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Oxygen-Controlled Regulation of the Flavohemoglobin Gene in *Bacillus subtilis*

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A gene, *hmp*, which encodes a ubiquitous protein homologous to hemoglobin was isolated among genes from *Bacillus subtilis* that are induced under anaerobic conditions. The *hmp* protein belongs to the family of two-domain flavohemoproteins, homologs of which have been isolated from various organisms such as *Escherichia coli*, *Alcaligenes eutrophus*, and *Saccharomyces cerevisiae*. These proteins consist of an amino-terminal hemoglobin domain and a carboxy-terminal redox active site domain with potential binding sites for NAD(P)H and flavin adenine dinucleotide. The expression of *hmp* is strongly induced upon oxygen limitation, and the induction is dependent on a two-component regulatory pair, ResD and ResE, an anaerobic regulator, FNR, and respiratory nitrate reductase, NarGHJI. The requirement of FNR and NarGHJI for *hmp* expression is completely bypassed by the addition of nitrite in the culture medium, indicating that *fnr* is required for transcriptional activation of *narGHJI*, which produces nitrite, leading to induction of *hmp* expression. In contrast, induction of *hmp* was still dependent on *resDE* in the presence of nitrite. A defect in *hmp* in *B. subtilis* has no significant effect on anaerobic growth.

The gram-positive bacterium *Bacillus subtilis* was shown to grow anaerobically in the presence of nitrate but not nitrite as a terminal electron acceptor (9, 11, 16, 32, 34, 36, 38). Genes involved in nitrate respiration in *B. subtilis* have been identified, and components of a regulatory pathway governing anaerobic gene expression are proposed in the accompanying paper (27). According to this model, the two-component signal transduction proteins, a histidine kinase, ResE; and a response regulator, ResD (17, 38); are required for nitrate respiration in part to activate transcription of *fnr*, an anaerobic gene regulator, from an *fnr*-specific promoter upon oxygen limitation (27). FNR thus produced activates the *narK-fnr* operon from a promoter located upstream of *narK*, encoding a protein with nitrite extrusion activity (9, 27). *fnr* is also required for the transcriptional activation of the *narGHJI* operon (9, 16), which encodes proteins homologous to the three subunits of *Escherichia coli* respiratory nitrate reductase and a polypeptide required for the assembly of the reductase complex (2–4). Putative FNR-binding sites were identified in the promoter region of *narK* and *narGHJI* (9). Mutations in *narGHJI* (16) as well as *moaA* which functions in the biosynthesis of molybdopterin, a precursor of the molybdenum cofactor of assimilatory and respiratory nitrate reductases (11), abolished nitrate respiration. In an attempt to uncover the regulation of gene expression responding to oxygen limitation, we have screened promoter activities specifically induced under anaerobic conditions. In this paper, we describe the isolation and characterization of one of these promoters. The activity identified is associated with a gene encoding a putative flavohemoglobin-like protein, the expression of which is induced by nitrite and requires the two-component regulators ResD and ResE.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture method. All *B. subtilis* strains used in this work are derivatives of JH642 (Table 1). The *E. coli* strains used were AG1574 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galUK* r^- m^+ *strA recA56 srl*] for general propagation of plasmids and MV1190 [Δ(*lac proAB*)*thi supE* Δ(*srl-recA*) *306*::Tn*10* (Tet^r)F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15] for α-complementation experiments. Integration plasmids pMMN13 (24) and pJDC9 (6), as well as a promoter-probe vector, pTKlac (21), were previously described. *fnr* and *resDE* mutants were constructed as previously described (9, 27, 38). Anaerobic growth on Luria-Bertani (LB) agar plates supplemented with 1% glucose and 0.2% KNO_3 was performed in an anaerobic jar with a gas-generating system (Becton Dickinson, Cockeysville, Md.). Anaerobic liquid culture was achieved by filling screw-cap tubes to the top with cells grown in 2× YT (yeast extract-tryptone) medium (25) with 1% glucose and 0.2% KNO_3.

