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# Nitric Oxide, Nitrite, and Fnr Regulation of *hmp* (Flavohemoglobin) Gene Expression in *Escherichia coli* K-12

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***Escherichia coli* possesses a soluble flavohemoglobin, with an unknown function, encoded by the *hmp* gene. A monolysogen containing an *hmp-lacZ* operon fusion was constructed to determine how the *hmp* promoter is regulated in response to heme ligands ($O_2$, NO) or the presence of anaerobically utilized electron acceptors (nitrate, nitrite). Expression of the Φ(*hmp-lacZ*)*1* fusion was similar during aerobic growth in minimal medium containing glucose, glycerol, maltose, or sorbitol as a carbon source. Mutations in *cya* (encoding adenylate cyclase) or changes in medium pH between 5 and 9 were without effect on aerobic expression. Levels of aerobic and anaerobic expression in glucose-containing minimal media were similar; both were unaffected by an *arcA* mutation. Anaerobic, but not aerobic, expression of Φ(*hmp-lacZ*)*1* was stimulated three- to four-fold by an *fnr* mutation; an apparent Fnr-binding site is present in the *hmp* promoter. Iron depletion of rich broth medium by the chelator 2′2′-dipyridyl (0.1 mM) enhanced *hmp* expression 40-fold under anaerobic conditions, tentatively attributed to effects on Fnr. At a higher chelator concentration (0.4 mM), *hmp* expression was also stimulated aerobically. Anaerobic expression was stimulated 6-fold by the presence of nitrate and 25-fold by the presence of nitrite. Induction by nitrate or nitrite was unaffected by *narL* and/or *narP* mutations, demonstrating regulation of *hmp* by these ions via mechanisms alternative to those implicated in the regulation of other respiratory genes. Nitric oxide (10 to 20 μM) stimulated aerobic Φ(*hmp-lacZ*)*1* activity by up to 19-fold; *soxS* and *soxR* mutations only slightly reduced the NO effect. We conclude that *hmp* expression is negatively regulated by Fnr under anaerobic conditions and that additional regulatory mechanisms are involved in the responses to oxygen, nitrogen compounds, and iron availability. Hmp is implicated in reactions with small nitrogen compounds.**

*Escherichia coli* is generally considered to consume oxygen by using two membrane-bound terminal oxidases for aerobic respiration, cytochromes *bo′* and *bd* (15, 36). Cytochrome *bo′* is a member of the heme-copper superfamily of terminal oxidases; it is a proton pump (42) and has a moderately high affinity for oxygen, with a $K_m$ in the submicromolar range (11). In contrast, cytochrome *bd* uses a heme-heme binuclear center to bind oxygen as a surprisingly stable oxygenated form and reduce oxygen to water (20, 39). Cytochrome *bd* is believed not to be a proton pump but has an extraordinarily high apparent affinity for oxygen, with a $K_m$ in vivo as low as 5 nM (12). The distinct properties of these oxidases, and thus their suitability for growth under different aerobic conditions, requires that they be differentially regulated. Cytochromes *bo′* and *bd* are maximally synthesized during growth with high (4) or limited (14) aeration, respectively. Expression of operons comprising the oxidase structural genes (*cyoABCDE* and *cydAB*, respectively) are each affected by Fnr and ArcA/ArcB, although dissection of the direct or indirect roles of these regulators requires further study (15). Oxygen consumption by cytochrome *bd* is subject to substrate (i.e., oxygen) inhibition (12), so that regulation of oxidase activity may also modulate electron flux through this branch (36). A second cytochrome *bd*-type oxidase (*bd*-II) is encoded by *appBC* (51).

In addition to these respiratory membrane-bound oxidases, *E. coli* contains a soluble flavohemoglobin, encoded by the *hmp* gene (54). The amino-terminal domain of this protein is predicted, from DNA sequence analysis, to be homologous to the globins of animals, plants, and other microorganisms. Hmp binds oxygen to the high-spin ferrous heme to form an oxygenated complex (21) that is moderately stable (38). However, unlike most globins, Hmp consumes both NAD(P)H and oxygen and is thus a fourth oxidase in *E. coli*. We have proposed, on the basis of the redox behavior of heme and the intrinsic flavin adenine dinucleotide during oxygen depletion, that Hmp could act as an oxygen sensor (38). As required by this model, Hmp reduces Fe(III) (1, 13, 32) and cytochrome *c* (37), and reductase activity is modulated by the binding of oxygen (37). The ability of Hmp to generate superoxide anion during oxygen reduction (32) and to form readily a nitrosyl complex at the heme (21) may also be relevant to its physiological function. Two-domain flavohemoglobins strikingly similar to Hmp are also known in *Saccharomyces cerevisiae*, *Candida* spp., *Alcaligenes eutrophus*, and several other bacteria (1, 30, 36). Simpler, single-domain globins have also been reported in bacteria and protozoa (39). The function of none of these is known.

