INFECTION AND IMMUNITY, Nov. 1996, p. 4618–4629
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 64, No. 11

Lack of Expression of the Global Regulator OxyR in *Haemophilus influenzae* Has a Profound Effect on Growth Phenotype

ISOBEL MACIVER AND ERIC J. HANSEN*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048

Received 20 March 1996/Returned for modification 25 April 1996/Accepted 20 August 1996

A pBR322-based library of chromosomal DNA from the nontypeable *Haemophilus influenzae* TN106 was screened for the expression of transferrin-binding activity in *Escherichia coli*. A recombinant clone expressing transferrin-binding activity contained a 3.7-kb fragment of nontypeable *H. influenzae* DNA. Nucleotide sequence analysis of this insert revealed the presence of two complete open reading frames encoding proteins of approximately 26 and 34 kDa. Mini-Tn*10kan* transposon mutagenesis at different sites within the open reading frame encoding the 34-kDa protein resulted in the abolition of transferrin-binding activity in the recombinant *E. coli* clone. The deduced amino acid sequence of the 34-kDa protein had 70% identity with the OxyR protein of *E. coli*; this latter macromolecule is a member of the LysR family of transcriptional activators. When a mutated *H. influenzae oxyR* gene was introduced into the chromosome of the wild-type *H. influenzae* strain by allelic exchange, the resulting *oxyR* mutant still exhibited wild-type levels of transferrin-binding activity but was unable to grow on media containing the heme precursor protoporphyrin IX (PPIX) in place of heme. This mutant also exhibited reduced growth around disks impregnated with heme sources. Supplementation of the PPIX-based growth media with catalase or sodium pyruvate resulted in normal growth of the *H. influenzae oxyR* mutant. Provision of the wild-type *H. influenzae oxyR* gene in *trans* also permitted the growth of this mutant on a PPIX-based medium. Exogenously supplied catalase restored the growth of this mutant with heme sources to nearly wild-type levels. These results indicate that expression of a wild-type OxyR protein by *H. influenzae* is essential to allow this organism to protect itself against oxidative stresses in vitro.

Haemophilus influenzae is a bacterial pathogen which is distinguished from virtually all other facultatively anaerobic organisms by the fact that it has an absolute requirement for exogenously supplied heme and NAD for aerobic growth in vitro (16). This heme requirement is caused by the inability of *H. influenzae* to convert δ-aminolevulinic acid to protoporphyrin IX (PPIX), the immediate biosynthetic precursor of heme (23, 72). *H. influenzae* does possess the ability to take up PPIX and convert it to heme via the activity of the enzyme ferrochelatase, which inserts a single molecule of iron into the PPIX molecule (23, 40). Accordingly, PPIX can satisfy the heme requirement of *H. influenzae* in vitro if a utilizable form of iron is available in the growth medium (23, 72).

H. influenzae can utilize many different sources of iron in vitro, including both heme (51) and iron-loaded transferrin (32). In the latter instance, it would appear that *H. influenzae*, in a manner similar to *Neisseria gonorrhoeae* and *N. meningitidis* (5, 59), binds the iron-loaded transferrin molecule to its cell surface (58) and then, by an undefined mechanism(s), extracts the iron from the transferrin and transports it into the cell. Two *H. influenzae* outer membrane proteins (Tbp1 and Tbp2) essential for this process have recently been described (25, 41), and *H. influenzae* is known to express transferrin-binding activity in vivo (35).

In an attempt to clone *H. influenzae* genes encoding transferrin-binding outer membrane proteins, we isolated a recombinant plasmid which caused *Escherichia coli* to express a transferrin-binding phenotype. Interestingly, this clone contained an *H. influenzae* gene that encoded a protein with 70% identity to the *E. coli* transcriptional activator OxyR. This regulator of hydrogen peroxide-inducible genes in *E. coli* and *Salmonella typhimurium* is a member of the LysR family of transcriptional activators which have been found in both gram-positive and gram-negative organisms (31). In this study, we describe the identification of the *H. influenzae* OxyR homolog and demonstrate the essential role of this regulatory protein in the growth of *H. influenzae* on media containing PPIX in place of heme.

MATERIALS AND METHODS

Bacterial strains and culture media. Nontypeable *H. influenzae* (NTHI) TN106 has been described in detail (37, 54, 55). Mutants and transformants derived from this NTHI strain are listed in Table 1. The other four NTHI strains (N182, OC201, BO-2, and BF105) and five *H. influenzae* type b Hib strains (DL42, DL26, DL302, Eagan, and RM7004) used in this study have been described previously (9, 37, 49). *H. influenzae* strains were routinely grown in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) containing Levinthal's base (BHIs) (1); this medium was used for growing *H. influenzae* under iron-replete conditions. The medium used for growing *H. influenzae* under iron-restricted conditions was BHI agar containing NAD (10 μg/ml), PPIX (1 μg/ml), and the iron chelator Desferal (deferoxamine mesylate [Sigma Chemical Co., St. Louis, Mo.]) at a final concentration of either 30 or 80 μM.

For comparison of the abilities of wild-type and *oxyR* mutant strains to form individual colonies on certain media, BHI-NAD agar plates supplemented with heme or PPIX (20 μg/ml) were used. In certain experiments, freshly prepared solutions of catalase (final concentration, 5 μg/ml) or sodium pyruvate (final concentration, 10 mM) were spread onto the agar surface immediately before bacteria were inoculated onto the medium. The plates were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 24 h.

For comparison of the abilities of these strains to grow in broth, bacteria were cultured overnight on BHIs agar and then suspended to a final concentration of 10^8 CFU/ml in phosphate-buffered saline (PBS). A 1-ml portion of these cells was used to inoculate 10 ml of BHI-NAD broth containing PPIX (10 μg/ml). This culture was incubated with aeration at 37°C for 3 h, after which a 1-ml portion

