Organization of genes for tetrapyrrole biosynthesis in Gram-positive bacteria

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Clusters of genes encoding enzymes for tetrapyrrole biosynthesis were cloned from *Bacillus sphaericus*, *Bacillus stearothermophilus*, *Brevibacillus brevis* and *Paenibacillus macerans*. The sequences of all *hemX* genes found, and of a 6.3 kbp *hem* gene cluster from *P. macerans*, were determined. The structure of the *hem* gene clusters was compared to that of other Gram-positive bacteria. The *Bacillus* and *Brevibacillus* species have a conserved organization of the genes *hemAXCDBL*, required for biosynthesis of uroporphyrinogen III (UroIII) from glutamyl-tRNA. In *P. macerans*, the *hem* genes for UroIII synthesis are also closely linked but their organization is different: there is no *hemX* gene and the gene cluster also contains genes, *cysG*^B and *cysG*^A-*hemD*, encoding the enzymes required for synthesis of sirohaem from UroIII. *Bacillus subtilis* contains genes for three proteins, NasF, YlnD and YlnF, with sequence similarity to *Escherichia coli* CysG, which is a multi-functional protein catalysing sirohaem synthesis from UroIII. It is shown that YlnF is required for sirohaem synthesis and probably catalyses the precorrin-2 to sirohaem conversion. YlnD probably catalyses precorrin-2 synthesis from UroIII and NasF seems to be specific for nitrite reduction.

Keywords: sirohaem, haem synthesis, *Bacillus*, tetrapyrrole synthesis, *hem* genes

INTRODUCTION

Biosynthesis of tetrapyrroles from the common intermediate 5-aminolaevulinic acid (ALA) follows a pathway universal to all living organisms (Fig. 1). There are, however, two separate biosynthetic pathways leading to ALA (for a review see Avissar & Moberg, 1995). Uroporphyrinogen III (UroIII) is the first cyclic tetrapyrrole intermediate in the pathway and is a precursor both to oxidized tetrapyrroles, such as haem and chlorophylls, and to reduced tetrapyrroles, such as sirohaem, vitamin B₁₂ and haem *d*₁.

In the Gram-positive bacteria *Bacillus subtilis*, *Clostridium josui*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*, the genes for enzymes catalysing

the biosynthesis of UroIII are organized in operons (Hansson *et al.*, 1991; Fujino *et al.*, 1995; Cole *et al.*, 1998; Kafala & Sasarman, 1997). The gene order in the *hem* operons from these species is the same, but there are some differences in the genes present. *B. subtilis* and *S. aureus* have a *hemAXCDBL* operon, encoding the enzymes for UroIII synthesis from glutamyl-tRNA (Fig. 1). The *hemX* gene is not essential for haem synthesis in *B. subtilis* (Schröder *et al.*, 1994). HemX is a polytopic membrane protein which by an unknown mechanism down-regulates the level of HemA (Schröder *et al.*, 1994). It is notable that *Escherichia coli* does not contain a *hemX* gene (Blattner *et al.*, 1997).

The *hemE*, *hemH* and *hemY* genes in Gram-positive bacteria are required for protohaem IX synthesis from UroIII and tend to be clustered, e.g. in *B. subtilis* (Hansson & Hederstedt, 1992), *Mycobacterium* (GenBank accession no. L01536; Cole *et al.*, 1998), *Propionibacterium freudenreichii* (Hashimoto *et al.*, 1997) and *Deinococcus radiodurans* (sequence data obtained through early release from The Institute for Genomic Research at www.tigr.org).

The compact organization of *hem* genes in Gram-

Abbreviations: ALA, 5-aminolaevulinic acid; BGSC, *Bacillus* Genetic Stock Center; TBAB, Tryptose Blood Agar Base; UroIII, uroporphyrinogen III.

The GenBank accession numbers for the sequences determined in this work are AF064058 (*B. stearothermophilus hemX*), AF064059 (*Br. brevis hemX*), AF064060 (*B. sphaericus hemX*) and AF064061 (*P. macerans hemA cysG*^B *hemC cysG*^A-*hemD*).