The goal of this work was to understand how *hmp* expression is regulated in the belief that this will help to identify a physiological function. We report the use of a chromosomal operon fusion to study *hmp* expression under various conditions of oxygen supply and in the presence of electron acceptors other than oxygen. Our results demonstrate that *hmp* expression is

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TABLE 1. Strains, plasmids, and phages used in this study

| Strain, plasmid, or phage | Relevant genotype | Reference or source |
|---|---|---|
| *E. coli* strains | | |
| BW831 | *soxS3*::Tn*10* Δ*(argF-lac)*169 *rpsL sup* (Am) | 52 |
| BW949 | *soxR9*::*cat* Δ*(argF-lac)*169 *rpsL sup*(Am) | 52 |
| ECL585 | *arcA1 zjj*::Tn*10* | 23 |
| MK1010 | IN*(rrmD-rrnE)1 cya*::Km$^r$ | 28 |
| RK4353 | *araD139* Δ*(argF-lac)U169 gyrA219 non-9 rpsL150* | 47 |
| RK5278 | Same as RK4353 but *narL215*::Tn*10* | 47 |
| RKP2161 | XL1 Blue/pPL341 | This work |
| RKP2178 | Same as VJS676 but Φ *(hmp-lacZ)1* | This work |
| RKP2181 | Same as RKP2178 but *narP253*::Tn*10*d(Cm) | This work |
| RKP2182 | Same as RKP2178 but *narL215*::Tn*10* | This work |
| RKP2185 | Same as RKP2178 but *fnr-271*::Tn*10* | This work |
| RKP2193 | Same as RKP2178 but *narL215*::Tn*10* *narP253*::Tn*10*d(Cm) | This work |
| RKP2195 | Same as RKP2178 but *arcA1* | This work |
| RKP3965 | Same as RKP2178 but *soxS* | This work |
| RKP3966 | Same as RKP2178 but *soxR* | This work |
| RKP4018 | Same as RKP2178 but *cya* | This work |
| VJS676 | Δ*(argF-lacZ)U169* | Stewart collection |
| VJS1741 | *fnr-271*::Tn*10* | Stewart collection |
| VJS3046 | Φ*(frdA-lacZ)* | 43 |
| VJS4322 | *narP253*::Tn*10*d(Cm) | Stewart collection |
| XL-1 Blue | *recA1* | Stratagene |
| Plasmids | | |
| pPL341 | *hmp*$^+$ in 1.5-kb *Eco*RI-*Bam*HI fragment in pBR322, Ap$^r$ | 54 |
| pRS528 | Ap$^r$ *lacZ*$^+$ *lacY*$^+$ *lacA*$^+$ | 45 |
| Phages | | |
| λRKP1 | Φ*(hmp-lacZ)1* (gene fusion) | This work |
| λRS45 | *bla'-lacZ*$_{SC}$ *att*$^+$ *int*$^+$ *imm*$^{21}$ | 45 |

negatively regulated by the global transcriptional regulator Fnr. Although *hmp* expression is positively regulated by nitrite and nitric oxide, the mechanisms for regulation are distinct from those identified thus far.

## MATERIALS AND METHODS

**Strains, plasmids, and phage.** The *E. coli* K-12 strains, plasmids, and λ specialized transducing bacteriophage used in this study are described in Table 1. Genetic crosses were performed by bacteriophage P1-mediated transduction (33). Standard methods were used for restriction endonuclease digestion and ligation of DNA (31, 44). Plasmid DNA was isolated by using the Wizard column system from Promega (Madison, Wis.). DNA fragments were isolated from agarose gels with the Prep-a-Gene kit (Bio-Rad, Hercules, Calif.). Transformation of bacteria with plasmid DNA was done by the single-step method of Chung et al. (3). Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc. (Beverly, Mass.), or Promega. DNA polymerase (*pfu*) was from Stratagene (La Jolla, Calif.).

