JOURNAL OF BACTERIOLOGY, Oct. 1999, p. 5922–5929 Vol. 181, No. 19
0021-9193/99/$04.00+0

Transcriptional Control of *Bacillus subtilis hemN* and *hemZ*

GEORG HOMUTH,[1] ALEXANDRA ROMPF,[2] WOLFGANG SCHUMANN,[1] AND DIETER JAHN[2]*

Institut für Genetik, Universität Bayreuth, 95440 Bayreuth,[1] and Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, 79104 Freiburg,[2] Germany

Received 7 May 1999/Accepted 23 July 1999

Previous characterization of *Bacillus subtilis hemN*, encoding a protein involved in oxygen-independent coproporphyrinogen III decarboxylation, indicated the presence of a second *hemN*-like gene (B. Hippler, G. Homuth, T. Hoffmann, C. Hungerer, W. Schumann, and D. Jahn, J. Bacteriol. 179:7181–7185, 1997). The corresponding *hemZ* gene was found to be split into the two potential open reading frames *yhaV* and *yhaW* by a sequencing error of the genome sequencing project. The *hemZ* gene, encoding a 501-amino-acid protein with a calculated molecular mass of 57,533 Da, complemented a *Salmonella typhimurium hemF hemN* double mutant under aerobic and anaerobic growth conditions. A *B. subtilis hemZ* mutant accumulated coproporphyrinogen III under anaerobic growth conditions. A *hemN hemZ* double mutant exhibited normal aerobic and anaerobic growth, indicating the presence of a third alternative oxygen-independent enzymatic system for coproporphyrinogen III oxidation. The *hemY* gene, encoding oxygen-dependent protoporphyrinogen IX oxidase with coproporphyrinogen III oxidase side activity, did not significantly contribute to this newly identified system. Growth behavior of *hemY* mutants revealed the presence of an oxygen-independent protoporphyrinogen IX oxidase in *B. subtilis*. A monocistronic *hemZ* mRNA, starting 31 bp upstream of the translational start codon, was detected. Reporter gene fusions of *hemZ* and *hemN* demonstrated a fivefold anaerobic induction of both genes under nitrate ammonifying growth conditions. No anaerobic induction was observed for fermentatively growing *B. subtilis*. The *B. subtilis* redox regulatory systems encoded by *resDE*, *fnr*, and *ywiD* were indispensable for the observed transcriptional induction. A redox regulation cascade proceeding from an unknown sensor via *resDE*, through *fnr* and *ywiD* to *hemN*/*hemZ*, is suggested for the observed coregulation of heme biosynthesis and the anaerobic respiratory energy metabolism. Finally, only *hemZ* was found to be fivefold induced by the presence of H_2O_2, indicating further coregulation of heme biosynthesis with the formation of the tetrapyrrole enzyme catalase.

During bacterial heme biosynthesis, coproporphyrinogen III is oxidatively decarboxylated to protoporphyrinogen IX (3, 22, 24). In bacteria, two structurally unrelated enzymatic systems catalyzing this reaction were identified (37, 40, 41, 47, 48). One type of coproporphyrinogen III oxidase (HemF) requires molecular oxygen (42, 43, 47). Since it obviously cannot function in the absence of molecular oxygen, a structurally different oxygen-independent enzyme (designated HemN and HemZ) is required for anaerobic growth and heme biosynthesis (42, 43, 47, 48). Up to now, genes coding for oxygen-independent enzymes (*hemN* and *hemZ*) were found only in bacteria (13, 34). Expression of the *Rhodobacter sphaeroides hemN* gene in *Escherichia coli* led to an increase in coproporphyrinogen III oxidase activity which was dependent on the presence of methionine, ATP, $NADP^+$, NADH, and Mg^{2+} (4).

In *Bacillus subtilis*, the anaerobic energy metabolism via nitrate ammonification requires oxygen-independent heme biosynthesis (14, 15, 26, 30, 31). The anaerobic enzymatic systems are induced via a regulatory cascade. Low oxygen tension is sensed by an unknown system, and the signal generated is transferred to the pleiotropic two-component regulatory system ResDE. ResDE is responsible, directly or indirectly, for induction of the gene encoding the redox regulator Fnr (32). Fnr in turn induces genes of nitrate respiration and the regulatory gene *ywiD* (5, 27). Subsequently, *ywiD* activates anaerobic respiratory and fermentative loci (27). Oxygen tension-dependent coregulation of energy metabolism and heme biosynthesis has been described for various bacteria (6, 18–21, 33). The genes encoding coproporphyrinogen III oxidases were found to be key redox regulatory targets in *Pseudomonas aeruginosa* (34). Fnr binding sites were located in the promoter sequences of coproporphyrinogen III oxidase genes from *Alcaligenes eutrophus*, *P. aeruginosa*, *Pseudomonas stutzeri*, and *R. sphaeroides* (25, 34, 49).

A *hemF* gene encoding the oxygen-dependent enzyme has not been found in the genome sequence of *B. subtilis* (13, 23). Recently, we described the identification and functional characterization of the *B. subtilis hemN* gene located upstream of the *B. subtilis dnaK* operon (13, 16). It is not part of the two *hem* operons in *B. subtilis*, *hemAXCDBL* at 244° and *hemEHY* operon at 94° of the genomic map, encoding almost all enzymes required for the formation of protoheme from glutamyl-tRNA (9, 11). However, mutation of *B. subtilis hemN* had no obvious consequences for aerobic and anaerobic growth, suggesting the presence of a second *hemN* type gene (13). Here, we describe the identification and functional characterization of the second *hemN*-type gene, *hemZ*. Both, *hemN* and *hemZ* are subject to redox regulation mediated by *resDE*, *fnr*, and *ywiD*. Peroxide stress regulation is limited to *hemZ*.

MATERIALS AND METHODS

Bacterial strains, plasmids and DNA manipulations. Bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* and *E. coli* strains were grown aerobically and anaerobically in complex and minimal media with additions as detailed before (14, 30, 36). Cloning procedures were carried out by standard protocols (36). PCR products were generated with Deep Vent DNA polymerase (New England Biolabs, Schwalbach, Germany). PCR primers were obtained from ARK Scientific GmbH Biosystems (Darmstadt, Germany). For