Fig. 1. Overview of tetrapyrrole biosynthesis, with gene products indicated. In animals, fungi and proteobacteria of the α -subdivision, 5-aminolaevulinic acid (ALA) is synthesized from succinyl-coenzyme A and glycine. In plants, most bacteria and archaea, ALA is synthesized from glutamyl-tRNA by the action of glutamyl-tRNA reductase (HemA) and glutamate-1-semialdehyde aminotransferase (HemL). Uroporphyrinogen III (UroIII) is formed from ALA in three steps, catalysed by ALA dehydratase (HemB), porphobilinogen deaminase (HemC) and UroIII synthase (HemD), respectively. Synthesis of sirohaem, vitamin B₁₂ and haem d_i involves an S-adenosylmethionine-dependent methylation of UroIII to form precorrin-2, which is catalysed by UroIII methyltransferase. Oxidation of precorrin-2 to sirohdrochlorin and chelation of sirohdrochlorin with iron results in sirohaem. Two of the enzymes for the synthesis of protohaem IX from UroIII are common to all organisms: UroIII decarboxylase (HemE) and ferrochelatase (HemH). Bacteria of the family *Enterobacteriaceae* have an aerobic and an anaerobic coproporphyrinogen III oxidase, HemF and HemN respectively, and protoporphyrinogen IX oxidase, HemG. Gram-positive bacteria and some Gram-negative bacteria have a HemY protoporphyrinogen IX oxidase but do not contain the HemF/HemN coproporphyrinogen III oxidase. The *B. subtilis* HemY protein can oxidize coproporphyrinogen III to coproporphyrin III and protoporphyrinogen IX to protoporphyrin IX (Dailey *et al.*, 1994; Hansson & Hederstedt, 1994) but does not resemble HemG (Hansson *et al.*, 1997). A third group of Gram-negative bacteria have HemF/HemN but do not contain either HemY or HemG.

positive bacteria is probably important for regulation. It may also be of evolutionary significance and could be related to the metabolism of the different bacteria. The aim of the present work was to better understand the organization of *hem* genes and to analyse the occurrence and sequence of the *hemX* gene in Gram-positive bacteria. *hem* gene clusters from *Bacillus* species, *Brevibacillus brevis* and *Paenibacillus macerans* were cloned and the gene organization analysed. The bacteria chosen represent different phenotypes ranging from strictly aerobic to facultatively anaerobic and also include a thermophilic species (*Bacillus stearothermophilus*). From this work a role of the *B. subtilis* YlnF protein in sirohaem synthesis was also identified.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Media and growth of bacteria. *B. subtilis* strains, *P. macerans* (previously *Bacillus macerans*: Ash *et al.*, 1993) and *B. sphaericus* were grown on Tryptose Blood Agar Base (TBAB; Difco), in LB medium (Sambrook *et al.*, 1989) or in Spizizen's minimal medium (Spizizen, 1958) with required amino acids added at 20 mg l⁻¹. *E. coli* strains were grown on TBAB, in LB medium or in M9 minimal medium (Sambrook *et al.*, 1989). *B. stearothermophilus* was grown on LA plates or in LB medium modified by exclusion of NaCl. *Br. brevis* (previously *Bacillus brevis*: Shida *et al.*, 1996) was grown on TBAB or in Todd-Hewitt Broth. *B. stearothermophilus* was grown at 55 °C, *Br. brevis* and *B. sphaericus* at 30 °C, and all other bacteria at 37 °C. The following antibiotics were used: ampicillin (75 mg l⁻¹), chloramphenicol (*E. coli*, 12.5 mg l⁻¹; *B. subtilis* 5 mg l⁻¹), erythromycin (3 mg l⁻¹), kanamycin (20 mg l⁻¹). Media containing haemin (liquid media, 0.5 mg l⁻¹; plates, 2.5 mg l⁻¹) were supplemented with cysteine (20 mg l⁻¹) and 0.5% (w/v) bovine serum albumin fraction V (Sigma). Haemin stock solutions were prepared as described previously (Petrick *et al.*, 1990).

Complementation of *B. subtilis* and *E. coli* mutations.

Complementation of the ALA requirement of the *B. subtilis* strain KA11 was assayed on Spizizen's minimal medium plates. The other *B. subtilis* and *E. coli* *hem* mutants were tested for complementation on TBAB plates. *B. subtilis* *ynlF* and *nasF* mutants were tested for complementation on Spizizen's minimal medium plates with nitrate as nitrogen source. *E. coli* *cysG* mutants were tested for complementation on M9 minimal medium plates. Chromosomal DNA isolated from *Br. brevis*, *B. stearothermophilus* and *P. macerans* was partially cleaved with *Sau3A*. DNA from *B. sphaericus* was partially cleaved with the isoschizomer *MboI* since it was resistant to cleavage by *Sau3A* (indicating that the C residue of the GATC recognition sequence is methylated in *B. sphaericus*). The cleaved DNA was size-fractionated and fragments of 5–10 kbp were ligated to *Bam*HI-cleaved *B. subtilis*–*E. coli* shuttle plasmids pHP13 or pHB201. pHB201 has a σ^A -type promoter in front of the cloning cassette to facilitate expression of cloned genes in *B. subtilis* (Haima *et al.*, 1990). *B. subtilis* strains were transformed with the ligation mixtures and haem prototrophs were selected. The colonies obtained were transferred to plates containing chloramphenicol to confirm that they carried the respective plasmids. Plasmid DNA was then extracted from the transformants and used in retransformation of the *B. subtilis* *hem* mutants. Finally, the cloned fragments were used in Southern blot analysis to verify that they represented continuous segments of the chromosome of the respective bacterial species.