**Culture media.** Cells were grown in LB or TY broth at an initial pH of 7.0 (33) or in 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered minimal medium with 40 mM glucose as the sole carbon source (48). Other carbon sources, specified in Results, were added to 40 mM. The initial pH of MOPS-buffered medium was 7.8. The *arcA* phenotype was determined on solid medium containing toluidine blue (22). Kanamycin, chloramphenicol, and ampicillin were used at final concentrations of 50, 25, and 150 μg/ml, respectively. Agar and other dehydrated media were from Difco or Oxoid Ltd. Other components were generally from Sigma.

**Culture conditions.** Culture turbidities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a no. 66 (red) filter. Alternatively, culture optical density was measured with a Pye-Unicam SP6-550 spectrophotometer at 600 nm by, using culture samples diluted with medium to bring the optical density at 600 nm to below 0.7 when measured in cells with a 1-cm path length. All cultures were grown at 37°C. Aerated cultures were grown with shaking (200 r.p.m.) in conical flasks containing 1/25 to 1/10 of their own volume of medium. Anaerobic cultures for β-galactosidase assays were grown in screw-cap glass tubes (48) filled to the brim and containing two glass beads (approximately 1-mm diameter) to aid resuspension of cells that had sedimented during static culture. All cultures were grown to the exponential phase of growth, unless otherwise specified. At the required culture density (about 50 Klett units or the equivalent optical density at 600 nm), chloramphenicol (100 μg/ml) or spectinomycin (for chloramphenicol-resistant strains; 300 μg/ml) was added and incubation was continued for a further 5 min before harvest to prevent adaptation to anaerobiosis or other change.

**Culture medium supplements.** $NaNO_3$ or $NaNO_2$ was added as an autoclaved solution to MOPS-buffered media at a final concentration of 40 or 5 mM, respectively.

Nitric oxide (NO) was generated in a reaction train that had previously been thoroughly purged with nitrogen gas. A concentrated solution of sodium nitrite was dropped slowly from a funnel into concentrated sulfuric acid. The NO thus generated was passed through a series of three Drechsel bottles with sintered glass bubblers; the first two contained 10 M-NaOH (to absorb any acid carried over with the gas), and the third contained distilled water (to wash the NO). The NO was then bubbled into water until the solution was saturated. From calibration experiments, this was known to be complete in 30 min. The container was fitted with a Suba-Seal closure with a turnover rubber flange (Merck Ltd.; Poole, United Kingdom) under nitrogen, and samples were removed with a Hamilton syringe as required. The concentration of the NO was noted from tables of concentrations of saturated solutions at various temperatures (approximately 1.8 mM at room temperature). Solutions were used within 2 days of preparation. The treatment of aerobic cultures with NO followed broadly that described by Nunoshiba et al. (35). Erlenmeyer flasks (250 ml) fitted with Suba-Seals contained 25 ml of TY medium. An overnight culture in the same medium was diluted 1 in 100 into the flasks, and growth was allowed to proceed for 45 min until cells were growing exponentially. NO solution was injected, and growth was allowed to continue for a further 1 h. The treatment of anaerobic cultures with NO was done by injection of a solution of NO into a culture in a full tube capped with a Suba-Seal closure. Cultures were treated with chloramphenicol for 10 min before harvest as described above.

**β-Galactosidase assay.** Assays were carried out at room temperature, around 21°C. Cell pellets were suspended in 2.5 to 4 ml of Z buffer (33) and stored on ice. β-Galactosidase activity was measured in $CHCl_3$- and sodium dodecyl sulfate-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in terms of the optical density at 600 nm of cell suspensions by using the formula of Miller (33). Each culture was assayed at least in triplicate; typically, these values gave a coefficient of variation (mean divided by the standard deviation) of <5%. All results were confirmed in at least two independent experiments.

**Construction of an *hmp* operon fusion.** An operon fusion of *hmp* to *lacZ*, Φ*(hmp-lacZ)1*, was constructed on a plasmid and then transferred to λ phage by recombination in vivo by the method of Simons et al. (45). A 635-bp DNA fragment was excised from pPL341 by using *Bam*HI and *Sma*I and ligated into the site created by digestion of pRS528 with *Sma*I and then *Bam*HI. The required recombinant plasmid was isolated by transformation of strain RK4353 (Δ*lac*). The fusion was recombined onto λRS45 to make λRKP1. Several single-copy fusions to the chromosome of VJS676 (Δ*lac*) were isolated and verified by using β-galactosidase assays and Ter tests as described before (50). One such fusion strain (RKP2178) was used for the experiments described in this report, but other fusions gave similar results. Mutant alleles transduced into RKP2178 (Table 1) were *arcA* (ECL585), *narL* (RK5278), *fnr* (VJS1741), *narP* (VJS4322), *soxR* (BW949), and *soxS* (BW831).

