The *hemX* gene of the *Bacillus subtilis* *hemAXCDBL* operon encodes a membrane protein, negatively affecting the steady-state cellular concentration of Hema (glutamyl-tRNA reductase)

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The *Bacillus subtilis* *hemAXCDBL* operon encodes enzymes for the biosynthesis of uroporphyrinogen III from glutamyl-tRNA. The function of the *hemX* gene product was studied in this work. The deduced amino acid sequence suggests HemX to be an integral 32 kDa membrane protein. This was confirmed by experiments using *Escherichia coli* minicells and *hemX-phoA* gene fusions. Deletion of the *hemX* gene from the *Bacillus subtilis* chromosome demonstrated that this gene is not required for haem synthesis. However, the deletion strain was found to overexpress the *hemA* gene product, glutamyl-tRNA reductase. A combination of results obtained with *B. subtilis* *hemA* and *hemX* in *Escherichia coli* and *Bacillus subtilis* shows that HemX negatively affects the steady-state cellular concentration of Hema protein. The mechanism by which HemX affects the Hema concentration is unclear.

Keywords: 5-aminolaevulinic acid biosynthesis, protohaem IX synthesis, *Bacillus subtilis*, *hem* genes

INTRODUCTION

5-Aminolaevulinic acid (ALA) is an early precursor in the synthesis of haem. Eight molecules of ALA are used for the synthesis of one protohaem IX molecule in a seven-step pathway. Little is known about regulation of ALA and protohaem IX synthesis in bacteria. In the Gram-positive strictly aerobic bacterium *Bacillus subtilis*, ALA is synthesized from glutamate by the C₅ pathway (O'Neill *et al.*, 1989). This pathway is found in plants and algae (Beale & Weinstein, 1990) and in most bacteria, e.g. *Escherichia coli* (O'Neill *et al.*, 1989), *Salmonella typhimurium* (Elliott, 1989), *Clostridium thermoaceticum* (Oh-hama *et al.*, 1988) and *Methanobacterium thermoautotrophicum* (Friedmann *et al.*, 1987). The pathway involves three enzymic steps. Glutamate is first activated by ligation to tRNA^{Glu}, a reaction catalysed by glutamyl-tRNA synthase (EC 6.1.1.17). The tRNA and the synthase are the same as used in protein synthesis (Jahn *et al.*, 1992). The glutamyl-tRNA is then reduced by glutamyl-tRNA reductase in an

NADPH-dependent reaction to yield glutamate 1-semialdehyde (GSA). In the last step a GSA aminotransferase (EC 5.4.3.8) converts GSA to ALA.

In *B. subtilis* there are two clusters of *hem* genes. The *hemAXCDBL* operon, located at about 244° on the chromosomal genetic map (Petricek *et al.*, 1990; Hansson *et al.*, 1991), encodes enzymes for the early steps of haem synthesis, i.e. the synthesis of ALA and uroporphyrinogen III (UroIII). The other cluster, the *hemEHY* operon at about 94°, encodes enzymes for late steps, i.e. UroIII to protohaem IX (Hansson & Hederstedt, 1992). The *hemA* and *hemL* genes encode glutamyl-tRNA reductase and GSA aminotransferase, respectively (Hansson *et al.*, 1991; Schröder *et al.*, 1992). The *hemB*, *hemC* and *hemD* genes encode the three enzymes required for the synthesis of UroIII from ALA: porphobilinogen synthase (EC 4.3.1.8), hydroxymethylbilane synthase (EC 4.2.1.75) and UroIII synthase (EC 4.1.1.37), respectively (Petricek *et al.*, 1990; Hansson *et al.*, 1991).

The function of the *hemX* gene of the *hemAXCDBL* operon has not been elucidated. The *B. subtilis* strain 3G18Δ404R has the *hemA* and *hemX* genes deleted from the chromosome. If this strain is transformed with a plasmid containing *hemA* it grows without supplemen-

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Abbreviations: ALA, 5-aminolaevulinic acid; UroIII, uroporphyrinogen III; GSA, glutamate 1-semialdehyde; ALP, alkaline phosphatase.

tation of ALA (Schröder *et al.*, 1992). This shows that *hemX* is not essential for the synthesis of UroIII from ALA. We have not found any apparent homologue to HemX in protein or DNA sequence databases. The *hemX* gene has coding capacity for a protein of 276 amino acids (32.0 kDa) (Petricek *et al.*, 1990). Inspection of the deduced amino acid sequence reveals two features: several uncharged segments of more than 18 amino acid residues and four consecutive lysine residues in the middle part of the deduced polypeptide. The length and amino acid composition of some of the uncharged segments indicate that they could be transmembrane segments, suggesting that HemX is an integral membrane protein (see Fig. 1a).

In this work we will show that HemX is a membrane-bound protein. Furthermore, although HemX is not essential for any step in tetrapyrrole synthesis, our experiments indicate that it plays a role in controlling the steady-state concentration of HemA in the cell.