Construction of *B. subtilis* 168 Δ YlnF. The last part of *B. subtilis* *ynlF* (601 bp) was amplified by PCR, using primers Yln3.5 and Yln4 (Table 2), and cloned in pBluescript II KS(–) giving plasmid pYLN2. The *cat* gene from pHV32 was inserted in pYLN2 giving plasmid pYLN3. The first part of *B. subtilis* *ynlF* (277 bp) was amplified by PCR, using primers YLN10 and YLN20, and cloned in pYLN3 giving plasmid pYLN30. By transforming *B. subtilis* 168 with pYLN30 and selecting for Cm^r, an internal region of the *ynlF* gene (nucleotides 1635115–1635381) was replaced with the *cat* gene, as confirmed by Southern blot analysis.

DNA techniques. Plasmids were propagated in *E. coli* XL-1 Blue. Large-scale preparations of plasmid DNA were carried out as described by Ish-Horowitz & Burke (1981). Small-scale preparations of plasmid DNA were performed by the boiling method (Sambrook *et al.*, 1989) or by using the Quantum purification system (Bio-Rad). Plasmid DNA was sequenced using the Sequenase version 2.0 kit and [³⁵S]dATP α S (Amersham). DNA amplification was achieved by PCR using *Taq* polymerase (Boehringer Mannheim). Chromosomal DNA was prepared as described by Marmur (1961). Chromosomal DNA fragments were size-fractionated by sucrose gradient centrifugation. Southern blot analysis of chromosomal DNA was performed using a random priming kit and [α -³²P]dCTP (Amersham). The programs used for sequence analysis were part of the Wisconsin Package Version 8.1 (Genetics Computer Group, Madison, WI, USA).

Other methods. SDS-PAGE was performed according to Neville (1971). Western blotting was performed according to Towbin *et al.* (1979) using a semi-dry electroblotter (Ancos) and Immobilon P filters. The antiserum against *B. subtilis* HemA was described by Schröder *et al.* (1994). Goat anti-rabbit IgG conjugated to alkaline phosphatase (Clontech) was used as secondary antibody. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Cloning of *hem* genes

The *B. subtilis* strains 1A589 (*hemB1*) and 1A590 (*hemC33*) have a strict growth requirement for external haem and give essentially no background growth when 10⁷–10⁸ cells are spread on TBAB plates (Anderson & Ivánovics, 1967). Since the *hemB* and *hemC* genes are well conserved in many bacteria (Rhie *et al.* 1996; Kafala & Sasarman, 1997), the above *B. subtilis* mutant strains were used to isolate *hem* genes from other bacteria by complementation as described in Methods.

DNA fragments which complemented *B. subtilis* *hemB1* and *hemC33* mutations were obtained from *B. sphaericus* and *P. macerans*. Only *hemB1*-complementing fragments were found for *Br. brevis* and *B. stearothermophilus* in a primary screen. However, when the *hemB1*-complementing plasmids were isolated and used to transform the *hemC33* mutant to chloramphenicol resistance, the plasmids could then complement the *hemC33* mutation (Table 3). It appears that *hemC* from these species is either not efficiently expressed in *B. subtilis* or is, for other reasons, not fully functional.

A *P. macerans* chromosomal DNA fragment that complemented *B. subtilis* *hemH* (strain 3G18 Δ 8) and a *B. sphaericus* DNA fragment that complemented *B. subtilis* *hemA* (strain KA11) were also isolated. Use of the various fragments cloned by complementation as probes permitted the isolation of additional fragments which cover a larger portion of the respective chromosomes.

Organization of *hem* genes for synthesis of UroIII from glutamyl-tRNA in *Br. brevis*, *B. sphaericus* and *B. stearothermophilus*

A *hemAXCDBL* gene cluster with the same compact organization as in *B. subtilis* was found in *Br. brevis*, *B. sphaericus* and *B. stearothermophilus* as determined by sequence analysis and complementation experiments (Fig. 2, Table 3). The spacing between the proposed stop codon of one gene and the proposed start codon of the next gene ranged from 210 bp (between *hemA* and *hemX* in *B. sphaericus*) to 12 bp (for several genes).

