Regulation of the *Salmonella typhimurium* Flavohemoglobin Gene

A NEW PATHWAY FOR BACTERIAL GENE EXPRESSION IN RESPONSE TO NITRIC OXIDE*

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Flavohemoglobins, a family of two-domain proteins with homology to vertebrate hemoglobins, are found in a variety of prokaryotic and eukaryotic microorganisms. Recent studies suggest a role for these proteins in nitrogen oxide metabolism. We now show that nitric oxide donors positively regulate a chromosomal flavohemoglobin (*hmp*)/lacZ operon fusion in *Salmonella typhimurium*. *hmp* gene expression in the presence of NO is independent of the SoxS, OxyR, and FNR transcription factors and instead relies on inactivation of the iron-dependent Fur repressor. Other Fur-repressed promoters in *S. typhimurium* are also activated by an NO donor. In contrast to the wild-type strain, an *hmp* mutant requires markedly lower concentrations of NO to induce the *hmp*/lacZ fusion, whereas its response to iron chelation is equivalent to wild type. These data unveil a new pathway for NO-dependent gene expression in *S. typhimurium*.

In addition to its function as a regulatory molecule in organisms ranging from humans to slime molds, nitric oxide possesses potent and broad spectrum antimicrobial activity (1). The cellular targets responsible for the cytotoxic and/or cytostatic action of NO include lipids, thiols, DNA, and transition metals. A growing body of evidence indicates that bacteria are capable of inducing protective enzymes in the presence of NO and other reactive nitrogen intermediates, a process recently termed the nitrosative stress response (2). The SoxRS system, which is activated by superoxide generators, will stimulate antioxidant genes in reaction to NO and defend *Escherichia coli* from the NO-dependent bactericidal activity of macrophages (3). OxyR, a transcription factor involved in stimulation of peroxide detoxification genes, is directly modified by NO via S-nitrosylation and assists in protecting the bacterium from the NO donor S-nitrosocysteine (2). Alkyl hydroperoxide reductase subunit C, one of the enzymes influenced by OxyR, has been shown to protect *Salmonella typhimurium* from another NO donor, S-nitrosogluthathione (GSNO) (4).

Deletion of flavohemoglobin, a two-domain protein with N-terminal homology to hemoglobins and C-terminal homology to oxidoreductases, also results in hypersensitivity of *S. typhimurium* to nitrosative stress (5). The mutant strain is equivalent to wild-type in sensitivities to superoxide and hydrogen peroxide, suggesting the existence of a nitrosative stress response that is independent of the oxygen-related stress pathways. Flavohemoglobins have been isolated from phylogenetically distant organisms, including *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *E. coli* (6–8). Although the promoter activities of some flavohemoglobins are influenced by oxygen availability (7, 9), nitric oxides are known to induce the transcription of these proteins in other organisms. Flavohemoglobin in the fungus *Fusarium oxysporum* is present only during denitrification, an anaerobic electron transport pathway that reduces nitrate to dinitrogen via nitrogen oxide intermediates (10). A flavohemoglobin mutant strain of the denitrifying bacterium *Alcaligenes eutrophus* is deficient in the transient production of nitrous oxide (N2O), an intermediate that is immediately downstream of NO in the denitrification pathway (11). Many other flavohemoglobin-containing organisms do not have the denitrifying capability of *F. oxysporum* or *A. eutrophus*. Some, such as *E. coli* and *B. subtilis*, will reduce nitrite to ammonia or ammonium rather than nitric oxide (12, 13). Nevertheless, purified NO was found to be a major inducer of flavohemoglobin (*hmp*) promoter activity in *E. coli* (14). An *hmp*/lacZ operon fusion was stimulated approximately 20-fold by 20 mM nitric oxide, while requiring 8 mM nitrite and 40 mM nitrate to give similar results. Unlike the nitrite and nitrate effects, which are escalated anaerobically, the NO induction is largely independent of O2 concentration. The *B. subtilis hmp* is also induced by comparable levels of nitrite, but the influence of nitric oxide was not elucidated (7). Recently, purified *E. coli* flavohemoglobin was demonstrated to oxidize NO to the less toxic nitrate (15), thus assigning an enzymatic activity to the nitrosative stress protection shown by phenotypic analyses (5).