* Corresponding author. Mailing address: Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, Albertstr. 21, 79104 Freiburg, Germany. Phone: 49(0)761-2036060. Fax: 49(0)761-2036096. E-mail: jahndiet@ruf.uni-freiburg.de.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
B. subtilis		
1A594	*trpC2 hemG321*	BGSC[a]
168	Wild type	BGSC
3G18Δ2	*trpC2 met ade* Δ*hemY*::*ble*	9
1012	*leuA8 metB5 trpC2 hsrM1*	35
AM01	1012 with *amyE*::*cat*	28
AM7	Promoterless *bgaB* inserted at *amyE*	28
AM10	*lepA*::*bgaB* transcriptional fusion at *amyE*	28
ARB1	HZ04 with *resDE* mutant from MH5081	This work
ARB2	HZ04 with *fnr* mutant from THB2	This work
ARB3	HZ04 with *ywiD* mutant from THB99	This work
ARB4	AM10 with *resDE* mutant from MH5081	This work
ARB5	AM10 with *fnr* mutant from THB2	This work
ARB6	AM10 with *ywiD* mutant from THB99	This work
ARB7	AM7 with *resDE* mutant from MH5081	This work
ARB8	AM7 with *fnr* mutant from THB2	This work
ARB9	AM7 with *ywiD* mutant from THB99	This work
ARB10	HZ03 with *hemY* mutation from ARB11, *hemN hemZ hemY* triple mutant	This work
ARB11	1012 carry *hemY* mutant introduced by using pMUTIN-hemY	This work
HZ01	1012 with 501-bp in-frame deletion in *hemN*, previously described as *hemN*Δ	17
HZ02	1012 with replacement of a part of *hemZ* by a *cat* cassette	This work
HZ03	HZ01 with part of *hemZ* replaced by a *cat* cassette, *hemN hemZ* double mutant	This work
HZ04	1012 with *hemZ*::*bgaB* transcriptional fusion inserted at *amyE*	This work
JH642	*pheA1 trpC2*	BGSC
MH5081	*trpC2 pheA1* Δ*resDE*::*tet*	39
THB2	*trpC2 pheA1 fnr*::*spec*	14
THB99	*trpC2 pheA1 ywiD*::*spec*	27
E. coli		
DH10B	Host in cloning experiments	Gibco BRL
DH5α	*hsdR recA1 lacZYA f80 lacZDM15 gyrA96*	36
S. typhimurium TE3006	*env-53 hemN704*::Mud-J(b) *hemF707*::Tn*10d*-Tet	47
Plasmids		
pALF01	pBluescript SK$^+$II with 783-bp PCR product carrying the complete former *yhaW* and the 5′ end of former *yhaV*	This work
pBgaB	Promoter-test vector containing the *bgaB* gene	28
pBluehemZ	pBluescript SK$^+$II with 1,706-bp PCR product carrying *hemZ* and its promoter	This work
pBlueSalISmaIΔ	pBluescript SK$^+$II with deletion of the polylinker region between and including *Sal*I and *Sma*I	17
pBluescript SK$^+$II	Cloning vector	Stratagene
pBluescript KS$^+$II	Cloning vector	Stratagene
pGH02	1,256-bp *Cla*I fragment in pBluescript KS$^+$ containing *lepA* and its 5′ region	17
phemNΔ	PCR product of the mutated *hemN* gene from *B. subtilis hemN*Δ in pBluescript KS$^+$II	This work
phemZ-PEX	pBluescript SK$^+$ carrying 677-bp PCR product of former *yhaW* and its 5′ region	This work
phemZ1RPT3	pBluescript SK$^+$II carrying 500-bp PCR product of former *yhaW*	This work
phemZ2RPT3	pBluescript SK$^+$II carrying 877-bp PCR product of former *yhaV*	This work
phemZΔ01	Fusion of a 149-bp PCR product carrying the 5′ region of former *yhaV* with a 153-bp fragment of the 3′ end of *hemZ* in pBluescript SK$^+$II	This work
phemZΔ02	316-bp *Bam*HI fragment from phemZΔ01 in pBlueSalISmaIΔ01	This work
phemZ::cat	*cat* cassette integrated in *Eco*RV site of the phemZΔ02 insert	This work
pBluehemN	*B. subtilis hemN* in pBluescript SK$^+$II	13
pBluehemZ::cat	PCR product of the tagged *hemZ* gene from *B. subtilis hemZ*::*cat* in pBluescript KS$^+$II	This work
pHZ01	175-bp *hemZ* promoter fused to *bgaB* in pBgaB	This work
pMUTIN-hemY	pMUTIN4 with 324-bp fragment of *hemY*	This work
pMUTIN4	Integration vector	44
pOKBShemN	1,302-bp fragment containing the *B. subtilis hemN* gene in pOK12	13
pSKCAT	*cat* cassette cloned into *Eco*RV site of pBluescript SK$^+$II	This work

[a] BGSC, Bacillus Genetic Stock Center.

DNA sequence determination of the *hemZ* locus from *B. subtilis* 1012, four different plasmids were constructed by PCR. The PCR products generated with primers containing artificial *Bam*HI restriction sites were inserted into the *Bam*HI site of pBluescript SK$^+$II. Cloning of a DNA fragment corresponding to the former *yhaW* gene (nucleotides 180 to 681 in Fig. 1), generated by PCR using primers YHAW5 (5′-GGCCATGGATCCTTGCAAATTAAAATAGAAGGCATA-3′; *Bam*HI restriction site underlined) and YHAW3 (5′-GGCCATGGATCCTTACTCGGTACAAATCCGGCACTG-3′), resulted in plasmid phemZ1RPT3. The former open reading frame (ORF) *yhaV* carrying plasmid (nucleotides 813 to 1685 in Fig. 1), generated via PCR using primers YHAV5 (5′-GGCCATGGATCCAT

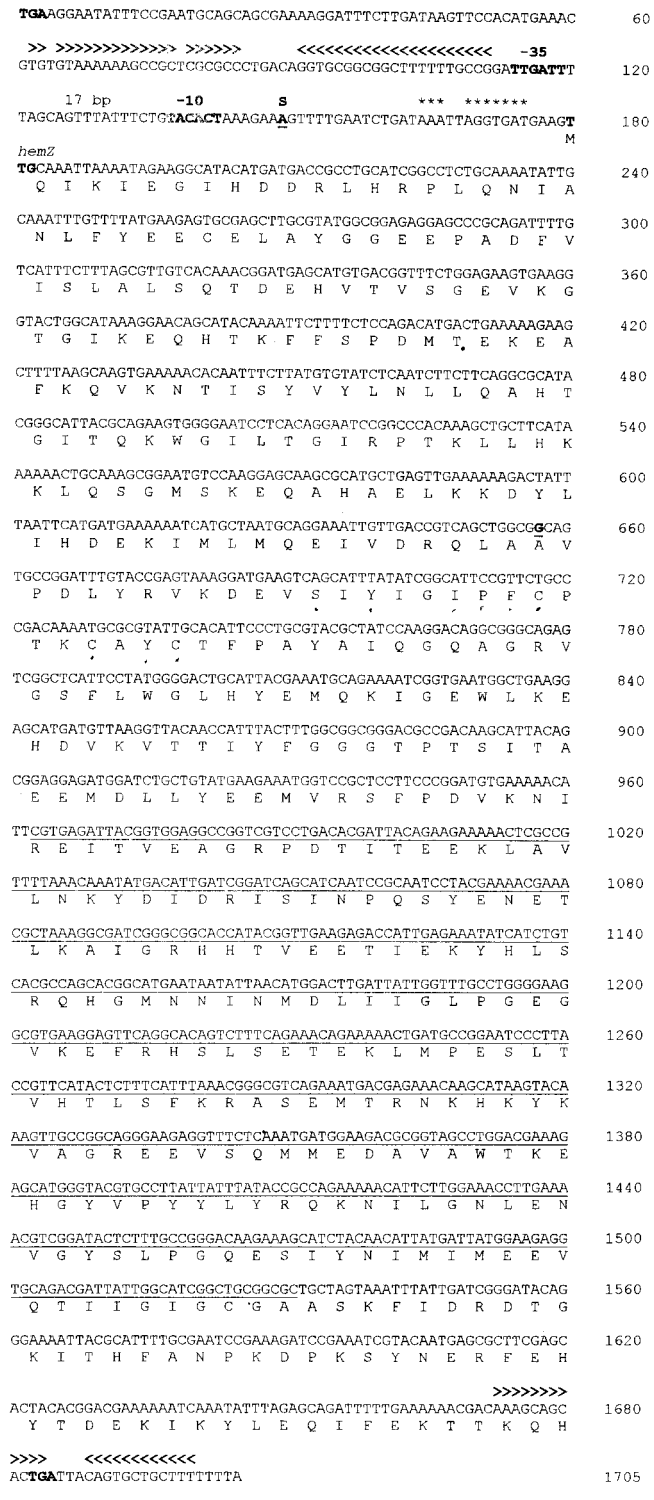

FIG. 1. Nucleotide sequence of *hemZ* and deduced protein sequence. The putative translational start and stop codon of *hemZ* and the stop codon of the upstream-located *yhaX* are shown in boldface. The stem-loop structures upstream of *hemZ* and at its 3′ end are indicated by arrowheads above the sequence. The potential *hemZ* promoter is shown in boldface, and the −35 and −10 regions of the potential σ^A promoter are indicated above the sequence. The mapped transcriptional start point (S) and the G residue at position 657, that is absent in the published genome sequence are underlined and in boldface. The *hemZ* region replaced by a *cat* cassette in various mutants is underlined.