Isolation of anaerobically induced (*ane*) genes. Promoter activities that are induced anaerobically were identified by screening transductants of *B. subtilis* ZB278 with an SPβ phage library containing *B. subtilis* chromosomal DNA fused to a promoterless *lacZ* gene (28, 44). Transductants were replica plated to duplicated LB agar plates supplemented with 1% glucose, 0.2% KNO_3 and 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. One set of plates was incubated in an anaerobic jar, and the other set was aerobically incubated. To score the Lac phenotype of cells grown anaerobically, agar plates were placed at 4°C for 0.5 to 1 h before detection of blue color. Alternatively, the plates were sprayed with the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside and were examined with a long-wavelength UV lamp (42). Among 3,000 transductants screened, four strains were shown to contain *lacZ* fusions that were induced under anaerobic conditions. One of these strains was used in this work as a donor to transfer the *B. subtilis* DNA fused to *lacZ* from SPβ to a plasmid replicon by a procedure described previously (45).

Isolation of *narGHJI* mutants. *narGHJI* mutants lacking respiratory nitrate reductase activity were identified among mutants defective in anaerobic growth (*ang*), which were isolated by transformation of *B. subtilis* JH642 with two plasmid libraries carrying *B. subtilis* chromosomal DNA. One library was constructed by a plasmid, pJPM1, (chloramphenicol resistance [Cm^r]) into whose *Sal*I site partial *Sau*3AI fragments (0.5 to 2.0 kb) of the *B. subtilis* chromosomal DNA were inserted (37). The other contains *Alu*I fragments (around 0.45 kb) inserted into the *Eco*RV site of pPS34 (erythromycin resistance [Erm^r]) (35). Transformants were replica plated to duplicated LB agar plates supplemented with 1% glucose, 0.2% KNO_3, and appropriate antibiotics. Integration of the plasmids into the *B. subtilis* chromosome by homologous recombination results in gene disruption when the cloned fragments contain an internal segment of a transcription unit. Chromosomal DNA from transformants which did not grow in an anaerobic jar was prepared and used to retransform JH642 to ensure the *ang* phenotype was due to the plasmid integration. Two thousand transformants with the pJPM1 library were tested, and 12 *ang* mutants were isolated. Eight *ang* mutants were isolated by screening 500 transformants with the pPS34 library.

TABLE 1. Characteristics of *B. subtilis* strains used in this study

Strain	Relevant characteristic	Source or reference
JH642	*trpC2 pheA1*	J. Hoch
ZB278	*trpC2* SPβ cured	45
LAB1703	*trpC2 pheA1* pMMN178 integrated to *hmp* region	This study
LAB1758	*trpC2 pheA1* pML1 integrated to *hmp* region	This study
LAB1845	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML6	This study
LAB1886	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML9	This study
LAB1938	*trpC2 pheA1 nar-2*	This study
LAB1940	*trpC2 pheA1 nar-4*	This study
LAB1955	*trpC2 pheA1 nar-15*	This study
LAB1962	*trpC2 pheA1 nar-21*	This study
LAB1992	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML25	This study
LAB2000	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML26	This study
LAB2026	*trpC2 pheA1* Δ*hmp*::Cm^r	This study
LAB2029	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML32	This study
LAB2111	*trpC2 pheA1 nar-15* SPβ*c2del2*::Tn*917*::pML26	This study
LAB2137	*trpC2 pheA1* Δ*resDE*::Tet^r SPβ*c2del2*::Tn*917*::pML26	This study
LAB2140	*trpC2 pheA1 fnr*::Spc^r SPβ*c2del2*::Tn*917*::pML26	This study
LAB2216	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML67	This study
LAB2237	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML70	This study
LAB2239	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML71	This study

Chromosomal DNA isolated from strains resulting from the second transformation was digested with restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, or *Pst*I) whose recognition sites were located on either side of the cloning sites to isolate flanking sequences of the original insert DNA. The restriction enzyme digests were ligated at a low DNA concentration to allow self-ligation, and the ligation mixtures were used to transform *E. coli* AG1574 to isolate plasmids carrying the *ang* DNA. These plasmids were subjected to double-stranded DNA sequence analysis by using reverse and T7 primers outside of the inserts.

Assay for nitrate reductase activity. For the respiratory nitrate reductase assay, cells were incubated aerobically until the optical density at 600 nm was 0.2 to 0.3, and 1 ml of cells was harvested (aerobic cultures). The remainder of the cultures were transferred to anaerobic conditions and were further incubated for 5 h without shaking before being harvested. Cells were washed with 0.2 M MOPS (morpholinopropanesulfonic acid) buffer (pH 7.0) and assayed for nitrate reductase activity as described previously (11).

