JOURNAL OF BACTERIOLOGY, May 1997, p. 3164–3170 Vol. 179, No. 10
0021-9193/97/$04.00+0

Paraquat Regulation of *hmp* (Flavohemoglobin) Gene Expression in *Escherichia coli* K-12 Is SoxRS Independent but Modulated by σ^S

JORGE MEMBRILLO-HERNÁNDEZ,[1] SUNG OOG KIM,[1] GREGORY M. COOK,[1] AND ROBERT K. POOLE[1]*

Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, United Kingdom[1]

Received 9 December 1996/Accepted 18 March 1997

We report the first example of a gene, *hmp,* encoding a soluble flavohemoglobin in *Escherichia coli* K-12, which is up-regulated by paraquat in a SoxRS-independent manner. Unlike what is found for other paraquat-inducible genes, high concentrations of paraquat (200 μM) were required to increase the level of *hmp* expression, and maximal induction was observed only after 20 min of exposure to paraquat. Neither a mutation in *soxS* nor one in *soxR* prevented the paraquat-dependent increase in Φ(*hmp-lacZ*) expression, but either mutant allele delayed full expression of Φ(*hmp-lacZ*) activity after paraquat addition. Induction of *hmp* by paraquat was demonstrated in aerobically grown cultures during exponential growth and the stationary phase, thus revealing two Sox-independent regulatory mechanisms. Induction of *hmp* by paraquat in the stationary phase was dependent on the global regulator of stationary-phase gene expression, RpoS (σ^s). However, a mutation in *rpoS* did not prevent an increase in *hmp* expression by paraquat in exponentially growing cells. Induction of σ^s in the exponential phase by heat shock also induced Φ(*hmp-lacZ*) expression in the presence of paraquat, supporting the role of σ^s in one of the regulatory mechanisms. Mutations in *oxyR* or *rob,* known regulators of several stress promoters in *E. coli,* had no effect on the induction of *hmp* by paraquat. Other known superoxide-generating agents (plumbagin, menadione, and phenazine methosulfate) were not effective in inducing *hmp* expression.

Although the functions of globins in higher organisms as facilitators of oxygen transport and storage are well established, the function(s) of microbial hemoglobins remains unclear. In 1986, Wakabayashi and coauthors reported the presence of a bacterial hemoglobin (Vgb) in *Vitreoscilla* cells, challenging the view that the distribution of hemoglobin was restricted to eukaryotes (for a review, see reference 37). Other hemoglobins found in many bacteria are two-domain proteins (6, 11, 27, 44). The N terminus contains the heme domain, whereas the C terminus contains a reductase domain with potential binding sites for flavin (flavin adenine dinucleotide) and NAD(P)H (9). Because of the additional domain, proteins in this class are called flavohemoglobins. The flavohemoglobin in *Escherichia coli* is encoded by the gene *hmp*, which lies at min 57.7 of the chromosome map (44). Hmp binds oxygen to the high-spin ferrous heme to form an oxygenated complex (18). Previous work in our laboratory has attempted to define the function(s) of Hmp by studying how *hmp* expression is regulated (31, 38) and the catalytic capabilities of Hmp (30, 39). The expression of *hmp* is strongly induced by nitrite and nitric oxide, suggesting that Hmp may be involved in the metabolism of these compounds (38) or is responsive to oxidative stress. Other evidence demonstrates that Hmp contributes to oxidative stress in vivo by producing superoxide anion (30, 39).

Paraquat (radical, PQ^{2+}; 1,1′-dimethyl-4,4′-bipyridinium dichloride; methyl viologen) and other superoxide-generating agents induce a global response leading to the expression of about 40 proteins in addition to at least another 40 proteins induced in response to hydrogen peroxide (13, 16). The former group includes about 10 proteins whose genes have been firmly identified as components of the *soxRS* regulon and are necessary for dealing with oxidative stress, including *sodA*, *ompF*, *fumC*, and *zwf* (2, 35, 45). The induction of the SoxRS modulon occurs in two steps. An unidentified intracellular signal of oxidative stress changes the conformation of the SoxR protein into a transcriptional activator of the *soxS* gene. The SoxS protein then activates the transcription of genes belonging to the modulon (2, 35, 46). The SoxRS regulon is activated by redox cycling agents, such as paraquat, plumbagin, menadione, and phenazine methosulfate, all of which generate intracellular superoxide (10, 13), and by nitric oxide (35, 36).

Unlike the induction of other nitric oxide-inducible genes, whose expression has been shown to be SoxRS dependent, the induction of *hmp* is SoxRS independent (38). In this paper, we report the regulation of *hmp* expression by paraquat. We demonstrate that paraquat strongly increases *hmp* expression, but unlike the regulation of all other known paraquat-inducible genes, the regulation of *hmp* is SoxRS independent. σ^s was shown to be the dominant regulator of *hmp* expression in the presence of paraquat during the stationary phase.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* K-12 strains used in this study are described in Table 1. Strain RKP2178 harboring an *hmp-lacZ* chromosomal operon fusion was constructed with plasmid pRS528 and recombined onto λRS45 (41). A single-copy fusion to the chromosome of VJS676 (Δ*lac*) was isolated and verified with Ter tests and β-galactosidase assays as described by Poole et al. (38). Expression from such fusion constructs derives almost exclusively from the unperturbed expression signals of the cloned segment and not, for example, from the induction of the bacteriophage λ (41). The stability of the lysogen was tested regularly on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The mutant alleles transduced into RKP2178 (Table 1) were *arcA* (ECL585), *narL* (RK5278), *fnr* (VJS1741), *narP* (VJS4322), *fur* (QC1732), *soxR* (BW949), *soxS* (BW831), *rpoS* (GS015), *oxyR* (GS08), *cya* (MK1010), *himA* (GS019), and *rob* (MK107). Genetic crosses were performed with bacteriophage P1*vir*-mediated transduction (32). Standard methods were used for restriction endonuclease digestion and ligation of DNA (40). Plasmid DNA was isolated

