Characterization of *Bacillus subtilis* hemN

BIRGIT HIPPLER,1 GEORG HOMUTH,2 TAMARA HOFFMANN,3,4 CHRISTOPH HUNGERER,4 WOLFGANG SCHUMANN,2 AND DIETER JAHN1,3,4*

Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, 79104 Freiburg,3 Laboratorium für Mikrobiologie, Fachbereich Biologie, Philippus-Universität Marburg,2 and Abteilung Biochemie, Max-Planck-Institut für Terrestrische Mikrobiologie,1 35032 Marburg, and Institut für Genetik, Universität Bayreuth, 95440 Bayreuth,2 Germany

Received 30 July 1997/Accepted 11 September 1997

A recently cloned *Bacillus subtilis* open reading frame (hemN) upstream of the *dnaK* operon was identified as encoding a protein involved in oxygen-independent coproporphyrinogen III decarboxylation. *B. subtilis* hemN functionally complemented two *Salmonella typhimurium* hemF hemN double mutants under aerobic and anaerobic conditions. Interestingly, growth experiments using the *B. subtilis* hemN mutant revealed normal aerobic and anaerobic growth, indicating the presence of an alternative oxygen-independent enzymatic system. Northern blot experiments identified hemN mRNA as part of an approximately 7-kb pentacistronic transcript consisting of *lepA*, hemN, *hrcA*, *grpE*, and *dnaK*. One potential start site for aerobic and anaerobic transcription was located 37 bp upstream of the translational start codon of *lepA*. Comparable amounts of hemN transcript were observed under aerobic and anaerobic growth conditions. No experimental evidence for the presence of hemF in *B. subtilis* was obtained. Moreover, *B. subtilis* hemY did not substitute for hemF hemN deficiency in *S. typhimurium*. These results indicate the absence of hemF and suggest the presence of a second hemN-like gene in *B. subtilis*.

During heme biosynthesis protoporphyrinogen IX is formed from coproporphyrinogen III in a reaction in which the propionyl groups on rings A and B are oxidatively decarboxylated to vinyl groups (12, 13, 16). Two enzymatic systems have been identified for the catalysis of this reaction. The aerobic coproporphyrinogen III oxidase (HemF) requires molecular oxygen for the oxidative decarboxylation of coproporphyrinogen III. For anaerobic heme biosynthesis a different oxygen-independent enzyme (HemN) was identified (16, 18, 19). The genes for the oxygen-independent enzyme (HemN) were found only in bacteria (1–3, 20, 21, 28, 29; GenBank accession no. D90901, D90904, D90912, and Z81368). Overexpression of the Rhodobacter sphaeroides hemN gene in Escherichia coli led to an increase of oxygen-independent coproporphyrinogen III oxidase activity (1). In *Bacillus subtilis* most known genes involved in heme biosynthesis were found clustered in two operons. The hemAXCDBL operon at 244° of the genomic map and the hemEHY operon at 94° encode all enzymes required for the formation of heme from glutamyl-tRNA, with the exception of obvious analogs to hemF or hemN (4–6). The oxygen-dependent protoporphyrinogen IX oxidase encoded by hemY carried out an enzymatic conversion of coproporphyrinogen III (5). Since the detected product of this activity was coproporphyrin III and not protoporphyrinogen IX, the question for hemF and hemN in *B. subtilis* remained open. Moreover, the recently discovered anaerobic life of *B. subtilis* via nitrate ammonification requires an oxygen-independent decarboxylation of coproporphyrinogen III for cytochrome formation (7, 8). Recently, an open reading frame encoding a protein sharing some amino acid sequence homology to bacterial HemN proteins was discovered upstream of the *B. subtilis* dnaK operon (9). Clear differences from the otherwise highly conserved amino acid sequences and deduced molecular weights of other known HemN proteins raised the question of functional identity.