METHODS

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

Media and growth of bacteria. *E. coli* strains were grown on LA plates or in LB medium (Sambrook *et al.*, 1989); M63 minimal medium (Pardee *et al.*, 1959) was used in the experiments on turnover of HemA in *E. coli*. *B. subtilis* strains were grown on Tryptose Blood Agar Base (Difco), in Spizizen's minimal medium (Spizizen, 1958) with required amino acids added at 25 mg l⁻¹, in LB or in nutrient sporulation medium with phosphate (NSMP) (Fortnagel & Freese, 1968). To media containing haemin (2.5 mg l⁻¹), cysteine (20 mg l⁻¹) and 0.5% (w/v) bovine serum albumin fraction V (Sigma) were also added. Stock solutions of haemin were prepared as described previously (Petricek *et al.*, 1990). The following antibiotics and concentrations were used: phleomycin (*E. coli*, 10 mg l⁻¹; *B. subtilis*, 0.4 mg l⁻¹), ampicillin (50 mg l⁻¹), chloramphenicol (*E. coli*, 20 mg l⁻¹; *B. subtilis*, 5 mg l⁻¹), kanamycin (30 mg l⁻¹ or 300 mg l⁻¹ in the initial screening of *phoA* gene fusions). Competent *E. coli* and *B. subtilis* were prepared as previously described (Mandel & Higa, 1979; Arwert & Venema, 1973).

DNA techniques. Large- and small-scale preparations of plasmid DNA were done as described by Ish-Horowitz & Burke (1981) or using the MagicMinipreps DNA purification system (Promega); *B. subtilis* cells were treated with lysozyme (5 mg l⁻¹ at 37 °C for 30 min) prior to the alkaline lysis. *B. subtilis* chromosomal DNA was extracted by the procedure of Marmur (1961). General DNA techniques were as described by Sambrook *et al.*, (1989). Southern blot analysis was done with digoxigenin-labelled probe DNA using the DNA Labelling and Detection Kit from Boehringer. DNA sequence analysis was done by the dideoxy-chain-termination method (Sanger *et al.*, 1977), using modified T7 DNA polymerase (Sequenase version II; US Biochemical) and [³⁵S]dATPαS (Amersham) on double-stranded DNA.

Analysis of plasmid-encoded gene products. *E. coli* D07 is a minicell-producing strain which was used to analyse plasmid-encoded proteins. The minicells were purified from early stationary phase cultures as described by Dougan & Kehoe (1984). Labelling medium for the *E. coli* D07 minicells consisted of 5 ml Methionine Assay Medium (Difco), 15 ml M9 (Sambrook *et al.*, 1989), 0.4 ml 50% (w/v) glucose, pyridoxine (0.025 ml l⁻¹), and adenine (0.025 mg l⁻¹); 10 µCi L-[³⁵S]methionine (800 Ci mmol⁻¹, Amersham; 1 Ci = 37 GBq) was used to

label every 0.5 ml of the minicells. The labelling of proteins with [³⁵S]methionine was done according to Dougan & Kehoe (1984). After labelling for 2 h at 37 °C, 0.5 mg non-radioactive methionine was added and the cells were incubated for another 5 min. The labelled minicells were centrifuged at 5000 g for 10 min at room temperature, washed twice with 50 mM Tris/HCl pH 8.0 and resuspended in 0.25 ml 10 mM Tris/HCl pH 8.0. The cells were broken by sonication and the membranes were separated from the soluble fraction by centrifugation at 100000 g for 60 min at 4 °C.

Construction of a *B. subtilis hemX* deletion-substitution mutant. The *B. subtilis* mutant 3G18ΔHemX was constructed by transforming *B. subtilis* 3G18 with pLUUII linearized by *ScaI* cleavage. Plasmid pLUUII (Table 1) consists of pUC18 containing a *ble* gene flanked by *hemA* chromosomal DNA (bp 90–1656) and *hemXC* DNA (bp 2144–2809), basepair numbering according to Petricek *et al.* (1990). Deletion mutants were selected on plates containing phleomycin and haemin.

Generation and analysis of *PhoA* fusions. *E. coli* CC118/pLUP513 was infected with λ::Tn*phoA*-1 and selection for transfectants was made on LA-plates supplemented with antibiotics and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine (X-Phosphate) (40 mg l⁻¹) as described by Manoil & Beckwith (1985). Plasmid DNA of *PhoA*⁺ clones was analysed by cleavage with restriction enzymes and the fusion points were determined by nucleotide sequence analysis using an internal 17-mer primer complementary to *phoA*: 5'-CACGCAGAGCGG-CAGTC-3'. The stable *hemX-phoA* fusions were subcloned into pUC18 using the *Bam*HI-*Sal*GI site. The transposase and kanamycin-resistance genes of λ::Tn*phoA*-1 were thereby eliminated. Alkaline phosphatase activity was measured as described by Von Wachenfeldt & Hederstedt (1990).