* Corresponding author. Mailing address: Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom. Phone: (0114) 222 4447. Fax: (0114) 272 8697. E-mail: r.poole@sheffield.ac.uk.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
BW831	*soxS3*::Tn*10* Δ(*argF-lac*) *rpsL*$^+$ *sup*(Am)	46
BW949	*soxR9*::*cat* Δ(*argF-lac*)*rpsL*$^+$*sup*(Am)	46
ECL585	*arcA1 zjj*::Tn*10*	19
GS08	*oxyR*::*kan*	1
GS019	*himA*::*cat*	1
GS015	*rpoS*::Tn*10*	1
MK1010	IN(*rrmD-rrnE*)*1 cya*::Km^r	23
MK107	Δ(*argF-lac*)*U169 araD rpsL relA flbB deoC ptsF rbsR rob*::*kan*	23
QC772	Δ*lacU rpsL* Φ(*sodA-lacZ*)	5
QC1732	F$^-$Δ(*argF-lac*)*U169 rpsL* Δ*fur*::*kan*	43
RK5278	*araD139* Δ(*argF-lac*)*U169 gyrA219 non-9 rpsL150 narL215*::Tn*10*	Valley Stewart
RKP2178	As VJS676, but Φ(*hmp-lacZ*)*1*	38
RKP2181	As RKP2178, but *narP253*::Tn*10*d(Cm)	38
RKP2182	As RKP2178, but *narL215*::Tn*10*	38
RKP2185	As RKP2178, but *fnr-271*::Tn*10*	38
RKP2195	As RKP2178, but *arcA1*	38
RKP3978	As RKP2178, but *soxS*::Tn*10*	38
RKP3979	As RKP2178, but *soxR*::*cat*	38
RKP3980	As RKP2178, but *rpoS*::Tn*10*	This work
RKP3982	As QC772, but *soxS*::Tn*10*	30
RKP3983	As QC772, but *soxR*::*cat*	This work
RKP3990	As RKP2178, but *himA*::*cat*	30
RKP4017	As RKP2178, but *oxyR*::*kan*	This work
RKP4018	As RKP2178, but *cya*::*kan*	38
RKP4023	As RKP2178, but Δ*fur*::*kan*	This work
RKP4024	As RKP2178, but *rob*::*kan*	This work
VJS676	Δ(*argF-lacZ*)*U169*	Valley Stewart
VJS1741	*fnr-271*::Tn*10*	Valley Stewart
VJS4322	*narP253*::Tn*10*d(Cm)	Valley Stewart

with the Wizard column system from Promega (Madison, Wis.). DNA fragments were isolated from agarose gels with the Bio-Rad Prep-a-Gene kit (Hercules, Calif.). Transformation of bacteria with plasmid DNA was done by the single-step method (4). 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 and growth conditions. Cells were grown in Luria-Bertani (LB) broth (initial pH 7.0) (32). 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 optical density (OD) was measured with a Pye-Unicam SP6-550 spectrophotometer at 600 nm, with culture samples diluted with medium to bring the OD_{600} to below 0.7 when measured in cells of a 1-cm path length. All cultures were grown at 37°C with shaking (200 rpm) in conical flasks containing 1/25 to 1/10 of their own volume of medium. After growth to an OD_{600} of 0.5 (for exponential-phase cultures) or about 3.0 (for stationary-phase cultures), chloramphenicol (100 μg/ml) or spectinomycin (for chloramphenicol-resistant strains; 300 μg/ml) was added, and incubation continued for 5 min before harvest.

For heat shock experiments, an overnight culture was diluted 1 in 100 in LB broth and shaken (200 rpm) at 37°C. After growth to an OD_{600} of 0.4, cells were transferred to 42°C, either 20 min or 5 min prior to exposure to 200 μM paraquat for 45 min at 37°C. Control experiments used untreated cells or cells subjected to a heat shock but not paraquat.

Viable counts for checking the *soxR* and *soxS* phenotypes. Cultures of strains RKP2178, RKP3978 (*soxS*), and RKP3979 (*soxR*) were grown aerobically in LB broth at 37°C to an OD_{600} of 0.5. The cultures were then treated with different concentrations of paraquat. After 45 min of further incubation at 37°C, dilutions of the culture in medium were spread on LB solid media containing the appropriate antibiotic. Results are expressed as percentages of the viable counts in cultures not exposed to paraquat.

β-Galactosidase assays. β-Galactosidase activity was measured as described by Miller (32) and Giacomini et al. (12). Assays were carried out at room temperature, around 21°C. Cell pellets were suspended in 2.5 to 4 ml of Z buffer (32) and stored on ice. β-Galactosidase activity in $CHCl_3$-and sodium dodecyl sulfate-permeabilized cells was measured by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in terms of the OD_{600} of cell suspensions by the formula of Miller (32). Each culture was assayed in triplicate; results were confirmed in at least two or three independent experiments.