Transformation and PBS1 transduction and β-galactosidase assay. Preparation of competent cells (12) and PBS1 transduction (15) were performed as previously described. Measurement of β-galactosidase activity was done as described in the accompanying paper, and the activity was expressed as specific activity per milligram of protein (27).

Primer extension analysis. Aerobically cultured JH642 cells were harvested at mid- to late-log growth phase (optical density at 600 nm of 0.8) to isolate RNA. Half of the culture was transferred to the anaerobic conditions and further incubated for 5 h before isolation of RNA. Isolation of RNA and primer extension analysis were performed as previously described (26). A 20-mer primer (5′-TCGATTGTTTTGTTATCTAA-3′) corresponding to just downstream of the ATG start codon of *hmp* was used for the primer extension analysis.

Construction of *hmp* deletion. A 2-kb fragment of pMMN179 isolated by digestion with *Bam*HI (followed by fill-in reaction) and *Hin*dIII was inserted into pJDC9 (Erm^r) to generate pML27 (Fig. 1). The 230-bp *Bgl*II-*Eco*RI fragment of *hmp* in pML27 was replaced with a Cm^r marker to generate pML31. A Cm^r Erm^s transformant (LAB2026) was chosen to ensure that the double-crossover recombination event occurred after transformation of JH642 with pML31.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank-EMBL-DDBJ nucleotide sequence databases under accession number D78189.

RESULTS

Isolation of *hmp*. In order to understand how gene expression is induced by anaerobiosis in *B. subtilis*, genes containing anaerobically induced promoters were identified by screening members of a *lacZ* fusion library. One anaerobically-expressed gene, *ane3*, which was the subject of this work, was found to encode flavohemoglobin, as shown later, and was named *hmp*. A plasmid containing the *hmp* promoter fused to *lacZ* was obtained by recombination in *B. subtilis* and then transformed into *E. coli* as described in Materials and Methods. Restriction enzyme analysis showed that the resultant plasmid, pMMN176, contains a 3-kb fragment of *B. subtilis* chromosome DNA (Fig. 1). The 3-kb insert DNA generated by digestion with *Xba*I, located in the multiple cloning site, was subcloned into pMMN13 (24) to give two plasmids, pMMN178 and 179, which have the insert fragment in both orientations. pMMN178 was used to transform JH642 cells, and a Cm^r transformant (LAB1703) was obtained by Campbell-type integration of the plasmid into the *hmp* locus. Chromosomal mapping of *hmp* was performed by PBS1 transduction with the Cm^r marker in *hmp*. *hmp* was found to reside at 118° on the genomic map with 91% linkage to *arg342*. Three factor crosses by transformation showed that the order of the genes in this region is *metC-arg342-hmp* (data not shown).

DNA sequence analysis of the 3-kb DNA revealed that the insert DNA in pMMN176 contains two intact open reading frames and a part of one open reading frame, all of which are transcribed in the same direction (Fig. 1). The regions between *orf1* and *orf2*, as well as *orf2* and *orf3*, contain putative factor-independent transcription terminators. *orf1* showed similarity to the gene encoding P14 of *E. coli* (31), which is adjacent to *tonB*, the product of which functions in siderophore-iron uptake.

orf2 (*hmp*) has strong similarity to the hemoglobin-like proteins from *E. coli* (40), *Alcaligenes eutrophus* (7), *Erwinia chrysanthemi* (10), *Vibrio parahaemolyticus* (23), and *Saccharomyces cerevisiae* (43) (Fig. 2). The N termini of these proteins also showed high levels of similarity to hemoglobin-like protein from *Vitreoscilla* sp. (22, 41). The amino acid sequence of the *orf3* product showed no significant similarity to known proteins.