* Corresponding author. Mailing address: Department of Microbiology, University of Texas Southwestern Medical Center, Hamon Biomedical Research Bldg., NA6.200A, 6000 Harry Hines Blvd., Dallas, TX 75235-9048. Phone: (214) 648-5974. Fax: (214) 648-5905. Electronic mail address: hansen01@utsw.swmed.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
H. influenzae strains		
TN106	Wild-type nontypeable strain	37, 55
TN106.13	Kanamycin-resistant *oxyR* mutant constructed by transforming TN106 with the mutated *oxyR* gene from pIM126	This study
TN106.13T	Kanamycin-sensitive transformant strain containing the wild-type NTHI *oxyR* gene, obtained by transforming TN106.13 with wild-type NTHI TN106 chromosomal DNA	This study
DL302	Wild-type type b strain	37
DL302.22	Chloramphenicol-resistant *oxyR* mutant constructed by transforming DL302 with the mutated *oxyR* gene from pIM127	This study
DL302.22T	Chloramphenicol-sensitive transformant containing the wild-type NTHI *oxyR* gene, obtained by transforming DL302.22 with wild-type NTHI TN106 chromosomal DNA	This study
E. coli strains		
RR1	Host for construction of NTHI TN106 genomic DNA library in pBR322	53
HB101	Host for cloning experiments	53
MC1060	Host for mini-Tn*10kan* transposon-mediated mutagenesis	G. J. Barcak (70)
DM1	*dam* and *dcm* methylase-deficient host used for mutagenesis of the *oxyR* gene in pIM120	GIBCO-BRL
Plasmids		
pBR322	Cloning vector, Amp^r Tet^r	53
pIM100	pBR322 with a 3.7-kb *Pst*I fragment of chromosomal DNA from NTHI TN106 that contains the *H. influenzae oxyR* gene; confers transferrin-binding activity on *E. coli*	This study
pIM110	pIM100 containing a mini-Tn*10kan* insertion that inactivates transferrin-binding activity in *E. coli*	This study
pBluescript II SK+	Cloning vector, Amp^r	43
pIM120	pBluescript II containing a 2.8-kb *Pst*I-*Bgl*II fragment from the NTHI DNA insert in pIM100; confers transferrin-binding activity on *E. coli*	This study
pIM122	pBluescript II SK+ containing a 1.9-kb *Hin*dIII fragment from the NTHI insert in pIM120; confers transferrin-binding activity on *E. coli*	This study
pIM123	pBluescript II SK+ containing a 1.8-kb *Alw*NI-*Bgl*II fragment from the NTHI DNA insert in pIM120; does not confer transferrin-binding activity on *E. coli*	This study
pIM126	pIM120 containing a kanamycin resistance cassette (*kan*) inserted at the *Bcl*I site in the *oxyR* gene	This study
pIM127	pIM120 containing a chloramphenicol resistance cassette (*cat*) inserted at the *Bcl*I site in the *oxyR* gene	This study
pLS88	Shuttle vector capable of replication in *E. coli* and *H. influenzae*; Kan^r, Str^r, Sulf^r	14, 73
pIM130	pLS88 with the 1.9-kb *Hin*dIII fragment from pIM120 containing *oxyR*	This study
pAQ17	pUC12 containing a 1.5-kb fragment of *E. coli* DNA including the *oxyR* gene	8

was transferred into 10 ml of fresh BHI-NAD broth containing PPIX (10 μg/ml) with or without catalase (5 μg/ml). Growth of this final culture was monitored by measuring the turbidity for 8 h.

E. coli DM1, which is both *dam* and *dcm* methylase deficient, was obtained from GIBCO-BRL (Gaithersburg, Md.). *E. coli* strains were grown in Luria-Bertani medium (43) supplemented with antimicrobial agents as required. Luria-Bertani medium containing 200 μM ethylenediamine-*N*,*N*′-diacetic acid (EDDA) was used for growth of *E. coli* under iron-limited conditions. Antimicrobial agents were used at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 15 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 1 μg/ml.

Plasmids. The plasmid shuttle vector pLS88, which is capable of replication in both *E. coli* and *H. influenzae*, has been described previously (14, 73), as have the *E. coli*-based cloning vectors pBR322 and pBluescript II SK+ (43, 53). Plasmid pAQ17 containing the *E. coli oxyR* gene was kindly provided by Gisela T. Storz, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, and has been described previously (8).

Genetic techniques. Standard genetic techniques including plasmid isolation, restriction enzyme digestions, DNA modifications, ligation reactions, and *E. coli* transformation were performed as described previously (43, 53, 70). A genomic DNA library was constructed from the wild-type NTHI TN106 in pBR322, using the *Pst*I site in this vector for cloning purposes. *H. influenzae* was transformed with linear DNA as described (33), and Hib DL302 was transformed with plasmids by a plate transformation method (55).

Mutagenesis techniques. Mini-Tn*10kan* transposon insertion mutagenesis of cloned DNA inserts in recombinant *E. coli* strains was performed as described previously (11, 28, 70). The *cat* gene, encoding chloramphenicol acetyltransferase and lacking an internal *Eco*RI site, was derived from the plasmid pUC4DE*cat*, which was kindly provided by Bruce A. Green, Lederle-Praxis Biologicals, West Henrietta, N.Y. The kanamycin resistance cassette (*kan*) was obtained from Pharmacia-LKB (Piscataway, N.J.).

Nucleotide sequence and Southern blot analyses. Nucleotide sequence analysis of the 2.8-kb *Pst*I-*Bgl*II fragment from pIM120 was performed by using nested deletions and other standard methods. Both strands were sequenced in their entirety. DNA sequence information was analyzed by using the IntelliGenetics Suite package and programs from the University of Wisconsin Genetics Computer Group software sequence analysis package (13). Southern blot analysis of chromosomal DNA purified from various *H. influenzae* wild-type strains was performed as described previously (43).

PCR. PCR was performed with the GeneAmp kit (Perkin-Elmer, Branchburg, N.J.). All reactions were carried out as specified by the manufacturer. To amplify products from total genomic DNA, 1 μg of chromosomal DNA and 100 ng of each primer were used in each 100-μl reaction mixture. Direct amplification of DNA fragments from single colonies was performed as described previously (24, 56).

Detection of transferrin-binding activity. The ability of bacterial strains to bind human transferrin was assessed by the dot blot method of Schryvers (57). Briefly, cell paste from an agar plate culture grown overnight was spotted onto Whatman no. 40 filter paper and allowed to dry at 37°C for 1 h. These filters were then incubated for 1 h at room temperature in Tris-buffered saline (TBS) (pH 7.4) containing 0.5% (wt/vol) skim milk and then for 4 h in TBS-skim milk containing horseradish peroxidase-coupled human transferrin (Jackson Immunoresearch Labs, West Grove, Pa.). After three 15-min washes with TBS, the blots were developed by incubating the filters in TBS containing 4-chloro-1-naphthol (2 mg/ml) and 0.01% (vol/vol) hydrogen peroxide.

Hydrogen peroxide sensitivity tests. Bacteria were plated as described immediately above but on BHIs agar plates. Filter paper disks impregnated with different concentrations (0.125 to 2.0%) of hydrogen peroxide in PBS were placed on the surface of the agar, and the plates were incubated overnight at 37°C.

Heme utilization assay. To determine the ability of wild-type and mutant *H. influenzae* strains to utilize heme sources for growth, bacteria grown overnight on BHIs agar plates were suspended in PBS (pH 7.4) to a final concentration of 10^8 CFU/ml. Portions (100 μl) of this suspension were spread onto BHI-NAD agar plates containing PPIX (1 μg/ml) and 80 μM Desferal. Sterile filter paper disks containing 5 μl of heme (5 mg/ml), hemoglobin (5 mg/ml), catalase (5 mg/ml), or PBS or various combinations thereof were placed on the surface of the plates, and the plates were incubated at 37°C overnight.

Measurement of catalase activity. Catalase activity in whole bacterial cells

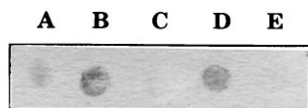

FIG. 1. Transferrin-binding activity expressed by wild-type NTHI and recombinant *E. coli* strains. Cells were spotted on filter paper and used in the transferrin-binding dot blot assay as described in Materials and Methods. Lanes: A, TN106 cells grown under iron-replete conditions; B, TN106 cells grown under iron-restricted conditions; C, *E. coli* RR1 containing pBR322; D, *E. coli* RR1 containing pIM100; E, *E. coli* RR1 containing pIM110.

harvested in the stationary phase of growth (7) was measured by a modification of the method described by Beers and Sizer (2). Briefly, whole cells (10 μg of protein) of wild-type and mutant *H. influenzae* strains were added to 1.5 ml of PBS containing 0.06 mM freshly diluted hydrogen peroxide. The optical density (A_{240}) of this suspension was monitored every 10 s for 200 s; catalase activity was evidenced by a decrease in optical density resulting from the degradation of the hydrogen peroxide. Solutions of crystalline bovine catalase (Sigma) were used to standardize this assay mixture.