RESULTS

Expression of *hmp* is increased by addition of paraquat to exponentially growing cultures. Previous work with a monolysogen Φ(*hmp-lacZ*) operon fusion has shown that expression of this flavohemoglobin gene is low during exponential growth, but expression can be increased dramatically (greater than 20-fold) if the cells are challenged with nitric oxide (38). Because paraquat has been shown to increase expression of other genes that respond to nitric oxide (36), we investigated the effect of paraquat on the expression of *hmp*. When exponentially growing cells were challenged with low concentrations of paraquat (up to 100 μM), the level of *hmp* expression did not increase above the basal level (around 50 Miller units [Table 2]). However, if the concentration was raised to 200 μM, there was a 10-fold increase in the expression of *hmp*. Furthermore, addition of paraquat did not increase the level of *hmp* expression significantly. In contrast, a known paraquat-inducible gene, i.e., *sodA*, responded to paraquat at lower concentrations and with maximal expression at about 100 μM (Table 2). To investigate whether *hmp* transcription was increased in response to superoxide generated by the reaction of paraquat with oxygen, we studied the effect of other superoxide-generating agents (10, 35), namely menadione, plumbagin, and phenazine methosulfate, on *hmp* expression. Cells challenged with menadione did not increase the level of *hmp* expression, even if 400 μM menadione was added. Similarly, cells did not increase the level of *hmp* expression if challenged with plumbagin (up to 40 μM) or phenazine methosulfate (5 μM) (Table 2). Higher concentrations of these compounds did not increase *hmp* expression (data not shown). In contrast, Φ(*sodA-lacZ*) expres-

TABLE 2. Effect of redox cycling agents on expression of Φ(*hmp-lacZ*) in strain RKP2178 and Φ(*sodA-lacZ*) in strain QC772 grown aerobically

Inhibitor (μM)[a]	β-Galactosidase sp act (Miller units)[b]	
	Φ(*hmp-lacZ*)	Φ(*sodA-lacZ*)
Control	51	120
Paraquat		
50	49	520
100	55	680
200	520	720
400	620	680
Menadione		
200	62	560
400	59	620
Plumbagin		
20	56	650
40	52	730
Phenazine methosulfate		
0.5	52	610
5	61	560

[a] All inhibitors were added to exponentially growing cultures (OD_{600}, <0.5). For the inhibitors dissolved in 100% ethanol, the equivalent volume of ethanol was added to the control flasks without an effect on β-galactosidase activities.

[b] Values expressed are for cells in the exponential phase of growth, 45 min after the addition of the redox cycling agent. Each result is the mean of three independent determinations in a typical experiment, and the values did not differ by more than 15%.

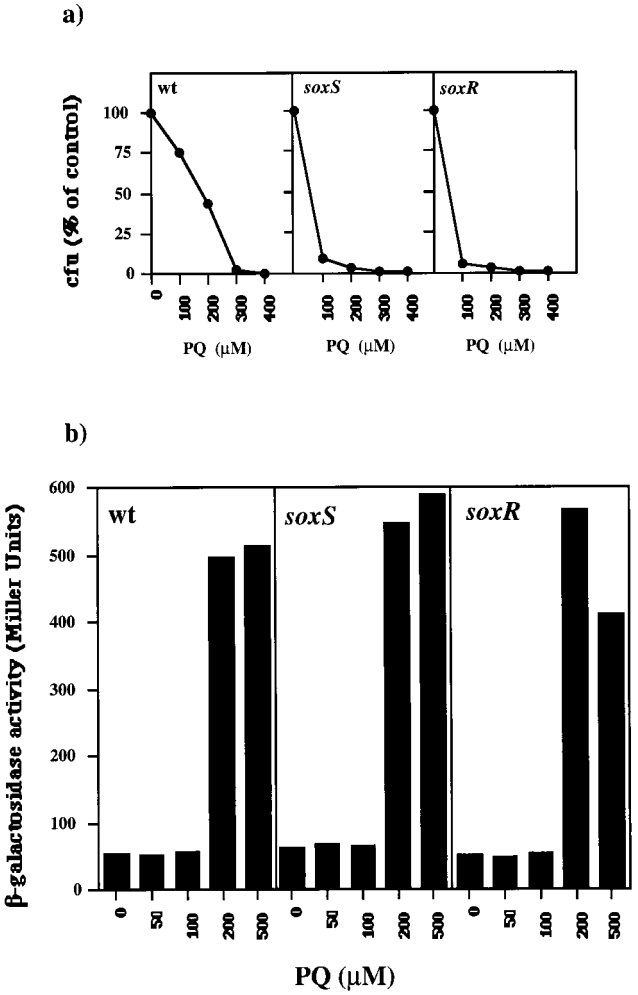

FIG. 1. (a) Effect of increasing concentrations of paraquat (PQ) on the viability (CFU) of wild-type (wt), *soxS*, and *soxR* strains. Paraquat was added during exponential growth (OD_{600}, <0.5), and samples for the determination of viability were taken 45 min after this addition and plated. (b) Effect of increasing concentrations of paraquat on the expression of Φ(*hmp-lacZ*) in wild-type (wt), *soxS*, and *soxR* genetic backgrounds during exponential growth. Paraquat was added during exponential growth (OD_{600}, <0.5), samples were taken 45 min after this addition, and β-galactosidase activity was determined in triplicate. The experiment described above was repeated more than three times, and the same pattern of Φ(*hmp-lacZ*) expression was consistently observed.

sion was increased at all concentrations of these compounds tested, indicating that these agents were acting by a common mechanism, presumably superoxide generation. Under the growth conditions tested, these compounds significantly reduced the growth rate (by greater than 50%) for both fusion-harboring strains.