## RESULTS

**Effects of medium composition and mutant alleles on aerobic Φ*(hmp-lacZ)1* expression.** Cellular levels of Hmp are too low for it to be detected spectrally under most conditions, and there is no known specific assay for the protein's activity. Therefore, a *hmp-lacZ* operon fusion was used to monitor *hmp* expression under various growth conditions. Strain RKP2178 carries a single-copy operon fusion that comprises 339 bp of the coding region of *hmp* and the promoter region up to 296 bp upstream of the translational start site. Aerobic expression of Φ(*hmp-lacZ)1* in MOPS defined medium was similar when glycerol, maltose, or sorbitol were present as the sole carbon source (Table 2). Expression was slightly but reproducibly lower when glucose was provided as the carbon source. Furthermore, deletion of *cya* (encoding adenyl cyclase) had no

TABLE 2. Expression of Φ(*hmp-lacZ*)*1* in cultures grown aerobically in various media[a]

| Carbon source | β-Galactosidase sp act[b] with: | | |
|---|---|---|---|
| | $O_2$ | $O_2 + NO_3^-$ | $O_2 + NO_2^-$ |
| Glucose | 46 | 44 | 74 |
| Maltose | 56 | ND[c] | ND |
| Sorbitol | 64 | ND | ND |
| Glycerol | 64 | ND | ND |

[a] Strain RKP2178 was cultured aerobically in MOPS defined medium containing the indicated carbon source (see Materials and Methods for details).
[b] Miller units.
[c] ND, not determined.

significant effect on Φ(*hmp-lacZ*)*1* expression during growth in either complex or defined media (data not shown).

The effects of culture pH were investigated by using LB medium adjusted to different initial pH values between 5 and 9. After 3 h of growth, the corresponding pH values were 5 to 8.3. In all cultures, β-galactosidase activity did not vary by more than 10% of the mean value (data not shown).

The addition of nitrate to aerobic cultures was without effect on expression of Φ(*hmp-lacZ*)*1* in either defined (glucose; Table 2) or complex (data not shown) medium, but 5 mM nitrite enhanced aerobic expression (Table 2). These data were confirmed with a second, independently isolated, single-copy lysogenic derivative of VJS676.

**Effects of electron acceptors and regulatory mutations on anaerobic Φ(*hmp-lacZ*)*1* expression.** Under anaerobic conditions (Table 3), the expression of Φ(*hmp-lacZ*)*1* was elevated by about 30% above the aerobic level (Tables 2 and 3). The effects of the alternative electron acceptors nitrate and nitrite were marked, elevating the anaerobic levels of expression 6- and 25-fold, respectively. To determine the effects on *hmp* expression of regulatory genes involved in response to oxygen and alternative electron acceptors, we transduced null alleles of *fnr, arcA, narL,* and *narP* into strain RKP2178.

Table 3 shows that in the absence or presence of nitrate or nitrite, the *fnr* mutation caused a three- to six-fold elevation in Φ(*hmp-lacZ*)*1* expression. The *arcA* and *narP* alleles were without significant effect on expression under any condition tested (Table 3). The *narL* allele, whether introduced alone or with the *narP* allele, reduced Φ(*hmp-lacZ*)*1* expression in cells grown with nitrite by about one-third but had slight and variable effects on expression in cells grown with nitrate (Table 3).