Preparation of cell extracts for determination of HemA. *B. subtilis* strains were grown overnight and then used to inoculate 100 ml fresh medium in 1 litre indented flasks to an OD₆₀₀ of 0.05. The cultures were grown at 37 °C (200 r.p.m.) and harvested at different times during growth or when entering stationary phase. The cells were sedimented by centrifugation at 6000 g for 10 min at 4 °C, washed once in 50 mM Tris/HCl, pH 7.2 and resuspended in 1 ml of the same buffer containing 0.1 mg lysozyme. The cells were incubated at 37 °C for 30 min and then sonicated while chilled on ice. Whole-cell extract was the supernatant obtained after centrifugation at 3000 g for 10 min at 4 °C. *E. coli* strains were grown in 100 ml medium in 500 ml flasks or in 1 litre medium in 5 litre flasks at 37 °C (200 r.p.m.). The cells were harvested and whole-cell extracts were prepared as described above except for the lysozyme treatment.

Turnover of *B. subtilis* HemA in *E. coli*. *E. coli* MM294 carrying pHp13, pHEMA2 or pHEMA5 was grown aerobically at 37 °C in M63 minimal medium supplemented with 18 of the common amino acids (methionine and cysteine were excluded). When the cultures reached mid-exponential growth (OD₆₀₀ = 1) they were pulsed with 10 µCi ml⁻¹ L-[³⁵S]methionine (1500 Ci mmol⁻¹, Amersham) and 30 min later a chase of 5 µg ml⁻¹ unlabelled methionine was added. Samples were taken at intervals from the cultures. Preparation of cell extracts and immunoprecipitation of HemA followed the procedures described by Anderson & Blobel (1983) using polyvalent rabbit anti-HemA antibodies (raised against denatured HemA) bound to protein A-Sepharose CL-4B (Pharmacia). Immunoabsorbed proteins were eluted by incubation at 55 °C for 20 min, in the presence of SDS-PAGE preparation buffer excluding dithiothreitol. The eluted proteins were analysed by SDS-PAGE and labelled protein quantified using a Phosphorimager (Fuji). Titrations were done to assure that antibodies against HemA and protein A-Sepharose were in excess relative to HemA

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
Strains		
<i>B. subtilis</i>		
3G18	<i>ade met trpC2</i>	G. Venema, University of Groningen
3G18Δ404R	<i>ade met trpC2 Δ hemAX::cat</i>	Petricek <i>et al.</i> (1990)
3G18ΔHemX	<i>ade met trpC2 Δ hemX::ble</i>	This work
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
D07	<i>F⁻ purE his ilv pdxC met rpsL tonA minB xyl</i>	Levy <i>et al.</i> (1977)
CC118	<i>araD139 Δ(ara leu)7697 ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argE_{am} recA1</i>	Manoil & Beckwith (1985)
MM294	<i>endA1 thi pro hsdR17 supE44</i>	L.-O. Hedén, University of Lund
Plasmids		
pUC18	Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pUC19	Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pHP13	Cm ^r Em ^r	Haima <i>et al.</i> (1987)
pACYC177	Ap ^r Km ^r	Chang & Cohen (1978)
pBLE1	Ap ^r Pm ^r ; <i>ble</i> in pUC18	Fridén & Hederstedt (1990)
pLUP512	<i>hemA</i> in pUC19	Petricek <i>et al.</i> (1990)
pLUP513	<i>hemX</i> in pUC19	Petricek <i>et al.</i> (1990)
pLUP515	pLUP513 deleted of the first 51 bp of <i>hemX</i>	Petricek <i>et al.</i> (1990)
pLUP330	<i>hemA</i> , <i>hemX</i> , <i>hemC</i> in pHV32	Petricek <i>et al.</i> (1990)
pLUIII	0.66 kbp <i>Hind</i> III fragment from pLUP515 and 1.6 kbp <i>Eco</i> RI fragment from pLUP512, in pBLE1	This work
pHEMAX	3.6 kbp <i>Bam</i> HI- <i>Eco</i> RI <i>hemAX</i> fragment from pLUP330, in pHP13	This work
pHEMA2	1.4 kbp <i>Mlu</i> I- <i>Eco</i> RI <i>hemX</i> fragment deleted from pHEMAX	This work
pHEMX	654 bp <i>hemA</i> in-frame <i>Sal</i> GI fragment deleted from pHEMAX	This work
pHEMX513	1.9 kbp <i>Bam</i> HI- <i>Eco</i> RI <i>hemX</i> fragment from pLUP513 in <i>Bam</i> HI- <i>Eco</i> RI site of pHP13	This work
pAHEMA2	2.2 kbp <i>Bam</i> HI- <i>Fsp</i> I <i>hemA</i> fragment from pHEMA2 in <i>Bam</i> HI- <i>Hind</i> II site of pACYC177	This work

antigen. Extracts of MM294/pHP13 served as negative control, i.e. lacked HemA antigen.

Other methods. SDS-PAGE was done according to Neville (1971). Proteins were stained with Coomassie Brilliant Blue R250, followed by autoradiography of the gel when required. The following proteins were used as markers: β -galactosidase (130 kDa), BSA (68 kDa), catalase (58 kDa), fumarase (48 kDa), carbonic anhydrase (29 kDa) and myoglobin (17 kDa). Antiserum against *E. coli* alkaline phosphatase (Pharmacia LKB Biotechnology) was prepared from rabbits. Preparation of antisera against native and denatured HemA, and immunoblotting, have been described previously (Schröder *et al.*, 1992). Rocket immunoelectrophoresis was done as described by Rutberg *et al.* (1978) and the relative amounts of antigen were determined from the areas under the precipitate arcs (Owen, 1981). The antiserum directed against native HemA was

absorbed with cell extract from an *E. coli* HemA mutant strain (AN344) (MacGregor, 1976) before being used to determine the amount of *B. subtilis* HemA expressed in *E. coli* JM109. Protein was determined by the Lowry method.