*B. subtilis* hemN complemented two *Salmonella typhimurium* hemF hemN double mutants under aerobic and anaerobic conditions. In order to test for hemN function the gene was cloned in the vector pOK12 and complementation experiments with two heme-requiring *S. typhimurium* hemF hemN mutants were performed. Primers hemN sacI start (5′-CCGGCTATGAAAGCCTGGCTTCAATTAGGCCC-3′) and hemNba (5′-GATACCTTGTGCTAATTAGCGAC-3′) corresponding to positions 1877 to 1911 and 3180 and 3204, respectively, of the recently cloned *B. subtilis* genomic region containing hemN (9; GenBank accession no. X91655) were used in a PCR reaction to generate a 1,325-bp fragment containing the complete 1,096-bp *B. subtilis* hemN and 141 bp of its 5′ and 88 bp of its 3′ region. The fragment containing SacI and XbaI restriction sites introduced by the primer sequences was cut with the appropriate enzymes, and the resulting 1,302-bp fragment was cloned into pOK12 (24) to generate the 3,369-bp pOKBshemN. Heme-deficient *S. typhimurium* TE3006 [env-53 hemN704::MudJ-b hemF707::Tn10k-Tet (27)] and TE 2849 [env-53 hemN704::MudJ-b hemF705 (27)] lacking the intact genes for the oxygen-dependent coproporphyrinogen III oxidase (hemF) and the oxygen-independent enzyme (hemN) were transformed with the newly constructed pOKBshemN containing *B. subtilis* hemN, pBlueN7 containing *E. coli* hemN (21), and pHem13 containing *E. coli* hemF (22) as positive controls and the vectors pOK12 and pBlueScript KS+ as negative controls via triparental mating. Transformants were subsequently screened aerobically and anaerobically for the recovery of heme sufficiency by plating on Luria-Bertani medium supplemented with 20 µg of kanamycin per ml and 10 µg of tetracycline per ml but without further addition of hemin (7, 21, 22). The cloned
B. subtilis open reading frame (pOKBhemN) was able to complement the S. typhimurium double mutants under aerobic and anaerobic conditions, indicating that the encoded protein is functionally related to the oxygen-independent coproporphyrinogen III decarboxylating enzyme. Therefore, the open reading frame will be referred to as hemN.

A B. subtilis hemN mutant accumulates coproporphyrinogen III under anaerobic conditions. To demonstrate the involvement of B. subtilis hemN in the metabolism of coproporphyrinogen III, the cellular porphyrin profiles of B. subtilis wild-type strain 1012 (leuA8 metB5 trpC2 hisM [14]) and the previously constructed hemN mutant (1012 with hemN:cat [9]), grown under aerobic and anaerobic conditions, were compared. The porphyrins were extracted, modified, and separated via high-performance liquid chromatography as described before (17). B. subtilis wild-type cells did not accumulate any significant amounts of porphyrins (Fig. 1). Porphyrins extracted from the hemN:cat mutant grown under anaerobic conditions showed a clear peak in the elution position of coproporphyrinogen III, indicating its accumulation in the cell (Fig. 1). These results demonstrated the direct involvement of the B. subtilis hemN gene product in the oxygen-independent metabolism of coproporphyrinogen III. The aerobically grown mutant did not accumulate significant amounts of any porphyrins, indicating the presence of an alternative coproporphyrinogen III oxidase system.