Analysis of recombinant proteins. Whole-cell lysates from bacterial cells were prepared as described previously (49, 50). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins present in whole-cell lysates was performed as described previously (49, 50), and the proteins were stained with Coomassie brilliant blue. Purified plasmids were used as templates in a prokaryotic DNA-directed translation kit (Amersham Corp., Arlington Heights, Ill.); [^{3}H]leucine was used to radiolabel plasmid-encoded proteins synthesized in vitro, with all the procedures being carried out as specified by the manufacturer. Radiolabeled proteins were resolved by SDS-PAGE and visualized by fluorography as described previously (27).

Nucleotide sequence accession number. The sequence of the ORFs encoding the 26- and 34-kDa proteins was submitted to GenBank and assigned accession number U49355.

RESULTS

The majority of *H. influenzae* strains express an inducible transferrin-binding activity when grown under iron-restricted conditions (29, 45, 58). Similarly, NTHI TN106 expressed readily detectable transferrin-binding activity when grown in an iron-restricted environment (Fig. 1, lane B) whereas transferrin-binding activity was barely detectable in cells grown under iron-replete conditions (Fig. 1, lane A).

Cloning of a NTHI locus that confers transferrin-binding activity on *E. coli*. In an attempt to clone NTHI genes encoding outer membrane proteins involved in transferrin binding, a genomic library of TN106 DNA was constructed in the plasmid vector pBR322. Preliminary screening of *E. coli* RR1(pBR322) confirmed that this strain did not detectably bind transferrin in this system when grown under iron-replete conditions on LB medium (Fig. 1, lane C). In addition, the growth of this strain under iron-restricted conditions (i.e., in LB medium containing 200 μM EDDA) did not result in any detectable binding of transferrin (data not shown).

Screening of 40,000 recombinants in the dot blot assay resulted in the identification of a single clone (Fig. 1, lane D) that bound transferrin at a readily detectable level. The recombinant plasmid in this strain was designated pIM100 and shown to contain a 3.7-kb *Pst*I fragment of NTHI TN106 chromosomal DNA (Fig. 2). In an attempt to localize the NTHI gene(s) responsible for the binding of transferrin by *E. coli* RR1(pIM100), the recombinant plasmid pIM100 was subjected to mini-Tn*10kan* transposon insertion mutagenesis. When the positions of the transposon insertions were mapped,

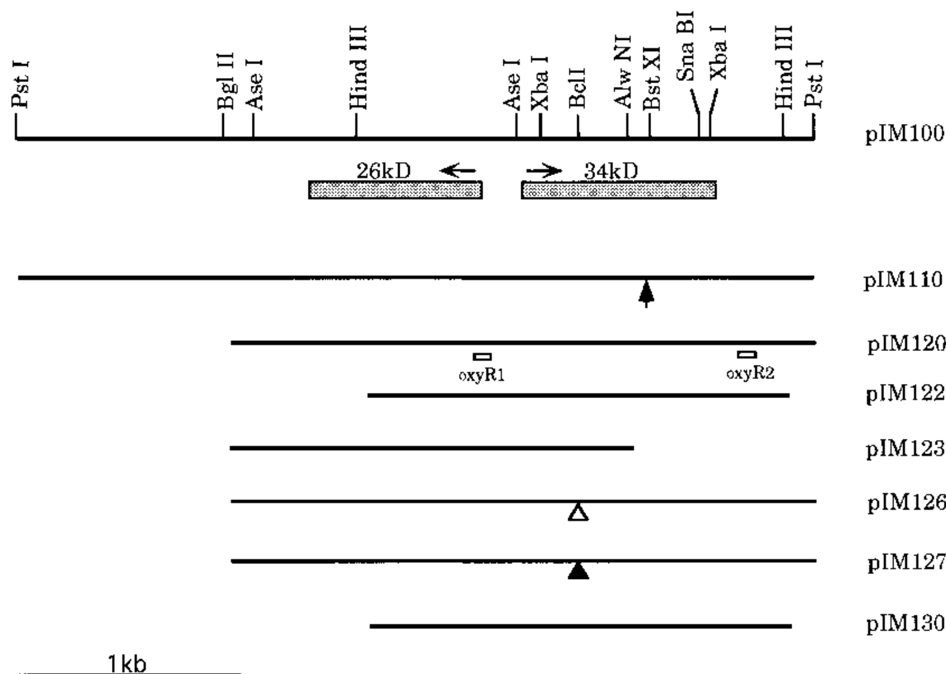

FIG. 2. Partial restriction enzyme map of the NTHI DNA insert in pIM100 and related plasmids. The divergent ORFs for the 26- and 34-kDa proteins are depicted by boxes underneath pIM100; the arrows indicate the predicted direction of transcription. The black arrowhead indicates the position of a mini-Tn*10kan* transposon insertion within the *oxyR* gene in pIM110. The 2.8-kb *Pst*I-*Bgl*II fragment from pIM100 was subcloned into pBluescript II SK+ to generate pIM120. The 1.9-kb *Hin*dIII fragment from pIM120 was subcloned into pBluescript to generate pIM122, which contains all of the ORF for the 34-kDa protein. The 1.8-kb *Bgl*II-*Alw*NI fragment from pIM120 was subcloned into pBluescript to generate pIM123, which contains all of the ORF for the 26-kDa protein. The open triangle indicates the position of a *kan* cartridge inserted into the *Bcl*I site in the *oxyR* gene in pIM126, and the solid triangle indicates the position of a *kan* cartridge inserted at the same site in the *oxyR* gene in pIM127. The 1.9-kb *Hin*dIII fragment from pIM120 was subcloned into shuttle vector pLS88 to construct pIM130. The bars labelled oxyR1 and oxyR2 beneath pIM120 indicate the positions of the oligonucleotide primers used to amplify *oxyR* genes from pIM126 and pIM127 and from total genomic DNA derived from wild-type and mutant *H. influenzae* strains.

it was found that all of the insertions that inactivated transferrin-binding activity, as typified by that in pIM110 (Fig. 1, lane E), were located within the 2.8-kb *Pst*I-*Bgl*II fragment in pIM100 (Fig. 2). This 2.8-kb fragment from pIM100 was subcloned into pBluescript II SK+, yielding the recombinant plasmid pIM120 (Fig. 2).

Features of the nucleotide sequence of the 2.8-kb *Pst*I-*Bgl*II fragment. Nucleotide sequence analysis of the 2.8-kb insert in pIM120 revealed the presence of two complete open reading frames (ORFs) and one incomplete ORF. The two complete ORFs encoded predicted proteins with calculated molecular weights of approximately 26,000 and 34,000 (Fig. 3). Interestingly, these two ORFs appeared to be transcribed from divergent, overlapping promoters (Fig. 3).

The likely translational start site of the larger complete ORF encoding the 34-kDa polypeptide was located at nucleotides 938 to 940 (Fig. 3); this ATG codon is located 7 nucleotides downstream from a sequence (5′-AGGGA-3′) with homology to ribosomal binding sites (60). Putative −10 and −35 consensus sequences were also identified upstream from this start codon (Fig. 3). The incomplete ORF containing 480 bp was located immediately downstream from this larger ORF and was truncated at the *Pst*I site (data not shown). The likely translational start site for the smaller complete ORF (on the opposite strand) encoding the 26-kDa polypeptide was located at nucleotides 813 to 815 and was preceded by a putative ribosomal binding site as well as by possible −10 and −35 regions (Fig. 3).

