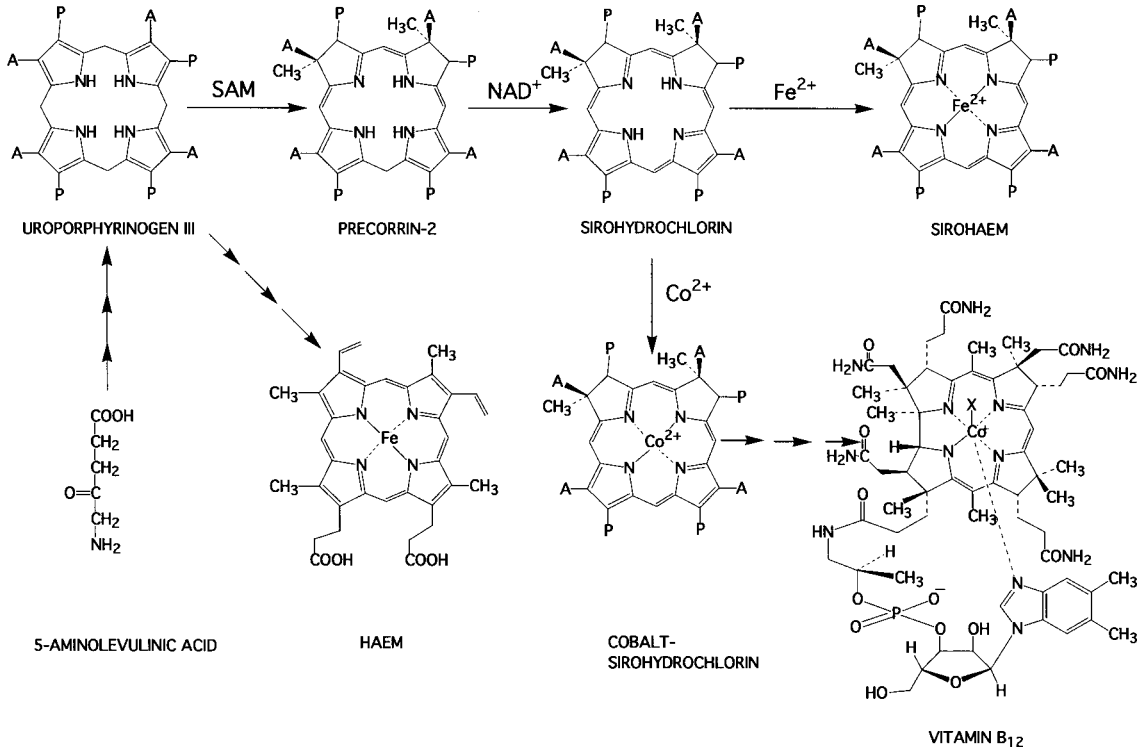

Scheme 1 Branched biosynthetic pathway of the modified tetrapyrroles

The biosynthesis of modified tetrapyrroles found in *B. megaterium* is outlined, including haem, vitamin B_{12} and sirohaem. Of particular interest is the importance of metal chelation, since this predetermines the direction that the intermediate takes along the pathway. A, acetic acid side chain; P, propionic acid side chain.

similarity between the proteins, and they are likely to adopt a similar three-dimensional structure to a cobalamin biosynthetic methyltransferase called CbiF [8]. Likewise, the comparable activities of $CysG^B$ and Met8p are also reflected in a level of sequence similarity between the two proteins, indicative of a common structure. In this respect it is interesting to note that the structure of Met8p has recently been determined, revealing a novel topology where the two enzyme activities, dehydrogenation and ferrochelation, are located within the same active site [9].

In *B. subtilis* it has been demonstrated recently that two gene products, YlnD and -F, located in a small operon containing *ylnD*, *-E* and *-F*, are required for sirohaem synthesis, where YlnD is thought to be a uroporphyrinogen III methyltransferase with similarity to $CysG^A$, and YlnF is believed to be a potential dehydrogenase/chelatase that has some similarity to both $CysG^B$ and Met8p [10]. In this paper we describe the isolation of a short cluster of three genes, *sirA*, *-B* and *-C* from *B. megaterium*, which are homologues of the *B. subtilis ylnD*, *-E* and *-F*, and demonstrate that all three gene products are required in the conversion of uroporphyrinogen III into sirohaem: where SirA acts as a methyltransferase, SirC acts as a dehydrogenase and SirB acts as a ferrochelatase. Moreover, SirB displays similarity to CbiX, a protein previously identified in the *B. megaterium* cobalamin biosynthetic operon as a putative cobaltochelatase [11,12]. Indeed it appears that both SirB and CbiX are functionally equivalent to the CbiK, a cobaltochelatase that has been characterized from *Salmonella enterica* [13,14]. The functions of all gene products associated with *B. megaterium* tetrapyrrole biosynthesis are outlined in Scheme 2.

EXPERIMENTAL

Chemicals and reagents

Cyanocobalamin, cobinamide and most other chemicals were purchased from Sigma. Other materials were purchased from manufacturers as follows: restriction and modification enzymes (Promega, Chilworth, Southampton, U.K.), pKK223.3, chelating Sepharose fast flow resin and gel filtration columns (Amersham Biosciences, Little Chalfont, Bucks., U.K.), pACYC184 (New England Biolabs, Hitchin, Herts., U.K.), pET14b (Novagen, Madison, WI, U.S.A.), tryptone and yeast extract (Oxoid, Basingstoke, U.K.) and primers (Bioline, 16 The Edge Business Centre, Humber Road, London, U.K. and Invitrogen, Carlsbad, CA, U.S.A.). The Vectorette system was provided by Sigma/Genosys, Europe, Cambridge, U.K.

Bacterial strains, media and growth conditions

The majority of strains and plasmids used in this study are listed in Table 1. Strains were routinely grown in LB (Luria–Bertani) broth or LB agar at 37 °C, with 100 mg/l of ampicillin, 34 mg/l of chloramphenicol, as required. To assess production of sirohaem in a cysteine-deficient environment, strains were grown on minimum medium (NaCl 0.5 g/l, Na_2HPO_4 6 g/l, KH_2PO_4 3 g/l, NH_4Cl 1 g/l, glucose 4 g/l, $MgSO_4$ 2 mM, $CaCl_2$ 0.1 mM), supplemented with 5 μM $CoCl_2$ to study the effect of the cobalt on cell growth. For control plates, cysteine at 50 mg/l was added to the medium. To assess cobalamin production the strains were grown in minimal medium supplemented with 0.1 g/l