RESULTS

HemX is membrane bound

The calculated molecular mass of HemX is 32.0 kDa. Hydropathy plots of the predicted HemX amino acid sequence indicate several hydrophobic stretches, with up to seven possible membrane-spanning segments (Fig. 1a). A protein of 26 kDa encoded by *hemX* has been detected using an *E. coli* *in vitro* transcription-translation system (Petricek *et al.*, 1990). To determine the size of HemX

Fig. 1. Hydropathy plots of the *B. subtilis* *hemX* gene product (a), products of *hemX* and *hemX-phoA* gene fusions (b), and the *hemX-phoA* out-of-frame fusion point in pPHOA128 (c). (a) The GCG sequence analysis software package, version 7 (Devereux *et al.*, 1984) was used to generate the hydropathy plots; a window of 9 was used. The continuous line represents the Kyte/Doolittle hydrophobicity plot and the dashed line the Goldman/Engelman/Steitz plot. (b) Schematic drawings of the deduced primary structures of wild-type HemX and two HemX-PhoA fusion proteins. The numbers refer to residues in HemX. The shaded parts represent PhoA sequences. (c) Close-up of the fusion point in the PhoA128 hybrid protein and the corresponding nucleotide sequence (the non-coding strand is shown).

synthesized *in vivo* and to analyse the cell localization of the *hemX* gene product, we made use of an *E. coli* minicell system. *E. coli* D07 was transformed with pLUP513 (pUC19 containing *hemX* under the *lac* promoter) (see Fig. 2c). As controls, *E. coli* D07 was also transformed with pLUP512 (pUC19 containing the *hemA* gene) and pUC19. Minicells from these transformants predominantly contain plasmid DNA and plasmid-encoded proteins can be identified after labelling with [35 S]methionine. We have previously shown that *hemA* encodes a soluble protein of 50 kDa (Schröder *et al.*, 1992). Analysis by SDS-PAGE revealed that a 26 kDa polypeptide is expressed from pLUP513 in minicells. This polypeptide is not expressed from pUC19 or pLUP512 (Fig. 3). We conclude that this polypeptide is the *hemX* gene product. Fractionation of labelled minicells showed that the 26 kDa HemX polypeptide copurifies with the particulate (membrane) fraction, whereas the 50 kDa *hemA* gene product is found in the soluble fraction as expected (Fig. 3).

HemX-PhoA fusion protein analysis

To confirm that HemX is an integral membrane protein and to analyse its topology we used the *phoA* fusion system developed by Manoil & Beckwith (1985). This

Fig. 2. Genetic map of the proximal part of the *B. subtilis* *hemAXCDBL* operon (a), deletion-substitutions of *hemX* in the chromosome of two different *B. subtilis* mutants (b), and DNA fragments present in different plasmids (c). (a) P indicates the location of the *hemAXCDBL* promoter; the restriction enzyme sites indicated are the ones which were used in the construction of plasmids. (b) *cat* is a chloramphenicol-resistance gene and *ble* is a bleomycin-resistance gene which replaces the deleted region. (c) DNA fragments present in different plasmid constructs; the plasmid vector used is given underneath the name of each plasmid.

system is based on the fact that *E. coli* alkaline phosphatase (ALP), encoded by the *phoA* gene, is a periplasmic enzyme which is active only after translocation from the cytoplasm to the periplasm. Translocation of ALP across the cytoplasmic membrane requires an N-terminal signal sequence, which can be either a cleaved leader sequence or a transmembrane segment of a membrane protein where the C-terminal of the segment is oriented towards the periplasmic side.

Fusions between *B. subtilis* *hemX* and *phoA* were generated *in vivo* using phage λ :Tn $phoA$ and plasmid pLUP513 in *E. coli* CC118 ($\Delta phoA$). This phage contains Tn5 with a modified *phoA* gene lacking its native signal sequence. Colonies with ALP activity were detected using plates containing the chromogenic substrate X-Phosphate. Six clones with *hemX-phoA* fusions were identified among about 50 ALP-positive clones analysed. Sequencing across the fusion points in plasmids from these six clones revealed that two clones had an in-frame fusion point at a site corresponding to amino acid number two of HemX (PhoA2, see Fig. 1b) and four clones had an out of frame fusion point at a site corresponding to amino acid 128 in HemX (PhoA128, see Fig. 1b, c).