Regulation of *hmp* expression by paraquat is independent of SoxRS. All genes described so far as inducible by paraquat in *E. coli* have been shown to be under the control of the SoxRS modulon (8, 16), but the induction of *hmp* by nitric oxide is SoxRS independent (38). Therefore, we tested the possibility that SoxRS is involved in the paraquat regulation of *hmp* expression. Strain RKP2178 was transduced with *soxR*::*cat* and *soxS*::Tn*10* mutations from strains BW949 and BW831, respectively. The transductants RKP3978 [Φ(*hmp-lacZ*) *soxS*::Tn*10*] and RKP3979 [Φ(*hmp-lacZ*) *soxR*::*cat*] were tested for their sensitivity to paraquat to demonstrate that these strains were indeed defective in these loci (Fig. 1a). When the wild-type strain, RKP2178, was challenged with 100 μM paraquat for 45 min, there was only a 25% reduction in the CFU (Fig. 1a). In contrast, the strains defective in *soxS* (RKP3978) and *soxR* (RKP3979) showed a 95% reduction in the CFU at 100 μM paraquat, thus confirming the *soxR* and *soxS* phenotypes (2). Wild-type cells showed an increase in *hmp* expression with increasing concentrations of paraquat (Fig. 1b and Table 2). Surprisingly, the *soxS* mutant also showed an increase in *hmp* expression when challenged with increasing concentrations of paraquat during exponential growth (Fig. 1b). The maximum level of *hmp* expression was approximately the same as that in wild-type cells, and *hmp* was induced at the same concentration of paraquat (200 μM) in both strains. Similar results were obtained with the *soxR* mutant, indicating that neither SoxS nor SoxR is required for induction of *hmp* by paraquat (Fig. 1b).

These experiments were performed by challenging cells with paraquat 45 min prior to measurement of *hmp* expression. This protocol does not elucidate the kinetics of induction. Therefore, samples were taken every 3 min after paraquat addition, and the level of Φ(*hmp-lacZ*) activity was determined (Fig. 2). The expression of *hmp* in wild-type cells began to increase 6 min after paraquat addition, and maximal expression was observed after 20 min (Fig. 2a). However, strains defective in *soxR* did not respond until 12 min following paraquat addition, and only after 30 min was the maximal level of *hmp* expression observed (Fig. 2a). A *soxS* mutant also responded slowly to paraquat addition, and maximal expression of *hmp* under these conditions was not observed until after about 33 min (Fig. 2a). A Φ(*sodA-lacZ*) fusion was also monitored under these conditions and responded within 3 min of paraquat addition; maximal expression was observed after 20 min (Fig. 2b). The induction of Φ(*sodA-lacZ*) was entirely dependent on *soxR* and *soxS*, because mutations in these genes prevented induction of *sodA* by paraquat (Fig. 2b). A clear distinction was observed between the paraquat-mediated levels of expression of Φ(*hmp-lacZ*) and Φ(*sodA-lacZ*) in *soxR* and *soxS* backgrounds, even though for both strains these mutations result in rapid loss of viability after addition of paraquat.

Induction of *hmp* by paraquat is regulated by σ^s in the stationary phase. From the results presented above, we conclude that *hmp* is the first gene described to be induced by paraquat but that its induction is independent of the global regulatory system for oxidative stress, SoxRS. During aerobic growth, Φ(*hmp-lacZ*) expression increases twofold as the cells enter the stationary phase, and this increase is dependent on the stationary-phase-specific sigma factor σ^s (31). Because σ^s is induced as cells enter the stationary phase (15), we analyzed the effect of paraquat on the regulation of *hmp* in stationary-phase cultures. Exponential-phase (Fig. 3A) and stationary-phase (Fig. 3B) cultures challenged with 200 μM paraquat showed an approximately fourfold increase in the level of *hmp* expression. In both cases, this induction was observed at 200 μM paraquat, and Φ(*hmp-lacZ*) activity remained high at higher paraquat concentrations.

An *rpoS* mutant (RKP3980) grown in the absence of paraquat or at up to 100 μM paraquat (Fig. 3C) exhibited levels of Φ(*hmp-lacZ*) activity that were indistinguishable from those in an exponential-phase culture of the wild type (Fig. 3A). Furthermore, 200 μM paraquat caused an approximately 10-fold increase in expression (Fig. 3C), as in the wild type. However, in the stationary phase, the *rpoS* mutant did not show the elevated level of *hmp* induction (about 130 Miller units; Fig. 3D) observed for wild-type cells at zero or low concentrations of paraquat (Fig. 3B). Although paraquat did enhance Φ(*hmp-lacZ*) expression in stationary-phase cultures of the *rpoS* mu-

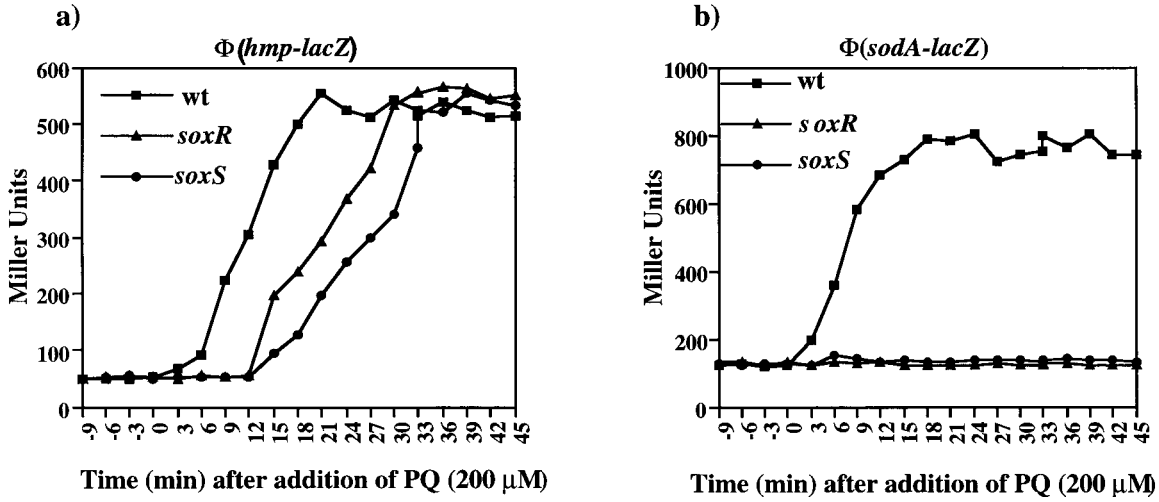

FIG. 2. Kinetics of gene transcription in response to added paraquat (PQ). Cultures of strains harboring single-copy Φ(*hmp-lacZ*) (a) and Φ(*sodA-lacZ*) (b) fusions were used. In panel a, the strains used were RKP2178 (wild type [wt]), RKP3979 (*soxR*), and RKP3978 (*soxS*). In panel b, the strains used were QC772 (wild type), RKP3983 (*soxR*), and RKP3982 (*soxS*). Cultures were grown to mid-exponential phase (OD_{600}, ~0.5) and then supplemented with paraquat to a final concentration of 200 μM. Samples were removed at 3-min intervals, and β-galactosidase activities in permeabilized cell extracts were measured.