GCAGAAAATCGGTGAATGGCTG-3′) and YHAV3 (5′-GGCCATGGATCCT CAGTGCTGCTTTGTCGTTTTTTC-3′), was designated phemZ2RPT3. The third construct, pALF01, contains the complete former *yhaW* gene, the 5′ end of postulated *yhaV*, and the region between these potential ORFs (nucleotides 180 to 962), generated by PCR using primers YHAW5 (see above) and ALF (5′-G GCCATGGATCCAATGTTTTTCACATCCGGGAAGGA-3′). Using the same primers (YHAWS and ALF), PCR products were generated from genomic DNA isolated from *B. subtilis* 168 and JH642 and cloned as outlined above. The fourth construct, phemZ-PEX, containing the promoter region upstream of *hemZ* and the complete former *yhaW* (nucleotides 4 to 681 in Fig. 1), was generated via PCR using primers HEMZPEX5 (5′-GGCCATGGATCCAGGAATATTTCC GAATGCAGCAGC-3′) and YHAW3 (see above). Complete DNA sequence determination of all cloned fragments was performed with an ALFexpress automatic DNA sequencer (Amersham Pharmacia, Freiburg, Germany).

RNA manipulations. Preparation of total RNA of *B. subtilis* and Northern blot analysis were performed as described before (17). Hybridizations specific for *hemZ* were carried out with digoxigenin (DIG)-labeled RNAs synthesized in vitro with T3 RNA polymerase from *Eco*RI-linearized plasmids phemZ1RPT3 and phemZ2RPT3. In vitro RNA labeling was accomplished according to the manufacturer's instructions (DIG-RNA-labeling kit; Boehringer, Mannheim, Germany). Primer extension experiments were carried out with the ^{32}P-labeled primer *hemZ*-PEX (5′-CTGCGGGCTCCTCTCCGCCA-3′) as outlined before (46). DNA sequencing reactions utilizing the same primer and plasmid phemZ-PEX as template were performed, and the sequencing products were separated on the same gel.

Construction of reporter gene fusions. The intercistronic region between *yhaX* and *hemZ* carrying the *hemZ* promoter (nucleotides 4 to 179 in Fig. 1) was amplified by PCR using primers HEMZPEX5 (see above) and HEMZTF3 (5′-GGCCATGAATTCCTTCATCACCTAATTTATCAGATT-3′). The *hemZ*-distal primer carried a *Bam*HI restriction site at its 5′ end; the *hemZ*-proximal primer carried an *Eco*RI site. The PCR product was inserted in *Bam*HI-*Eco*RI-digested pBgaB vector (28). The reporter gene *bgaB* encodes a thermostable β-galactosidase from *Bacillus stearotheromophilus*. In the resulting plasmid, pHZ01, the *bgaB* reporter gene is under control of the *hemZ* promoter region. *B. subtilis* AM01 carrying a *cat* cassette in *amyE* (28) was transformed with pHZ01 (28). Double-crossover integration of the fusion at *amyE* was selected by neomycin resistance and screened for the loss of chloramphenicol resistance of *B. subtilis* (named HZ04). Reporter gene fusions were tested under the indicated growth conditions as described earlier (28). The construction of the P$_{lepA}$-*bgaB* transcriptional fusion (AM10) has been described before (28). Mutations in the regulatory loci *resDE*, *fnr*, and *ywiD* were introduced into HZ04 (*hemZ*::*bgaB* fusion), AM10 (*hemN*::*bgaB* fusion), and AM7 (*bgaB*) via transformation using genomic DNA prepared from MH5081 (*resDE*), THB2 (*fnr*), and THB99 (*ywiD*), respectively, as described previously (14, 27, 39).

Construction of a *B. subtilis hemZ* single mutant and a *hemN hemZ* double mutant. A PCR product containing the 5′-terminal region of former *yhaV* was generated with primers YHAV5 (see above) and YHAV5EV (5′-GGCCATGA TATCAATGTTTTTCACATCCGGGAAGGA-3′) (nucleotides 813 to 962 in Fig. 1). This fragment was designed to carry a promoter-proximal *Bam*HI site and a promoter-distal *Eco*RV site. A second PCR product enclosing the 3′ end of *hemZ* (nucleotides 1532 to 1685 in Fig. 1), generated with primers YHAVEV (5′-GGCCATGATATCTGCTAGTAAATTTATTGATCGGGA-3′) and YHAV3 (see above) carried an *Eco*RV site at its promoter-proximal end and a *Bam*HI site at its promoter-distal end. Both fragments were fused and ligated into the *Bam*HI site of pBluescript SK$^+$II. The resulting plasmid p*hemZ*Δ01 was digested with *Bam*HI, and the 316-bp fragment obtained was subcloned into the *Bam*HI site of pBlueSalIISmaIΔ, a derivative of pBluescript SK$^+$II in which the polylinker region between the *Sal*I and *Sma*I site was deleted, to generate p*hemZ*Δ02 (17). With this strategy, phemZΔ02 carried only one *Eco*RV site, the one between the two *hemZ* fragments. A *cat* cassette was liberated from pSKCAT (*cat* cassette cloned into the *Eco*RV site of pBluescript SK$^+$II) by using *Eco*RV and inserted into phemZΔ02 after linearization with *Eco*RV, generating phemZ::cat. In phemZ::cat, transcription of *cat* occurs in the same direction as the *hemZ* reading frame. phemZ::cat was linearized with *Sca*I and transformed into the chromosome of the *B. subtilis hemN* mutant HZ01 (16) and wild-type *B. subtilis* 1012. After selection on plates containing neomycin and 5 μg of hemin per ml, several transformants were obtained and checked by PCR for correct double-crossover integration of the *cat* cassette into the chromosomal copy of *hemZ*. The resulting mutant strains were designated *B. subtilis* HZ03 (*hemN hemZ* double mutant) and *B. subtilis* HZ02 (*hemZ* mutant). In both cases, the part of *hemZ* underlined in Fig. 1 is replaced by the *cat* cassette.

Construction of a *hemY* and a *hemN hemZ hemY* triple mutant. An internal fragment of the *B. subtilis hemY* gene corresponding to positions 1088210 to 1088509 of the *B. subtilis* genome sequence (GenBank accession no. A1009126) was amplified by PCR using primers HEMY5 (5′-GGCCATGAATTCATTGA CAAGCTCAGCCTGATGTCG-3′), carrying a *Bam*HI restriction site at its 5′ end, and HEMY3 (5′-GGCCATGGATCCTGAATCAGCATCAAGTGTGAC GCC-3′), carrying an *Eco*RI restriction site at its 5′ end. The 324-bp PCR product was digested with *Bam*HI and *Eco*RI and inserted into *Bam*HI-*Eco*RI-restricted pMUTIN4 (44). The resulting plasmid, pMUTIN-hemY, was used to transform *B. subtilis* 1012 and the *hemN hemZ* double mutant HZ03. Transformants were selected on plates containing erythromycin plus 5 μg of hemin per ml

and checked via PCR as described above. The obtained *hemY* and the *hemN hemZ hemY* triple mutant were designated *B. subtilis* ARB11 and ARB10, respectively.

Complementation experiments. To test for the *hemN*-like function, we cloned the *B. subtilis hemZ* gene into pBluescript SK^+II and performed complementation experiments with a heme-auxotrophic *S. typhimurium hemF hemN* double mutant TE3006 (47). For this purpose, primers HEMZPEX5 (see above) and YHAV3 (see above), corresponding to positions 1056984 to 1057007 and 1058641 to 1058664 of the recently cloned *B. subtilis* genomic region containing *hemZ* (GenBank accession no. AL009126), were used in a PCR to generate a 1,706-bp fragment containing the complete 1,506-bp *B. subtilis hemZ* and 188 bp of its 5′ region including its promoter. The fragment containing *Bam*HI restriction sites introduced by the primer sequences was cut with *Bam*HI, and the resulting 1,692-bp fragment was cloned into pBluescript SK^+II to generate pBluehemZ. *S. typhimurium* TE3006 lacking the intact genes for the oxygen-dependent coproporphyrinogen III oxidase (*hemF*) and a component of the oxygen-independent enzyme (*hemN*) was transformed via electroporation with the newly constructed pBluehemZ containing *B. subtilis hemZ*, pBluehemN containing *B. subtilis hemN* as positive control, and the cloned PCR products of the inactivated *hemN* gene from *B. subtilis* HZ01(phemNΔ) and the tagged *B. subtilis hemZ* from HZ02(phemZ::cat) as negative controls. Transformants were subsequently screened aerobically and anaerobically for the recovery of heme sufficiency by plating on Luria-Bertani medium supplemented with 100 μg of ampicillin per ml and 10 μg of tetracycline per ml but without further addition of hemin. For comparison of the complementation efficiency of *B. subtilis hemN* and *hemZ*, growth experiments with the indicated strains in liquid medium under aerobic and anaerobic conditions followed by optical density measurements were performed.