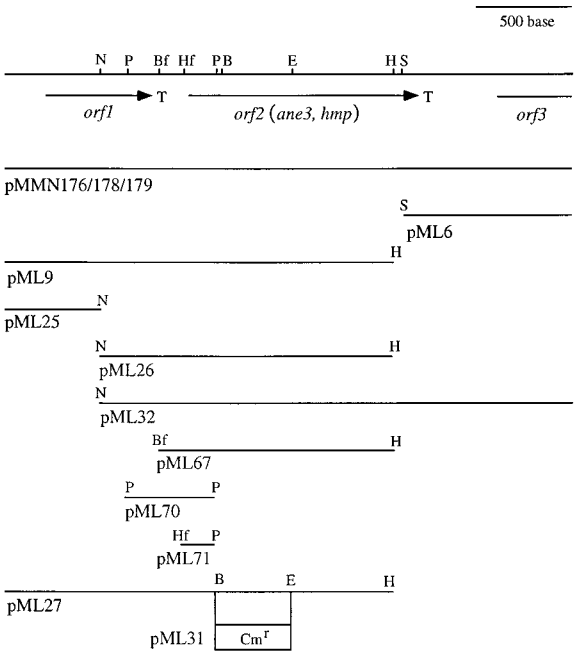

FIG. 1. Physical map of the *hmp* region. The restriction enzyme map is shown above an illustration depicting open reading frames. Putative transcription terminators are indicated as T. Also shown below by bars are inserts of plasmids. A box with Cm^r is chloramphenicol resistance marker used for replacement of an internal *hmp* fragment. B, *Bgl*II; Bf, *Bfa*I; E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; N, *Nco*I; P, *Pst*I; S, *Sac*I. Only relevant *Bfa*I and *Hin*fI sites are shown.

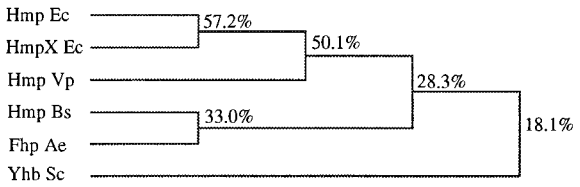

FIG. 2. Phylogenic analysis of the relationship of flavohemoglobin from *B. subtilis* (Hmp Bs), *A. eutrophus* (Fhp Ae), *E. coli* (Hmp Ec), *E. chrysanthemi* (HmpX Ec), *V. parahaemolyticus* (Hmp Vp), and *S. cerevisiae* (Yhb Sc). An automatic multiple alignment by Higgins-Sharp (14) was used, and calculated matching percentages are indicated at each branch point of the dendrogram.

Localization of the *hmp* promoter. The DNA sequencing analysis of the fragment bearing the *hmp* promoter activity showed that the fragment contains three open reading frames as described above. First, we determined with which coding region the *hmp* promoter activity is associated. In an attempt to localize the *hmp* promoter, various DNA fragments from the insert of pMMN176 were placed in front of a promoterless *lacZ* gene in plasmid pTKlac (Fig. 1). The plasmids were integrated into the SPβ prophage locus, and phage lysates carrying *lacZ* fusions were used to lysogenize JH642 cells as described in a previous paper (45). Table 2 shows the β-galactosidase activities of each strain before and 5 h after a shift to anaerobic conditions. There was very little, if any, oxygen-independent promoter activity detected in the insert of pML6 that contains the upstream region of *orf3*. Strains carrying pML9, pML26, and pML32, which contain the upstream DNA of *orf2*, showed promoter activity that was significantly induced by oxygen limitation. A promoter activity located upstream of *orf1* (pML25) was weakly induced by oxygen limitation. From these results, it is clear that the *hmp* promoter resides in front of *orf2*. β-Galactosidase activity was 30-fold reduced in the strain carrying pML32 compared with that of pML26 under anaerobic conditions. This indicates that most of the transcription initiating from the *hmp* promoter ends at the putative transcription terminator located between *hmp* (*orf2*) and *orf3* and that the original *lacZ* activity fused to *orf3* (in pMMN176) is the result of the read-through transcription that traverses the terminator sequence.

Primer extension analysis. To determine the transcriptional start site of *hmp*, primer extension analysis was performed with RNA isolated from cells cultured under aerobic and anaerobic conditions. Weak signals were observed with RNA from both aerobic and anaerobic cultures, and strong induction of transcription from another start site was detected under anaerobiosis (Fig. 3). The −10 region of the *hmp* promoter showed similarity to the −10 hexamer recognized by σ^A (four matches out of six), but no consensus −35 sequence was detected when an optimal spacing of 16 bp between −10 and −35 was assumed. There is a sequence similar to −35 (four matches out of six) created by extension of the spacing to 23 bp (Fig. 4).