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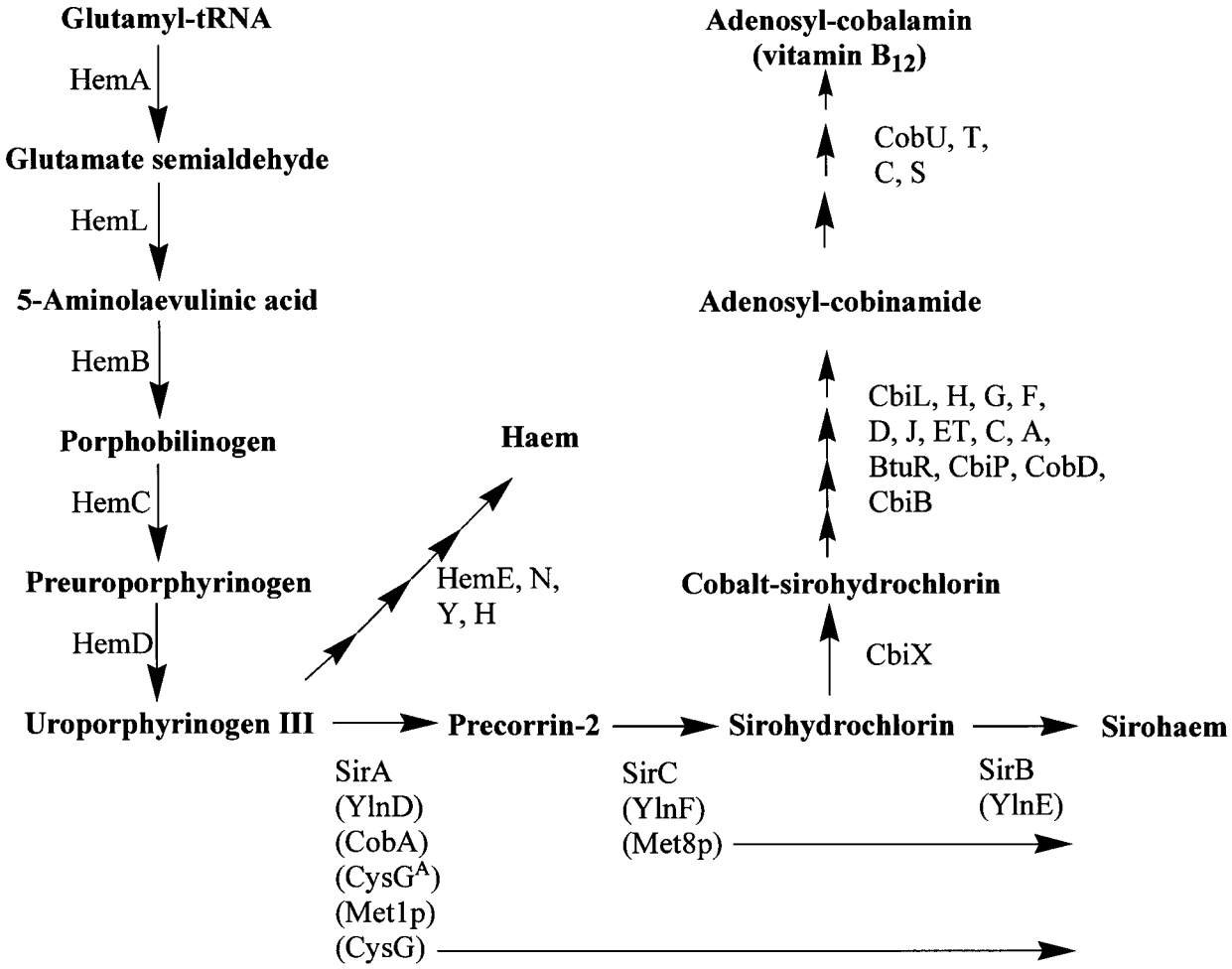

Scheme 2 Proteins associated with the biosynthesis of modified tetrapyrroles in *B. megaterium*

Haem proteins are associated with the biosynthesis of uroporphyrinogen III and its transformation into haem, Sir proteins are associated with the transformation of uroporphyrinogen III into sirohaem, whilst the Cbi and Cob proteins are associated with the transformation of uroporphyrinogen III into cobalamin.

yeast extract and 50 mg/l cysteine under anaerobic conditions (induced by placing a layer of mineral oil on the top of the medium).

Estimation of cobyric acid production

Bioassay plates were prepared as described previously with the strain AR3612 (*Salmonella typhimurium metE cysG*) [15]. Cobyric acid was quantified from the *S. typhimurium* indicator strain by estimation of growth obtained from the cell-extract sample after 18–24 h at 37 °C.

Isolation of a *hemA–D* operon from *B. megaterium*

The complete 5000 bp of the *B. megaterium hemAXCDBL* locus was cloned by a combination of complementation experiments using *E. coli hemA*, *-B* and *-L* mutants with a *B. megaterium* genomic library and a chromosome walking strategy using the Vectorette kit (Genosys/Sigma). After complete DNA sequence determination (Genbank accession number AJ508220) it was observed that the gene arrangement was identical to that in *B. subtilis* [16].

Overproduction of HemC and -D as His-tagged enzymes

To allow overproduction of the *B. megaterium* HemC and -D, the *hemC* and *hemD* genes were cloned separately into the plasmid pET14b after they had been amplified from genomic DNA with 5′ and 3′ primers containing *Nde*I and *Hin*dIII sites respectively. Once transformed into *E. coli* BL21 pLys, the recombinant protein was overproduced with an N-terminal hexa-His tag by induction with 0.4 mM isopropyl β-D-thiogalactoside (IPTG).

Cloning of *cbiX* and truncations of the His-tail

The *B. megaterium cbiX* gene was initially cloned into pKK223.3 by a *Ssp*I/*Sna*BI digestion of pAR8766 (the plasmid that harbours the complete *B. megaterium cob* operon), resulting in pAR8882 (Table 1b). The DNA fragment containing *cbiX* was subsequently subcloned by *Eco*RI/*Hin*dIII restrictions into pUC18, giving pUC.*cbiX*.

The truncated *cbiX* variant, encoding just 6 histidines of the tail, was generated by PCR using pUC.*cbiX* as template. The 5′ primer used in this reaction was the pUC(−21) primer (5′-AAC AGC TAT GAC CAT G-3′), whereas the 3′ primer was designed

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Table 1 Bacterial strains and plasmids used in this study

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

(a)

Strains	Genotype and/or phenotype	Description	Reference or source
Bacillus megaterium			
DSM509			DSM
Salmonella enterica			
AR3612	*metE cysG*	*metH* B_{12} dependent and B_{12} deficient	[15]
Escherichia coli			
302Δa	*E.coli cysG*; NirS, Lac +		[21]
ER171 = 302Δa (pCIQ)	*E.coli cysG*; NirS, Lac +	*cysG* deleted strain & pACYC184 harbouring the *lacI*^{q} gene	[13]
BL21(DE3)pLysS	F$^-$ *ompT gal*[dcm][ion]	IPTG inducible T7 promoter	Novagen

(b)

Plasmids	Inserted genes	Description	Reference or source
pAR8766	*B. megaterium cbiW-H*$_{60}$*-X-J-C-D-ET-L-F-G-A-cysG*^{A}*-cbiY-btuR-* > < -ORF1-ORF2	16.3 kb *B. megaterium Sau* 3AI fragment cloned into *Bam*HI site of pKK223.3 (the *tac* promoter has been deleted)	[11]
pAR8882	*B. megaterium cbiX*	pKK223.3 derived	[12]
pER119	*P. denitrificans cobA*	pKK223.3 derived	[13]
pER126K$^\Delta$	*S. enterica cbiA-C-D-E-T-F-G-H-J-K*$^\Delta$*-L-M-N-Q-O-P*	pACYC184 derived, deletion in the *cbiK* gene	[13]
pER179	*P. denitrificans cobA* and *B. megaterium cbiX*	*Eco*RI-*Sca*I fragment from pAR8882 cloned into pER119 in 3′ of *cobA*	This study
pER193	*P. denitrificans cobA* and *B. megaterium cbiX*^{6H}	*Pst*I-*Sca*I fragment from pRBKX6 cloned into pER179 cut with the same enzymes	This study
pER242	*P. denitrificans cobA*	cloned by PCR into pET14b	[5]
pER328	*P. denitrificans cobA* and *B. megaterium cbiX*^{0H}	*Pst*I-*Sca*I cbiX^{0H} PCR fragment cloned into *Pst*I-*Sca*I of pER179	This study
pET14b	His-tag fusion protein vector with T7 promoter		Novagen
PHL97	*B. megaterium sirA*	Cloned by PCR into pKK223.3	This study
PHL98	*B. megaterium sirA-B*	Cloned by PCR into pKK223.3	This study
PHL100	*B. megaterium sirA-C*	Cloned by PCR into pKK223.3	This study
PHL99	*B. megaterium sirA-B-C*	Cloned by PCR into pKK223.3	This study
PHL101	*B. megaterium sirB*	Cloned by PCR into pET14b	This study
PHL103	*B. megaterium sirC*	Cloned by PCR into pET14b	This study
pKK223.3		Overexpression vector derived from pBR322 with *tac* promoter	Pharmacia
pRBKX6	*B. megaterium cbiX*^{6H}	*cbiX*^{6H} PCR fragment cloned into pKK223.3	This study
pSD1	*B. megaterium hemC*	Cloned by PCR into pET14b	This study
PSD2	*B. megaterium hemD*	Cloned by PCR into pET14b	This study
pUC.*cbiX*	CbiX	*Eco*RI/*Hin*dIII insert from pAR8882	This study