**Effects of an iron chelator on Φ(*hmp-lacZ*)*1* expression.** Hmp reduces Fe(III), raising the possibility that the protein

TABLE 3. Effects of electron acceptors and regulatory mutations on Φ(*hmp-lacZ*)*1* expression[a]

| Strain | Genotype[b] | β-Galactosidase sp act[c] | | | |
|---|---|---|---|---|---|
| | | With $O_2$ | Without $O_2$ | Without $O_2$, with $NO_3^-$ | Without $O_2$, with $NO_2^-$ |
| RKP2178 | Wild type | 46 | 59 | 370 | 1,500 |
| RKP2185 | *fnr* | 56 | 210 | 2,400 | 5,900 |
| RKP2195 | *arcA* | 43 | 59 | 320 | 1,100 |
| RKP2182 | *narL* | 48 | 60 | 280 | 930 |
| RKP2181 | *narP* | 48 | 63 | 390 | 1,400 |
| RKP2193 | *narL narP* | 40 | 60 | 450 | 930 |

[a] Strains were cultured in glucose-MOPS defined medium in the presence of the indicated electron acceptors (see Materials and Methods for details).
[b] All strains carry Φ(*hmp-lacZ*)*1*.
[c] Miller units.

TABLE 4. Effects of 2′2′-dipyridyl on Φ(*hmp-lacZ*)*1* and Φ(*frdA-lacZ*) expression[a]

| 2′2′-Dipyridyl concn (mM) | β-Galactosidase sp act[b] | | | |
|---|---|---|---|---|
| | Φ(*hmp-lacZ*)*1* | | | Φ(*frdA-lacZ*) without $O_2$, stationary |
| | With $O_2$, exponential | With $O_2$, stationary | Without $O_2$, stationary | |
| 0 | 33 | 120 | 98 | 500 |
| 0.1 | 150 | 483 | 3,900 | 84 |
| 0.2 | 550 | 2,100 | 3,000 | 100 |
| 0.4 | 1,120 | 5,200 | 2,700 | 110 |

[a] Strains RKP2178 and VJS3046 were cultured in complex medium (LB) with or without aeration, as indicated (see Materials and Methods for details).
[b] Miller units.

plays a role in iron metabolism and acquisition, for example, by mobilizing siderophore-bound iron. We therefore examined the effects of diminished iron concentrations on Φ(*hmp-lacZ*)*1* expression. The addition to broth medium (LB) of low concentrations of the Fe(II) chelator 2′2′-dipyridyl strongly induced Φ(*hmp-lacZ*)*1* expression under aerobic conditions in the exponential phase of growth (Table 4). Expression was enhanced about 5-fold at a 0.1 mM chelator concentration and about 30-fold at 0.4 mM. Expression of Φ(*hmp-lacZ*)*1* was very sensitive to the phase of growth at which samples were withdrawn for assay. Aerated cultures that were allowed to enter the stationary phase showed elevated levels of expression. In the presence of the iron chelator, *hmp* expression levels were further elevated relative to the values observed in the mid-exponential phase. Anaerobically grown cells were particularly sensitive to the effects of 2′2′-dipyridyl: addition of only 0.1 mM chelator raised levels of Φ(*hmp-lacZ*)*1* expression by 40-fold. We attempted to demonstrate reversal of the effects of the iron chelator by addition of iron [provided as a solution of Fe(III) in EDTA, pH 7.0]. However, growth of strain RKP2178 in LB medium was highly sensitive to the addition of <0.05 mM iron (results not shown).

Iron chelators have been shown (34) to alter expression of Fnr-regulated genes, so we tested the effects of 2′2′-dipyridyl on Φ(*frdA-lacZ*) expression under these growth conditions. Table 4 shows that levels of *frdA* expression were high anaerobically but, in marked contrast to the effects on *hmp* expression, were reduced by about 80% on addition of 0.1 mM chelator, an effect we attribute to inactivation of Fnr. These data suggest that the stimulatory effects of iron chelation on *hmp* expression are, in part, the result of Fnr inactivation.

**Regulation by NO.** Since nitrite induction was observed aerobically (Table 2) and both nitrate and nitrite enhanced Φ(*hmp-lacZ*)*1* expression in a NarL- and NarP-independent manner (Table 3), we considered the possibility that reduction of these ions to NO might be the cause of enhanced expression. Addition of NO as a solution to exponentially growing cultures strongly enhanced Φ(*hmp-lacZ*)*1* expression after 1 h of exposure (Table 5). The increase in expression in the wild-type strain was dose dependent within the range of NO concentrations examined; at 10 μM NO, aerobic induction was 9-fold, and at 20 μM NO, aerobic induction was 19-fold. NO has previously been shown to activate expression of genes involved in response to oxidative stress; this effect is mediated via the SoxR-SoxS sensor-regulator system (35). However, null alleles of either *soxR* (encoding the response component of the system) or *soxS* (encoding the regulator component) did not prevent significant NO stimulation of Φ(*hmp-lacZ*)*1* expression (Table 5). The lower levels of expression observed at 20 μM