High-performance liquid chromatography analysis of porphyrins. Porphyrins were extracted, modified, and separated as described before (13, 38).

RESULTS AND DISCUSSION

Identification and sequence analysis of a second *hemN*-like gene *hemZ* containing the previously proposed ORFs *yhaV* and *yhaW*. Recently, we provided experimental evidence for the existence of a second HemN-type enzyme in *B. subtilis* (13). To identify the corresponding gene, we analyzed the completed and published DNA sequence of the *B. subtilis* genome with the program SubtiList and the BLAST algorithm, using the protein sequence deduced from *B. subtilis hemN* (16, 23, 29). One ORF encoding a protein with significant amino acid sequence identity to HemN on the protein level (26% identity and 51% similarity) was identified. This ORF, designated *yhaV*, encoded a putative protein of 290 amino acids with a calculated molecular mass of 33,556 Da. YhaV revealed a high degree of amino acid sequence similarity to numerous HemN proteins in the database, the strongest to HemN of *B. subtilis*. However, it was significantly smaller than other known HemN proteins. Initial inspection of the published *yhaV* sequence first led to the assumption that *yhaV* forms a bicistronic operon with the promoter-distal gene *yhaW*. The potential *orf yhaW* encoded a protein of 166 amino acids with a calculated molecular mass of 19,024 Da. This hypothetical protein showed no homology to any other protein in the SwissProt database. No obvious ribosome binding site was present upstream of the predicted *yhaV* coding sequence. Moreover, the 130-bp intercistronic sequence between *yhaW* and *yhaV* seemed to be unusually large. These observations suggested a sequencing error in the published genome sequence (23). To substantiate this hypothesis, we resequenced the whole *yhaV* locus of *B. subtilis* 1012, using four independently generated PCR products from this locus (phemZ1RPT3, phemZ2RP3, pALF01, and phemZ-PEX). The four products covered the complete region of the postulated bicistronic operon. Three of the cloned fragments contained the 3′ end of *yhaW*, and they all contained a G residue 25 bp upstream of the 3′ end of the postulated *yhaW* coding sequence (Fig. 1). This G was lacking in the published genome sequence (23). No additional sequencing errors were detected. Identical results were obtained for a PCR-based analysis of the same genomic region of *B. subtilis* 168 and *B. subtilis* JH642. After correction of the DNA sequence, the coding region of the former reading frames *yhaW* and *yhaV* were fused to one large ORF of 1,506 bp, which was designated *hemZ* (Fig. 1). The newly identified *hemZ* gene encodes a protein of 501 amino acids with a calculated molecular mass of 57,526 Da. The molecular mass was confirmed by in vivo expression experiments in *E. coli* using T7 RNA polymerase-driven transcription and radioactive protein labeling (data not shown). The protein deduced from *hemZ* showed significant homology to numerous HemN proteins in the database, the strongest to *B. subtilis* HemN (28% identity and 51% similarity) and to the *Aquifex aeolicus* HemN (28% identity and 50% similarity). However, alignment of the currently known approximately 30 HemN and HemZ proteins revealed the phylogenetically most distant position for *B. subtilis* HemZ in the HemN/HemZ protein family tree (data not shown). *B. subtilis* HemZ carries a unique approximately 100-amino-acid residue N-terminal extension, while 50 amino acid residues present in all other HemN/HemZ proteins are missing close to the C terminus. However, all highly conserved amino acid residues of HemN/HemZ proteins, like the potential iron-sulfur cluster signature sequence (CXXXCXXC) or the glycine-rich box (GGGIP), are present. A biochemical characterization of recombinant *B. subtilis* HemZ is under way.

The *hemZ* gene is preceded by a distinct ribosome binding site (AAAUUAGGUGAU) with a high degree of identity to the consensus ribosome binding sequence of *B. subtilis* (AAAGGAGGUGAU). Further upstream of *hemZ*, a potential vegetative σ^A-dependent promoter (TTGATT-17 bp-TACACT) with sequence similarity to the consensus sequence of vegetative *B. subtilis* promoters (TTGACA-17 bp-TATAAT) was identified; 2 bp upstream of this potential promoter, a strong secondary structure ($\Delta G = -29$ kcal/mol) can be predicted at the RNA level. The observed structure could serve as transcriptional terminator for the upstream located *yhaX* gene. Immediately downstream of *hemZ* and partially overlapping with the 3′ end of its coding sequence a second potential RNA secondary structure ($\Delta G = -18$ kcal/mol) was located followed by a 7-U stretch. We assume that *hemZ* transcription is terminated here (Fig. 1).

***B. subtilis hemZ* complemented a *Salmonella typhimurium hemF hemN* double mutant under aerobic and anaerobic conditions.** *S. typhimurium* TE3006 (*hemZ hemN* double mutant) is unable to grow in the absence of heme both under aerobic and anaerobic conditions (47). To determine whether the *B. subtilis hemZ* gene is able to complement the mutant strain TE3006 for growth without added heme, it was transformed with plasmid pBluehemZ. Transformants were able to grow both under aerobic and anaerobic conditions, indicating that the HemZ protein is involved in the oxygen-independent coproporphyrinogen III decarboxylation. It had already been shown that the *B. subtilis hemN* gene was able to complement strain TE3006 in a similar way (13). In contrast, plasmids carrying inactivated *hemZ* (phemZ::cat) or *hemN* (phemNΔ) failed to complement TE3006 under these growth conditions. Comparison of the growth behavior of *Salmonella* mutants complemented with *B. subtilis hemN* and *hemZ* revealed significant differences. Strain TE3006 carrying either *hemZ* (doubling time [T_d] = 2.5 h) or *hemN* (T_d = 2 h) grew faster under anaerobic ammonifying growth conditions than under aerobic conditions. Under aerobic growth conditions, mutants complemented with *hemN* (T_d = 3.2 h) grew significantly faster than their *hemZ* (T_d = 11.0 h)-complemented counterparts. The amounts of *hemN* and *hemZ* mRNA isolated from the complemented *Salmonella* mutants and analyzed via RNA dot blot experiments were found to be approximately identical under all analyzed conditions (data not shown).

A *B. subtilis hemZ* mutant accumulates coproporphyrinogen III under anaerobic conditions. To demonstrate the involvement of *B. subtilis hemZ* in the metabolism of coproporphyrinogen III, the cellular porphyrin profiles of wild-type *B. subtilis*, the *hemZ* mutant HZ02 (construction described in Materials and Methods), and the previously described *hemN* mutant HZ01 were compared. All three strains were grown aerobically and anaerobically in the presence of 10 mM nitrate. The porphyrins were extracted, modified, and separated via high-pressure liquid chromatography as described before (13, 38). *B. subtilis* wild-type cells did not accumulate any significant amounts of porphyrins. In agreement with our previous findings, porphyrins extracted from the *hemN* mutant HZ01 grown under anaerobic conditions exhibited a clear peak of coproporphyrin III. A coproporphyrin III accumulation of approximately 40 nmol/g (dry weight) was deduced. Similar to these results, the *hemZ* mutant HZ02 accumulated coproporphyrin III only under anaerobic conditions. However, only 25% of the coproporphyrin III amount (10 nmol/g [dry weight]) detected with the *hemN* mutant were observed. The amount of coproporphyrin III (55 nmol/g [dry weight]) accumulated by the *hemN hemZ* double mutant almost exactly corresponded to the sum of precursors detected in the two single mutants. These results demonstrated the involvement of the *B. subtilis hemZ* gene product in the oxygen-independent metabolism of coproporphyrinogen III.