The above two types of *hemX-phoA* fusions were subcloned by transferring the *Bam*HI-XbaI fragment from the original plasmids to the *Bam*HI-SalGI site in pUC18. The resulting plasmids (pPHOA2 and pPHOA128) lack the major part of Tn5 encoding

Fig. 3. Analysis of *hemX* and *hemA* expression in *E. coli* minicells. Proteins synthesized in minicells were labelled with [35 S]methionine, then fractionated by SDS-PAGE (12%, w/v) and detected by autoradiography (see Methods). Lanes 1, 5 and 9, *E. coli* D07/pUC19 (negative control); lanes 2, 6, and 10, *E. coli* D07/pLUP512 (*hemA*); lanes 3, 7 and 11, *E. coli* D07/pLUP513 (*hemX*). Total lysates were loaded in lanes 1–3, membrane fractions in lanes 5–7, and soluble fractions in lanes 9–11. Lanes 4 and 8 contain non-radioactive size-marker proteins.

kanamycin resistance and transposase functions. The gene fusions in the subclones were confirmed by sequence analysis.

Cells of *E. coli* CC118 containing pPHOA2 or pPHOA128 were analysed for PhoA protein and ALP activity. Cells containing pPHOA2 showed a 30-fold higher ALP activity than those containing pPHOA128. However, the

Fig. 4. Immunoblot analysis of HemX–PhoA fusion proteins. Whole cell lysate, periplasm and membrane fractions were prepared as described in Methods and analysed by SDS-PAGE (10%, w/v) followed by Western blotting using antiserum directed against *E. coli* ALP. Lanes 1 and 3, 10 μ g protein of whole-cell lysates from *E. coli* CC118 containing pUC18 and pPHOA128, respectively; lane 2, 2 μ g protein of whole-cell lysate from *E. coli* CC118 containing pPHOA2; lanes 4, 5 and 6, 1 μ g protein of periplasmic fraction from *E. coli* CC118 containing pUC18, pPHOA2 and pPHOA128, respectively; lanes 7, 8 and 9, 2.5 μ g protein of membrane fraction from *E. coli* CC118 containing pUC18, pPHOA2, and pPHOA128, respectively.

ALP activity of CC118/pPHOA128 was significantly above the background (CC118/pUC18) (Table 2). The ALP activity and PhoA hybrid protein of CC118/pPHOA2 was found predominantly in the periplasm (Table 2, Fig. 4). The mass of this hybrid protein was 51 kDa as determined by Western blot analysis using anti-ALP serum. This mass is close to that of purified *E. coli* ALP (50 kDa), confirming that the hybrid protein only contains a few amino acids of HemX. In contrast, the ALP activity and PhoA hybrid protein of CC118/pPHOA128 was localized in the membrane fraction (Table 2, Fig. 4). The mass of the HemX–PhoA protein expressed from pPHOA128 was approximately 60 kDa, which demonstrates that the out-of-frame fusion produces a full-length hybrid protein of a mass expected from an in-

Table 2. ALP activity of *E. coli* CC118 containing *hemX*–*phoA* gene fusions

ALP activities were measured as described in Methods and are given in μ mol (mg protein) $^{-1}$ min $^{-1}$. The mean values shown were derived from three independent experiments and are given with standard deviations.

	ALP activity		
	CC118/pPHOA2	CC118/pPHOA128	CC118/pUC18
Permeabilized cells	98 $\pm$ 17	4 $\pm$ 2	< 0.1
Periplasm	87 $\pm$ 8	3 $\pm$ 3	< 2
Membrane	3 $\pm$ 4	35 $\pm$ 14	< 1

Table 3. Steady-state relative concentrations of HemA in *B. subtilis* strains containing different plasmids

The different bacteria were grown in LB to early stationary phase (OD₆₆₀ about 2) and whole-cell extracts were prepared as described in Methods. The relative amount of HemA antigen per mg total cell protein was determined by rocket immunoelectrophoresis using antiserum directed against native HemA. The experiment was repeated three times; mean values with standard deviations are given. ND, Not determined.

Plasmid	3G18 (wild-type)	3G18ΔHemX (ΔhemX)	3G18Δ404R (ΔhemAX)
pHP13	< 0.005	0.2 ± 0.1	ND
pHEMA2	1.0 ± 0.4	1.7 ± 0.8	2.5 ± 1.1
pHEMAX	0.8 ± 0.5	1.1 ± 0.1	0.2 ± 0.1
pHEMX	< 0.005	< 0.005	< 0.005

frame fusion at a position corresponding to about residue 128 of HemX. PhoA antigen of about 50 kDa was found in extracts of CC118/pPHOA128. This 50 kDa protein probably corresponds to PhoA protein proteolytically cleaved from the PhoA128 protein.

To confirm that PhoA128 is an integral membrane protein, the following experiment was done. The membrane fraction was isolated from CC118/pPHOA128. After centrifugation at 100000 *g* for 60 min, more than 90% of both ALP activity and PhoA128 protein was found in the pellet. When the membranes were treated with 2% (v/v) Triton X-100 before centrifugation, more than 95% of ALP activity and PhoA128 protein was found in the supernatant fraction.