tant (Fig. 3D), the increase was only 2.5-fold, resulting in a maximal level of expression that was still 3-fold lower than that in the wild type (Fig. 3B). These results suggest that RpoS is a positive regulator of *hmp* induction by paraquat in stationary-phase cells.

To obtain further evidence that RpoS is involved in paraquat regulation in the stationary phase, we exploited the recent findings of Muffler et al. (34) and Jishage and Ishihama (20), who showed that σ^s can be induced by an upshift in temperature during the exponential phase, reaching maximum levels 20 min after the heat shock. Unlike the induction of σ^{32}, the heat shock sigma factor, which reaches its maximum level 5 to 10 min after the upshift (42), the induction of σ^s is not a transient response. To examine further the involvement of σ^s in the activation of *hmp* by paraquat, cells grown in the exponential phase were pretreated with a heat shock at 42°C and then challenged with paraquat at 37°C (Fig. 4). When cells of strain RKP2178 were pretreated with heat shock for 5 min, there was no difference in the level of induction of *hmp* by paraquat (Fig. 4D). However, when cells were pretreated with heat shock for 20 min, the level of expression of Φ(*hmp-lacZ*) was increased 1.4-fold in the presence of paraquat (Fig. 4E), indicating that σ^s is involved in the activation of *hmp*.

Effect of regulatory mutations on the induction of *hmp* by paraquat. The regulation of *hmp* expression during exponential growth by paraquat was independent of SoxRS and RpoS. These results suggest that other regulators must operate to control the expression of *hmp* by paraquat during exponential growth. To investigate this possibility further, we determined the effect of other known regulators on *hmp* expression. The global regulator of anaerobic gene expression, Fnr, has been shown to be a negative regulator of *hmp* expression under anaerobic conditions, but it had no effect under aerobic conditions (38). Neither a mutation in *arcA* nor one in *fnr* was able to prevent the induction of *hmp* by paraquat during exponential or stationary phases of growth (data not shown). Mutations in *narL* or *narP* did not decrease the induction of *hmp* by paraquat under any growth condition tested. Similarly, mutations in the global regulators OxyR, Fur, IHF (HimA), and adenylate cyclase (*cya*) did not significantly alter the level of *hmp* expression by paraquat. These experiments were also performed with stationary-phase cells, and again the only positive transcriptional activator of *hmp* in the presence of paraquat was σ^s. Neither a mutation in *soxR* nor one in *soxS* prevented the induction of *hmp* by paraquat in stationary-phase cultures (data not shown).

The protein Rob (right of the origin binding protein) has

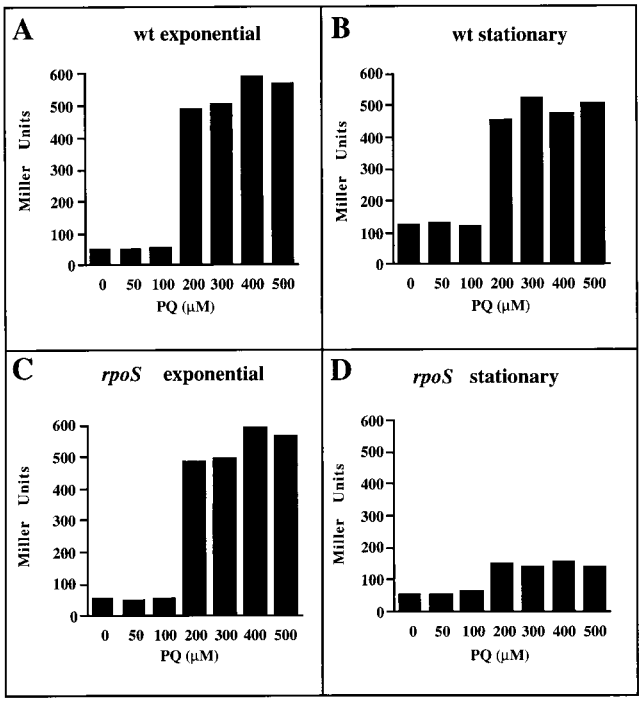

FIG. 3. Effect of increasing concentrations of paraquat (PQ) on the expression of Φ(*hmp-lacZ*) during exponential growth (A and C) and the stationary phase (B and D). The experiments were performed with a wild-type (wt) strain (A and B) or in an *rpoS* background (C and D). Paraquat was added during exponential growth (OD_{600}, <0.5) and the stationary phase (OD_{600}, >3.0). Samples were taken 45 min after this addition, and β-galactosidase activity was determined in triplicate.