Analysis of the protein products of these ORFs. The calculated molecular weight of the protein encoded by the smaller complete ORF was 26,756. A search of the available databases did not reveal any bacterial proteins with significant homology to this 26-kDa polypeptide aside from the hypothetical 26-kDa protein (predicted ORF HI0570) encoded by the *H. influenzae* Rd genome (18). The protein encoded by the larger complete ORF had a calculated molecular weight of 33,763 and was found to be strikingly similar (70% identity) to OxyR, a protein that controls a regulon for an adaptive response to oxidative stress in *E. coli* (66) and is a member of the LysR family of transcriptional activators (31). The similarity between this NTHI protein and other members of the LysR family was reinforced by comparing the N-terminal region of this 34-kDa NTHI molecule with those of several LysR family members including OxyR. This NTHI protein was shown to possess a helix-turn-helix motif near its N terminus, which is typical of members of the LysR family (Fig. 4). In addition, this 34-kDa *H. influenzae* protein contains a Cys residue at position 199 (Fig. 3). In the *E. coli* OxyR protein, this Cys is critical for maintenance of the redox-sensitive nature of this transcriptional regulator (39). Because of these properties, this 34-kDa *H. influenzae* protein has been designated OxyR. This OxyR protein from NTHI TN106 was identical to that (ORF HI0571) encoded by the *H. influenzae* Rd genome (18). The 18-kDa truncated protein product of the incomplete ORF was shown to have 69% identity to a hypothetical 26.6-kDa *E. coli* protein of unidentified function which is also part of the *oxyR-trmA* region of the *E. coli* chromosome (26), and it was not analyzed further.

Identification of the ORF responsible for conferring transferrin-binding activity on *E. coli*. All of the mini-Tn*10kan* transposon insertions that inactivated transferrin-binding activity in pIM100 mapped within the *oxyR* gene (data not shown). To confirm directly that the *H. influenzae* OxyR protein was involved in conferring the transferrin-binding phenotype on *E. coli*, two different subclones were constructed in pBluescript II SK+. Plasmid pIM122 contained the 1.9-kb *Hin*dIII fragment from pIM120; this insert contained all of the ORF encoding the 34-kDa protein and only part of the ORF for the 26-kDa molecule (Fig. 2). Plasmid pIM123 contained the 1.8-kb *Bgl*II-*Alw*NI fragment from pIM120; this insert contained the whole ORF for the 26-kDa protein and only part of the ORF for the 34-kDa protein (Fig. 2). When *E. coli* HB101 was transformed with pIM120, the resultant transformants bound transferrin, as expected (Fig. 5, panel 2, lane B). Control transformants containing only the pBluescript vector (panel 2, lane A) did not bind transferrin detectably. *E. coli* HB101 transformants carrying pIM123 with the complete ORF for the 26-kDa protein did not exhibit detectable transferrin-binding activity (panel 2, lane D). In contrast, transformants carrying pIM122 with the intact ORF for the 34-kDa protein did bind transferrin (panel 2, lane C).

Analysis of whole-cell lysates from these transformant strains revealed that the recombinant strain HB101(pIM120) (Fig. 5, panel 1, lane B) expressed 26- and 76-kDa proteins not present in the strain containing only the vector (panel 1, lane A). HB101(pIM122) (panel 1, lane C) expressed a 76-kDa protein that migrated at the same rate as that expressed by HB101 (pIM120), whereas HB101(pIM123) (panel 1, lane D) expressed only the 26-kDa protein. Expression of the 34-kDa OxyR protein could not be detected by Coomassie blue staining, so that plasmid pIM120 was used in an in vitro DNA-directed translation system. Under these conditions, expression of both the 26- and 34-kDa products was readily detectable; however, no 76-kDa protein was visible in the fluorograph, even after long-term exposure (data not shown). Taken together, these results indicated that production of the *H. influenzae* OxyR protein in *E. coli* HB101 caused the expression of both a novel 76-kDa *E. coli* protein and transferrin-binding activity.

Construction of an isogenic *H. influenzae oxyR* mutant. The possible involvement of the OxyR protein in regulating the expression of transferrin-binding activity in *H. influenzae* was investigated by mutant analysis. The mutated plasmid pIM110 with a mini-Tn*10kan* insertion in the *oxyR* gene (Fig. 2) was linearized and used to transform the wild-type strain TN106. Kanamycin-resistant transformants were screened for their ability to bind transferrin. A number of these transformants were unable to bind transferrin, but Southern blot analysis indicated that proper allelic exchange (i.e., replacement of the wild-type *oxyR* allele with the mutated *oxyR* gene) had not occurred in these transformants. Numerous additional attempts to introduce this particular mutated *oxyR* gene into the proper site in the NTHI chromosome were also unsuccessful.

Next, plasmid pIM120 (Fig. 2) was used to transform *E. coli* DM1, which is *dam* and *dcm* methylase deficient. Propagation of pIM120 in this strain allowed cleavage of the *H. influenzae oxyR* gene at the methylase-sensitive *Bcl*I site (Fig. 2). Cassettes encoding either kanamycin or chloramphenicol resistance were then ligated into pIM120 at this position, forming the *kan*-containing plasmid pIM126 and the *cat*-containing plasmid pIM127 (Fig. 2), which were then transformed into *E. coli* HB101. After selection on Luria-Bertani medium containing appropriate antimicrobial compounds, single colonies containing the mutated plasmid pIM126 or pIM127 were picked and found to be unable to grow again on the same medium (52). Therefore, the original colony material was used as the source of DNA for PCR-based amplification with oligonucleotide primers flanking the *oxyR* gene (oxyR1 and oxyR2; Fig. 2 and 3). This approach yielded a large quantity of PCR-derived product containing each mutated *oxyR* gene and its flanking sequences.