Deletion of the *hemX* gene

Part of the *B. subtilis* chromosomal *hemX* gene corresponding to amino acids 17 to 180 of HemX was deleted in strain 3G18 and replaced with a *ble* gene, generating strain 3G18ΔHemX (Fig. 2b). The deletion-substitution, confirmed by Southern blot analysis (data not shown), was designed such that *hemCDBL* would also in the mutant be transcribed from the promoter in front of *hemA*. Strain 3G18ΔHemX does not have a growth requirement for ALA or haemin and it has the same growth rate as wild-type cells in broth and minimal medium (data not shown).

hemX affects the steady-state concentration of HemA

The steady-state concentration of HemA in wild-type *B. subtilis* seems very low (Schröder *et al.*, 1992). Surprisingly, a more than 15-fold higher concentration of HemA was found in strain 3G18ΔHemX compared to strain 3G18 (Table 3). This difference in HemA cellular concentration was found in all media tested: NSMP, minimal medium containing 0.5% glucose and 0.05% Casamino

acids, and minimal medium with 0.5% glutamate and 0.05% Casamino acids.

To study the effect of HemX on the cellular concentration of HemA protein, plasmids pHP13, pHEMX (contains *hemX*), pHEMA2 (contains *hemA*) and pHEMAX (contains both *hemA* and *hemX*) were introduced into *B. subtilis* strains 3G18 and 3G18ΔHemX (Fig. 2). HemA protein was quantified in total cell extracts from these strains by rocket immunoelectrophoresis; the results obtained (Table 3) can be summarized as follows. No HemA protein was detected in 3G18ΔHemX/pHEMX, supporting the idea that HemX is involved in regulating HemA protein levels. Increasing the copy number of *hemA* by placing it on a plasmid leads to increased levels of HemA protein also in the presence of the chromosomal copy of the *hemX* gene. A significant effect of HemX on the HemA protein concentration was not seen in strains 3G18 and 3G18ΔHemX when both *hemA* and *hemX* were present on the plasmid.

These experiments were extended by introducing pHEMX, pHEMA2 and pHEMAX into the *B. subtilis* strain 3G18Δ404R, which is deleted for both *hemA* and *hemX* (Fig. 2b). This strain has a non-defined promoter allowing expression of *hemCDBL* and requires ALA for growth (Petricek *et al.*, 1990). Strain 3G18Δ404R carrying either pHEMA2 or pHEMAX does not require ALA for growth. The steady-state amount of HemA protein was 10-fold higher in 3G18Δ404R/pHEMA2 compared to 3G18Δ404R/pHEMAX (Table 3).

A growth-phase-related quantification of HemA protein was next done for the following *B. subtilis* strains: 3G18ΔHemX containing pHP13 or pHEMX (Fig. 5a), pHEMA2 or pHEMAX (Fig. 5b); 3G18 containing pHEMA2 or pHEMAX (Fig. 5c); and 3G18Δ404R containing pHEMA2 or pHEMAX (Fig. 5d). The results confirmed the data presented in Table 3 and indicated that the steady-state concentration of HemA protein in these strains is not dependent on the growth phase.

Effect of HemX on *B. subtilis* HemA in *E. coli*

B. subtilis HemA protein can be expressed at high levels from the *hemA* gene with its native promoter in *E. coli* (Schröder *et al.*, 1992). It was of interest to analyse if the effect of HemX on HemA protein can be observed also in a heterologous system like *E. coli*.

E. coli JM109 carrying pHEMA2 or pHEMAX was grown overnight in LB supplemented with chloramphenicol and the concentrations of HemA protein in whole-cell extracts were quantified as before. The steady-state concentration of *B. subtilis* HemA was significantly lower if the *B. subtilis* *hemX* gene was present (Table 4). It is important to note that *E. coli* HemA is not detectable with antisera against *B. subtilis* HemA (Schröder *et al.*, 1992). Next, extracts were prepared from samples taken at different times of growth. The steady-state concentration of HemA in JM109/pHEMA2 remained essentially constant, whereas that in JM109/pHEMAX decreased further after the cells had entered stationary phase (Fig. 6).

Fig. 5. Steady-state relative concentrations of HemaA in different *B. subtilis* strains carrying the *hemA* gene or both *hemA* and *hemX* on a plasmid. The *B. subtilis* strains were grown in LB supplemented with chloramphenicol; samples were taken at different times in the growth curve (OD_{660}) and whole-cell extracts were prepared as described in Methods. The amount of HemaA antigen per mg protein was analysed by rocket immunoelectrophoresis using antiserum directed against native HemaA and the relative amounts are presented in the bar plot. (a) *B. subtilis* 3G18 Δ HemX carrying pHP13 (open bars) or pHEMX (no HemaA protein was detectable in this strain; see Table 3); (b) *B. subtilis* 3G18 Δ HemX carrying pHEMA2 (open bars) or pHEMAX (hatched bars); (c) *B. subtilis* 3G18 carrying pHEMA2 (open bars) or pHEMAX (hatched bars); (d) *B. subtilis* 3G18 Δ 404R carrying pHEMA2 (open bars) or pHEMAX (hatched bars).

Table 4. Relative concentrations of *B. subtilis* HemaA in *E. coli* JM109 containing the *B. subtilis* *hemA* and *hemX* genes on plasmids

The *E. coli* strains were grown overnight in LB and cell extracts were prepared as described in Methods. The relative amount of HemaA per mg total cell protein compared to that of JM109/pHEMA2 was determined by rocket immunoelectrophoresis using antiserum directed towards native HemaA; the results are given as mean values, with standard deviations, of *n* experiments.