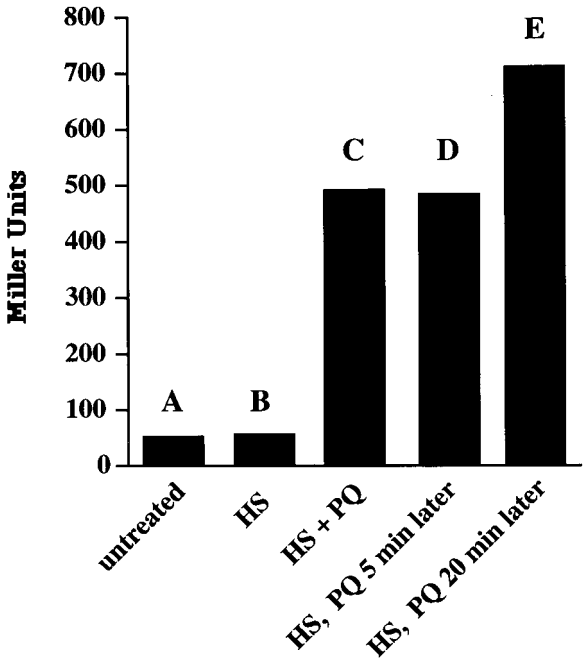

FIG. 4. Effect of two different heat shock (HS) pretreatments on Φ(*hmp-lacZ*) expression. Strain RKP2178 was grown in LB medium at 37°C. In the exponential phase (OD_{600}, 0.4), cells were shifted to 42°C for 0 (C), 5 (D), or 20 (E) min prior to exposure to 200 μM paraquat (PQ) at 37°C for 45 min. Control experiments consisted of untreated cells (A) and cells exposed only to the upshift of temperature (B). The values shown are the means of triplicate assays, and a repeat experiment gave essentially similar results.

been shown to induce some oxidative stress promoters when overexpressed by a multicopy plasmid (3, 21). The promoters shown to be affected to date are *inaA*, *fumC*, and *sodA* (3). This pattern of activation was distinct from that found for SoxS, and so we investigated the effect of Rob on the expression of *hmp*. When the mutation *rob*::*kan* was transduced into strain RKP2178, there was no effect on the expression of *hmp* during exponential growth or the stationary phase (data not shown). Furthermore, there was no effect on the induction of *hmp* by paraquat in exponentially growing or stationary-phase cells in the *rob* mutant (data not shown).

DISCUSSION

The electron-accepting properties of oxygen make its use as a terminal electron acceptor potentially dangerous for living systems; superoxide anion radical (O_2^-) and other reactive by-products of oxygen reduction are produced in all aerobic cells and, if not scavenged, can damage critical biomolecules during oxidative stress (14). During aerobic growth of *E. coli*, for example, reduction of oxygen leads to the production of superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) (10). In *E. coli*, two oxidative stress regulons are triggered, respectively, by H_2O_2 (the *oxyR* regulon) and by superoxide-generating (redox cycling) agents (the *soxRS* regulon) (10, 13, 16). Superoxide dismutases disproportionate O_2^-, while catalases remove H_2O_2. Paraquat is one of several redox-active compounds that catalyze a cyclic single-electron reduction of O_2 with the formation of O_2^- (10). In *E. coli*, exposure to paraquat triggers the oxidative response controlled by the SoxRS regulon (8, 16), presumably as a result of superoxide generation. Rereduction of the paraquat radical (PQ^+) by intracellular diaphorases regenerates the fully reduced species (28), leading to continued superoxide generation (redox cycling). In *E. coli*, many paraquat-inducible genes (encoding about 40 proteins) have been reported (2, 22, 24, 25, 33), but very few have been characterized in detail. These genes include those encoding endonuclease IV, glucose-6-phosphate dehydrogenase, superoxide dismutase, fumarase C, *micF* (an antisense RNA), and the gene encoding the NADPH:ferredoxin oxidoreductase (Fpr). A recent study described a novel paraquat-inducible gene that encodes a membrane protein of unknown function (*pqi-5*) (25). This gene is also a member of the SoxRS regulon (26).

Several lines of evidence point to complex mechanisms for *hmp* regulation and implicate the flavohemoglobin Hmp in oxidative stress. First, use of a Φ(*sodA-lacZ*) fusion to monitor intracellular superoxide production demonstrated that Hmp is capable of generating superoxide in vivo when overexpressed (30). Second, oxygen is bound at a single heme in Hmp which accepts electrons from NADH via flavin adenine dinucleotide (18, 37, 39), facilitating single-electron reduction of bound O_2 to superoxide anion. Indeed, studies of purified Hmp in vitro have demonstrated that Hmp generates superoxide which reduces Fe(III) to Fe(II) (30), although the reduction of other substrates (e.g., cytochrome *c*) by Hmp is not superoxide dependent (39). Third, Φ(*hmp-lacZ*) expression is regulated in a complex manner (38). Expression is similar in minimal glucose-containing media under aerobic and anaerobic conditions, yet anaerobic expression is stimulated three- to fourfold by an *fnr* mutation. An apparent Fnr-binding site is present in the *hmp* promoter (38), as was subsequently confirmed by in vitro studies of Fnr binding to the *hmp* promoter (29). Also, high concentrations (0.4 mM) of 2′2′-dipyridyl, an iron chelator, stimulate expression anaerobically and aerobically. Of particular note is that Φ(*hmp-lacZ*) expression is increased dramatically in the presence of nitric oxide and nitrite, and this induction is independent of SoxRS (38). Expression levels of Φ(*hmp-lacZ*) are low in aerobic, exponentially growing cultures, but increase twofold in the stationary phase in rich media in an *rpoS*-dependent fashion (31). The absence of integration host factor is also necessary for maximal Φ(*hmp-lacZ*) expression (31). Thus, in addition to Fnr, several other regulatory mechanisms are involved in the responses to oxygen, nitrogen compounds, iron availability, and the onset of the stationary phase. Such complex regulation is reminiscent of the regulation of *sodA*, which is subject to regulation by six global transcriptional regulators (5).