The B. subtilis hemN mutant showed unaffected aerobic and anaerobic growth. The hemN mutant (9) was investigated in comparison to its parental strain for growth capacities under aerobic and anaerobic conditions. The B. subtilis strains were grown aerobically and anaerobically at 37°C on Luria-Bertani medium supplemented with 20 mM K2PO4, pH 7, 2 mM (NH4)2SO4, 1 mM L-glutamic acid, 1 mM L-tryptophan, 0.8 mM L-phenylalanine, 0.005% (wt/vol) ammonium iron(III) citrate, 1 mM glucose, and 10 mM nitrate (7, 8). To our surprise the hemN mutant showed normal growth under aerobic and anaerobic conditions. The growth behavior of the B. subtilis hemN mutant was clearly different from that observed for other bacterial hemN mutants. The hemN mutants from S. typhimurium, Pseudomonas aeruginosa, and Alcaligenes eutrophus failed to grow under anaerobic conditions (11, 28). In B. subtilis additional enzymatic activities seemed to substitute for the mutated HemN under aerobic and anaerobic conditions. Since B. subtilis most likely does not possess any hemF analog (see below), a second hemN copy could substitute for the mutated hemN copy. For Helicobacter pylori, R. sphaeroides, and Synechocystis sp. the existence of two potential hemN genes has been described (1, 20, 29; GenBank accession no. D90904). For Rhizobium etli, disruption of hemN had no symbiotic effect (23). The complete DNA sequence of the B. subtilis chromosome might provide further insights into the nature of the supplementing oxygen-independent coproporphyrinogen III-metabolizing system.

Transcriptional analysis of hemN expression. The observed aerobic and anaerobic function of hemN requires appropriate expression of the hemN gene. The expression of E. coli and P. aeruginosa hemN was induced at the transcriptional level under anaerobic growth conditions (11, 21). To understand B. subtilis hemN transcription in detail, we first analyzed for the presence of hemN transcripts. In Northern blot experiments total RNA prepared from B. subtilis wild-type strain 1012 (14) was hybridized to digoxigenin-labelled hemN riboprobe RNA and revealed a weak band of about 7 kb (Fig. 2B, lane 1; for technical details see references 9, 10, and 25). When a lepA riboprobe was used, the same transcript and a very strong signal at about 1.8 kb were detected (Fig. 2C, lane 1). Where do these two transcripts start; where do they end? The potential transcriptional organization of the lepA operon and of the downstream dnaK operon is outlined in Fig. 2A (here, only the first four genes of the heptacstrionic operon are shown) (10). The lepA gene is preceded by a putative vegetative σ70-dependent promoter (denoted P1 in Fig. 2A); a potential rho-independent terminator T1 is located between lepA and hemN. This genomic organization can explain the origin of the 1.8-kb transcript which starts at P1 and is terminated at T1 (9). There is no obvious transcriptional terminator between hemN and hrcA, the first gene of the dnaK operon, and the next terminator T2 is located immediately downstream of dnaK (Fig. 2A). The distance between P1 and T1 is about 7 kb, and therefore, this transcript might result from a readthrough at the terminator structure T1 separating lepA from hemN. To prove these assumptions, total RNA was prepared from five different mutant strains (1012 with lepA::cat [9]; lepA = lepA::cat with replacement of lepA::cat by lepAΔ [9]; 1012 with hemN::cat [9]; hemNΔ = hemN::cat with replacement of hemN::cat by hemNΔ [9]; AS01 = 1012 with ΔhrcA [15]) and hybridized separately to hemN and lepA riboprobes. First, RNA from a hemN deletion strain (a 0.6-kb internal fragment has been removed) was analyzed. When the RNA preparation was probed with hemN only a very weak signal at 6.4 kb was detected (Fig. 2B, lane 2). The same signal was found stronger with the lepA riboprobe and, in addition, a strong band at 1.8 kb was observed (Fig. 2C, lane 2). Hybridization signals at the regions where the 16S and 23S RNA migrated occurred due to the trapping of mRNA degradation products by the rRNA rather than by nonspecific hybridization (10). Analysis of RNA
from a strain with a 0.7-kb deletion within hrcA (Fig. 2) shortened the 7-kb transcript to 6.3 kb, detectable with both riboprobe (lanes 3), while the 1.8-kb signal detected with lepA remained unchanged. These results are in perfect agreement with the assumptions made above. When the lepA gene was inactivated by insertion of a 1.3-kb cat cassette flanked by two strong rho-independent terminators no transcript was detected with either probe (lanes 4). Deletion of an internal 1.3-kb fragment from lepA shortened the long transcript detected by the hemN probe to 5.7 kb (Fig. 2B, lane 5), while the lepA riboprobe corresponding to the deleted fragment failed to detect any transcript (Fig. 2C, lane 5). Insertion of the cat cassette into hemN (Fig. 2) resulted in two signals revealed with the lepA probe, the strong one at 1.8 kb and a somewhat larger transcript terminated within the insertion (Fig. 2C, lane 6).