***B. subtilis hemZ* and *hemN hemZ* double mutants have no obvious growth phenotype.** The consequences of *hemZ* inactivation for the growth of *B. subtilis* were studied. No obvious growth phenotype under aerobic and anaerobic nitrate respiratory or fermentative conditions was observed for the *B. subtilis hemZ* mutant (Fig. 2). To investigate whether *hemN* supplemented for *hemZ* inactivation, a *B. subtilis hemN hemZ* double mutant was constructed. For this purpose, the *hemZ* mutation was introduced into the *hemN* deletion strain *B. subtilis* HZ01 (16). *B. subtilis* HZ01 contains in the *hemN* gene an in-frame deletion reducing the size of the HemN protein from 363 to 167 amino acid residues. As described above, the knockout phenotype of this deletion was verified by the absence of complementation of the *S. typhimurium hemN hemF* double mutant with the cloned, partially deleted *hemN* gene (phemNΔ). The newly constructed *hemN hemZ* double mutant was designated *B. subtilis* HZ03. Surprisingly, mutation of both genes did not abolish aerobic and anaerobic respiratory growth. However, reduction of aerobic and anaerobic growth was observed (Fig. 2). These results point to the existence of an additional third oxygen-independent enzymatic system for the conversion of coproporphyrinogen III to protoporphyrinogen IX. One potential candidate, at least for aerobic heme formation, was the product of the *hemY* gene in combination with a yet unknown enzyme.

Analysis of *B. subtilis hemZ hemN hemY* triple mutant. Hansson et al. described the oxidation of coproporphyrinogen III to coproporphyrin III as a side activity of the oxygen-dependent protoporphyrinogen oxidase encoded by *hemY* (8). It was postulated that in combination with a new type of enzyme, a coproporphyrin III decarboxylase which converts coproporphyrin III into protoporphyrin IX, HemY could convert coproporphyrinogen III into protoporphyrin (8, 10). However, due to the oxygen dependence of HemY, this pathway would be limited to aerobic conditions. First, the effect of *hemY* mutation on aerobic and anaerobic growth was tested in the newly constructed *hemY* mutant ARB11. Surprisingly, reduced growth of the *B. subtilis hemY* mutant under aerobic and anaerobic conditions was observed (Fig. 2). To reconfirm this observation, the *B. subtilis hemY* deletion mutants 3G18Δ2 and 1A594 were subjected to similar growth experiments (9, 10). Almost identical growth behaviors to ARB11 were observed (data not shown). The differences of these observations from the previously detected clear detrimental effects of the *hemY* mutation are possibly caused by differences in the growth media used (9, 10). We concluded from our results that a still unknown oxygen-independent protoporphyrinogen IX oxidase partially compensated under aerobic and anaerobic conditions for *hemY* mutation. In *S. typhimurium* and *P. aeruginosa*, oxygen-independent HemN compensated for the loss of the oxygen-dependent HemF under aerobic and anaerobic growth conditions (34, 48). Due to the anaerobic respiratory energy metabolism of *B. subtilis*, an oxygen-independent protoporphyrinogen IX oxidase is required at least for anaerobic heme biosynthesis.

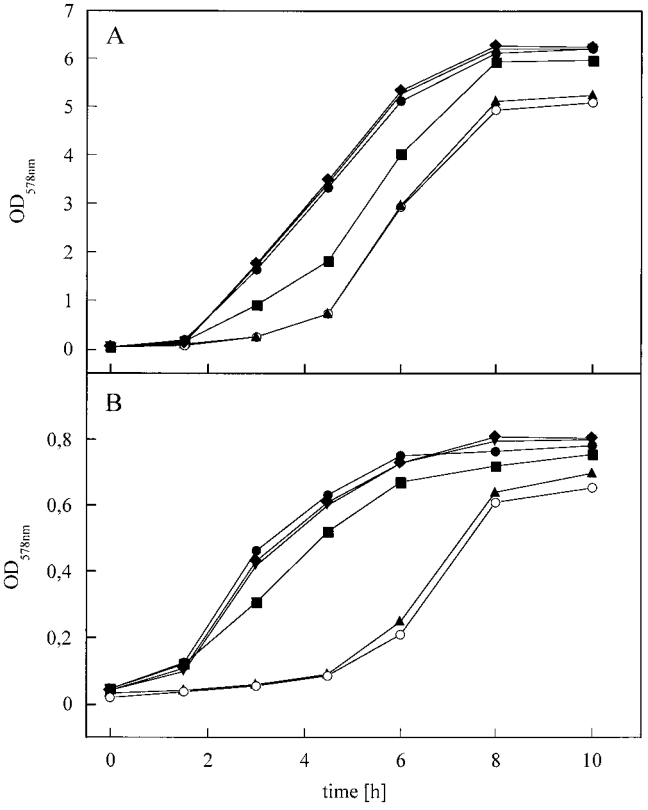

FIG. 2. Growth behavior of *B. subtilis* 1012 (●), the *hemN* mutant HZ01 (◆), the *hemZ* mutant HZ02 (▼), the *hemY* mutant ARB11 (▲), the *hemN hemZ* double mutant HZ03 (■), and the *hemN hemZ hemY* triple mutant ARB10 (○) in minimal medium under aerobic (A) and anaerobic nitrate ammonifying (B) conditions. OD, optical density.

Moreover, the growth experiment indicated an unexpected anaerobic role for HemY. Similar observations were made for *P. aeruginosa hemF*, encoding the oxygen-dependent coproporphyrinogen III oxidase. Chromosomal deletion of *hemF* resulted in reduced aerobic and anaerobic growth of *P. aeruginosa* (34). In agreement with this observation, a strong anaerobic induction of *hemF* transcription was observed (34). The potential anaerobic roles for both oxygen-dependent enzymes remain to be elucidated.

To test for the *hemY* function in in vivo conversion of coproporphyrinogen III into protoporphyrin IX a *hemN hemZ hemY* triple mutant (ARB10) was constructed and tested for aerobic and anaerobic growth. The aerobic and anaerobic growth phenotype of this mutant was identical to the pheno-

type of the *hemY* mutant ARB10 (Fig. 2). These experiments indicated that *hemY* is not directly responsible for the viability of the *hemN hemZ* double mutant.

From our results, we concluded the existence of a third oxygen-independent enzymatic system for coproporphyrinogen III oxidation and an oxygen-independent system for protoporphyrinogen IX oxidation in *B. subtilis*.

Analysis of *hemZ* transcriptional unit. The existence of two genes (*hemN* and *hemZ*) encoding two structurally highly related proteins with similar functions could provide the cell with a regulatory tool to differentially respond to variations in the cellular heme requirement as a consequence of changing environmental conditions. Regulated gene expression in response to changes in oxygen tension was previously observed for *hemN* and *hemF* from *P. aeruginosa* and for *E. coli hemN* (34, 42). Moreover, highly conserved oxygen tension regulator Fnr binding sites have been identified in the 5′ region of the *A. eutrophus hemN* and *R. capsulatus hemZ* (25, 49).