Here we describe a further link between Hmp and oxidative stress: *hmp* expression is induced by paraquat during both exponential growth and the stationary phase. The minimal concentration of paraquat (200 μM) required to elicit this induction was two- to fourfold higher than that for previously described paraquat-inducible genes, and hence *hmp* may have been overlooked in previous studies that sought paraquat-activated genes (24–26, 33). Other superoxide-generating agents used in this study, such as menadione, plumbagin, and phenazine methosulfate did not induce *hmp* expression even at high concentrations. We can only speculate on why certain oxidative chemicals other than nitric oxide and paraquat do not appear to increase *hmp* expression. Nitrite also induces *hmp* expression (38), although this may be due to nitric oxide formation in aqueous solutions. In the case of nitric oxide, *hmp* expression may be related to the fact that nitric oxide is a ligand for Hmp (18). Detailed studies with the purified protein will be needed in vitro to determine the binding kinetics, the dissociation constant, and the possibility that Hmp reduces NO, but it seems plausible that the effects of NO are related to its status as an intermediate in denitrification or a product of nitrite

reduction rather than its ability to interact with the Fe-S cluster in SoxR (16). Metabolic dysfunction caused by redox-cycling agents cannot always be attributed to superoxide (17), and other interactions between paraquat and *E. coli* should be considered. Paraquat has a redox potential considerably lower (E_m, −446 mV, 30°C, pH independent) than those of menadione (E_m, 0 mV, 25°C, pH 7) and phenazine methosulfate (E_m, +80 mV, 30°C, pH 7) (7), for example, and may reduce Hmp directly or interact with redox-sensitive regulators of *hmp*.

The kinetics of *hmp* induction in wild-type cells indicated that cells responded to paraquat within 3 min, and maximal *hmp* expression was observed after 20 min. However, when the kinetics of *hmp* induction were studied in both a *soxS* mutant and a *soxR* mutant, there was a 12- to 15-min lag period before the level of *hmp* expression increased. This delay suggests that a product of the Sox regulon may normally facilitate induction. Further work is needed to elucidate regulators of *hmp* expression during exponential growth.

The induction of *hmp* by paraquat was significantly reduced by a mutation in *rpoS*, but only during the stationary phase, suggesting that σ^s modulates induction of *hmp* by paraquat. Other paraquat-inducible genes, e.g., *pqi-5*, have also been shown to be regulated by σ^s (26). However, *pqi-5* was induced by paraquat only during exponential growth (SoxS/Eσ^{70}), and no induction was seen in the stationary phase even at high concentrations (770 μM) of paraquat. Koh and Roe (26) hypothesized that SoxS activation of *pqi-5* during the stationary phase does not occur because SoxS does not activate the Eσ^s holoenzyme. The level of *hmp* expression also increases in the stationary phase, and this induction is dependent on RpoS (31). The results of the present study clearly demonstrate that RpoS only affects induction of *hmp* by paraquat in stationary-phase cells. The ability of exponentially growing cells to induce *hmp* in the presence of paraquat was unaffected by mutations in *rpoS*. Moreover, when exponentially growing cells were pretreated with heat shock to increase the concentration of σ^s (34), the magnitude of induction by paraquat was 1.4-fold higher than that in cells not treated with heat shock. While this increase is lower than that (three- to fivefold) reported (34) for the elevated level of cellular σ^s, it does suggest that RpoS plays a role in the induction of *hmp* by paraquat. We conclude that RpoS is a regulator of *hmp* expression by paraquat during the stationary phase, but *hmp* transcription in exponentially growing cells may be regulated by other, unknown factors.

ACKNOWLEDGMENTS

We thank Muna F. Anjum and Vincent Wood for valuable preliminary experiments and Bernard Weiss, Daniélle Touati, Gisela Storz, Valley Stewart, Carmen Gómez-Eichelmann, Chiharu Ueguchi, and Bruce Demple for sending strains used in this work.

This work was supported by BBSRC research grant P05184 (awarded to R.K.P.).