to substitute the aspartate codon (located 3′ to the sixth histidine) with a stop codon (underlined) as well as to introduce a *Bam*HI (in bold) restriction site (5′-CGC **GGA TCC** TCA ATG ATC GTG ATG ATG-3′). The second *cbiX* truncation required the complete removal of all the histidines from the tail. This was also generated by PCR using pAR8882 as the DNA template. The pKK-forward primer (5′-CAT CGG CTC GTA TAA TGT G-3′) was used as the 5′ primer whilst the 3′ primer was designed to substitute the first histidine of the tail for a stop codon (underlined) as well as to introduce a *Hin*dIII (in bold) site (5′-CG**A AGC TTC** AAT CAA TAT GCT CCA TAA TGC C-3′). The three *cbiX* derived fragments, *cbiX*, *cbiX*^{6H} and *cbiX*^{0H}, were subsequently cloned into a pKK223.3-derived plasmid, 3′ of the *Pseudomonas denitrificans cobA* gene, as described in Table 1b.

Isolation of the *sirA,B,C* operon

From the *B. megaterium cobA* sequence [17] a Vectorette (Genosys/Sigma) approach was taken to isolate the downstream genes in *B. megaterium* DSM509. This resulted in the isolation of a 2.5 kb fragment which was completely sequenced, and the genetic information has been deposited with the Genbank accession number AJ509159. Primers were designed to allow the cloning of the three genes within this fragment, which were termed *sirA–C*, such that constructs containing *sirA*, *sirA–B* and *sirA–B–C* within pKK223.3 were generated. These three constructs were subsequently used to study the complementation of an *E. coli cysG* deleted strain (302Δa/pCIQ).

Overproduction and purification of CbiX, SirB, SirC, HemC and HemD

For overproduction of CbiX, the corresponding gene was cloned into pKK223.3 as described above. After transformation of *E. coli* 302Δa (pCIQ), protein production was induced by the addition of 0.4 mM IPTG when the cells reached an A_{600} of 0.6, then the cells were left to grow for a further 2 h. Due to the presence of a naturally occurring histidine-rich region within CbiX, the protein could be purified by passing through a His-bind column charged with $NiSO_4$. The purification was performed as described in the pET system manual instructions

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(Novagen). The protein was further purified by gel filtration (G75) in (50 mM Tris/HCl, pH 8.0, 100 mM NaCl).

The *B. megaterium hemC*, *-D*, *sirB* and *-C* were individually cloned into pET14b, which allowed the encoded proteins to be produced as N-terminal His-tagged fusions. Recombinant protein overproduction was achieved after the corresponding plasmids were transformed into BL21(DE3)pLysS (Novagen) and induced by IPTG when the cells reached an A_{600} of 0.6. The proteins were purified on His-bind columns as described in the pET system manual instructions (Novagen). SirB and -C were purified further by gel filtration using the same buffer as for CbiX.

In vitro dehydrogenase and chelatase assays

SirB, CbiX and SirC were overproduced and purified as described previously, and were diluted to between 1.0 and 0.2 mg/ml for the assay in 50 mM Tris/HCl, pH 8.0, and 100 mM NaCl. Purified recombinant *B. megaterium* porphobilinogen deaminase (HemC), uroporphyrinogen III synthase (HemD), *P. denitrificans* uroporphyrinogen III methyltransferase (CobA) and the *B. megaterium* precorrin-2 dehydrogenase (SirC) were subsequently dialysed against 50 mM Tris/HCl buffer, pH 8.0, containing 100 mM NaCl. Porphobilinogen (PBG) was synthesized from 5-aminolaevulinic acid using purified 5-aminolaevulinic acid dehydratase [18].

Precorrin-2 was generated *in situ* under an atmosphere of nitrogen in a glove box (Belle Technology), with less than 2 ppm oxygen. This was accomplished by incubating 2.5 mg PBG in a total volume of 40 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 5 mg of purified PBG deaminase, 1 mg purified uroporphyrinogen III synthase, 5 mg of purified uroporphyrinogen III methyltransferase and 10 mg of SAM. The reaction was left overnight at 22 °C to allow it to reach completion and was filtered prior to use. Sirohydrochlorin was generated using the same enzyme cocktail as described above, except that 5 mg of SirC and 13 mg of NAD were added to the incubation. The rate for the dehydrogenase reaction was calculated by monitoring the appearance of sirohydrochlorin at a λ_{max} of 376 nm, using a molar absorption coefficient of $2.4 \times 10^5\ M^{-1} \cdot cm^{-1}$ [9]. The rate of the chelatase reaction for cobalt insertion into sirohydrochlorin was calculated by measuring the rate of disappearance of sirohydrochlorin, using the molar absorption coefficient given above. The rate of the chelatase reaction for cobalt insertion into precorrin-2 was calculated by measuring the appearance of cobalt-precorrin-2 at λ_{max} of 418 nm, using a molar absorption coefficient of $1.6 \times 10^5\ M^{-1} \cdot cm^{-1}$. This molar absorption coefficient was calculated from the complete conversion of a known amount of precorrin-2 into cobalt-precorrin-2.

The dehydrogenase assay was monitored by incubating precorrin-2 (2.5 μM) with 100 μg of SirC in a reaction volume of 1 ml with 1 mM NAD in 50 mM Tris/HCl buffer, pH 8. The chelatase activity was measured with sirohydrochlorin (2.5 μM), Co^{2+} (20 μM) and 2.5 μg of either SirB or CbiX in a 1 ml reaction volume in 50 mM Tris/HCl buffer, pH 8. For all reactions, initial rates were recorded on a Hewlett–Packard (Boise, ID, U.S.A.) 8452A photodiode array spectrophotometer and assays were performed in duplicate.

RESULTS

Isolation of the *B. megaterium* hemAXBCDL genes from *B. megaterium*

In order to gain some understanding into how the genes for tetrapyrrole biosynthesis are organized and regulated within *B. megaterium*, the operon encoding the enzymes for the transformation of glutamyl-tRNA into uroporphyrinogen III was isolated from a *B. megaterium* genomic library. The genes were found within a 5 kb fragment of DNA and were found in the order *hemAXBCDL*, highly similar to that found in *B. subtilis* [16], and the sequence data have been deposited with the accession number AJ508220. The *hemA*, *-B* and *-L* genes were shown to be functional through their ability to complement defined *E. coli* mutants (results not shown). No role has yet been ascribed to *hemX*. The products of *hemC* and *-D* were shown to be functional after recombinant protein overproduction in *E. coli* as His-tagged enzymes, which facilitated a simple purification by metal chelate chromatography, permitting their direct enzymic assay. HemC was shown to contain the dipyrromethane cofactor, as the protein was shown to react positively with Ehrlich's reagent (results not shown) and had a specific activity of 30 μmol of PBG utilized/h/mg, similar to the specific activity of other porphobilinogen deaminases [19]. Purified recombinant *B. megaterium* uroporphyrinogen III synthase had a specific activity of 750 μmol of uroporphyrinogen synthesized/h/mg, again similar to the activity of the enzyme purified from other sources [20]. The recombinant overproduction of HemC and -D allowed the isolation of about 20 mg of each homogeneous protein from a litre of the respective *E. coli* cultures, thereby providing a sufficient source of enzyme to permit the large scale generation of uroporphyrinogen III from porphobilinogen *in vitro*. The synthesis of uroporphyrinogen III is required in the assay of later enzymic steps in the pathway.