To verify the presence of promoter P_A1 and to demonstrate whether this promoter is used under both aerobic and anaerobic conditions, primer extension experiments were carried out using the primer lep-PEX (5'-TCGTTGAGTGATTGCAGCG-3') as outlined before (26). DNA sequencing reactions utilizing the same primer and plasmid pGH02 as the template (9) were carried out, and the sequencing products were separated on the same gel. One potential transcriptional start was mapped 37 bp upstream of the putative start codon of lepA and at an appropriate distance from a DNA sequence matching the consensus sequence for vegetative s^A-dependent promoters (Fig. 3, lane 1). This 5' end turned out to be identical under both aerobic and anaerobic growth conditions (compare lanes 1 and 2).

Taking all these results together, we concluded that indeed the lepA operon is preceded by a vegetative promoter, P_A1, from which expression of the operon is initiated. Most of the transcripts are terminated at the potential terminator structure (T_1) located between lepA and hemN, and a low amount of readthrough transcript extends into the downstream dnaK operon and is finally terminated between dnaK and dnaJ. There is only one potential transcription start site from which both transcripts originate, and this start site is used under aerobic and anaerobic growth conditions. To further substantiate the findings made by the primer extension experiments, namely that both the potential transcriptional start site and the amount of transcript remained unchanged during aerobic and anaerobic growth conditions, the amount of lepA- and hemN-specific transcripts were analyzed by slot blotting. It turned out that, with both riboprobes, the amounts of transcripts were the same (data not shown). In good agreement with the results obtained by primer extension and by slot blot experiments, immunoblotting using polyclonal anti-HemN antibodies revealed no signif-

FIG. 2. Northern blot analysis of transcripts from the B. subtilis hemN-containing operon. (A) Schematic representation of the chromosomal organization of the hemN-containing operon in B. subtilis wild type (row 1) and different mutants analyzed here (rows 2 to 6). The expected transcripts detected using the lepA probe (upper arrows) or the hemN probe (lower arrows) are indicated below each strain description. The regions of the mRNA molecules complementary to the riboprobes used are indicated by black bars. The chromosomal arrangement of the following strains is shown: B. subtilis wild type (row 1), hemNΔ (row 2), hrcAΔ (row 3), lepA::cat (row 4), lepAΔ (row 5), and hemN::cat (row 6). P_A1 indicates the promoter upstream of lepA responsible for hemN transcription; P_A2 indicates the position of the promoter upstream of hrcA; T labels potential rho-independent transcription terminators. (B) Hybridization of the RNA prepared from the strains described in panel A with the hemN-specific probe. (C) Hybridization of the same RNA preparations with the lepA-specific probe. On the left side of the blots, the positions of RNA molecules of the RNA molecular weight standard (Gibco BRL, Eggentein, Germany) run on the same gel are shown. Furthermore, nonspecific signals resulting from hybridization of the riboprobes with 16S and 23S rRNA are indicated; these were explained before in detail (17, 18). Lanes are labelled corresponding to the RNA sources described in panel A: 1, B. subtilis wild type; 2, hemNΔ; 3, hrcAΔ; 4, lepA::cat; 5, lepAΔ; 6, hemN::cat.
significant differences in the amount of HemN protein in cells grown under both growth conditions (data not shown). It has to be concluded that there is oxygen tension-independent expression of \( B. \) \textit{subtilis} \textit{hemN}.