REFERENCES

1. **Altuvia, S., M. Almirón, G. Huisman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^s in stationary phase. Mol. Microbiol. **13:**265–272.
2. **Amábile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res. **19:**4479–4484.
3. **Ariza, R. R., Z. Li, N. Ringstad, and B. Demple.** 1995. Activation of multiple antibiotic resistance and binding of stress-inducible promoters by *Escherichia coli* Rob protein. J. Bacteriol. **177:**1655–1661.
4. **Chung, C. T., S. L. Niemala, and R. H. Miller.** 1989. One-step transformation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
5. **Compan, I., and D. Touati.** 1993. Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. J. Bacteriol. **175:**1687–1696.
6. **Cramm, R., R. A. Siddiqui, and B. Friedrich.** 1994. Primary sequence and evidence for a physiological function of the flavohaemoprotein of *Alcaligenes eutrophus*. J. Biol. Chem. **269:**7349–7354.
7. **Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones.** 1986. Data for biochemical research, 3rd ed., p. 350–377. Clarendon Press, Oxford, United Kingdom.
8. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. **25:**315–337.
9. **Ermler, U., R. A. Siddiqui, R. Cramm, and B. Friedrich.** 1995. Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 angstrom resolution. EMBO J. **14:**6067–6077.
10. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
11. **Favey, S., G. Labese, V. Vouille, and M. Boccara.** 1995. Flavohaemoglobin HmpX: a new pathogenicity determinant in *Erwinia chrysanthemi* strain 3937. Microbiology **141:**863–871.
12. **Giacomini, A., B. Corich, F. J. Ollero, A. Squartini, and M. P. Nuti.** 1992. Experimental conditions may affect reproducibility of the β-galactosidase assay. FEMS Microbiol. Lett. **100:**87–90.
13. **Greenberg, J. T., and B. Demple.** 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. J. Bacteriol. **171:**3933–3939.
14. **Halliwell, B., and J. M. C. Gutteridge.** 1989. Free radicals in biology and medicine, p. 86–187. Clarendon Press, Oxford, United Kingdom.
15. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell **72:**165–168.
16. **Hidalgo, E., and B. Demple.** 1996. Adaptive responses to oxidative stress: the soxRS and oxyR regulons, p. 435–452. *In* E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Company, Austin, Tex.
17. **Imlay, J. and I. Fridovich.** 1992. Exogenous quinones directly inhibit the respiratory NADH dehydrogenase in *Escherichia coli*. Arch. Biochem. Biophys. **296:**337–346.
18. **Ioannidis, N., C. E. Cooper, and R. K. Poole.** 1992. Spectroscopic studies on an oxygen-binding haemoglobin-like flavohaemoprotein from *Escherichia coli*. Biochem. J. **288:**649–655.
19. **Iuchi, S., and E. C. C. Lin.** 1988. *arcA* (*dye*), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. USA **85:**1888–1892.
20. **Jishage, M., and A. Ishihama.** 1995. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of σ^{70} and σ^{38}. J. Bacteriol. **177:**6832–6835.
21. **Kakeda, M., C. Ueguchi, H. Yamada, and T. Mizuno.** 1995. An *Escherichia coli* curved DNA binding protein whose expression is affected by the stationary phase-specific sigma factor σ^s. Mol. Gen. Genet. **248:**629–634.
22. **Kao, S. M., and H. M. Hassan.** 1985. Isolation of paraquat-resistant mutants of *Escherichia coli*: lack of correlation between resistance and the activity of superoxide dismutase. FEMS Microbiol. Lett. **28:**93–97.
23. **Kawamukai, M., R. Utsumi, K. Takeda, A. Higashi, H. Matsuda, Y.-L. Choi, and T. Komano.** 1991. Nucleotide sequence and characterization of the *sfs1* gene: *sfs1* is involved in CRP*-dependent *mal* gene expression in *Escherichia coli*. J. Bacteriol. **173:**2644–2648.
24. **Kogoma, T., S. B. Farr, K. M. Joice, and D. O. Natvig.** 1988. Isolation of gene fusions (*soi*::*lacZ*) inducible by oxidative stress in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**4799–4803.
25. **Koh, Y.-S., and J.-H. Roe.** 1995. Isolation of a novel paraquat-inducible (*pqi*) gene regulated by the *soxRS* locus in *Escherichia coli*. J. Bacteriol. **177:**2673–2678.
26. **Koh, Y. S., and J. H. Roe.** 1996. Dual regulation of the paraquat-inducible gene *pqi-5* by SoxS and RpoS in *Escherichia coli*. Mol. Microbiol. **22:**53–61.
27. **LaCelle, M., M. Kumano, K. Kurita, K. Yamane, P. Zuber, and M. M. Nakano.** 1996. Oxygen-controlled regulation of the flavohemoglobin gene in *Bacillus subtilis*. J. Bacteriol. **178:**3803–3808.
28. **Liochev, S. I., and I. Fridovich.** 1994. Paraquat diaphorases in *Escherichia coli*. Free Radical Biol. Med. **16:**555–559.
29. **Membrillo-Hernández, J., J. Green, and R. K. Poole.** Unpublished data.
30. **Membrillo-Hernández, J., N. Ioannidis, and R. K. Poole.** 1996. The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide *in vitro* and causes oxidative stress *in vivo*. FEBS Lett. **382:**141–144.
31. **Membrillo-Hernández, J., G. M. Cook, and R. K. Poole.** Roles of σ^s, IHF and ppGpp in the expression of *hmp* encoding the flavohemoglobin (Hmp) of *Escherichia coli* K-12. Mol. Gen. Genet., in press.
32. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Mito, S., Q.-M. Zhang, and S. Yonei.** 1993. Isolation and characterization of *Escherichia coli* strains containing new gene fusions (*soi*::*lacZ*) inducible by superoxide radicals. J. Bacteriol. **175:**2645–2651.
34. **Muffler, A., M. Barth, C. Marschall, and R. Hengge-Aronis.** 1997. Heat shock regulation of σ^s turnover: a role for DnaK and relationship between stress responses mediated by σ^s and σ^{32} in *Escherichia coli*. J. Bacteriol. **179:**445–452.

35. **Nunoshiba, T., E. Hidalgo, C. F. Amábile-Cuevas, and B. Demple.** 1992. Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. J. Bacteriol. **174:**6054–6060.
36. **Nunoshiba, T., T. de Rojas-Walker, J. S. Wishnok, S. R. Tannenbaum, and B. Demple.** 1993. Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. Proc. Natl. Acad. Sci. USA **90:**9993–9997.
37. **Poole, R. K.** 1994. Oxygen reactions with bacterial oxidases and globins: binding, reduction and regulation. Antonie Leeuwenhoek **65:**289–310.
38. **Poole, R. K., M. F. Anjum, J. Membrillo-Hernández, S. O. Kim, M. N. Hughes, and V. Stewart.** 1996. Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. **178:**5487–5492.
39. **Poole, R. K., N. J. Rogers, R. A. M. D'mello, M. N. Hughes, and Y. Orii.** *Escherichia coli* flavohaemoglobin (HMP) reduces cytochrome *c* and Fe(III)-hydroxamate K by electron transfer from NADH via FAD: sensitivity of oxidoreductase activity to haem-bound oxygen. Microbiology, in press.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based vectors for protein and operon fusions. Gene **53:**85–96.
42. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ^{32}. Nature **329:** 348–351.
43. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:** 2305–2314.
44. **Vasudevan, S. G., W. L. F. Armarego, D. C. Shaw, P. E. Lilley, and R. K. Poole.** 1991. Isolation and nucleotide sequence of the *hmp* gene that encodes a hemoglobin-like protein in *Escherichia coli* K-12. Mol. Gen. Genet. **226:** 49–58.
45. **Walkup, L. K. B., and T. Kogoma.** 1989. *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. J. Bacteriol. **171:** 1476–1484.
46. **Wu, J., and B. Weiss.** 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. J. Bacteriol. **173:**2864–2871.