Plasmid(s) in <i>E. coli</i> JM109	Amount of HemaA	<i>hemX</i> /HemX
pHEMA2	1	Not present
pHEMAX	0.17 ± 0.02 (<i>n</i> = 3)	Present/intact
pHP13	< 0.005 (<i>n</i> = 3)	Not present
pAHEMA2	0.15 ± 0.05 (<i>n</i> = 3)	Not present
pAHEMA2+pLUP513	< 0.005 (<i>n</i> = 3)	Present/intact
pAHEMA2+pHEMX513	< 0.005 (<i>n</i> = 1)	Present/intact
pAHEMA2+pLUP515	0.18 ± 0.06 (<i>n</i> = 2)	Present/lacks N-terminal 17 residues
pAHEMA2+pPHOA2	0.17 ± 0.06 (<i>n</i> = 2)	Present/HemX (residue no. 2)–PhoA fusion protein
pAHEMA2+pPHOA128	0.16 ± 0.06 (<i>n</i> = 2)	Present/HemX (residue no. 128)–PhoA fusion protein
pAHEMA2+pUC19	0.16 ± 0.06 (<i>n</i> = 3)	Not present
pAHEMA2+pHP13	0.18 (<i>n</i> = 1)	Not present

Fig. 6. *B. subtilis* Hema content of *E. coli* JM109 carrying pHEMA2 (*hemA*) or pHEMAX (*hemAX*). The *E. coli* strains were grown in LB supplemented with chloramphenicol, and samples were taken at different times in the growth curve. Cell extracts were prepared as described in Methods and analysed for relative concentrations of HemaA antigen per mg protein by rocket immunoelectrophoresis using antiserum directed against native HemaA preabsorbed with an *E. coli* (*hemA*) cell lysate (see Methods). The two *E. coli* strains showed practically identical growth curves (OD_{660}). The relative amounts of HemaA are shown in the bar plot: open bars, JM109/pHEMA2; hatched bars, JM109/pHEMAX.

Fig. 7. Analysis of the stability of HemaA protein depending on the presence of HemX. *E. coli* MM294 carrying pHEMA2 (filled symbols) or pHEMAX (open symbols) was grown in M63 minimal medium supplemented with chloramphenicol and 18 of the common amino acids (excluding methionine and cysteine). Growth was followed by the OD_{660} (circles). The cultures were pulse-labelled for 30 min with [35 S]methionine in the mid-exponential growth phase (indicated by arrows). Samples were taken at intervals from the cultures and radioactive 50 kDa HemaA (triangles) was quantified after immunoprecipitation as described in Methods. The amounts of radioactive HemaA antigen in different samples are presented relative to that of the first (5.0 h) MM294/pHEMA2 sample.

In the above experiments *hemA* and *hemX* were present on the same high-copy-number vector. The *hemA* gene of plasmid pHEMA2 was cloned into the low-copy-number vector pACYC177, resulting in pAHEMA2 (Fig. 2c). This plasmid is compatible with pUC derivatives. In *E. coli* JM109/pAHEMA2, HemaA protein was found at a concentration corresponding to about 20% of that in *E. coli* JM109/pHEMA2, consistent with the copy number difference (Table 4). To study the effect of HemX on *B. subtilis* HemaA expressed in *E. coli* JM109/pAHEMA2, we transformed this strain with plasmid pLUP513 (a derivative of pUC19 with *hemX* under control of the *lac* promoter). The resulting strain was analysed for HemaA antigen. As seen in Table 4, the presence of pLUP513 caused a strong decrease of the HemaA concentration. The *hemX* gene from pLUP513 was transferred to pHP13, resulting in pHEMX513, which was transformed into *E. coli* JM109/pAHEMA2. The effect of HemX on HemaA was observed in this strain also (Table 4). To analyse if the complete *B. subtilis* HemX protein is needed to obtain the effect on HemaA concentrations, *E. coli* JM109/pAHEMA2 was transformed with pLUP515 (the first 17 amino acids of HemX deleted), pPHOA2 (encodes the HemX-PhoA fusion protein with PhoA fused to the second amino acid of HemX), and pPHOA128 (encodes the HemX-PhoA fusion protein containing 128 amino acids of HemX) (Figs 1b and 2c). None of these truncated versions of *hemX* affected the cellular concentration of HemaA (Table 4).

A σ^A -type promoter positioned 170 nucleotides upstream of *hemA* has been identified as the only (or major)

promoter for the *hemA* operon (M. Petricek, unpublished data). We have constructed two *hemA-lacZ* transcriptional and translational fusions containing the first 34 and 300 codons, respectively, of *hemA*. The amount of β -galactosidase produced from these fusions in *E. coli* measured both as enzyme activity and as antigen was not influenced by the presence or absence of HemX, indicating that HemX does not directly affect the activity of the *hemA* promoter or *hemA* translation initiation (data not shown). In order to test if HemX affects the stability of the HemaA protein the following experiment was done. Exponentially growing *E. coli* MM294 harbouring pHEMA2 or pHEMAX was pulse-labelled with [35 S]methionine and samples were taken at intervals. HemaA was immunoprecipitated with an excess of specific antibodies and radioactive HemaA was quantified as described in Methods. The results of this experiment revealed no difference in the stability of the HemaA protein in the two strains (Fig. 7). Control experiments on the steady-state concentration of HemaA antigen in MM294/pHEMA2 and MM294/pHEMAX grown in supplemented M63 minimal medium gave results very similar to those shown in Fig. 6.