The elements responsible for the NO-dependent expression of flavohemoglobins are not known. Although the ResDE two-component system accounts for some of the anaerobic expression of *hmp* in *B. subtilis*, factors contributing to the nitrite induction were not elucidated (7). *E. coli* strains harboring null alleles of *narL* and *narP*, which regulate nitrate and nitrite reductases, do not substantially alter *hmp* expression (14). FNR, a transcription factor known to positively regulate a flavohemoglobin homolog in the bacterium *Vitreoscilla* as well as many denitrification enzymes in other species, actually represses anaerobic expression of the *E. coli hmp* (14, 16, 17). The NO expression is also largely independent of the SoxRS system in *E. coli* (14). In addition to nitrogen oxides, the iron chelator 2,2'-dipyridyl significantly enhances *E. coli hmp* expression. This induction was tentatively attributed to deactivation of FNR, which requires iron for function (14).

In this study, we have found that the *S. typhimurium hmp* gene expression is induced by the NO donors and that the transcriptional repressor Fur is the primary factor responsible for *hmp* regulation. Nitric oxide causes a general derepression

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1 The abbreviations used are: NO, nitric oxide; GSNO, S-nitrosoglutathione; NONOate, 2,2′-(hydroxyuratosylhydrazono)bisethanamine; FURTA, fur titration assay.
of other Fur-regulated genes, suggesting a new mechanism of NO action on bacterial gene expression.

**EXPERIMENTAL PROCEDURES**

Reagents Used—Spermine NONOate (2,2’-hydroxynitrosospermine) was obtained from Alexis Biochemicals (San Diego, CA). MacConkey agar and LB broth were acquired from Difco. Other chemicals were purchased from Sigma. S-Nitrosoglutathione (GSNO) was made as described previously (18).

Bacterial Strains and Plasmids—The strains in this study are listed in Table I. To obtain an integrated hmp::lacZ operon fusion, a 1-kilobase pair SalI genomic fragment containing approximately 950 base pairs of hmp upstream sequence was isolated from pMC71 and ligated into the SalI site of pFUSE, a suicide plasmid that harbors the promoterless lacZYA operon (19). The resultant plasmid was propagated in E. coli strain S17-1 pir (20) and transformed into S. typhimurium (GSNO) was made as described previously (18).

**TABLE I**

| Strains and plasmids used in this study |
|----------------------------------------|
| Plasmids                               | Relevant characteristics or genotype | Reference or Source |
| pMC71                                  | 4.4-kilobase pair SalI genomic fragment containing hmp::Kan in pBSKII+ | 5 (Stratagene, La Jolla, CA) |
| pSKO1/2                                | 300 bp of hmp promoter sequence in pBSKII+ | 5 |
| pSKO3/4                                | 350 bp of hmp ORF in pBSKII+ | 5 |

S. typhimurium

14026 Wild-type strain
14028 hmp+ (MCS2A) 14028 with hmp::Kan 5
14028-oxoS oxoS::pBR10 44
DJ1444 oxyR::Tn10 45
Tn2336 oxoA2 (fur::Tn10) 46
JF2904 irxA1::MudJ fur1 thr::Zs1572::Tn10 (60% to fur-1) 18
AJB27 14028 Nal’ with irxC::lacZYA J. Foster 18
MCS32 AJB27 with pSKO1/2 This study
MCS33 AJB27 with pSKO3/4 This study
MCS34 14028 with pSKO1/2 This study
MCS38 14028 with pSKO3/4 This study
MCS39 MCS38 with hmp::Kan This study
MCS40 MCS38 with fur-1 thr::Zs1572::Tn10 This study
MCS41 MCS38 with oxyR::Tn10 This study
MCS43 MCS38 with oxoS::pBR10 This study
MCS45 MCS38 with fur::Tn10 This study
MCS46 14028 with irxA1::MudJ This study

E. coli

XLI-Blue recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lacI(q+); F’ proAB lacY18ZDM15, Tn10| Stratagene (La Jolla, CA)
S17–1 pir prp Prp thi recA hsdR A96 supE44 relA1 lacI(q+); F’ proAB lacY18ZDM15, Tn10| Stratagene (La Jolla, CA)

a bp, base pair.
b ORF, open reading frame.

**RESULTS**

The NO Donor Spermine NONOate Is Able to Induce hmp through a Novel Pathway—To facilitate gene expression studies on hmp, we used an S. typhimurium strain harboring a single copy of an hmp::lacZ operon fusion. The expression construct contains 920 base pairs of hmp upstream sequence fused to the promoterless lacZYA operon in the suicide plasmid pFUSE (19). As confirmed by Southern as well as by phenotypic analyses using NO donors (not shown), the endogenous hmp gene was not affected by this intervention event. Spermine NONOate (SPER/NO), a well characterized NO donor (25, 26), was employed to determine sensitivities of wild-type and hmp- strains as well as hmp promoter activity in response to nitric oxide. Using 1 mM SPER/NO, a growth delay is observed for both strains but is much more pronounced in the hmp- mutant (Fig. 1). Addition of SPER/NO induces the hmp promoter ap-
approximately 20-fold (Fig. 2). We examined the transcription factors known to respond to nitric oxide (SoxS and OxyR) or to influence hmp transcription in E. coli (Fnr) for their contribution to aerobic hmp expression in S. typhimurium (2, 15, 27).