These two different PCR products, each containing a mutated *oxyR* gene, were purified and used to transform the wild-

```
                              ———►    ◄———
TTTGCAATAAATTCGTACAAATGAAAAGGGCGCTTAATGCGCCCTTTAAATTACATTCTCAAATAATAATCAGCTAAATACTTGCCTGCT   90
AAACGTTATTTAAGCATGTTTACTTTTCCCGCGAATTACGCGGGAAATTTAATGTAAGAGTTTATTATTAGTCGATTTATGAACGGACGA

TATGCAAAGTATTTTTCCAAATCGTCGCTACCGCCAATGTGTTTACCACCGATAAACACTTGTGGAACAGTAGCACGACCTGAAACTGCA   180
ATACGTTTCATAAAAAGGTTTAGCAGCGATGGCGGTTACACAAATGGTGGCTATTTGTGAACACCTTGTCATCGTGCTGGACTTTGACGT
*  A  F  Y  K  E  L  D  D  S  G  G  I  H  K  G  G  I  F  V  Q  P  V  T  A  R  G  S  V  A

CGTACGCTCACGATTGTTGCATCGTGACCTAATATGATTTCTTCAAAGCTTAAGCCTTTATCGTGTAAAAGTTGTTTTGCTTTTGCACAG   270
GCATGCGAGTGCTAACAACGTAGCACTGGATTATACTAAAGAAGTTTCGAATTCGGAAATAGCACATTTTCAACAAAACGAAAACGTGTC
R  V  S  V  I  T  A  D  H  G  L  I  I  E  E  F  S  L  G  K  D  H  L  L  Q  K  A  K  A  C

AAAGGACAGCCAGGTTTTGTAAAGATTGAAATAGACTCTTGCACTTGGTGTTGTGGTGCAAGGTATTTCAACATAGTGTCAGCATCGGAT   360
TTTCCTGTCGGTCCAAAACATTTCTAACTTTATCTGAGAACGTGAACCACAACACCACGTTCCATAAAGTTGTATCACAGTCGTAGCCTA
F  P  C  G  P  K  T  F  I  S  I  S  E  Q  V  Q  H  Q  P  A  L  Y  K  L  M  T  D  A  D  S

ACTTTGAACGGATCGCCTGGTTCGTTTGGTTCGATAAACATTTTTTCAACTACGCCGTTTTTCACAAGCATAGAATAACGCCATGAACGT   450
TGAAACTTGCCTAGCGGACCAAGCAAACCAAGCTATTTGTAAAAAAGTTGATGCGGCAAAAAGTGTTCGTATCTTATTGCGGTACTTGCA
V  K  F  P  D  G  P  E  N  P  E  I  F  M  K  E  V  V  G  N  K  V  L  M  S  Y  R  W  S  R

TTACCGAAGCCTAAATCTTCTTTACCAACTAACATACCCATGCCTTCGGTAAATTCACCATTACCATCTGGAATGAAAGTGATGTTTTCA   540
AATGGCTTCGGATTTAGAAGAAATGGTTGATTGTATGGGTACGGAAGCCATTTAAGTGGTAATGGTAGACCTTACTTTCACTACAAAAGT
K  G  F  G  L  D  E  K  G  V  L  M  G  M  G  E  T  F  E  G  N  G  D  P  I  F  T  I  N  E

GATTTTTCATCTTCTTTCCATGCGTTCATTACGAAAGTATCATTTACAGGATACACAAGAATATCGTCTACACCGTATTTTTTGAATACT   630
CTAAAAAGTAGAAGAAAGGTACGCAAGTAATGCTTTCATAGTAAATGTCCTATGTGTTCTTATAGCAGATGTGGCATAAAAAACTTATGA
S  K  E  D  E  K  W  A  N  M  V  F  T  D  N  V  S  V  V  L  I  D  D  V  G  Y  K  K  F  V

GGCACTAATTCGTTGTAACGTGGTAAGTGTGATGATGAGCAAGTTGGAGTGAATGCGCCCGGTAATGAGAACACGATCACTGTTTTGTTA   720
CCGTGATTAAGCAACATTGCACCATTCACACTACTACTCGTTCAACCTCACTTACGCGGGCCATTACTCTTGTGCTAGTGACAAAACAAT
P  V  L  E  N  Y  R  P  L  H  S  S  S  C  T  P  T  F  A  G  P  L  S  F  V  I  V  T  K  N

                                                        oxyR1
TCAAATAACTCTGAGGTAGTTACATCAACCCATTTATCACCCTGACGAGTC[CGGAATGTCACTTGAGGG]ACTTTTTTTCCTTCCATACTA   810
AGTTTATTGAGACTCCATCAATGTAGTTGGGTAAATAGTGGGACTGCTCACGCCTTACAGTGAACTCCCTGAAAAAAAGGAAGGTATGAT
D  F  L  E  S  T  T  V  D  V  W  K  D  G  Q  R  T  R  F  T  V  Q  P  V  K  K  G  E  M  S

                      -35                    -10
GACATTGTTTTTTCTCCTATTGGTTGTTAATTTAAATTTTACGTGCTGTATTATAGGGAAAATTATGATATAGTCATAATCAATTAATTC   900
CTGTAACAAAAAAGAGGATAACCAACAATTAAATTTAAAATGCACGACATAATATCCCTTTTAATACTATATCAGTATTAGTTAATTAAG
S  M          S.D.                           -10                          -35
◄—— 26 kD                        ——► 34 kD
                    S.D.          M  N  I  R  D  L  E  Y  L  V  A  L  S  E  Y  K  H  F
TATTCTTTTAATTGAATTTTTCTATAGGGAAAATCTCATGAATATCCGTGATCTAGAATATCTTGTTGCCTTATCTGAATATAAACATTT   990
ATAAGAAAATTAACTTAAAAAGATATCCCTTTTAGAGTACTTATAGGCACTAGATCTTATAGAACAACGGAATAGACTTATATTTGTAAA

  R  R  A  A  D  S  C  N  V  S  Q  P  T  L  S  G  Q  I  R  K  L  E  D  E  L  G  I  I  L  L
CCGCCGTGCTGCCGATTCTTGTAATGTGAGTCAACCAACATTAAGTGGGCAAATTCGTAAGTTAGAAGATGAGCTTGGTATTATTTTGTT   1080
GGCGGCACGACGGCTAAGAACATTACACTCAGTTGGTTGTAATTCACCCGTTTAAGCATTCAATCTTCTACTCGAACCATAATAAAACAA

  E  R  T  S  R  K  V  L  F  T  Q  S  G  M  L  L  V  D  Q  A  R  T  V  L  R  E  V  K  L  L
ATAACGTACTAGCCGTAAAGTGTTGTTTACTCAATCTGGGATGTTATTGGTTGATCAGGCTCGTACAGTTCTTCGAGAAGTAAAATTATT   1170
TCTTGCATGATCGGCATTTCACAACAAATGAGTTAGACCCTACAATAACCAACTAGTCCGAGCATGTCAAGAAGCTCTTCATTTTAATAA
```

type NTHI TN106 and the wild-type Hib DL302. Kanamycin-resistant transformants of TN106 and chloramphenicol-resistant transformants of DL302 were screened for insertion of the appropriate antibiotic resistance cartridge into the *oxyR* gene by PCR analysis of total genomic DNA, using the oligonucleotide primers oxyR1 and oxyR2 (Fig. 2 and 3). The PCR product generated from total genomic DNA of the mutant strain TN106.13 (Fig. 6, lane F) was 1.3 kb larger than the PCR