Deletion analysis of *hmp* promoter. For the purpose of identifying a sequence required for induction of *hmp* transcription, DNA carrying the *hmp* promoter was further deleted from the 5′ or 3′ direction. The resulting deleted DNA was fused to *lacZ* as described above. β-Galactosidase activity of the deletion-mutated promoter derivatives showed that removal of DNA to −118 bases from the transcription start site (*Bfa*I site [pML67]) has no significant effect on anaerobic *hmp* expression compared with promoters carrying more upstream sequences (pML26), although aerobic expression was increased in pML67, which may indicate that a *cis* site for aerobic repression was removed by the deletion (Table 2 and Fig. 4). A deletion to −49 (*Hin*fI [pML71]) caused a severe reduction in *hmp* expression under anaerobiosis, although the basal level of expression was not significantly affected. These results suggest that the sequence between −118 and −49 shown in Fig. 4 is primarily responsible for anaerobic induction of *hmp* expression.

Regulation of *hmp* expression. A two-component regulatory pair, *resD* and *resE* (38), an anaerobic gene transcription reg-

TABLE 2. β-Galactosidase activity of *lacZ* fused to DNA fragment from *hmp* region

Strain	Plasmid	β-Galactosidase sp act/mg of protein[a]		Induction ratio[b]
		Aerobic	Anaerobic	
LAB1845	pML6	3.7	4.7	1.3
LAB1886	pML9	13.4	10,335	771
LAB1992	pML25	64.6	189.7	2.9
LAB2000	pML26	9.2	8,773	954
LAB2029	pML32	2.6	296.7	114
LAB2216	pML67	75.7	10,928	144
LAB2237	pML70	14.6	8,659	593
LAB2239	pML71	26.4	197.8	7.5

[a] The specific β-galactosidase activity was determined under aerobic or anaerobic (5 h after the shift) conditions as previously described (27). Each value is the average of duplicate samples from three independent cultures. The average standard error is 25%.

[b] Induction ratio shows β-galactosidase activities under anaerobic conditions divided by those under aerobic conditions.

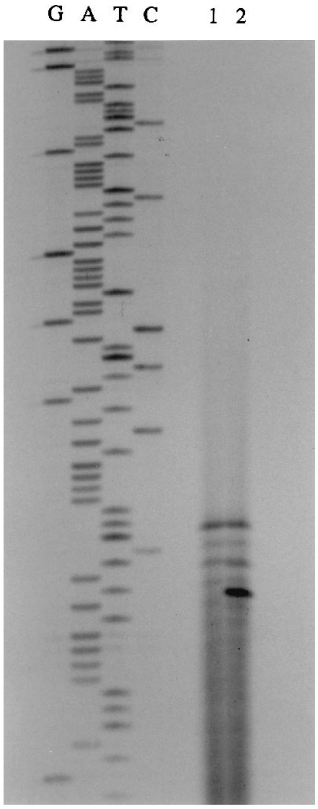

FIG. 3. Determination of the transcription initiation site of *hmp* by primer extension analysis. Total RNA was isolated from aerobic cultures (lane 1) and from anaerobic cultures (lane 2). Also shown is a sequence ladder (GATC) of noncoding DNA determined with the primer used for primer extension analysis.

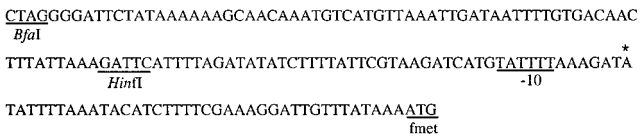

FIG. 4. DNA sequence of the *hmp* promoter region. The transcription start site determined by primer extension analysis in Fig. 3 is shown as an asterisk. The −10 region is underlined, and a putative translation initiation site (fmet) is shown. Two restriction enzyme sites, *Bfa*I and *Hin*fI, used for deletion analysis in Fig. 1 and Table 2 are marked.