DISCUSSION

The *B. subtilis* *hemX* gene is located in the *hemAXCDBL* operon, which encodes enzymes required for the synthesis of uroporphyrinogen III from glutamyl-tRNA (Petricek *et al.*, 1990; Hansson *et al.*, 1991). However, *hemX* is not

directly involved in ALA or tetrapyrrole synthesis since deletion of the gene does not lead to a growth requirement for ALA or haemin or otherwise impair haem synthesis. Furthermore HemX mutants do not accumulate or excrete tetrapyrroles (unpublished results), as has been observed, for example, for *E. coli* mutants blocked in different steps of haem biosynthesis (Cox & Charles, 1973).

We have not found any obvious similarity between the predicted amino acid sequence of the HemX protein and sequences available in databases. Some weak similarity has been observed between HemX and predicted chloroplast-encoded proteins of unknown function, e.g. the protein encoded by ORF313 of tobacco chloroplasts (Shinozaki *et al.*, 1986). The significance of this is uncertain. Hydropathy plots of the HemX sequence show it to be a hydrophobic protein with the potential to form up to seven membrane-spanning segments. Another notable feature of HemX is a lysine-rich region KKKKWGK (residues 164–170) which may be functionally relevant.

hemX has the potential to code for a protein of 32 kDa. However, SDS-PAGE of HemX produced in an *E. coli* *in vitro* transcription-translation system (Petricek *et al.*, 1990) or in *E. coli* minicells expressing *hemX* has yielded a protein of only 26 kDa. This difference probably reflects the hydrophobic character of HemX; hydrophobic proteins often show aberrant mobility in SDS-PAGE. Fractionation of *E. coli* minicells producing HemX reveals that the protein is located in the membrane fraction, supporting the notion that it is an intrinsic membrane protein.

We have used *hemX-phoA* fusions (Manoil & Beckwith, 1985) to gain some information about the topology of HemX and we have so far obtained two types of active fusions. One type had *phoA* fused in-frame to a position corresponding to the second amino acid of HemX. This fusion produces high amounts of hybrid protein in *E. coli* as shown by ALP activity measurements and the presence of a 51 kDa protein which reacts with anti-ALP antibodies. Most of this hybrid protein was found in the periplasmic fraction and therefore it does not provide any information on HemX topology. It is however surprising that this protein is secreted since the deduced N-terminal sequence of the fusion protein, MIADSYTQVASWTE-PFPFCP (residues from HemX are underlined) does not have the features of a typical bacterial signal sequence (Von Heijne & Abrahamsen, 1989). The other type of fusion was an out-of-frame fusion at a site corresponding to residue 128 of HemX. It produces a fusion protein of the expected size for an in-frame fusion, i.e. an ALP immunoreactive protein of 60 kDa. This protein copurifies with the membrane fraction and can be solubilized with detergent, indicating that it is a membrane protein. Similar +1 out-of-frame fusions producing active ALP have been described for *E. coli glpT-phoA* (Gött & Boos, 1988). Since this *hemX-phoA* fusion produces a full-length (60 kDa) hybrid protein there must be readthrough (translational slipping) in the region corresponding to amino acids 95–128 of HemX. A +1 frame shift before residue 95 results in a stop codon at this position.

The results of the *hemX-phoA* fusion experiments support a membrane localization of HemX. Since we obtained only two types of fusion variants, detailed predictions of HemX membrane topology cannot be made. However, the PhoA part of the fusion protein produced from the out-of-frame fusion is probably located on the outside of the membrane. This indicates that the predicted hydrophilic segment around residue 120 is exposed on the outer side of the cytoplasmic membrane. This would place the lysine-rich region around position 165 on the inside, in compliance with the 'positive-inside rule' (Von Heijne, 1992). Thus the hydrophobic segment containing residues 130–160 probably spans the membrane.

HemX does not seem to be directly involved in tetrapyrrole synthesis, but the present work has established that it negatively affects the steady-state concentration of *B. subtilis* HemA in both *B. subtilis* and *E. coli*. This in turn suggests that HemX may play a role in controlling tetrapyrrole synthesis in *B. subtilis*. HemX may act to convey information about the environment to the cell interior, but we have found no similarity between the predicted amino acid sequence of HemX and the sequences or conserved motifs of known membrane-spanning bacterial signal transduction proteins (Stock *et al.*, 1990). The mechanism by which HemX affects the steady-state concentration of HemA is not understood. The rate of turnover of HemA does not seem to be affected by HemX, indicating that HemX influences the rate of synthesis of HemA. However, results with *hemA-lacZ* fusions have not revealed an effect of HemX on the (major) *hemA* promoter or on translation.

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