Identification of a locus for sirohaem synthesis in *B. megaterium*: *sirA, -B and -C*

There have been two publications reporting the cloning of a uroporphyrinogen III methyltransferase gene from *B. megaterium* [11,17]. However, a comparison of the encoded proteins from these reports reveals only a 63 % identity at the amino acid level, indicating that the proteins are independent and presumably encoded on separate regions of the genome. One of these genes, $cysG^A$, is found within the same biosynthetic operon as *cbiX* [11], a gene which, on the basis of the fact that its encoded protein contains a highly unusual histidine-rich region, is thought to encode a cobaltochelatase. The other *B. megaterium* uroporphyrinogen methyltransferase gene ($cobA^{Bm}$) was isolated from a 2.5 kb piece of genomic DNA that was also shown to complement a *cysG E. coli* strain [17]. This latter observation implied that a chelatase-encoding gene must be adjacent to the $cobA^{Bm}$ gene, although this was not sequenced.

In order to identify this chelatase, primers were designed to allow extension by Vectorette PCR downstream of the $cobA^{Bm}$ sequence published in [17]. This resulted in the amplification of a 1.5 kb fragment from *B. megaterium* genomic DNA, sequencing of which revealed two further open reading frames downstream (3′) of the *cobA* gene. Furthermore, an error (frameshift) was found in the sequencing of the original *cobA* sequence resulting in an early termination to the predicted CobA primary structure. The correct sequence revealed that the *B. megaterium* CobA actually contains 259 amino acids, an extension of 21 amino acids on the C-terminus of the original sequence. The $cobA^{Bm}$ and the two downstream genes are highly similar to the *ylnD*, *-E* and *-F* genes from *B. subtilis* [10]. The *B. subtilis ylnD* and *-F* genes had previously been implicated in sirohaem biosynthesis on the basis of their ability to complement defined mutants [10]. We suggest that these three genes, *ylnD*, *-E* and *-F*, now be called *sirA*, *-B* and *-C*, where the *sir* prefix reflects the role of the genes

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Table 2 The genes associated with sirohaem synthesis and cobalt chelation in cobalamin biosynthesis

Gene name	Previously defined as	Orthologues	Encoded enzyme function
sirA	*ylnD* [10] *cobA* [17]	*cobA*, *cysG*^{A}, *corA*, *MET1*	Uroporphyrinogen III methyltransferase
sirB	*ylnE* [10]	Found in a wide range of bacteria as a gene of unknown function (is similar though distinct to *cbiX*)	Ferrochelation of sirohydrochlorin
sirC	*ylnF* [10]	Is similar to the 5′ region of *cysG* (corresponding to CysG^{B}) and *MET8*	Precorrin-2 dehydrogenase. Unlike Met8p and CysG^{B}, SirC has no chelatase activity.
cbiX	–	Found in a wide range of bacteria as a gene encoding a protein of unknown function Protein is similar to SirB. Some CbiX proteins contain a histidine-rich region	Cobaltochelatase, responsible to the chelation of Co^{2+} into sirohydrochlorin

Table 3 Complementation of *E. coli cysG* deletion strain

Genes used to complement *E. coli cysG* strain	Growth on minimum medium and cysteine	Growth on minimum medium	Growth on minimum medium and $CoCl_2,6H_2O$ at 1 mg/l
sirA	+ + +	–	–
sirA and *sirB*	+ + +	+ + +	+ +
sirA and sirB and sirC	+ + +	+ +	+ +
pAR8766: *cbi*W-H_{60}-X-J-C-D-ET-L-F-G-A-*cys*G^{A}-*cbi*Y-*btu*R	+ + +	+ + +	–
cobA^{Pd}	+ + +	–	–
cobA^{Pd} *and cbiX*	+ + +	+ +	–
cobA^{Pd} *and cbiX*^{6H}	+ + +	+ +	–
cobA^{Pd} *and cbiX*^{0H}	+ + +	+ +	+ +

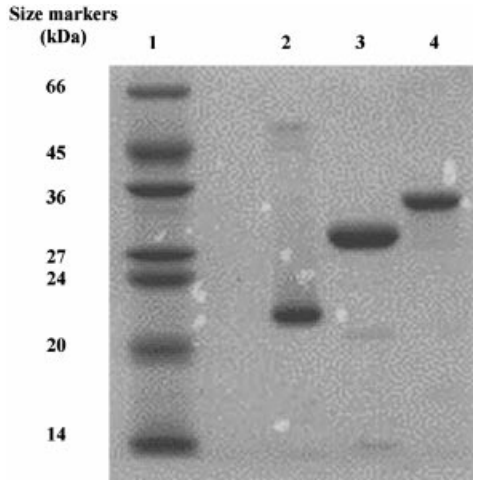

Figure 1 SDS/PAGE of purified SirC, -B and CbiX

Purified SirC, -B and CbiX were isolated as described in the Experimental section and analysed on a 12% gel. Lane one contains the molecular mass standards, sizes as marked on the left-hand side, whilst lanes 2–4 contain 5 μg of purified SirC, -B and CbiX respectively.

in sirohaem biosynthesis. An outline of the names of these genes, their homologues and orthologues and encoded enzyme function is given in Table 2. Thus, in *B. megaterium*, SirA (YlnD) corresponds to the previously characterised CobA^{Bm} [17] and is a uroporphyrinogen III methyltransferase. SirB (YlnE) is similar to CbiX and is likely therefore to be a chelatase, although it does not contain the histidine-rich region found in CbiX [11]. Finally, SirC (YlnF) bears some similarity with both the CysG^{B} domain of CysG and Met8p, although the level of identity is greatest around the putative NAD^+ binding site suggesting that it is a dehydrogenase [9]. Thus the likely function of SirA–C is to convert uroporphyrinogen III into sirohaem.

To demonstrate that all three genes within the *B. megaterium sirA–C* cluster are required for sirohaem synthesis, the genes were used in complementation experiments with the *E. coli cysG* deletion strain 302Δa [21]. In summary, it was found that plasmids containing *sirA* and *-B* were able to complement the cysteine auxotrophy of the *E. coli* mutant, consistent with their predicted roles as a uroporphyrinogen III methyltransferase and a ferrochelatase respectively (Table 3). In contrast with the results with *cbiX* (see below), the presence of exogenous cobalt did not prevent the complementation, suggesting that SirB had a higher specificity for iron over cobalt, as would be expected with a defined role within sirohaem synthesis. The presence of SirC had no real effect upon the complementation, presumably due to the spontaneity of the non-enzymatic oxidation of precorrin-2 to sirohydrochlorin (Table 3).

Identification of CbiX as a chelatase; complementation of an *E. coli cysG*^{B} strain

When *E. coli* 302Δa was transformed with a plasmid carrying the *B. megaterium* cobalamin biosynthetic genes responsible for the conversion of uroporphyrinogen III into the cobalamin precursor cobyrinic acid *a*,*c*-diamide (the *cobI* operon, housed in pAR8766: *cbi*W,-H_{60},-X,-J,-C,-D,-ET,-L,-F,-G-A,*cys*G^{A},*cbi*Y,*btu*R) [12], complementation of the cysteine auxotrophy was observed (Table 3). The presence of *cysG*^{A} on plasmid pAR8766 permits the synthesis of precorrin-2 so the transformation of this intermediate into sirohaem must be reliant on another gene product(s) within the *B. megaterium cobI* operon. To identify the gene responsible for this activity, various plasmids with deletions/mutations in the *cob* operon were made and these were subsequently tested for their ability to restore cysteine biosynthesis within the *E. coli cysG* deletion strain. In summary, only plasmids containing a functional *cysG*^{A} and *cbiX* were found to complement *E. coli* 302Δa, thereby identifying *cbiX* as the gene encoding an enzyme responsible for the completion of sirohaem synthesis (results not shown). To confirm that CbiX has the ability to act as the final enzyme in sirohaem biosynthesis, it was cloned in tandem with