We started our transcriptional analysis of *B. subtilis hemZ* with the definition of the transcriptional unit by Northern blot hybridization. Computer analysis of the *hemZ* locus suggested a monocistronic transcriptional organization of *hemZ* (see above). Total RNA was isolated from exponentially growing *B. subtilis* 1012 cultivated under aerobic conditions. We used two different riboprobes in the Northern blot hybridizations, one with specificity to the 5′-terminal part of *hemZ* (the former *yhaW*) and the second complementary to the 3′-terminal part (the former *yhaV*). Northern blot analysis detected one transcript of around 1.6 kb with both probes, indicating a monocistronic transcriptional unit for *hemZ* (Fig. 3A). To determine the 5′ end of the *hemZ* transcript, we performed a primer extension analysis. Total RNA isolated from exponentially aerobically growing *B. subtilis* 1012 was hybridized to a ^{32}P-labeled oligonucleotide primer (*hemZ*-PEX) complementary to the 5′ region of the *hemZ* mRNA and was extended by using avian myeloblastosis virus reverse transcriptase. The resulting autoradiograph revealed one single transcriptional start site which corresponded to an A on the RNA level located 31 bp upstream the translational start of *hemZ* (Fig. 3B). This putative transcriptional start site was within the appropriate distance (7 bases) from the postulated σ^A-dependent promoter mentioned above (Fig. 1). No obvious differences in transcript length and transcriptional start site were detected with RNA prepared from anaerobically grown cells (data not shown).

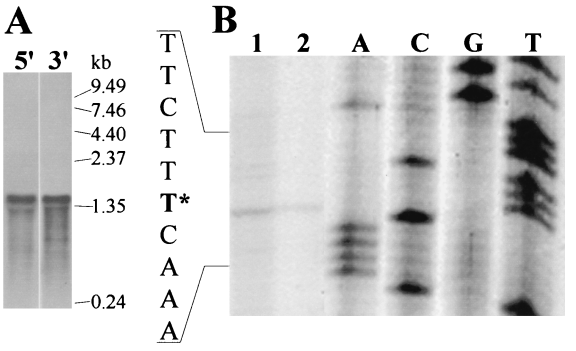

FIG. 3. (A) Characterization of *hemZ* transcriptional unit by Northern blot analyses. RNA was isolated from *B. subtilis* wild-type strain 1012 grown under aerobic conditions. After electrophoresis in 1.2% denaturing agarose gels and transfer to nylon membranes, hybridization was performed with riboprobes directed against the 5′ end (5′) and 3′ end (3′) of *hemZ*. We used 5 μg of RNA for the assay shown in lane 5′ and 10 μg of RNA for the assay shown in lane 3′. The positions of RNA size markers are indicated. (B) Mapping of the 5′ end of the *hemZ* mRNA. Primer extension analyses were carried out with the oligonucleotide *hemZ*-PEX and 20 μg (lane 1) or 5 μg (lane 2) of RNA prepared from exponentially growing *B. subtilis* 1012 cells under aerobic conditions. DNA sequencing reactions utilizing the same primer and plasmid p*hemZ*PEX were performed in parallel, and the reaction products were separated on the same gel (lanes A, C, G, and T). The mapped 5′ end of *hemZ* mRNA is denoted by an asterisk.

Regulation of *hemZ* and *hemN* expression by oxygen tension, nitrate, and H_2O_2. After identification of the *hemZ* promoter region, we analyzed the regulation of *B. subtilis hemZ* and *hemN* expression by reporter gene fusions using the *bgaB* system (28). For this purpose, the promoter regions of *hemZ* and *hemN* were amplified by PCR and cloned into plasmid pBgaB immediately upstream of the promoterless *bgaB* gene (28). Since *hemN* forms an operon with the upstream-located *lepA*, the promoter region of *lepA* was fused to *bgaB* (13, 16). The *bgaB* gene encodes a thermostable β-galactosidase of *B. stearotheromophilus*. Using this construct, the transcriptional fusions P_{hemZ}-*bgaB* and P_{hemN}-*bgaB* were integrated into the chromosome at the *amyE* locus by double crossover to gener-

TABLE 2. Regulation of *B. subtilis hemN* and *hemZ*

Strain	β-Galactosidase activity (U/mg of protein)[a]			
	$+O_2$	$+H_2O_2$[b]	$-O_2$	$-O_2$ + 10 mM nitrate
HZ01(*hemZ*::*bgaB*)	9	52	4	47
ARB1(*hemZ*::*bgaB resDE*)	10	NT	3	10
ARB2(*hemZ*::*bgaB fnr*)	9	NT	2	4
ARB3(*hemZ*::*bgaB ywiD*)	14	NT	1	2
AM10(*hemN*::*bgaB*)	7	6	4	34
ARB4(*hemN*::*bgaB resDE*)	8	NT	4	8
ARB5(*hemN*::*bgaB fnr*)	9	NT	2	5
ARB6(*hemN*::*bgaB ywiD*)	9	NT	2	3
AM7(*bgaB*)	2	2	2	3
ARB7(*bgaB resDE*)	2	NT	2	2
ARB8(*bgaB fnr*)	3	NT	2	2
ARB9(*bgaB ywiD*)	2	NT	2	3

[a] Strains were grown aerobically or anaerobically as described in Materials and Methods. All cell extracts were prepared and assayed for β-galactosidase activity as described before (28). The results represent averages of three different experiments performed in triplicate. A standard deviation of approximately 1.5 U/mg of protein was observed. NT, not tested.

[b] Growing cultures were treated for 15 min with 0.1 mM H_2O_2 before assaying for reporter gene expression.

ate *B. subtilis* HZ04 and AM10. Both strains were grown under the indicated conditions into mid-exponential growth phase, and β-galactosidase activity was assayed as described before (28). As a control, *B. subtilis* AM07 carrying the *bgaB* gene without any promoter in *amyE* was analyzed in parallel. The results of the various BgaB assays are summarized in Table 2. A clear fivefold induction of *hemZ* and *hemN* transcription was observed under anaerobic growth conditions in the presence of the alternative electron acceptors nitrate and nitrite compared to aerobic growth conditions. Fermentative growth conditions failed to induce *hemZ* and *hemN* transcription, in agreement with the greatly reduced heme requirement for nonrespiratory fermentative growth. The observed anaerobic induction was visible only during growth on well-defined minimal medium. In our previous investigation of *hemN* expression via RNA slot blot experiments using RNA prepared from *B. subtilis* grown aerobically and anaerobically on complex medium, we failed to detect significant redox regulation (13). Similar results were obtained in this study for the *hemZ-bgaB* and *hemN-bgaB* fusion strains grown aerobically and anaerobically on complex medium (data not shown). The exact nature of the inhibitory compound contained in the complex medium remains to be elucidated.

A second regulatory factor, peroxide stress, was specific to *hemZ* expression. An approximately fivefold induction of the *hemZ-bgaB* fusion was observed in the presence of H_2O_2, while the expression of the *hemN-bgaB* fusion remained unchanged. The H_2O_2-neutralizing enzyme catalase possesses a heme cofactor. The catalase gene *katA* is also under the control of H_2O_2 (1, 7). A catalase-overproducing *B. subtilis* mutant was found to overproduce tetrapyrroles, too (7). A consensus sequence called the per box was detected upstream the promoter of *katA* and a second H_2O_2-regulated gene, *mrgA* (2). The same per box was present at two positions upstream of the *hemAXCDBL* operon, encoding the enzyme for initial steps of heme biosynthesis in *B. subtilis*, indicating peroxide stress regulation for this operon (2). However, no obvious DNA sequence matching the per box was found upstream of *hemZ*. Other examples of H_2O_2-regulated genes without a per box are known (7, 12, 45). The alternative sigma factor σ^B is responsible for the general stress response of *B. subtilis*, including the expression of the second catalase gene, *katE*, induced by oxidative stress (12). However, no obvious promoter sequence for σ^B was found in the 5′ region of *hemZ* (Fig. 1).