Strains harboring null alleles of soxS, oxyR, and fnr are still able to respond to SPER/NO (Fig. 2), indicating that a previously uncharacterized pathway is responsible for the NO-dependent expression of flavohemoglobin.

Low Iron-induced Promoters Are Stimulated by NO—Low iron conditions increase expression of E. coli hmp (14). Nitric oxide is known to react with transition metals and may therefore perturb cellular iron levels (28). To determine whether the SPER/NO effect on the hmp promoter is specific or due to a more general phenomenon of iron metabolism, we also tested iroA and iroC reporter fusions, which were originally isolated because of their induction under low-iron conditions (19, 29). In addition to induction by the iron chelator 2,2'-dipyridyl, the β-galactosidase activity driven by these promoters is also stimulated by 1 mM SPER/NO (Fig. 3). Therefore, NO appears to cause widespread alteration of iron-regulated gene expression in S. typhimurium.

The hmp Promoter Is Repressed by Fur—The iroA and iroC promoters are repressed by the Fur protein, a global transcriptional regulator that is active when bound to ferrous iron (19, 29). We tested whether a fur null allele could influence the hmp promoter (24). In this assay, a high copy number plasmid carrying a putative Fur-binding element is introduced into a strain that carries a Fur-repressed lacZ fusion. If the plasmid is capable of binding Fur, a titration of this repressor will occur, leading to expression of the lacZ reporter. A
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...3') found over the transcription start site in the hmp promoter that can be aligned to the consensus “Fur box” (5'-GATAAT-GATAATCATATC-3') (24). This level of identity to the consensus (10/19 matches) is equivalent to that of another FUR- TAp-positive clone (pFTE-1) found during a general screen for Fur-regulated genes in S. typhimurium (30).

The hmp Mutant Is Hypersensitive to NO and Not to Iron Chelation—The finding that Fur, well characterized for its regulation of iron scavenging (31), also influences the expression of hmp raises the possibility that flavohemoglobin may function in the processing of intracellular iron. Consequently, we tested the effects of 2,2'-dipyridyl on growth and hmp/lacZ activity in the hmp- strain. No differences in growth rates between wild-type and hmp- strains could be demonstrated for any concentration of the iron chelator (not shown). Also, the expression of the hmp/lacZ fusions as a function of 2,2'-dipyridyl concentration is equivalent in both wild-type and hmp- strains (Fig. 6a). In contrast, induction of the hmp/lacZ reporter in the hmp- strain is more sensitive to SPER/NO than wild type (Fig. 6b). S-Nitrosoglutathione (GSNO), another nitric oxide donor that causes an accentuated growth deficiency in the hmp- strain (5), is able to induce the hmp/lacZ fusion and provides a more dramatic (approximately 10-fold) decrease in the quantity needed for hmp gene induction in the hmp mutant background when compared with wild type (Fig. 6c). GSNO also causes a greater relative growth impairment in the hmp- strain than SPER/NO (not shown).

With increasing concentrations of NO donors, expression begins to decline after maximal gene induction (Fig. 6). This phenomenon is likely the result of toxicity to the strains, since cell growth diminishes at these concentrations. It is interesting to note that GSNO does not achieve the induction levels seen with SPER/NO. This may be due to the more complex chemistry of the nitric oxide species coming from GSNO. Unlike SPER/NO, which should provide a relatively pure source of the NO· radical (32), GSNO will donate both NO· and the nitrosonium ion (NO+·) (33). Therefore, NO· may be the major form of nitric oxide regulating hmp gene expression.