ulator, *fnr* (9), and respiratory nitrate reductase encoded by *narGHJI* (9, 16) were shown to be required for nitrate respiration in *B. subtilis*. Since *hmp* expression is strongly induced by oxygen limitation, we attempted to determine if the genes required for anaerobic growth are also necessary for anaerobic induction of *hmp*. Strains carrying the *resDE* deletion and *fnr* mutation were constructed as described in the accompanying paper (27). Respiratory nitrate reductase mutants were isolated as follows. With plasmid integration libraries, *ang* mutants unable to grow anaerobically in the presence of nitrate were isolated as described in Materials and Methods. Four mutants (LAB1938, 1940, 1955, and 1962) were found to have undergone a plasmid insertion into the *narGHJI* operon, as shown by sequence analysis and comparison with the reported DNA sequence of *narGHJI* (9, 16). It was shown that the original chromosomal DNA insert of the plasmid integrated into LAB1938 was a 573-bp *Sau*3AI fragment extending to the 3′ end of *narG* and the 5′ end of *narH*. The insert of the plasmid integrated into LAB1940 and was a 1,051-bp *Sau*3AI fragment from *narH* and *narJ*. In addition, the inserts of LAB1955 and LAB1962 were identified as a 591-bp *Alu*I fragment of *narG* and a 483-bp *Alu*I fragment, including *narH* and *narJ*. These results show that LAB1955 cells have an integration mutation in *narG*, LAB1938 has one in *narH*, and LAB1940 and LAB1962 have one in *narJ*. Respiratory nitrate reductase activity of the integration mutants and an isogenic wild-type strain (JH642) was measured (Table 3). No respiratory nitrate reductase activity was detected from any strain cultured under aerobic conditions, confirming the result previously shown (11). After a shift to an anaerobic condition, nitrate reductase activity was induced in JH642 cells, but very little activity (in LAB1940 and LAB1962) or no activity (in LAB1938 and LAB1955) was detected in the mutant cells.

To examine the effects of *resDE*, *fnr*, and *narGHJI* mutations on *hmp* expression, the mutant strains were lysogenized with an SPβ phage carrying pML26. Measurement of β-galactosidase activity indicated that *hmp-lacZ* expression in the wild-type strain (LAB2000) was induced around 30 min after anaerobic shift and that the maximal induction was 10-fold lower in the Δ*resDE* cells than that observed in wild-type cells (Fig. 5A). Mutations in *fnr* and *narG* completely abolished *hmp-lacZ* expression. The putative *cis*-regulatory region identified above does not contain a consensus FNR-binding site which is detected in the promoter region of the *narK-fnr* and *narGHJI* operons (9). This suggests that the requirement of *fnr* for *hmp* transcription is probably indirect. The defect in *hmp* induction in the *narG* mutant is due to the loss of respiratory nitrate reductase activity, since the mutation in *narA* (11) (a *moaA* homolog which is required for synthesis of nitrate reductase cofactor) also abolished *hmp-lacZ* expression (data not shown). This result prompted us to determine if nitrite, the product of nitrate reduction, can suppress the defect in *hmp* expression caused by loss of nitrate reductase activity. As shown in Fig. 5B, *hmp* expression in the *narG* mutant during anaerobic growth is completely restored by the addition of nitrite to 2× YT medium supplemented with glucose and nitrate. The same level of *hmp-lacZ* expression was observed when nitrite was added without nitrate (data not shown). No β-galactosidase activity was detected in a *narG* mutant carrying pML67, and the activity was again fully restored by the addition of nitrite (data not shown). This indicates that the sequence shown in Fig. 4 is sufficient for nitrite induction under anaerobiosis. Since we have shown in the accompanying paper that *narGHJI* expression is dependent on *resDE* and *fnr* (27), we attempted to determine if the function of *resDE* and *fnr* in *hmp* expression is to activate *narGHJI* expression. *hmp-lacZ* was induced at the wild-type level in the *fnr* mutant by addition of nitrite, but the expression was still dependent on *resDE* (Fig. 5B). The conclusion drawn from these experiments is that the function of *fnr* and *narGHJI* in *hmp* induction is to stimulate the production of nitrite, which itself or in combination with an unidentified regulator acts as an inducer. The additional function of *resDE* in *hmp* expression remains to be elucidated.

Next we addressed the possibility that nitrite is able to induce *hmp* expression in the presence of oxygen. A half hour after addition of nitrite, β-galactosidase activity was induced 6-fold (Fig. 5C) compared with the 1,000-fold induction of the same fusion observed under anaerobic conditions. The aerobic induction by nitrite is not dependent on *narG*, which is not expressed under aerobic conditions, as shown by the result that the same induction was observed in the *narG* mutant and that nitrate was unable to induce *hmp* in wild-type cells.

Mutant phenotype of *hmp*. In order to examine the function of *hmp*, LAB2026 cells carrying a deletion of part of the *hmp* gene were incubated anaerobically in the presence of nitrate. LAB2026 cells were observed to grow as well as JH642 cells anaerobically, and *hmp-lacZ* activity in the two strains showed no significant difference under both aerobic and anaerobic conditions (data not shown). This result shows that *hmp* is not required for either nitrate respiration or its own expression.