Deletion of *hemX* in *B. subtilis* causes the steady-state amount of HemA protein to increase (Schröder *et al.*, 1994). No other phenotype has been observed in such mutants. Expression of *hemX* from *Br. brevis*, on plasmid pXBRE411, or *B. subtilis* *hemX*, on plasmid pHEMX403, in the *B. subtilis* HemX deletion mutant 3G18 Δ HemX in both cases led to a decrease in the amount of HemA protein, as determined by Western blot analysis (data not shown). This demonstrated that the *B. subtilis* and *Br. brevis* HemX proteins are functionally interchangeable. The HemX proteins from *B. subtilis*, *B. stearothermophilus*, *Br. brevis* and *B. sphaericus* are of the same size (272–276 residues) and show about 22% sequence identity across the entire

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant description	Source
Strains		
<i>B. subtilis</i> 1A589	<i>trpC2 hemB1</i>	BGSC
<i>B. subtilis</i> 1A590	<i>trpC2 hemC33</i>	BGSC
<i>B. subtilis</i> 1A591	<i>trpC2 hemD11</i>	BGSC
<i>B. subtilis</i> 3G18ΔHemX	<i>ade met trpC2 ΔhemX::ble</i>	Schröder <i>et al.</i> (1994)
<i>B. subtilis</i> KA11	<i>ade met trpC2 hemA20</i>	Holmgren <i>et al.</i> (1979)
<i>B. subtilis</i> 3G18Δ7	<i>ade met trpC2 ΔhemEHY::ble</i>	Hansson & Hederstedt (1992)
<i>B. subtilis</i> 3G18Δ8	<i>ade met trpC2 ΔhemH::ble</i>	Hansson & Hederstedt (1994)
<i>B. subtilis</i> 168	<i>trpC2</i>	BGSC
<i>B. subtilis</i> 168ΔYlnF	<i>trpC2 ΔylnF::cat</i>	This study
<i>B. subtilis</i> LAB1926	<i>trpC2 pheA1 nasEF::pML16</i>	Ogawa <i>et al.</i> (1995)
<i>Br. brevis</i> ATCC 8246		BGSC
<i>B. sphaericus</i> ATCC 33203		BGSC
<i>B. stearothermophilus</i> 9A14	<i>thr-1</i>	BGSC
<i>P. macerans</i> DSM 24		Schirawski & Uden (1995)
<i>E. coli</i> XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F'[proAB⁺ lacI^q lacZΔM15Tn10(Tet^r)]</i>	Stratagene
<i>E. coli</i> 302Δa	<i>cysG</i>	Griffiths & Cole (1987)
<i>E. coli</i> AN344	<i>hemA pro leu</i>	MacGregor (1976)
<i>E. coli</i> SASZ31	<i>hisG1 rpsL9 xylA7 hemD31 mobA2 argH1 Δthi-1 met</i>	Chartrand <i>et al.</i> (1979)
Plasmids		
pBluescript II KS/SK(–)	Ap ^r <i>lacZ'</i>	Stratagene
pUC19	Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pHP13	Cm ^r Em ^r	Haima <i>et al.</i> (1987)
pHB201	Cm ^r Em ^r	S. Bron, University of Groningen
pACYC177	Ap ^r Km ^r	Chang & Cohen (1978)
pHV32	Ap ^r Cm ^r Tc ^r	Niaudet <i>et al.</i> (1982)
pLUM403	296 bp <i>HindIII</i> – <i>EcoRI</i> fragment containing the <i>B. subtilis</i> Orf4 promoter cloned in pAMB22	Holmberg <i>et al.</i> (1990)
pHPSK	301 bp <i>PvuII</i> fragment of pHP13 exchanged for 448 bp <i>PvuII</i> fragment of pBluescript II SK(–)	This study
pHEMX513	<i>B. subtilis</i> <i>hemX</i> in pHP13	Schröder <i>et al.</i> (1994)
pHEMX403	259 bp <i>PstI</i> – <i>BamHI</i> fragment containing the Orf4 promoter of pLUM403 cloned in pHEMX513	This study
pXBRE4	7.9 kbp <i>Sau3A</i> (partial) fragment from <i>Br. brevis</i> cloned in pHB201 (<i>BamHI</i>); complements <i>B. subtilis</i> 1A589	This study
pXBRE47	3 kbp <i>EcoRI</i> fragment from pXBRE4 cloned in pHB201	This study
pXBRE49	1.1 kbp <i>EcoRI</i> – <i>PvuI</i> fragment (<i>hemX</i>) of pXBRE47 cloned in pHP13	This study
pXBRE411	296 bp <i>HindIII</i> – <i>EcoRI</i> fragment containing the Orf4 promoter of pLUM403 cloned in pXBRE49	This study
pXSPH1	4.8 kbp <i>MboI</i> (partial) fragment from <i>B. sphaericus</i> cloned in pHP13 (<i>BamHI</i>); complements <i>B. subtilis</i> 1A589 and 1A590	This study
pXSPH10	7.5 kbp <i>MboI</i> (partial) fragment from <i>B. sphaericus</i> cloned in pHP13 (<i>BamHI</i>); complements <i>B. subtilis</i> 1A590 and KA11	This study

Table 1. (cont.)