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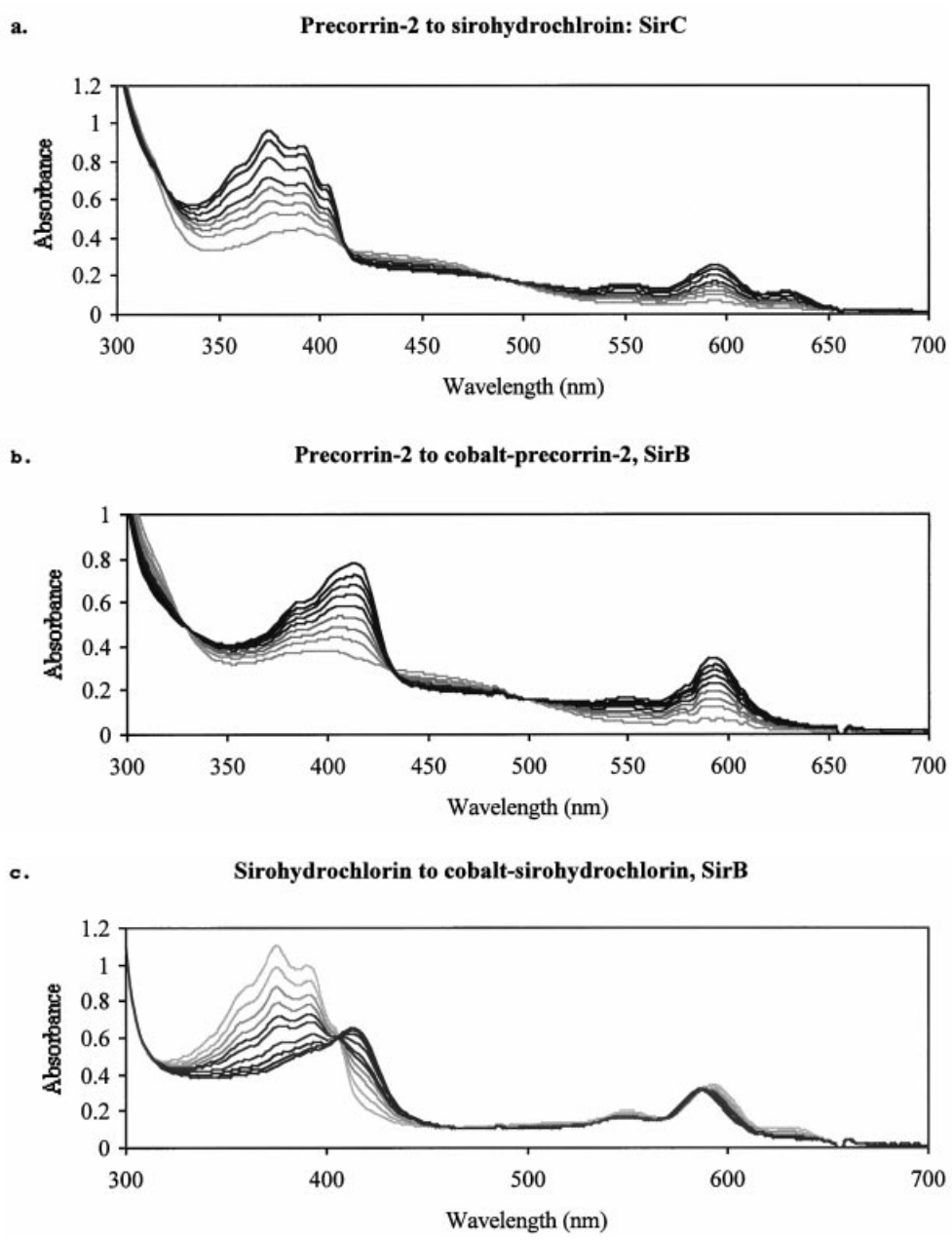

Figure 2 UV/visible spectra showing the conversion of precorrin-2 into sirohydrochlorin, cobalt precorrin-2 and cobalt-sirohydrochlorin

(**a**) Spectra obtained from the reaction of SirC with precorrin-2 and NAD^+ with time. The generation of sirohydrochlorin is characterized by the appearance of absorption maxima at 376 nm and 590 nm. (**b**) Spectra obtained from the reaction of SirB with precorrin-2 and Co^{2+} with time. The generation of cobalt-precorrin-2 is characterized by the appearance of absorption maxima at 418 nm and 596 nm. This reaction was only observed with SirB, as CbiX did not catalyse this chelation. (**c**) Spectra obtained from the reaction of SirB with sirohydrochlorin and Co^{2+} with time. The generation of cobalt-sirohydrochlorin is characterized by the appearance of absorption maxima at 414 nm and 590 nm. A similar reaction profile was also observed with CbiX.

the gene encoding the uroporphyrinogen III methyltransferase (*cobA*^{Pd}) from *P. denitrificans*, whose gene product is well characterized and is known to synthesize only precorrin-2 [22,23]. This plasmid, pER179 (*cobA*^{Pd} and *cbiX*), was also found to complement *E. coli* 302Δa, whereas a plasmid harbouring only *cobA*^{Pd} alone could not (Table 3).

In regard to metal ion specificity, it was interesting to note that when exogenous cobalt (5 μM) was added to the minimal media, complementation of the cysteine auxotrophy of *E. coli* 302Δa by pER179 was prevented (Table 3). This was in stark contrast with complementation experiments of the same auxotroph by *sirB* (see above), where excess exogenous cobalt did not prevent complementation. One interpretation of this result is that cobalt is able to out-compete iron within the active site of CbiX. This would be consistent with the hypothesis that CbiX is actually a cobaltochelatase, a credible interpretation since *cbiX* is located within the *cobI* operon of *B. megaterium*. A similar cobalt-dependent antagonism on the complementation of *E. coli* 302Δa has been observed with the *S. enterica cbiK* [14], a gene that is known to encode a cobaltochelatase.

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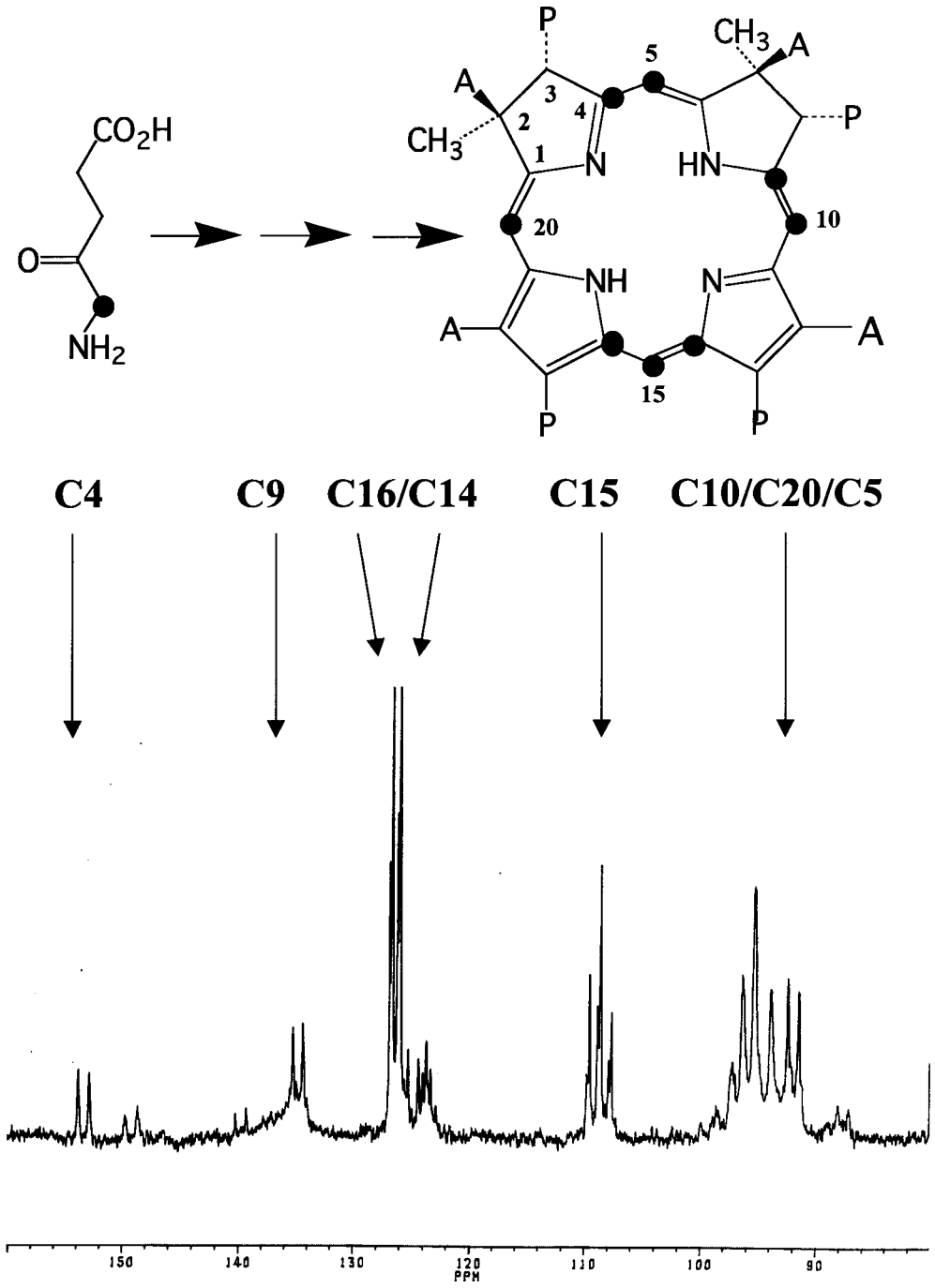