Anaerobic induction of *hemN* and *hemZ* is dependent on *resDE*, *fnr*, and *ywiD*. To investigate the molecular basis for the observed transcriptional activation of *hemN* and *hemZ*, we analyzed expression of the reporter gene constructs in mutants of the previously identified redox regulatory loci *resDE*, *fnr*, and *ywiD* (Table 2). While aerobic expression of *hemN* and *hemZ* remained mainly unaffected by the *resDE*, *fnr*, and *ywiD* mutants, anaerobic expression was found to be drastically reduced, indicating the importance of all three loci for anaerobic *hemN* and *hemZ* induction. Mutation in *ywiD* resulted in the most severe reduction of *hemN* and *hemZ* expression compared to the *fnr* and *resDE* mutation (Table 2). The observed low expression under anaerobic fermentative conditions remained unchanged. Previously, the requirement of *resDE* for efficient *fnr* expression was established (32). Furthermore, Fnr mediates the anaerobic induction of *ywiD* via a highly conserved consensus binding site (Fnr box) (27). Finally, YwiD activates anaerobic transcription of genes encoding nitrite reductase (*nasDE*), a potential flavohemoglobin (*hmp*), lactate dehydrogenase (*lctE*), and enzymes of acetoin fermentation (*alsSD*), all involved in anaerobic metabolism (27). A similar redox regulatory cascade is proposed for the regulation of *hemN* and *hemZ*. However, from our results a direct involvement of *resDE* in anaerobic *hemN* and *hemZ* cannot be excluded. A direct activation mediated by *fnr* is very unlikely since appropriate Fnr boxes are missing in the *hemN* and *hemZ* promoter regions.

Since heme requirements drastically vary between aerobic respiratory, anaerobic respiratory, and anaerobic fermentative conditions, heme biosynthesis is obviously coregulated via *hemN* and *hemZ* expression (33). The known regulators employed by *B. subtilis* for its general redox adaptation encoded by *resDE*, *fnr*, and *ywiD* are also used for the coordination of heme biosynthesis with the anaerobic energy metabolism. Moreover, the *resDE* genes are also involved in the regulation of *qcrABC* (encoding subunits of the cytochrome *bc* complex), *ctaA* (required for heme A synthesis), and genes important for cytochrome *c* biogenesis, loci all related to tetrapyrrole-associated processes (39).

ACKNOWLEDGMENTS

This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to D.J. and W.S., the Max-Planck-Gesellschaft, the Sonderforschungsbereich 388, and the Graduiertenkolleg "Biochemie der Enzyme" of the Albert-Ludwigs-Universität Freiburg to D.J. We are indebted to T. Elliott (West Virginia University, Morgantown) for the gift of several *S. typhimurium* strains and to M. Nakano (Louisiana State University, Shreveport), H. Cruz Ramos, and P. Glaser (Pasteur Institute, Paris, France) for the gift of *B. subtilis* strains. We thank T. Hoffmann (Max-Planck-Institut, Marburg, Germany) for helpful discussions and Antonio Espin for technical assistance. We thank R. K. Thauer (Max-Planck-Institut) for helpful discussions and continuous support. We are indebted to L. Hederstedt and M. Hansson (University of Lund, Lund, Sweden) for the gift of *B. subtilis* strains and helpful discussions and suggestions.

REFERENCES

1. **Bol, D. K., and R. E. Yasbin.** 1994. Analysis of the dual regulatory mechanisms controlling expression of the vegetative catalase gene of *Bacillus subtilis*. J. Bacteriol. **176:**6744–6748.
2. **Chen, L., L. Keramati, and J. D. Helmann.** 1995. Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. Proc. Natl. Acad. Sci. USA **92:**8190–8194.
3. **Ciba Foundation Symposia Staff.** 1994. The biosynthesis of the tetrapyrrole pigments. Ciba Foundation Symposia 180. John Wiley & Sons, New York, N.Y.
4. **Coomber, S. A., R. M. Jones, P. M. Jordan, and C. N. Hunter.** 1992. A putative anaerobic coproporphyrinogen III oxidase in *Rhodobacter sphaeroides*. I. Molecular cloning, transposon mutagenesis and sequence analysis of the gene. Mol. Microbiol. **6:**3155–3169.
5. **Cruz Ramos, H., L. Boursier, I. Moszer, F. Kunst, A. Danchin, and P. Glaser.** 1995. Anaerobic transcription activation in *Bacillus subtilis*: identification of distinct FNR-dependent and -independent regulatory mechanisms. EMBO J. **14:**5984–5994.
6. **Doss, M. O., and W. K. Philipp-Dornston.** 1971. Porphyrin and heme biosynthesis from endogenous and exogenous δ-aminolevulinic acid in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Achromobacter metalcaligenes*. Hoppe-Seyler's Z. Physiol. Chem. **352:**725–733.
7. **Dowds, B. C. A.** 1994. The oxidative stress response in *Bacillus subtilis*. FEMS Microbiol. Lett. **124:**255–264.
8. **Hansson, M., M. C. Gustafsson, C. G. Kannangara, and L. Hederstedt.** 1997. Isolated *Bacillus subtilis* HemY has coproporphyrinogen III to coproporphyrin III oxidase activity. Biochim. Biophys. Acta **20:**97–104.
9. **Hansson, M., and L. Hederstedt.** 1992. Cloning and characterization of the *Bacillus subtilis hemEHY* gene cluster, which encodes protoheme IX biosynthetic enzymes. J. Bacteriol. **174:**8081–8093.
10. **Hansson, M., and L. Hederstedt.** 1994. *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coproporphyrinogen III and protoporphyrinogen IX. J. Bacteriol. **176:**5962–5970.
11. **Hansson, M., L. Rutberg, I. Schröder, and L. Hederstedt.** 1991. The *Bacillus subtilis hemAXCDBL* gene cluster, which encodes enzymes of the biosynthetic pathway from glutamate to uroporphyrinogen III. J. Bacteriol. **173:**2590–2599.
12. **Hecker, M., and U. Völker.** 1998. Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the δ^B regulon. Mol. Microbiol. **29:**1129–1136.