Strain or plasmid	Genotype or relevant description	Source
pXSTE3	3.7 kbp <i>Sau3A</i> (partial) fragment from <i>B. stearotheophilus</i> cloned in pHP13 (<i>Bam</i> HI); complements <i>B. subtilis</i> 1A589	This study
pHMAC1	6.5 kbp <i>Sau3A</i> (partial) fragment from <i>P. macerans</i> cloned in pHP13 (<i>Bam</i> HI); complements <i>B. subtilis</i> 3G18Δ8	This study
pXMAC1	5.3 kbp <i>Sau3A</i> (partial) fragment from <i>P. macerans</i> cloned in pHP13 (<i>Bam</i> HI); complements <i>B. subtilis</i> 1A590	This study
pXMAC15	2.8 kbp <i>Sall</i> fragment from pXMAC1 cloned in pHP13	This study
pXMAC18	<i>Pst</i> I deletion derivative of pXMAC1	This study
pXMAC115	3 kbp <i>Hind</i> III– <i>Sall</i> (partial) fragment from pXMAC18 cloned in pHP13	This study
pXMAC116	3.3 kbp <i>Pvu</i> II (partial) fragment from pXMAC115 cloned in pACYC177 (<i>Hinc</i> II)	This study
pXMAC3	6.2 kbp <i>Sau3A</i> (partial) fragment from <i>P. macerans</i> cloned in pHP13 (<i>Bam</i> HI); complements <i>B. subtilis</i> 1A590	This study
pXMAC35	1700 bp <i>Pst</i> I fragment from pXMAC3 cloned in pBluescript II KS(–)	This study
pXMAC39	120 bp <i>Bam</i> HI– <i>Pst</i> I fragment (amplified by PCR, using MAC1 and MAC2 primers) from pXMAC3 cloned in pHPSK	This study
pXMAC310	2.5 kbp <i>Pst</i> I (partial) fragment from pXMAC3 cloned in pXMAC39	This study
pXMAC311	2.5 kbp <i>Pvu</i> II fragment from pXMAC310 cloned in pACYC177 (<i>Hinc</i> II)	This study
pXMAC350	1.7 kp <i>Bam</i> HI– <i>Xho</i> I fragment from pXMAC35 cloned in pHB201	This study
pYLN2	601 bp <i>Bam</i> HI– <i>Xho</i> I fragment from <i>B. subtilis</i> 168 (amplified by PCR, using YLN3.5 and YLN4 primers) cloned in pBluescript II KS(–)	This study
pYLN3	<i>Hind</i> III– <i>Bam</i> HI fragment from pHV32 containing the <i>cat</i> gene cloned in pYLN2	This study
pYLN30	277 bp <i>Eco</i> RV– <i>Hind</i> III fragment from <i>B. subtilis</i> 168 (amplified by PCR, using YLN10 and YLN20 primers) cloned in pYLN3	This study
pSCW1	<i>E. coli cysG</i> with the Gly224Ala mutation cloned in pUC19	Warren <i>et al.</i> (1994)
pSCW2	<i>E. coli cysG</i> ^A cloned in pSCW1	Warren <i>et al.</i> (1994)
pACYC177Δ <i>bla</i>	<i>Bam</i> HI– <i>Hinc</i> II deletion derivative of pACYC177 (removes part of the <i>bla</i> gene)	This study

Table 2. Oligonucleotides

Oligonucleotide	Sequence (5'–3')
MAC1	GGATCCAGGTGAACTTGCAATGC
MAC2	GCCGCTCCATCGCTTCC
YLN3.5	TACAAGCGGATCCAGT
YLN4	GTGCCTCTAGATTATAC
YLN10	GCATCTCGATATCCATCAGTCC
YLN20	GACAAGCTTTATATCAGTCCC

protein (two single-residue gaps in the alignment only; not shown). Pairwise, the HemX proteins have approximately 40% sequence identity. HemX shows sequence similarity to the CcsA protein, which is required for cytochrome *c* biogenesis in plants (Xie *et al.*, 1998). A *B. subtilis* *hemX* deletion strain (3G18ΔHemX) is, however, not impaired in cytochrome *c* synthesis (our unpublished data). A tryptophan-rich sequence motif, invariant in CcsA and other cytochrome *c* biogenesis proteins (cf. Goldman *et al.*, 1998; Thöny-Meyer, 1997), is not conserved in the HemX proteins.

Fig. 2. Physical map of chromosomal DNA fragments cloned from *B. stearothermophilus*, *B. sphaericus* and *Br. brevis*. The gene map shown at the top is that of *B. subtilis* and serves to illustrate the genes present on the different cloned fragments. Segments of the cloned DNA that span the *hemX* gene and which were sequenced continuously on both strands are indicated with thicker lines.

Table 3. Complementation of different *B. subtilis* tetrapyrrole synthesis mutants with plasmids carrying chromosomal DNA from *P. macerans* (pXMAC, pHMAC), *Br. brevis* (pXBRE4), *B. sphaericus* (pXSPH) and *B. stearothermophilus* (pXSTE3)

+, Forms single colonies; —, does not form single colonies; ND, not determined.