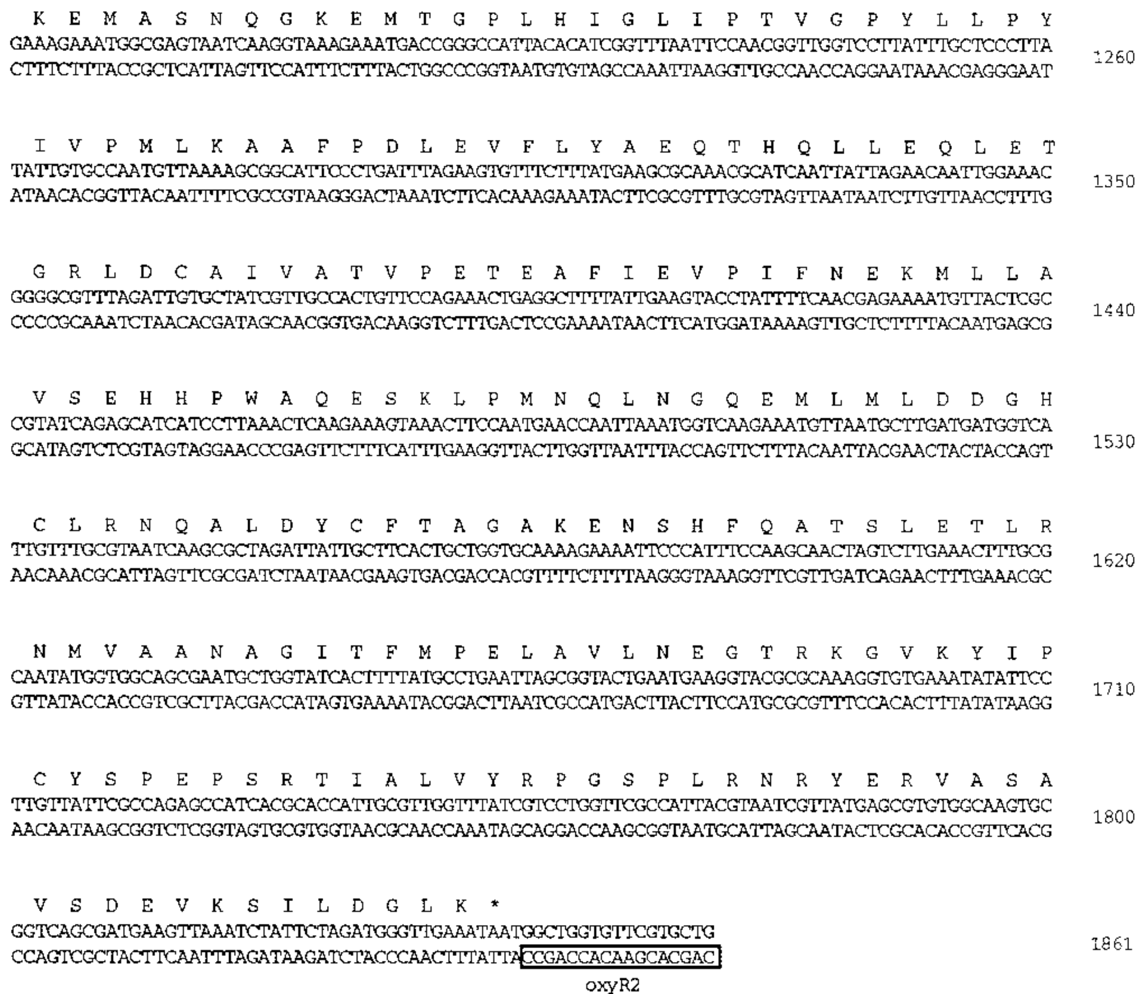

FIG. 3. Nucleotide sequence of the ORFs encoding the 26- and 34-kDa proteins, together with the deduced amino acid sequence. Putative −35 and −10 regions, as well as possible Shine-Dalgarno (S.D.) sites, are indicated. An inverted repeat located 3′ from the ORF encoding the 26-kDa protein is indicated by opposing arrows. The arrows next to the ORF designations indicate the predicted direction of transcription. The oxyR1 and oxyR2 oligonucleotide primers used for selected PCR experiments are enclosed in boxes.

product from the wild-type parent strain (lane E). This increase in size is consistent with insertion of the *kan* cartridge into the *oxyR* gene in the mutant strain. Similarly, the PCR product derived by the use of these primers with chromosomal DNA from the Hib mutant DL302.22 (lane C) was 1.4 kb larger than the product generated from chromosomal DNA derived from its wild-type parent (lane B), consistent with the presence of the *cat* cartridge in the *oxyR* gene of this mutant. In addition, Southern blot analysis was used to confirm that proper allelic exchange had occurred in these two mutants (data not shown).

Hydrogen peroxide sensitivity of isogenic *oxyR* mutants. The known ability of OxyR to regulate positively the expression of catalase in *E. coli* (67) prompted us to determine whether these *H. influenzae oxyR* mutants were more sensitive to killing by hydrogen peroxide. Approximately 10^7 CFU of the wild-type and mutant strains were spread onto BHIs agar plates, filter paper disks impregnated with various concentrations of

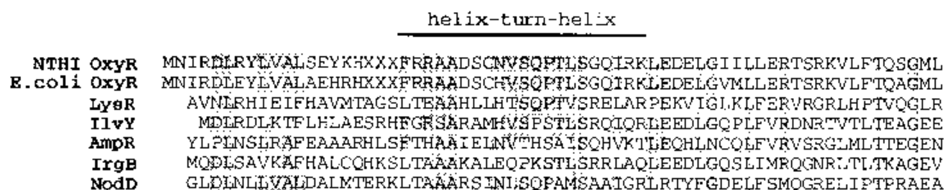

FIG. 4. N-terminal sequence homology between the *H. influenzae* OxyR protein and other selected members of the LysR family of transcriptional activators. Shading indicates conserved regions. This figure is a modification of that published by Goldberg et al. (21). The other proteins in this figure include OxyR from *E. coli* (8), IlvY from *E. coli* (71), IrgB from *Vibrio cholerae* (21), LysR from *E. coli* (64), NodD from *Rhizobium meliloti* (22), and AmpR from *Enterobacter cloacae* (36). The helix-turn-helix motif is indicated.

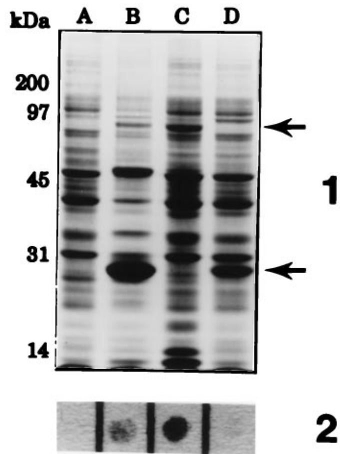

FIG. 5. Binding of transferrin by recombinant *E. coli* strains and analysis of the corresponding whole-cell lysates by SDS-PAGE. Proteins present in whole-cell lysates were resolved by SDS-PAGE and stained with Coomassie blue (panel 1). Cells of these strains were also tested in the transferrin-binding dot blot assay (panel 2). Lanes: A, *E. coli* HB101 containing pBluescript; B, HB101 containing pIM120; C, HB101 containing pIM122; D, HB101 containing pIM123. The lower arrow on the right of the figure indicates the position of the 26-kDa protein in lanes B and D. The upper arrow indicates the position of the 76-kDa protein in lanes B and C. Molecular mass markers (in kilodaltons) are given on the left of this figure.

hydrogen peroxide were applied to the surface of the agar, and the plates were incubated at 37°C overnight. The zones of growth inhibition caused by hydrogen peroxide were significantly larger for the *oxyR* mutants (Fig. 7, panels 2 and 4) than for their respective wild-type parent strains (panels 1 and 3).

Expression of catalase activity by the wild-type and *oxyR* mutant strains. When levels of catalase activity in whole cells of the wild-type strain TN106 and the isogenic *oxyR* mutant strain TN106.13 were measured, catalase activity (measured as the rate of disappearance of hydrogen peroxide) was readily detectable in the wild-type cells (Fig. 8A). In contrast, no catalase activity was detected in cells of the mutant (Fig. 8A). However, when catalase activity present in the wild-type Hib DL302 (Fig. 8B) was compared with that of its isogenic *oxyR* mutant DL302.22 (Fig. 8B), there was no appreciable difference.