TABLE 5. Expression of Φ(*hmp-lacZ*)*1* in cultures grown with NO and effects of *soxR* and *soxS* mutations[a]

| Strain | Genotype[b] | β-Galactosidase sp act[c] | | |
|---|---|---|---|---|
| | | Without NO | With 10 μM NO | With 20 μM NO |
| RKP2178 | Wild type | 52 | 470 | 984 |
| RKP3965 | *soxS* | 56 | 546 | 617 |
| RKP3966 | *soxR* | 45 | 341 | 570 |

[a] Strains were cultivated aerobically in complex medium (TY). After the 45 min of growth required to reach the mid-logarithmic phase, an aqueous solution of NO was added where indicated.
[b] All strains carry Φ(*hmp-lacZ*)*1*
[c] Miller units.

NO in the *soxS* and *soxR* mutants may be related to NO toxicity in these strains. NO also increased anaerobic Φ(*hmp-lacZ*)*1* expression in TY medium. In strain RKP2178, 10 μM NO stimulated expression 9-fold and 20 μM NO gave a 12-fold increase over control values.

## DISCUSSION

The functions of microbial globin-like proteins are uncertain. Understanding the regulation of globin synthesis may suggest possible physiological roles. Synthesis of the *Vitreoscilla* single-domain globin-like protein (Vgb protein) (9, 10) and of the flavohemoglobin proteins of *A. eutrophus* (Fhp protein) (41) and *Bacillus subtilis* (Hmp protein) (30) is increased dramatically in response to oxygen limitation. These observations have led to the concept that the Vgb and Fhp proteins may serve as oxygen stores (41, 56), although each globin molecule could presumably store only one oxygen molecule. A somewhat different view postulates that the Vgb protein might facilitate oxygen transport (27). Conversely, the flavohemoglobin of *S. cerevisiae* (Yhb protein, later called Yhg protein) is maximally synthesized during exponential growth and under oxygen-replete conditions (6). Transcription of the *YHG* gene is positively regulated by factors that respond to heme availability (Hap).

In contrast to these examples, our results indicate that expression of the *E. coli hmp* gene in wild-type strains is not markedly affected by oxygen supply. The maintenance of *hmp* transcription under both aerobic and anaerobic conditions appears to be consistent with a function for Hmp as an oxygen sensor (36, 38), in which continual presence in the cell of the sensor molecule would be required.

**Fnr control of globin synthesis.** In an *fnr* null strain, anaerobic expression of the Φ(*hmp-lacZ*)*1* fusion was increased about three- to fourfold over that in aerated cultures (Table 3). This suggests that Fnr is a negative regulator of *hmp* expression during anaerobiosis and that a distinct regulatory factor is responsible for anaerobic induction. Our evidence indicates that ArcA (24) is not involved in this anaerobic induction (Table 3).

Fnr is perhaps best known as an activator of anaerobic respiratory gene expression, but it also serves as a repressor for some genes, including *ndh* (17, 18). It is not known whether Fnr is a direct regulator of *hmp* expression, but inspection of the DNA upstream of *hmp* reveals two potential Fnr-binding sites. One site, 5′-TTGAG-$N_4$-ATCAA-3′, is centered 34 bp upstream of the *hmp* translation start codon and closely resembles the consensus sequence, 5′-TTGAT-$N_4$-ATCAA-3′ (18). This sequence may well serve as an Fnr-responsive operator site, and its position downstream of the putative −35 and −10 boxes is consistent with the Fnr inhibition described here. A second potential Fnr site, 5′-TTGAC-$N_4$-AGGAA-3′, is centered 222 bp upstream of the *hmp* translation start codon, but this site has a weaker resemblance to the consensus Fnr-binding sequence. Preliminary gel mobility shift studies of the *hmp* promoter region have revealed a retardation complex formed upon incubation with extracts from anaerobically grown cells. Such a complex was absent when an extract from aerobically grown cells or from an *fnr* mutant (32a) was used. There is some evidence that globin synthesis in other bacteria is regulated by Fnr. The sequence upstream of the *Vitreoscilla vgb* gene contains a potential Fnr binding site, and expression of a Φ(*vgb-xylE*) fusion in *E. coli* is positively regulated by Fnr (25). Analysis of the *A. eutrophus fhp* promoter region (5) also reveals a potential Fnr binding site. By contrast, the *B. subtilis* Fnr protein acts indirectly as a positive regulator of *hmp* gene expression (30). The Fnr requirement is bypassed by the addition of nitrite (see below), and potential Fnr binding sites are not apparent in the *hmp* promoter region (30).