**DISCUSSION**

In addition to its role in iron acquisition, Fur is postulated to regulate general metabolic processes as well as adaptation to acid stress (24, 29). The results presented here on the regulation of S. typhimurium flavohemoglobin demonstrate that the influence of Fur extends to the nitrosative stress response. We show that nitric oxide will derepress other iron-regulated promoters. The role of NO in control of iron metabolism has precedent in mammalian systems, although at a different level of regulation (34, 35). The translation factors IRP-1 and IRP-2, which are thought to sense iron levels through the gain or loss of Fe-S clusters, control protein expression from several mRNAs involved in the uptake of iron. Nitric oxide is thought to mimic or cause iron deficiency by destruction of these clusters or by complexing with free iron, leading to depletion of the cellular iron pool (36). A similar situation may occur in a bacterial cell under nitrosative stress. NO could induce the conversion of Fe-Fur to an inactive Fur by modification of the iron or protein moiety. Alternatively, iron can react with nitric oxide to form dinitrosyl iron complexes, which may effectively sequester cellular iron in an unusable form. The formation of these complexes occurs in macrophages upon induction of nitric oxide synthase (37, 38) and is thought to assist in creating the low iron environment found in these cells (39). We have shown that the hmp promoter is capable of binding Fur in vivo, and a putative Fur box is found over the start site of hmp transcription. We were, however, unable to find anything resembling a Fur box in either the E. coli or B. subtilis hmp promoters.

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**FIG. 6.** Hmp has no effect on the gene expression response to iron chelation but mutes the response to NO. Expression of the hmp/lacZ fusion in either a wild-type (MCS38) or hmp- (MCS39) background was monitored after introduction of increasing concentrations of 2,2'-dipyridyl (a), SPER/NO (b), or GSNO (c). Shown is a representative experiment done in triplicate. Similar results were obtained in subsequent experiments.

plasmid containing 213 base pairs upstream of the hmp start codon is able to derepress the iroC/lacZ fusion (Fig. 5) on MacConkey plates, whereas a plasmid with an insert containing a portion of the hmp open reading frame is not positive by FURTA. There is a sequence (5'-TCTAATGATGATATCAAA-
Therefore, if Fur is influencing hmp expression in these organisms, it might be through a novel binding site or by indirect means.

Sensitivity to nitric oxide (or NO donors) is the only known growth or gene expression difference between wild-type and hmp⁻ strains (5, 15). The hmp⁻ strain is no more sensitive than wild type to iron chelation, bolstering the hypothesis that Hmp is involved with nitrosative stress protection rather than with general iron maintenance. Control of iron uptake, however, is an elaborate and complex process (40). We currently cannot rule out the possibility that flavohemoglobin is involved with an aspect of iron metabolism that is particularly sensitive to nitric oxide. Like flavohemoglobin, the expression of superoxide dismutase, an enzyme involved in oxidative stress protection, is regulated by Fur (41). Therefore, in addition to SoxRS and OxyR (2, 3), nitrosative and oxidative stresses share a third pathway toward the induction of protective genes in enteric bacteria. Although these stresses share many common targets within the cell (2), an intriguing disparity exists in their interaction with iron. Whereas reactive oxygen intermediates will undergo Fenton chemistry with iron to create highly toxic metabolites (42), nitric oxide will simply bind iron, with the resultant complex creating unknown consequences for the cell (28). Despite the induction of superoxide dismutase, the E. coli fur mutant is hypersusceptible to oxygen metabolites and cannot grow aerobically without an efficient DNA repair mechanism (43). It will prove interesting to assess the contribution of intracellular iron levels to nitrosative stress and the role that Fur and Hmp play in this process.

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Additions and Corrections

Regulation of the *Salmonella typhimurium* flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide.

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**Page 34029, “Experimental Procedures”:** The greek symbols were not inserted so the *E. coli* strain listed as S17-1 1pir should be S17-1 λpir, and the concentrations of the antibiotics ampicillin, kanamycin, chloramphenicol, and tetracycline should read “μg/ml” rather than “mg/ml.”

**Page 34029, Table I:** “S17-1 1pir” should be “S17-1 λpir.” The reference for strain AJB27 should be 19 rather than 18 and for S17-1 λpir it should be 20 rather than 19.

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Strain MCS38 has been found to have the lacZ integration in the *iroC* locus instead of the *hmp* locus and therefore reflects regulation of the *iroC* gene.

Molecular characterization of the gerbil C5a receptor and identification of a transmembrane domain V amino acid that is crucial for small molecule antagonist interaction.

Stephen M. Waters, Robbin M. Brodbeck, Jeremy Steflik, Jianying Yu, Carolyn Baltazar, Amy E. Peck, Daniel Severance, Lu Yan Zhang, Kevin Currie, Bertrand L. Chenard, Alan J. Hutchison, George Maynard, and James E. Krause

PAGE 40622, LEFT COLUMN, LINE 45:
The last three words, “rat polymorphonuclear leukocytes,” should be replaced with “mouse polymorphonuclear leukocytes.”