Experimental approaches for identification of genes involved in alternative coproporphyrinogen III oxidase function. The \( \textit{hemN} \) mutant did not show any drastic growth phenotype indicating the presence of alternative enzymatic systems. Since most investigated heme-forming organisms contained a HemF-type coproporphyrinogen III oxidase, we first focused our attempts on the identification of a hem\( \text{F} \) analog from \( B. \) \textit{subtilis}. We aligned all known hem\( \text{F} \) sequences, deduced various primers from highly conserved amino acid sequence regions, optimized them by \( B. \) \textit{subtilis} codon usage for \( \textit{hem} \) genes, and used them in PCR reactions. While the PCR reactions yielded specific signals from a variety of bacteria, even with totally different codon usage, no specific signal was obtained with \( B. \) \textit{subtilis} genomic DNA (data not shown). Southern blot experiments under low stringent conditions using the \( E. \) \textit{coli} and \( P. \) \textit{aeruginosa} hem\( \text{F} \) genes as probes failed to detect any specific hybridization signal using \( B. \) \textit{subtilis} genomic DNA, while control experiments with an \( E. \) \textit{coli} hem\( \text{N} \) probe identified the known \( B. \) \textit{subtilis} \textit{hemN} gene (data not shown). In agreement with the Northern blot shown in Fig. 2 no obvious second hem\( \text{N} \) gene was detected with this method. Finally, searching the almost complete \( B. \) \textit{subtilis} genomic DNA sequence failed to reveal any obvious hem\( \text{F} \) analog. Interestingly, the very recently published \( H. \) \textit{pylori} genome does not contain any obvious hem\( \text{F} \) analog (20). On the basis of these experiments we would like to conclude that \( B. \) \textit{subtilis} does not possess a hem\( \text{F} \) analog. The potential second \( B. \) \textit{subtilis} hem\( \text{N} \) gene should possess structural differences responsible for the described Southern blot results.

To investigate whether the hem\( \text{Y} \) gene can substitute for hem\( \text{F} \) in vivo, complementation experiments with the \( S. \) \textit{typhimurium} hem\( \text{F} \) hem\( \text{N} \) double mutant were performed. Plasmid pBShemY containing the \( B. \) \textit{subtilis} hem\( \text{Y} \) gene with little of its 5' region and its 3' region in pBluescript KS+ (Stratagene) was constructed using PCR with the primers hem\text{Y}BSEcoRI (5'GCTGGAATTCCTTTGATGATTTGGCG-3') and hem\text{Y}BSBamHI (5'-GCAAAATGGATCTAACCTGTCGTTCAATT-3'). The obtained PCR fragment was digested with EcoRI and BamHI and cloned into pBluescript KS+ cut with the appropriate enzymes. After transformation of the strain with pBShemY and growth in the presence of heme, colonies were transferred to heme-deficient medium. Transformants failed to grow after 2 weeks of incubation at various temperatures. Control transformations with \( E. \) \textit{coli} hem\( \text{F} \) and hem\( \text{N} \) and \( B. \) \textit{subtilis} hem\( \text{N} \) lead to heme prototrophy and immediate bacterial growth. Obviously, \( B. \) \textit{subtilis} hem\( \text{Y} \) did not substitute for hem\( \text{F} \) or hem\( \text{N} \) in the \( S. \) \textit{typhimurium} background. However, these results do not exclude the participation of hem\( \text{Y} \) in coproporphyrinogen III metabolism in \( B. \) \textit{subtilis}.

This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to D.J. and W.S. and the Max-Planck-Gesellschaft, the Sonderforschungsbereich 395, and the Graduiertenkolleg Enzymchemie of the Philipps-Universität Marburg to D.J.

We are indebted to T. Elliott (West Virginia University, Morgantown) for the gift of several \( S. \) \textit{typhimurium} strains. We thank E. Bremer, R. Kappes, and M. Marahiel (Universität Marburg, Marburg, Germany) for many helpful discussions. We thank R. K. Thauer (Max-Planck-Institut Marburg, Marburg, Germany) for helpful discussions and continuous support.

REFERENCES