Iron chelators, such as 2′2′-dipyridyl, increase the synthesis of Mn-containing superoxide dismutase (19) and enhance Φ(*sodA-lacZ*) expression (40). These results are interpreted as revealing the involvement of an iron-containing repressor protein. This chelator was an effective inducer of Φ(*hmp-lacZ*)*1* expression, particularly under anaerobic conditions (Table 4). Even aerobically, 2′2′-dipyridyl enhanced Φ(*hmp-lacZ*)*1* expression four- to fivefold, suggesting that Fnr function is not essential for the iron effect. Under identical experimental conditions, 2′2′-dipyridyl also inhibited Φ(*frdA-lacZ*) expression about fivefold (Table 4). Niehaus et al. (34) previously reported that a different iron chelator, ferrozine, causes a fivefold decrease in Φ(*frdA-lacZ*) expression. This result is interpretable in view of the requirement of iron for Fnr function (16, 29). Thus, the observed effect of iron limitation further supports a role for Fnr inhibition of *hmp* expression. No sequence resembling the iron box (5′GATAATGATAATCAT TATC 3′) for the iron-responsive Fur regulatory protein (7) is apparent in the *hmp* promoter region. The effects of entering the stationary phase on Φ(*hmp-lacZ*)*1* expression will be discussed in detail elsewhere.

**Nitrite and NO control of globin synthesis.** Anaerobic Φ(*hmp-lacZ*)*1* expression was strongly induced by nitrate or nitrite (Tables 2 and 3). The *B. subtilis hmp* gene is also induced by nitrite, in this case, during either aerobic or anaerobic growth (30). We imagined that NO formed by nitrite reduction might be the actual signal molecule for *hmp* induction. Indeed, NO at micromolar concentrations was an effective inducer of *hmp* expression (Table 5).

NO also induces SoxR-mediated expression of several genes involved in oxidative stress responses (35), but NO induction of Φ(*hmp-lacZ*)*1* expression was largely independent of SoxR (Table 5). Furthermore, SoxR mediates an approximately fourfold induction of oxidative response gene expression (at 200 μl of NO gas per 2 ml of culture; 35), whereas Φ(*hmp-lacZ*)*1* expression was induced approximately 20-fold by only 20 μM NO in solution (Table 5).

The NarL and NarP response regulators mediate nitrate and nitrite induction of anaerobic respiratory gene expression in *E. coli* (49). However, *narL* and *narP* null alleles had only slight effects on Φ(*hmp-lacZ*)*1* expression (Table 3). The control elements that mediate induction of *hmp* gene expression by nitrogen compounds remain to be identified.

*E. coli* is not generally regarded as a denitrifying bacterium, although the organism can produce nitrous oxide ($N_2O$) from either nitrate or nitrite (46) and also reduce nitrous oxide to dinitrogen by an unidentified mechanism (26). What, then, is the source of NO in vivo? Several possible mechanisms exist,

including nonenzymatic reactions (57). In aqueous solution, particularly at low pH values, protonation of nitrite results in disproportionation to give NO and nitrate. Furthermore, the reduction of nitrite to NO might be catalyzed by an oxidase. Mitochondrial cytochrome *c* oxidase reduces nitrite to NO anaerobically (55), and *E. coli* cytochrome *bd* also reacts with nitrite (2).

The relationship between Hmp and the metabolism of nitrogen oxide compounds warrants further investigation. We have previously shown that a nitrosyl complex is readily formed at the ferrous heme of Hmp (21), and experiments are under way to study the reaction between NO and Hmp in vitro and in vivo. One possibility is that Hmp sequesters NO, as proposed for the salivary nitrophorins (NO-carrying hemoproteins) in various insects (e.g., see reference 53). An unusual hemoprotein capable of reversibly binding NO has been isolated from *Bacillus halodenitrificans* (8). Alternatively, Hmp might itself be an NO reductase. Indeed, an *fhp* mutant of *A. eutrophus* has a striking phenotype, namely, the absence of nitrous oxide as a detectable intermediate of denitrification. NO reductase activity of Fhp could not, however, be demonstrated (5).

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