13. **Hippler, B., G. Homuth, T. Hoffmann, W. Schumann, and D. Jahn.** 1997. Characterization of *Bacillus subtilis hemN*. J. Bacteriol. **179:**7181–7185.
14. **Hoffmann, T., N. Frankenberg, M. Marino, and D. Jahn.** 1998. Ammonification in *Bacillus subtilis* utilizing dissimilatory nitrite reductase is dependent on *resDE*. J. Bacteriol. **180:**186–189.
15. **Hoffmann, T., B. Troup, A. Szabo, C. Hungerer, and D. Jahn.** 1995. The anaerobic life of *Bacillus subtilis*. Cloning and characterization of the genes encoding the respiratory nitrate reductase system. FEMS Microbiol. Lett. **131:**219–225.
16. **Homuth, G., M. Heinemann, U. Zuber, and W. Schumann.** 1996. The genes *lepA* and *hemN* form a bicistronic operon in *B. subtilis*. Microbiology **142:** 1641–1649.
17. **Homuth, G., S. Masuda, A. Mogk, Y. Kobayashi, and W. Schumann.** 1997. The *dnaK* operon of *Bacillus subtilis* is heptacistronic. J. Bacteriol. **179:**1153–1164.
18. **Jacobs, N. J., and J. M. Jacobs.** 1976. Nitrate, fumarate, and oxygen as electron acceptors for a late step in microbial heme synthesis. Biochim. Biophys. Acta **13:**1–9.
19. **Jacobs, N. J., J. M. Jacobs, and B. A. Mills.** 1973. Role of oxygen in the late steps of heme synthesis in Pseudomonads and *Escherichia coli*. Enzyme **16:**50–56.
20. **Jacobs, N. J., J. M. Jacobs, and J. R. Morgan.** 1972. Comparative effect of oxygen and nitrate on protoporphyrin and heme synthesis from D-aminolevulinic acid in bacterial cultures. J. Bacteriol. **112:**1444–1445.
21. **Jahn, D., E. Verkamp, and D. Söll.** 1992. Glutamyl-transfer RNA: a precursor of heme and chlorophyll biosynthesis. Trends Biochem. Sci. **17:**215–218.
22. **Jordan, P. M.** 1991. Biosynthesis of tetrapyrroles. *In* A. Neuberger and L. L. M. van Deenen (ed.), New comprehensive biochemistry, volume 19. Elsevier, Amsterdam, The Netherlands.
23. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Briginell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, N. J. Cummings, R. A. Danie, F. Denizot, K. M. Devine, A. Dusterhoft, S. D. Ehrlich, P. T. Emmerson, K. D. Entian, J. Errington, C. Fabret, E. Ferrari, D. Foulger, C. Fritz, M. Fujita, Y. Fujita, S. Fuma, A. Galizzi, N. Galleron, S. Y. Ghim, P. Glaser, A. Goffeau, E. J. Golightly, G. Grandi, G. Guiseppi, B. J. Guy, K. Haga, J. Haiech, C. R. Harwood, Y. Hasahara, A. Henaut, H. Hilbert, S. Holsappel, S. Hoson, M. F. Hullo, M. Itaya, L. Jones, B. Joris, D. Karamata, M. Klaerr-Blanchard, C. Klein, Y. Kobayashi, P. Koetter, G. Koningstein, S. Krogh, M. Kumano, K. Kurita, A. Lapidus, S. Lardinois, J. Lauer, V. Lazarevic, S. M. Lee, A. Levine, H. Liu, S. Masuda, C. Mauel, C. Medigue, N. Medina, R. P. Medallo, M. Mizuno, D. Moestl, S. Nakai, M. Noback, D. Noone, M. O'Reilly, K. Ogawa, A. Ogiwara, B. Oudega, S. H. Park, V. Parro, T. M. Pohl, D. Portetelle, S. Porwollik, A. M. Prescott, E. Presecan, P. Pujic, B. Purnelle, G. Rapoport, M. Rey, S. Reynolds, M. Rieger, C. Rivolta, E. Rocha, B. Roche, M. Rose, Y. Sadaie, T. Sato, E. Scanlan, S. Schleich, R. Schroeter, F. Scoffone, J. Sekiguchi, A. Sekowska, S. J. Seror, P. Serror, B. S. Shin, B. Soldo, A. Sorokin, E. Tacconi, T. Takagi, H. Takahashi, K. Takemaru, M. Takeuchi, A. Tamakoshi, T. Tanaka, P. Terpstra, A. Tognini, V. Tosato, S. Uchiyama, M. Vandenbol, F. Vannier, A. Vassarotti, A. Viari, R. Wambutt, E. Wedler, H. Wedler, T. Weitzenegger, P. Winters, A. Wipat, H. Yamamoto, K. Yamane, K. Yasumoto, K. Yata, K. Yoshida, H. Yoshikawa, H. F. Yoshikawa, E. Zumstein, and A. Danchin.** 1998. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
24. **Leeper, F.** 1991. Intermediate steps in the biosynthesis of chlorophylls, p. 407–432. *In* H. Scheer (ed.), Chlorophylls. CRC Press, London, England.
25. **Lieb, C., R. A. Siddiqui, B. Hippler, D. Jahn, and B. Friedrich.** 1998. The *Alcaligenes eutrophus hemN* gene encoding the oxygen-independent coproporphyrinogen III oxidase is required for heme biosynthesis during anaerobic growth. Arch. Microbiol. **169:**52–60.
26. **Mach, H., M. Hecker, and F. Mach.** 1988. Physiological studies on cAMP synthesis in *Bacillus subtilis*. FEMS Microbiol. Lett. **52:**189–192.
27. **Marino, M., H. Cruz Ramos, T. Hoffmann, P. Glaser, and D. Jahn.** 1999. Unpublished results.
28. **Mogk, A., R. Hayward, and W. Schumann.** 1996. Integrative vectors for constructing single-copy transcriptional fusions between *Bacillus subtilis* promoters and various reporter genes encoding heat-stable enzymes. Gene **182:**33–36.
29. **Moszer, I., P. Glaser, and A. Danchin.** 1995. SubtiList: a relational database for the *Bacillus subtilis* genome. Microbiology **141:**261–268.
30. **Nakano, M. M., T. Hoffmann, Y. Zhu, and D. Jahn.** 1998. Nitrogen and oxygen regulation of *Bacillus subtilis nasDEF* encoding NADH-dependent nitrite reductase by TnrA and ResDE. J. Bacteriol. **180:**5344–5350.
31. **Nakano, M. M., and P. Zuber.** 1998. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). Annu. Rev. Microbiol. **52:**165–190.
32. **Nakano, M. M., P. Zuber, P. Glaser, A. Danchin, and F. M. Hulett.** 1996. Two-component regulatory proteins ResD-ResE are required for transcriptional activation of *fnr* upon oxygen limitation in *Bacillus subtilis*. J. Bacteriol. **178:**3796–3802.
33. **Philipp-Dornston, W. K., and M. O. Doss.** 1973. Comparison of porphyrin and heme in various heterotrophic bacteria. Enzyme **16:**57–64.
34. **Rompf, A., C. Hungerer, T. Hoffmann, M. Lindenmeyer, U. Römling, U. Groß, M. O. Doss, H. Arai, Y. Igarashi, and D. Jahn.** 1998. Regulation of *Pseudomonas aeruginosa hemN* and *hemF* by the dual action of the redox response regulators Anr and Dnr. Mol. Microbiol. **29:**985–997.
35. **Saito, H., T. Shibata, and T. Ando.** 1979. Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **170:**117–122.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Seehra, J. S., P. M. Jordan, and M. Akhtar.** 1982. Anaerobic and aerobic copro-porphyrinogen III oxidases of *Rhodopseudomonas spheroides*. Biochem. J. **269:**709–718.
38. **Straka, J. G.** 1986. High-performance liquid chromatography of porphyrin methylesters. Methods Enzymol. **123:**352–363.
39. **Sun, G., E. Sharkova, R. Chesnut, S. Birkey, M. F. Duggan, A. Sorokin, P. Pujic, S. D. Ehrlich, and F. M. Hulett.** 1996. Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. J. Bacteriol. **178:**1374–1385.
40. **Tait, G. H.** 1969. Coproporphyrinogenase activity in extracts from *Rhodopseudomonas spheroides*. Biochem. Biophys. Res. Commun. **37:**116–122.
41. **Tait, G. H.** 1972. Coproporphyrinogenase activities in extracts of *Rhodopseudomonas spheroides* and *Chromatium* strain D. Biochem. J. **128:**1159–1169.
42. **Troup, B., Hungerer, C., and D. Jahn.** 1995. Cloning and characterization of the *Escherichia coli hemN* gene encoding the oxygen-independent coproporphyrinogen III oxidase. J. Bacteriol. **177:**3326–3331.
43. **Troup, B., M. Jahn, C. Hungerer, and D. Jahn.** 1994. Isolation of the *hemF* operon containing the gene for the *Escherichia coli* aerobic coproporphyrinogen III oxidase by in vivo complementation of a yeast *HEM13* mutant. J. Bacteriol. **176:**673–680.
44. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
45. **Völker, U., S. Engelmann, B. Maul, S. Riethdorf, A. Völker, R. Schmid, H. Mach, and M. Hecker.** 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology **140:**741–752.
46. **Wetzstein, M., U. Völker, S. Dedio, U. Löbau, U. Zuber, M. Schiesswohl, C. Herget, M. Hecker, and W. Schumann.** 1992. Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. J. Bacteriol. **174:**3300–3310.
47. **Xu, K., J. Delling, and T. Elliott.** 1992. The genes required for heme synthesis in *Salmonella typhimurium* include those encoding alternative functions for aerobic and anaerobic coproporphyrinogen oxidation. J. Bacteriol. **174:**3953–3963.
48. **Xu, K., and T. Elliott.** 1994. Cloning, DNA sequence, and complementation analysis of the *Salmonella typhimurium hemN* gene encoding a putative oxygen-independent coproporphyrinogen III oxidase. J. Bacteriol. **176:**3196–3203.
49. **Zeilstra-Ryalls, J. H., and S. Kaplan.** 1995. Regulation of the 5-aminolevulinic acid synthesis in *Rhodobacter sphaeroides* 2.4.1: genetic basis of mutant H-5 auxotrophy. J. Bacteriol. **177:**2760–2768.