Growth phenotype of *oxyR* mutants on PPIX-based media. Growth experiments with the wild-type and *oxyR* mutants revealed an interesting dichotomy in the relative abilities of these strains to grow when PPIX, the immediate biosynthetic precursor of heme, was used in place of heme in BHI-NAD agar. Both the wild-type Hib DL302 (Fig. 9, panel 1A) and the Hib *oxyR* mutant DL302.22 (panel 2A) readily formed single colonies on BHI-NAD agar containing heme (20 μg/ml). Similarly, the wild-type strain DL302 (panel 1B) formed single colonies when PPIX (20 μg/ml) replaced heme in the medium. However, the *oxyR* mutant strain DL302.22 was unable to form single colonies on BHI-NAD medium containing PPIX (panel 2B). To determine whether this inability of the *oxyR* mutant to utilize PPIX as a porphyrin source could be complemented by provision of the wild-type NTHI *oxyR* gene in *trans*, the 1.9-kb *Hin*dIII fragment of pIM120 was subcloned into the shuttle vector pLS88 to construct pIM130 (Fig. 2). The recombinant Hib DL302.22(pIM130) (Fig. 9, panel 2C) grew readily on this PPIX-based medium, whereas the presence of the pLS88 vector alone had no effect on the growth phenotype of this mutant (panel 2D). Interestingly, incorporation of bovine catalase into the agar allowed growth of the *oxyR* mutant strain on this PPIX-containing medium (panel 2E). Provision of this quantity of catalase in BHI-NAD agar did not allow the development of individual colonies of either the wild-type or mutant *H. influenzae* strains (data not shown). Identical results were obtained with the wild-type strain TN106 and its isogenic *oxyR* mutant TN106.13 (data not shown).

Catalase is noted for its ability to degrade hydrogen peroxide. To confirm that hydrogen peroxide was the toxic factor preventing growth of the *oxyR* mutants on the PPIX-containing medium, 10 mM sodium pyruvate was added to the same BHI-NAD plates containing PPIX. Hydrogen peroxide is destroyed in the presence of pyruvic acid in a nonenzymatic reaction (46, 47). The *oxyR* mutant was shown to readily form single colonies on the PPIX-based medium when pyruvic acid was present (data not shown).

Growth phenotype of the *oxyR* mutant in broth. When cells of the wild-type NTHI TN106 and its isogenic *oxyR* mutant strain TN106.13 (Fig. 10) were inoculated into BHI-NAD broth containing PPIX, the *oxyR* mutant was unable to grow. However, supplementation of this broth medium with catalase resulted in growth of the *oxyR* mutant (Fig. 10).

Elimination of the *oxyR* mutation by transformation. The mutation in each of the isogenic *oxyR* mutants TN106.13 and DL302.22 was cured by transformation with chromosomal DNA from the wild-type strain TN106; the cured transformants were selected by plating the transformation reaction mixtures on BHI-NAD medium containing PPIX. These experiments yielded the cured transformant strains TN106.13T and DL302.22T. As expected, when the oxyR1 and oxyR2 primers were used to amplify the *oxyR* gene, the sizes of the PCR products from these two transformants (Fig. 6, lanes D and G, respectively) were equivalent to those of the wild types (lanes B and E, respectively). The cured transformant strain DL302.22T readily formed single colonies on PPIX-based media (Fig. 9, panel 3B), as well as on heme-based media (panel 3A); identical results were obtained with the transformant strain TN106.13T (data not shown).

Growth of *oxyR* mutants on heme. Both the wild-type and *oxyR* mutant strains were barely able to grow on BHI-NAD agar containing PPIX (1 μg/ml) and 80 μM Desferal, an iron chelator. Inclusion of Desferal in the growth medium was necessary to prevent overgrowth of the wild-type inoculum used in this experiment. Placement of filter paper disks impregnated with heme or hemoglobin on the surface of these plates allowed assessment of the ability of wild-type and mutant strains to utilize these heme sources for growth. Figure 11

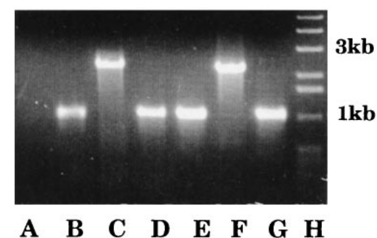

FIG. 6. Products generated by PCR amplification of the *oxyR* locus from chromosomal DNA purified from wild-type, mutant, and transformant strains of *H. influenzae*. The oligonucleotide primers oxyR1 and oxyR2 (Fig. 3) were used to amplify the *oxyR* gene from wild-type DL302 (lane B), mutant strain DL302.22 (lane C), transformant strain DL302.22T (lane D), wild-type TN106 (lane E), mutant strain TN106.13 (lane F), and transformant strain TN106.13T (lane G). Lane H contains DNA size standards, and DNA size markers (in kilobases) are shown on the right. *H. ducreyi* chromosomal DNA (lane A) was used as a negative control in this PCR experiment.

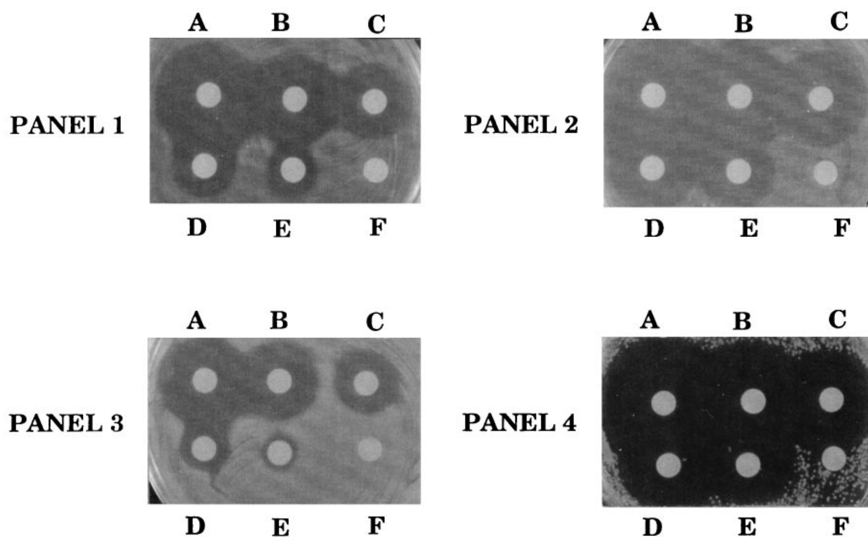

FIG. 7. Inhibition of the growth of wild-type and mutant *H. influenzae* strains by hydrogen peroxide. Approximately 10^7 CFU of wild-type NTHI TN106 (panel 1), isogenic NTHI *oxyR* mutant TN106.13 (panel 2), wild-type Hib DL302 (panel 3), and Hib *oxyR* mutant DL302.22 (panel 4) were spread onto BHIs agar plates. Sterile filter paper disks containing 2.0% (A), 1.0% (B), 0.5% (C), 0.25% (D), 0.125% (E) or 0% (F) hydrogen peroxide in PBS were then placed on the surface of the agar. The sensitivity to hydrogen peroxide is indicated by the clear zone around each disk after overnight incubation at 37°C.

shows growth of the wild-type Hib DL302 (panel 1) and the *oxyR* mutant strain DL302.22 (panel 2) around disks containing hemoglobin (disks D) and heme (disks F). The *oxyR* mutant grew less extensively than the wild type around disks containing either heme or hemoglobin. The addition of catalase (25 μg) to each of these disks (panel 2, disks C and E) resulted in the restoration of growth of the *oxyR* mutant strain DL302.22 to levels equivalent to those exhibited by the wild-type parent strain (panel 1, disks D and F). Both wild-type and *oxyR* mutant strains were able to form a very small zone of patchy growth around disks containing catalase alone (panels 1 and 2, disks A) even though catalase has been reported to be unable to serve as a source of heme for the growth of *H. influenzae* (65). Identical results were obtained with the wild-type NTHI TN106 and its isogenic *oxyR* mutant TN106.13 (data not shown).