DISCUSSION

The gene (*hmp*) which encodes flavohemoglobin was isolated from *B. subtilis* as a gene induced under oxygen limitation. Expression of the *B. subtilis hmp* gene is induced by limited oxygen supply, and the anaerobic induction was shown to be dependent on *fnr*. However, as we have shown in this paper, FNR probably does not directly activate *hmp* expression by binding to the promoter region; instead, *fnr* was shown to be required for expression of *narGHJI*, which results in the production of nitrite, the apparent inducer of *hmp* transcription. Nitrite can induce *hmp* expression even under aerobic conditions; however, the expression was shown to be further induced

TABLE 3. Respiratory nitrate reductase activity from *B. subtilis* strains

Strain	Nitrate reductase activity (nmol of nitrite/mg of protein/min)[a]	
	Aerobic	Anaerobic
JH642	<0.01	74.9
LAB1938	<0.01	<0.01
LAB1940	<0.01	1.4
LAB1955	<0.01	<0.01
LAB1962	<0.01	5.5

[a] Nitrate reductase activity was measured under aerobic or anaerobic (5 h after the anaerobic shift) conditions as previously described (11). Data shown are averages of those obtained from two independent experiments.

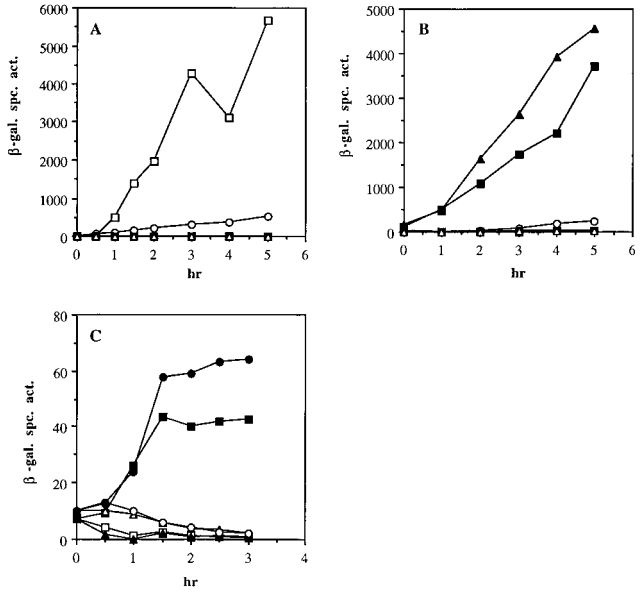

FIG. 5. (A) Effect of mutations on *hmp-lacZ* expression. SPβ phage carrying pML26 was lysogenized to various strains. Cells were cultured aerobically in 2× YT medium with 1% glucose and 0.2% KNO_3 and transferred to anaerobic conditions at zero hour. Samples were taken at intervals for measurement of β-galactosidase activity as previously described (27). □, LAB2000 (wild type); △, LAB2111 (*nar-15*); ○, LAB2137 (Δ*resDE*); ■, LAB2140 (*fnr*). (B) Effect of nitrite on *hmp-lacZ* expression. Cells were cultured aerobically in 2× YT medium with 1% glucose and 0.2% KNO_3 with or without 0.05% KNO_2 and transferred to anaerobic conditions at zero hour. ■ and □, LAB2111 (*nar-15*) with (■) or without (□) KNO_2; ● and ○, LAB2137 (Δ*resDE*) with (●) or without (○) KNO_2; ▲ and △, LAB2140 (*fnr*) with (▲) or without (△) KNO_2. (C) Effect of nitrite on *hmp-lacZ* expression under aerobic conditions. Cells were cultured in 2× YT medium with 1% glucose. KNO_3 (0.2%) or KNO_2 (0.05%) was added at zero hour when indicated. Symbols represent LAB2000 (wild type) without addition (□), with KNO_3 (▲), and with KNO_2 (■) and LAB2111 (*nar-15*) without addition (△), with KNO_3 (○), and with KNO_2 (●).