Figure 3 NMR spectrum of sirohydrochlorin derived from [5-^{13}C]aminolaevulinic acid

The labelled ALA was transformed into precorrin-2 with an enzyme cocktail consisting of recombinant 5-aminolaevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase and uroporphyrinogen III methyltransferase. The transformation of precorrin-2 into sirohydrochlorin was then catalysed by the addition of purified SirC and NAD^+. NMR signals corresponding to the specific enriched ^{13}C atoms are marked on the spectrum. A, acetic acid side chain; P, propionic acid side chain.

Overproduction and purification of CbiX, SirB and SirC

CbiX was overproduced in *E. coli* as a recombinant protein after the gene had been cloned under the control of a *tac* promoter within pKK223-3 (pAR8882) and transformed in *E. coli* 302a (pCIQ) cells. When induced, the strain produced CbiX to approx. 20% of the total cellular protein level. The protein was soluble and the presence of the histidine-rich region allowed CbiX to be purified by metal chelate chromatography as outlined in the Experimental section. When analysed by SDS/PAGE, the protein migrated with a molecular mass of 35 kDa (gene predicted molecular mass = 34.3 kDa) (Figure 1), although it eluted from gel filtration columns with a molecular mass corresponding to 70 kDa suggesting that it exists in its native state as a homodimer. An examination of the *cbiX* sequence revealed two possible ATG start codons each associated with a strong potential ribosome-

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binding site [11]. In order to identify which translation start site is used, at least in *E. coli*, the protein was subject to N-terminal sequencing, revealing the following sequence: Met-Lys-Ser-Val-Leu-Phe-Val-Gly. The N-terminal sequence, therefore, indicates that translation of CbiX starts at the first potential start codon at position 3802 within the *B. megaterium cobI* operon [11].

To facilitate the rapid purification of both the *B. megaterium* SirB and -C, their respective genes were cloned separately into pET14b to allow overproduction of His-tagged versions of the proteins, under the inducible control of the T7 promoter. The proteins were found to be soluble and were subsequently isolated by metal chelate chromatography to give preparations that were in excess of 90 % purity. SirB ran as a single band on SDS/PAGE with a molecular mass of 33 kDa (Figure 1), and eluted from gel filtration columns with a similar molecular mass, indicating that it exists as a monomer in its native state. SirC gives a large band on SDS/PAGE corresponding to a molecular mass of 22 kDa (Figure 1), although a band corresponding to a mass of 45 kDa can be observed with a smear down to the 22 kDa band. This observation could reflect a strong homodimer formation that is maintained even under denaturating conditions. Indeed, the sequence similarity between SirC and Met8p, the bifunctional dehydrogenase/chelatase from yeast, suggests that it adopts a similar three-dimensional fold, which is that of an intertwined homodimer [9].

SirC is a precorrin-2 dehydrogenase

When SirC was incubated with precorrin-2 under anaerobic conditions (less than 2 p.p.m. oxygen) in the presence of NAD^+, the yellow colour of the dipyrrocorphin solution was observed to change rapidly to a translucent mauve. This was accompanied by a change in the UV/VIS spectrum with the generation of a new absorption maximum at 376 nm, consistent with the generation of sirohydrochlorin [9] (Figure 2a). The enzyme had a specific activity of 60 nmol of sirohydrochlorin formed/min/mg. The assay was also repeated with precorrin-2 that had been generated from [5-^{13}C]aminolaevulinic acid and the NMR spectrum of the product recorded (Figure 3). Again, this was consistent with the generation of sirohydrochlorin, with a change in the aromaticity within ring C of the macrocycle and the alteration in the hybridization state of C-15 from sp^3 to sp^2 (Figure 3) [24,25]. No activity of SirC was observed in the absence of NAD^+ and little activity was observed with $NADP^+$. The enzyme was unable to oxidize precorrin-3, demonstrating an ability to discriminate against the methyl group added to position C-20 (results not shown). These results indicate that SirC is an NAD^+-dependent dehydrogenase that catalyses the conversion of precorrin-2 into sirohydrochlorin.

In vitro activity of *CbiX* and *SirB*

The activities of CbiX and SirB as chelatases were investigated by incubation of these enzymes with either precorrin-2 or sirohydrochlorin in the presence of cobalt. Cobalt is used in the reaction for two reasons. Firstly it is simpler to handle than the easily oxidizable ferrous species and, secondly it produces a greater change in the spectrum of the metal-chelated product [9]. A typical reaction of SirB with precorrin-2 and SirB/CbiX with sirohydrochlorin is shown in Figures 2(b) and 2(c). Generation of cobalt-precorrin-2 by chelation of cobalt resulted in absorption maxima at 418 nm and 595 nm (Figure 2b) [26]. Generation of cobalt sirohydrochlorin produced absorption maxima at 414 nm and 590 nm (Figure 2c) [27]. The specific activities of the reactions for SirB with precorrin-2 and sirohydrochlorin were 3.4 nmol of product formed $\cdot$ min^{-1} $\cdot$ mg^{-1} and 337 nmol of product formed $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. The specific activities of CbiX with precorrin-2 and sirohydrochlorin were 0 and 318 nmol of product formed $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. These results clearly suggest that both SirB and CbiX prefer to chelate metal into the oxidized tetrapyrrole framework of sirohydrochlorin rather than the more reduced precorrin-2 species. Thus, in sirohaem synthesis, it is likely that chelation takes places after the generation of sirohydrochlorin, whilst during cobalamin biosynthesis it is likely that cobalt is also inserted into sirohydrochlorin, consistent with previous data we have produced indicating that sirohydrochlorin is an intermediate during corrin ring synthesis [5].