Plasmid	Complementation of <i>B. subtilis</i> strain:						
	KA11 (<i>hemA</i>)	1A589 (<i>hemB</i>)	1A590 (<i>hemC</i>)	1A591 (<i>hemD</i>)	168ΔYlnF (<i>ylnF</i>)	LAB1926 (<i>nasF</i>)	3G18Δ7 (<i>hemEHY</i>)
pXMAC1	ND	+	+	+	ND	ND	ND
pXMAC3	+	ND	+	+	+	—	ND
pXMAC15	ND	ND	+	ND	+	ND	ND
pXMAC115	ND	ND	ND	+	ND	—	ND
pHMAC1	ND	ND	ND	ND	ND	ND	+
pXBRE4	ND	+	+	+	ND	ND	ND
pXSPH1	ND	+	+	+	ND	ND	ND
pXSPH10	+	ND	+	+	ND	ND	ND
pXSTE3	ND	+	+	+	ND	ND	ND

Organization of *hem* genes in *P. macerans*

An 8.1 kbp DNA segment containing genes for synthesis of UroIII and sirohaem from glutamyl-tRNA was cloned from *P. macerans*. Five complete open reading frames, ORF1 to ORF5, and the beginning of a sixth, ORF6, were identified in the cloned segment (Fig. 3). Located 59 bp upstream of the putative start codon of ORF1 is a typical σ^A -type —10 promoter sequence, TATAAT. The amino acid sequence deduced from ORF1 shows 52% sequence identity to *B. subtilis* HemA (Petricek *et al.*, 1990). pXMAC3 complemented *B. subtilis* *hemA*, while pXMAC311 complemented *E. coli* *hemA* (Fig. 3, Table 4). (The region preceding ORF1 is not tolerated in *E. coli*.) Thus, ORF1 is *hemA*, encoding glutamyl-tRNA reductase.

ORF2 shows 50% sequence similarity to the N-terminal part of *E. coli* CysG (Peakman *et al.*, 1990) (aa 1–201) (Fig. 4), an enzyme which catalyses sirohaem synthesis from UroIII (Fig. 1). The sequence similarity, together

with experimental results presented in the next section, shows that ORF2 corresponds to the CysG^B domain, which catalyses the conversion of precorrin-2 into sirohaem.

P. macerans *hem* genes were cloned by complementation of *B. subtilis* *hemC*, indicating that *hemC* is present in the cloned fragment. The protein encoded by ORF3 shows 59% sequence identity to *B. subtilis* HemC (Petricek *et al.*, 1990), identifying ORF3 as *hemC*.

The N-terminal part of the protein encoded by ORF4 (aa 1–245) is similar (54% identity) to CobA of *Bacillus megaterium* (Robin *et al.*, 1991) and the C-terminal part of *E. coli* CysG, indicating that it functions as an S-adenosylmethionine-dependent UroIII methylase, i.e. it catalyses the first step in sirohaem synthesis. The C-terminal part of the ORF4 protein (aa 246–511) shows only 27% identity to the HemD protein of *B. subtilis* (Hansson *et al.*, 1991), but expression of ORF4, on pXMAC115, was able to complement *B. subtilis* *hemD*

Fig. 3. Physical map of a *hem* gene cluster for UroIII and siroheme synthesis in *P. macerans*. A restriction enzyme map is provided for the part that was sequenced continuously on both strands (accession no. AF064061). Restriction site abbreviations: A, *Afl*III; B, *Bam*HI; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sc, *Scal*I; Sm, *Sma*I; X, *Xho*I. The lines below the map indicate *P. macerans* DNA fragments cloned in the indicated plasmids.

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Pmac : MPHYV VMLN EGRRCVIV VVTVARKAAALTEA VVTVISPGVTAWLQDRVREGIEAWLAREYREGDLKGAFLVF TDSRQV TDSIVKE : 93
Bsu : ---ML LHS EKKKVIA SI LRLKTVISE ADITLVSPDVEPEIKQMAEERRIKWEKRTIEKEDYLN AFFI TNDNAAV KEIAQS : 90
Eco : -MDHL IFCQ RDRDCLV VDV EPKARLLDA RLTVNALAFIPQFTAWADAGMLTLVEGPFDESLLDTCWLAT TDDDAL QVRVQA : 92
Nme : -MNYF IIFAN AGRPVLVV VAV ARKISLLKA EVRVAAKHLNAELSALAAENKILWLAE EFRAEHIRT VFLI TSSDQAL RRVFHL : 92
Cjo : -YGRF LFDI SGKKVLVV EIEI TRRVKTL LRF ADIYLVAPHLTSELQEMLNCKLINYREGYYESQDIQNMFLVIE TNDRET HKVYLD : 455