by anaerobiosis. This suggests that there are two regulatory pathways which activate *hmp* transcription. One is a nitrite-dependent pathway which requires ResDE, FNR, and NarGHJI under anaerobic conditions. Addition of nitrite in culture media can mimic this condition under aerobic conditions. *hmp* is also regulated by the FNR-independent, NarGHJI-independent, and ResDE-dependent pathway that is responsible for further anaerobic induction. The latter pathway is strictly dependent on oxygen limitation, and other conditions such as stationary growth phase are not able to induce *hmp* transcription (data not shown). Deletion of the *hmp* promoter up to position −49 caused a severe reduction in *lacZ* activity under anaerobic conditions (pML71 has 2% of the activity of that observed in pML67 and 70), indicating that the region between −118 and −49 is primarily important for anaerobic induction of *hmp*. However, pML71 still maintains a low but significant level of anaerobic regulation; therefore, there must be another *cis* region downstream of −49 for this residual regulation. Mutational analysis of the *cis*-acting sequences as well as isolation of a *trans*-acting factor(s) will further our understanding of the anaerobic regulation of *hmp* in *B. subtilis*. Most of the (flavo)hemoglobin genes in other bacteria, thus far examined, are also induced during growth under oxygen limitation. The anaerobic regulator FNR was shown to function in the induction of the expression of the *Vitreoscilla* hemoglobin gene in *E. coli* (20, 39). Two potential binding motifs for FNR and NARL (nitrate regulator) were identified upstream of *fhp* in *A. eutrophus* (7). There is a potential FNR-binding site downstream of the putative *hmp* promoter, suggesting that FNR may be a transcriptional repressor for *hmp* in *E. coli* (29), and a recent study showed that *hmp* expression is indeed negatively regulated by FNR under anaerobic conditions (30). In these studies, it was shown that *hmp* expression in *E. coli*, which was barely induced by oxygen limitation in wild-type cells, was stimulated by nitrate, nitrite, and nitric oxide (30).

Hemoglobin-like proteins have been isolated from many microorganisms, including *Vitreoscilla* sp. (22, 41), *E. coli* (40), *A. eutrophus* (7), *E. chrysanthemi* (10), *V. parahaemolyticus* (23), *S. cerevisiae* (43), and *Candida norvegenesis* (19). The hemoglobin-like proteins from these organisms, except *Vitreoscilla* sp., have an extra C-terminal domain homologous to the ferredoxin $NADP^+$ reductase proteins (1). The *B. subtilis hmp* protein also has conserved residues for binding of flavin adenine dinucleotide and NAD(P)H, as well as the glutamine residue at position 53 and the histidine residue at position 85, which are presumed to be the distal and proximal hem ligands, respectively. Several possible functional roles of this ubiquitous protein have been proposed; i.e., the protein may function in oxygen storage or diffusion (33, 41), or alternatively, the hem domain, by binding to oxygen, may undergo a change in its conformation, thereby activating functions of the C-terminal reductase domain of the protein (29, 43). An obvious way to uncover the physiological function of unknown genes is to examine the phenotype of a null mutant. Mutations in hemoglobin-like genes have been reported in several organisms. No phenotypic differences were found between *S. cerevisiae* wild-type and mutant strains (8). *hmpX* mutants of *E. chrysanthemi*, a plant pathogen, grew as well as wild-type cells under aerobic and microaerobic conditions, but the mutants are nonpathogenic (10). The *fhp* mutation in *A. eutrophus* was shown to have no effect on aerobic and anaerobic growth; however, the mutant did not show transient accumulation of nitrous oxide, as observed in the wild-type cells during denitrification with nitrite as an electron acceptor (7). The authors suggested that FHP acts as a nitric oxide reductase, even though known membrane-bound nitric oxide reductases (5, 13) clearly differ from hemoglobin-like proteins and the mutant strain can convert nitrite to dinitrogen even slightly faster. The involvement of flavohemoglobin in production of nitrous oxide requires further examination; however, it is intriguing that a recent report by Poole and coworkers has shown that nitric oxide induces *hmp* expression in *E. coli*, and they proposed that nitric oxide might be the actual signal molecule for *hmp* induction (30). Another observation which may indicate the involvement of *hmp* in formation of nitrous oxide came from the result that the hem domain was shown to react with nitrite in the oxidized form and that reduction of the nitrite complex in the absence of oxygen generates a hem-nitrosyl complex (18). It is not known, at present, if *B. subtilis*, which cannot use nitrite as an alternative electron acceptor, has a denitrification pathway; however, this possibility is worth examining, since *hmp* expression is induced by nitrite, as shown in this work.

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