Truncations of the His-tail of CbiX

The major difference between SirB and CbiX is the presence of a histidine-rich region at the C-terminus of CbiX (Figure 4a), a region that could be envisaged to bind cobalt cations. To investigate the role of the polyhistidine region of CbiX, two variants of *cbiX* were generated that encode proteins truncated at the C-terminus (Figure 5). They were both generated by PCR such that stop codons were introduced at positions 4627 bp ($CbiX^{6H}$) and 4606 bp ($CbiX^{0H}$) (Figure 5). These produced shortened genes, $cbiX^{6H}$ and $cbiX^{0H}$, which expressed proteins containing either 6 or 0 histidines respectively rather than the 18 histidines found in the native histidine-rich region of CbiX (Figure 5). Both truncated constructs were expressed as recombinant proteins within *E. coli* after cloning into pKK223-3. The removal of the histidine-rich region did not affect the solubility of the proteins and they were still found to complement the cysteine auxotrophy of 302Δa, demonstrating that they were active as chelatases (Table 3). Noteworthy, though, was the observation that both CbiX and $CbiX^{6H}$ are unable to complement the *E. coli cysG* strain in the presence of exogenous cobalt whereas $CbiX^{0H}$ does not display this cobalt-sensitive complementation (Table 3). On the basis of this result it is likely that the histidine-rich tail is involved in metal binding and deleting it results in the loss of the higher affinity of CbiX for cobalt over iron. $CbiX^{0H}$ might, therefore, be a more efficient ferrochelatase than CbiX. The histidine-rich tail could act either as a cobalt store or as a cobalt trap, delivering the cobalt to the active site of the enzyme. In this respect the histidine-rich region could be viewed as an in-built metal chaperone.

cbiX, *cysG*, *met8* and sirB can all substitute for *cbiK* in the *S. typhimurium cob* operon

Despite containing the *S. enterica cobI* operon, *E. coli* strain $ER185K^{\Delta}A$ is unable to synthesize cobyric acid as it does not contain either a functional *cbiK* or $cysG^B$ [13]. However, as observed from the results in Table 4, cobyric acid synthesis can be restored to this strain by addition of CysG, SirB or CbiK. Alternatively, cobyric acid synthesis can be restored by the addition of *cbiX* (Table 4). Thus CbiX can function as a cobalt chelatase in the *S. enterica* cobalamin biosynthetic pathway. All the sirohydrochlorin ferrochelatases tested (CysG, Met8p and SirB) are also able to complement this cobaltochelatase deficient strain and restore cobalamin biosynthesis. In general, it is observed that the presence of a precorrin-2 dehydrogenase, such as SirC, Met8p and CysG (the $CysG^{G21D}$ variant has zero dehydrogenase activity), leads to overall increases in the cobalamin levels (Table 4). This is probably due to the fact that sirohydrochlorin is more stable than precorrin-2, providing a more suitable platform for metal insertion. The

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a

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Bm SirB    1:                MHKKLTKEVDYMDAVLYVCHGSRVKEGADQAVAFIERCKKNLDVPIQ.: 47
Bst SirB   1:                      MAMEAVLYVSHGSRIAAARHEAARFVEQCRRAIDIPIQ.: 38
Bm CbiX    1:                   MGGHYMKSVLFVGHGSRDPEGND..REFISTMKHDWDASILV: 40
Bst CbiX   1:                        MRSILFVGHGSRDPEGNEQVRQFVDRLRPRLRDSFHI: 37
Syn CbiX   1:MTLTSVPAPVSLFPELELPPLPYHRPLLMIGHGTRDEDGRQTFLDFVAQYQ.ALDHSRPV: 59
Se CbiK  114:LSSHNDYVQLMQALRQQMPSLRQTEKVVFMGHGA....SHHAFAAYACLDHMMTAQRFPA:169

Bm SirB   48:EVCFLELASPTIEQGFEACIEQGATRIAIVPLLLLTAAHAKHDIPEEI.QKVYERY....:102
Bst SirB  39:ELCFVELAEPDIVTGVDRCVAQGATRVIVVPLLLLSAGHAKHDIPAAL.DIARRRH....: 93
Bm CbiX   41:ETCFLEFERPNVSQGIDTCVAKGAQDVVVIPIMLLPAGHSKIHIPAAI.DEAKEKY....: 95
Bst CbiX  38:ETSFLEFGRPSIGEGIERCAEAGAMEVAVIPLILLPAGHSKLHIPAEI.DEAKARY....: 92
Syn CbiX  60:IPCFLELTEPNIQAGVQQCVDQGFEEISALPILLFAARHNKFDVTNEL.DRSRQAH....:114
Se CbiK  170:RVGAVE.SYPEVDILIDSLRDEGVTGVHLMPLMLVAGDHAINDMASDDGDSWKMRFNAAG:228

Bm SirB  102:.PQVEVLYGEPFGVDERIVDILVERINE.TNVDKH.......EDSMVLLVGRGSSDPAVK:153
Bst SirB  93:.PSVDILCGAPFGVHEAMIDIMIDRISE.QSAPLD.......GESMILLVGRGSSDPDTK:144
Bm CbiX   95:.PHVNFVYGRPIGVHEEALEILKTRLQE.SGENLETPA....EDTAVIVLGRGGSDPDAN:149
Bst CbiX  92:.PHMVFRYGRPIGVHEQTFAILRERLQE.IGERPEEPS....DGTAVILLGRGGSDPDAN:146
Syn CbiX 114:.PQINFFYGRHFGITPAILDLWKARLNQ.LDSPEANPQGIDRQDTVLLFVGRGSSDPDAN:172
Se CbiK  229:IPATPWLSG..LGENPAIRAMFVAHLHQALNMAVEEAA                    :264

Bm SirB  154:RDLNEIAQLLKGKGAFKEVSTCYLAAASPNLKEGLHLAKRTSYKQVFVLPYLLFTGILMN:213
Bst SirB 145:RDMSAIAALLKEKTNVPHVDVCFLAAIRPTLDEGLERAHASAYRRVFVVPYLLFTGVLMK:204
Bm CbiX  150:SDLYKITRLLWEKTNYKIVETSFMGVTAPLIDEGVERCLKLGAKKVVILPYFLFTGVLIK:209
Bst CbiX 147:SDLYKIARLFWEQTGYALIEPAFMGVTTPSLDDAVHRCLMLGARRIVVLPYFLFTGILIK:206
Syn CbiX 173:GDVYKMARMLWEGSGYQTVETCFIGISHPRLEEGFRRARLYQPKRIIVLPYFLFMGALVK:232

Bm SirB  214:EIKEELEQLSTDAQ..QFILANYLGYHDGLAHILSHQV.KTLLSSKGNQYDVYRYA    :266
Bst SirB 205:TIERKLQGFSVSDK..QWHLCSYLGDHPRLVLLIQQQV.LSLSSVKKGA          :250
Bm CbiX  210:RLEEMVKQYKMQHENIEFKLAGYFGFHPKLQTILKERAEEGLEGEVKMNCDTCQYRLGIM:269
Bst CbiX 207:RLEQQVAQYQAEHSHISFALAGYFGFHPKLEEIVLDRLNEVLGQTVAMNCDICQYRL...:263
Syn CbiX 233:KIFTITEEQRATFPEIEIQSLSEMGIQPELLALVRERE IETQLGQVAMNCEACKFRLAFK:292

Bm CbiX  270:E..HIDHHHHHDHDHDHDHGHHHHDHHHDHHEDKVGELK     :306
Bst CbiX 263:...HAAHHHHHHHHH                            :275
Syn CbiX 293:NQGHGHDHGHGHHHHGHDHGHSHGEWVDTYIEPTAYHEKIWQAP:336
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b

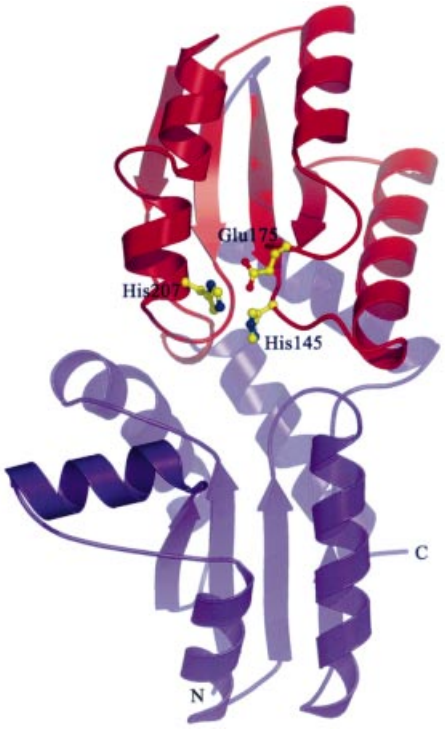

Figure 4 For legend, see facing page

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CbiX   241:RAEEGLEGEV KMNCDTCQYR LGIMEHIDHH HHHDHDHDHD HGHHHHDHHH DHHEDKVGEL K*