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Pmac : EMLGIPVNDTA GRRARKFYH HVVRGKLVIG TS AGPAAARELCREIDRRFGDTYEQYVEFLSLVTRVVKQVEDKERRKRLRLARG : 185
Bsu : SPFQL-VNCVS AELG-NVYM KIVKRGHVTVS TS ASPKHTKELAEENVDKLIDGDFVAE VNRLYQMRRKK----- : 162
Eco : EARRIFCN-VV APKAASFIM SIIDRSPLMVA TS GTSFVLARLLREKLESLLPLHLGQVAKYAGQLRGRVKQFATMGERRRFWEKLF : 183
Nme : ESCQKPVN-VV DRDHCSFIF SVIDRNPVQIA TS SAPVLARLLRERLEALLPPLSGDMAEISGRWRDAVKGLKSVTERRRFWEKQF : 183
Cjo : KEKGIQMS-IA CREECFSYF AIFEFDGIVGGL QN DNHSLVKSVAEQIR-----KIGQATD----- : 515

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Pmac : ELDILPSIRQGGFTRGANEIAAWIEEQRRNSG- : 218
Bsu : ----- : -
Eco : VNDRLAQSLANNDQK-AITETTEQLINEPLDHRG : 216
Nme : -NGRFAALVKNRQNTLAERELAGQLEQSRQNDQG : 216
Cjo : ----- : -

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Fig. 4. Sequence alignment between CysG^B proteins or domains from different bacteria. The *P. macerans* (Pmac) CysG^B protein was aligned to aa 1–216 of *E. coli* (Eco) CysG (Peakman *et al.*, 1990), aa 1–216 of *Neisseria meningitidis* (Nme) CysG (GenBank accession no. Y10177), aa 364–515 of *C. josui* (Cjo) Hema-CysG^B (Fujino *et al.*, 1995) and *B. subtilis* (Bsu) YlnF (GenBank accession no. AJ000974).

and *E. coli* *hemD* (Tables 3 and 4). These findings, together with results reported in the next section, identify ORF4 as *cysG*^A-*hemD*.

The protein deduced from ORF5 shows similarity to HemB proteins from various species (Rhie *et al.*, 1996). pXMAC1 complemented *B. subtilis* *hemB* (Fig. 3, Table 3) thus identifying ORF5 as *hemB*. The first part of ORF6 encodes a polypeptide which shows similarity to *B. subtilis* HemL.

Another *P. macerans* DNA fragment (plasmid pXMAC1) was isolated by complementation of *B.*

subtilis *hemH* (strain 3G18Δ8) and was found to also complement *B. subtilis* *hemEHY* (Table 3). This, together with partial sequence analysis of the cloned fragment, indicates that *P. macerans* contains a *hemEHY* operon very similar to that previously described in *B. subtilis* (Hansson *et al.*, 1992).

Genes for sirohaem synthesis in *B. subtilis*

Sirohaem is a cofactor for assimilatory nitrite and sulphite reductases and is therefore essential for growth on nitrate or nitrite as a source of nitrogen and sulphate

Table 4. Complementation of different *E. coli* tetrapyrrole synthesis mutants with plasmids carrying chromosomal DNA from *P. macerans* (pXMAC) and *E. coli* (pSCW2)

+, Forms single colonies; —, does not form single colonies; ND, not determined.

Plasmid	Complementation of <i>E. coli</i> strain:				
	AN344 (<i>hemA</i>)	SASZ31 (<i>hemD</i>)	302Δa (<i>cysG</i>)	302Δa/pXMAC116 (<i>cysG^B</i>)	302Δa/pSCW1 (<i>cysG^A</i>)
pXMAC116	ND	+	—	ND	+
pXMAC311	+	ND	—	—	ND
pXMAC350	ND	ND	—	—*	ND
pSCW2	ND	ND	+	ND	ND

* Cells produce a green pigment.

or sulphite as the sole sulphur source. *E. coli* CysG has several enzymic activities and catalyses all steps in the synthesis of sirohaem from UroIII (Warren *et al.*, 1994). The C-terminal part of CysG, the CysG^A domain, which is similar to, for example, *B. megaterium* CobA, catalyses S-adenosylmethionine-dependent methylation of UroIII to form precorrin-2, a common intermediate in sirohaem and vitamin B₁₂ synthesis (Fig. 1). The N-terminal part of CysG, the CysG^B domain, catalyses the NAD-dependent dehydrogenation of precorrin-2 to sirohydrochlorin and the subsequent chelation with iron to form sirohaem.