CbiX6H 241:RAEEGLEGEV KMNCDTCQYR LGIMEHIDHH HHHDH*

CbiX0H 241:RAEEGLEGEV KMNCDTCQYR LGIMEHID*
```

Figure 5 C-terminal truncations made to investigate the role of the histidine-rich region of CbiX

The wild-type protein (CbiX) sequence from position 241–301 is compared with the C-terminal truncated forms $CbiX^{6H}$ (amino acids 241–275) and $CbiX^{0H}$ (amino acids 241–268).

Table 4 Cobyric acid synthesis in derivatives of an *E. coli* strain containing all the genes necessary for cobyric acid synthesis except that encoding the cobaltochelatase, CbiK

The parent strain was 302Δa containing the pACYC184-*lacI*^{q} derived plasmid harbouring all *S. enterica cbi* genes but with a deletion in *cbiK* [pER126K$^{\Delta}$] [13].

Strain	Gene(s) cloned into pKK223.3	Cobyric acid (pmol/A_{600})	Reference
ER185K$^{\Delta}$A	*cobA*^{Pd}	0	[13]
ER185K$^{\Delta}$AK	*cobA*^{Pd} and *cbiK*	482	[13]
ER185K$^{\Delta}$G	*cysG*	544	[13]
ER278	*cysG*^{G21D}	170	[5]
ER252	*cobA*^{Pd} and *MET8*	290	[5]
HK97	*sirA*	0	This study
HK100	*sirA* and *sirC*	0	This study
HK98	*sirA* and *sirB*	34	This study
HK99	*sirA* and *sirB* and *sirC*	580	This study

generation of cobalt-sirohydrochlorin poses a biosynthetic problem, however, since the cobalamin biosynthetic pathway in *S. enterica* proceeds via cobalt-precorrin-3 [28,29]. Therefore cobalt-sirohydrochlorin would have to be reduced back to the level of a hexahydroporphyrin.

Structural similarity between CbiK, CbiX and SirB?

Since CbiX, SirB and CbiK appear isofunctional, it would be reasonable to assume that they may share a similar structure. Whilst there is obvious sequence identity between SirB and CbiX (37% amino acid identity; Figure 4a), there is little obvious sequence similarity between CbiX/SirB and CbiK. However, it is possible to align amino acids 142–265 of CbiK (corresponding roughly to the C-terminal domain that houses the main catalytic groups of CbiK) with the first 150 amino acids of CbiX (with 18% amino acid identity) and SirB (with 17% amino acid identity). An alignment of some SirB and CbiX sequences with CbiK is shown in Figure 4(a). There are reasons to believe that this alignment represents a genuine structural conservation. For instance, the two key histidine residues of CbiK, 145 and 207, are conserved, and likewise E175 is also maintained [14]. It is likely, therefore, that that both SirB and CbiX will adopt a similar fold to the C-terminal region of CbiK, which in turn has structural similarity to the protoporphyrin ferrochelatases of the haem branch of the tetrapyrrole pathway. A structure of CbiK is shown in Figure 4(b) with the region of similarity with SirB/CbiX highlighted in red, and the conserved catalytic groups shown in ball and stick format. It is possible that CbiX/SirB may represent an evolutionarily ancient form of the chelatases.

DISCUSSION

In this paper, we have described the isolation of three genes required for the biosynthesis of sirohaem, which we have termed *sirA*, *-B* and *-C*, which encode a uroporphyrinogen III methyltransferase, a sirohydrochlorin ferrochelatase and a precorrin-2 dehydrogenase respectively. *B. megaterium* has at least two uroporphyrinogen III methyltransferases, a $CysG^{A}$ and SirA ($CobA^{Bm}$), demonstrating enzyme multiplicity as an important control element in the biosynthesis of sirohaem and cobalamin. The identification of SirC as a precorrin-2 dehydrogenase is the first description of such an enzyme with sole precorrin-2 dehydrogenase activity, since all other reported precorrin-2 dehydrogenases appear as part of multifunctional enzymes (e.g. CysG and Met8p, [9]). The sequence similarity between SirC and Met8p, which has both dehydrogenase and chelatase activities, would suggest that both proteins adopt a similar structure. It is more likely, therefore, that the protein originally evolved as a dehydrogenase and that Met8p subsequently acquired a further activity as a chelatase utilizing the same dehydrogenase active site. The identification of SirB as a sirohydrochlorin ferrochelatase is also the first time an enzyme with a sole function of chelation has been described for the biosynthesis of sirohaem. However, the observation that SirB can chelate cobalt with similar efficiency to ferrous iron (results not shown) suggests that metal ion specificity may be governed by interaction with specific metal ion binding proteins, a likely scenario since transition metal ions are thought to be largely protein bound once inside the cell (for example see [30]).

SirB has a high level of similarity with CbiX, a cobaltochelatase found within the main cobalamin biosynthetic operon of *B. megaterium*. Indeed, CbiX was found to have the same activities as SirB in that it could act as a chelatase for both sirohaem and cobalamin biosynthesis. Similar functions have also been assigned to CbiK, the *S. enterica* cobaltochelatase. There would appear to be some slight sequence similarity between SirB/CbiX and CbiK, including the conservation of key catalytic groups, suggesting that the protein have evolved from a common ancestor. The area of sequence similarity relates to one domain of CbiK, indicating that this region represents the minimal chelatase structure. CbiX also contains a histidine-rich region towards its C-terminus. Deletion of this region does not interfere with its ability to act as

Figure 4 Similarity between amino acid sequences of CbiX, SirB and CbiK

(**a**) Sequence alignment of selected SirB and CbiX sequences in comparison with the sequence of CbiK. Bm, *Bacillus megaterium*; Bst, *Bacillus stearothermophilus*; Se, *Salmonella enterica*; Syn, *Synechocystis* sp. Conserved amino acids are boxed in black whilst amino acids with a high level of similarity are boxed in grey. (**b**) Schematic representation of the structure of CbiK. The region of CbiK that aligns to the SirB/CbiX family is highlighted in red with three conserved catalytic residues highlighted in ball-and-stick representation. The Figure was prepared with MolScript [35] and PyMOL (http://www.pymol.org; Delano Scientific, San Carlos, CA, U.S.A.).

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a chelatase. Histidine-rich regions have been reported on other proteins, most significantly on HypB [31,32], a protein required for the delivery of nickel to the hydrogenase, and in CooJ [33], nickel-insertion accessory protein of the carbon monoxide dehydrogenase of *Rhodospirillum rubrum*. Interestingly, deletion of the histidine-rich region from these proteins does not abolish their respective activities [33,34]. It is possible that the histidine-rich region of CbiX is used as a store for the metal ion after direct interaction with the metal ion importer, or it is used possibly to deliver excess metal to the metal-removal system. The histidine-rich region of CbiX contains 19 histidines in a stretch of 35 amino acids. These histidines are often interspaced by aspartate residues, which could provide a negative charge to counter the divalent metal cation. Even if one metal is bound via four histidines, this region alone could bind up to 4 cobalt ions. It would appear that this C-terminus region of the protein would represent a metal ion chaperone, which presumably would have arisen by fusion of the chelatase with a smaller metal binding protein.

One major question still to be addressed, with respect to all the tetrapyrrole-pathway chelatases, is how they get their metal. Answers to this question need to be sought urgently if we are to understand fully the physiological role played by these enzymes.

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