The *B. subtilis* genome (Kunst *et al.*, 1997) contains genes for two CysG^A homologues: the YlnD and NasF proteins. UroIII methyltransferase activity of these *B. subtilis* proteins has not yet been demonstrated. YlnD is of the same size as the *E. coli* CysG^A domain and *B. megaterium* CobA, while *B. subtilis* NasF has a C-terminal extension of 240 residues (Ogawa *et al.*, 1995). The *ylnD* gene is located at 140° on the chromosome together with genes encoding enzymes for sulphate reduction. The *nasF* gene is located at 30°, clustered with genes encoding subunits of nitrite reductase. A NasF null mutant, LAB1926, does not grow with nitrate as a nitrogen source but can use sulphate as the only sulphur source (Ogawa *et al.*, 1995), i.e. it apparently contains sirohaem. The phenotype of the mutant shows that a protein other than NasF, probably YlnD, catalyses the S-adenosylmethionine-dependent methylation of UroIII in sirohaem synthesis.

The *B. subtilis* YlnF protein shows 47% sequence similarity to the *E. coli* CysG^B domain (Fig. 4) and contains in the N-terminal part a consensus dinucleotide-binding sequence (GxGxxAxxxAxxxxxxxA) which most likely is involved in binding NADH (Warren *et al.*, 1994). The *ylnF* gene is located in the same gene cluster as *ylnD*. A *ylnF* deletion mutant, strain 168ΔYlnF, was constructed (see Methods) in order to analyse whether or not YlnF has a role in sirohaem synthesis. The mutant was unable to grow on minimal medium plates with nitrate as nitrogen source and growth was fully restored

only after addition of both cysteine and ammonia to the medium. Expression of the *P. macerans cysG^B* gene, from plasmid pXMAC3 or pXMAC15, in strain 168ΔYlnF complemented the defect (Fig. 3, Table 3). These results demonstrate that YlnF in *B. subtilis* is required for sirohaem synthesis and most likely catalyses the synthesis of sirohaem from precorrin-2.

Thus, genes for sirohaem synthesis in *B. subtilis* seem to be clustered but are not linked to the two *hem* gene clusters encoding enzymes for UroIII synthesis from glutamyl-tRNA and protohaem IX synthesis from UroIII, respectively.

Genes for sirohaem synthesis in *P. macerans*

In contrast to *B. subtilis*, genes for sirohaem synthesis in *P. macerans* are part of the gene cluster for UroIII synthesis (Fig. 3). *E. coli cysG* was not complemented by the *P. macerans cysG^A-hemD* or *cysG^B* or these genes together (Table 4). However, co-expression of *cysG^A-hemD* and *cysG^B* in the mutant resulted in the accumulation of a greenish pigment. The pigment is presumably derived from UroIII and some type of side-product from the sirohaem synthetic pathway. The *P. macerans cysG^A-hemD* gene complemented *E. coli cysG^A*, but *P. macerans cysG^B*, which complemented *B. subtilis ylnF*, did not complement *E. coli cysG^B*. Since the green pigment accumulated in *E. coli* only when both *P. macerans cysG* genes were present, it seems as if the *P. macerans CysG^B* protein is synthesized in *E. coli* but is functionally deficient. It is noted that in *E. coli* the chelatase deficiency of a CysG^B mutant can be complemented by the seemingly unrelated *S. typhimurium* and *B. megaterium* cobalt chelatases CbiX (Raux *et al.*, 1997; Beck *et al.*, 1997). In these two cases, precorrin-2 is probably non-enzymically oxidized to sirohydrochlorin (Raux *et al.*, 1997).

We conclude that *P. macerans cysG^A-hemD* encodes a protein with UroIII methylase activity while *P. macerans cysG^B* probably encodes a protein for sirohaem synthesis from precorrin-2.

The gene organization in *P. macerans* is similar to the *hemA-cysG^B hemC cysG^A-hemD hemB* and *hemA cysG^B hemC cysG^A-hemD hemL hemB* clusters found in strictly anaerobic *Clostridium* species, i.e. *C. josui* and *C. acetobutylicum* (Fujino *et al.*, 1995; pre-release by GTC, www.genomecorp.com). Perhaps it reflects the fact that *P. macerans* is a facultative anaerobe (Schirawski & Uden, 1995).

Organization of the genes for UroIII synthesis together with those for sirohaem synthesis in one operon ensures that the enzymes for the whole pathway from glutamyl-tRNA to sirohaem can be co-regulated at the transcriptional level. This would be especially important under conditions where most or all of the UroIII produced in the cell is used for synthesis of the reduced metallo-tetrapyrroles, sirohaem and vitamin B₁₂. A *cysG^A-hemD* gene like that of *P. macerans* and *Clostridium* species is found in evolutionarily very distant bacteria, e.g. in green non-sulphur bacteria (sequence data obtained through early release from The Institute for Genomic Research at www.tigr.org), cyanobacteria (Kaneko *et al.*, 1996) and *Mycobacterium* (GenBank accession no. U00018). Thus, it seems as if the *cysG^A-hemD* gene is of early origin in evolution and has been split into two widely separated genes in *Bacillus* and *Staphylococcus* species.

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