Binding of transferrin by wild-type and mutant strains. Binding of horseradish peroxidase-labelled transferrin was identical in the wild-type strain DL302 and the *oxyR* mutant strain DL302.22 (data not shown). The TN106 *oxyR* mutant strain TN106.13 exhibited a very slight reduction in transferrin-binding activity under iron-restricted conditions when compared with its wild-type parent (data not shown). These results indicate that the OxyR protein does not exert a direct regulatory effect on expression of transferrin-binding activity in *H. influenzae.*

Identification of *oxyR* genes in other *H. influenzae* strains. To determine the distribution of the *oxyR* gene among other

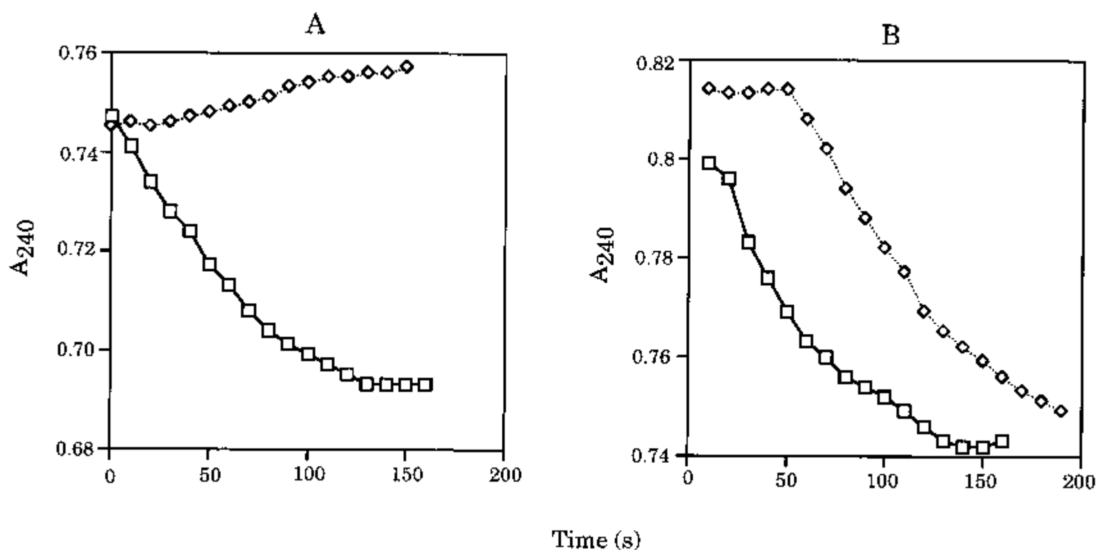

FIG. 8. Catalase activity present in wild-type and *oxyR* mutant strains of *H. influenzae*. Catalase activity in whole cells of the wild-type NTHI TN106 (open squares) and the *oxyR* mutant TN106.13 (open diamonds) (A) and the wild-type Hib DL302 (open squares) and the *oxyR* mutant DL302.22 (open diamonds) (B) was evidenced by a decrease in optical density (A_{240}) over time as described in Materials and Methods.

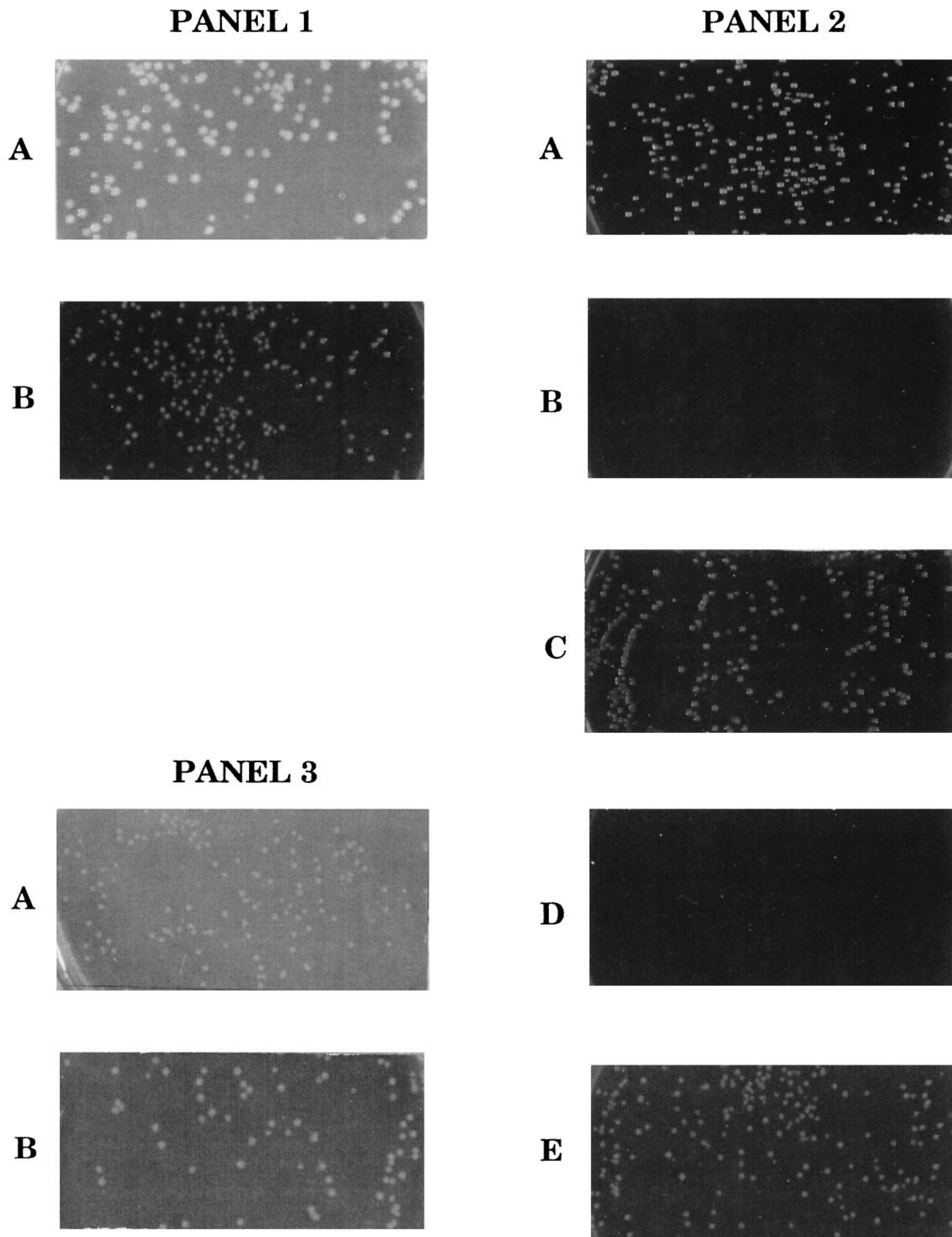

FIG. 9. Formation of single colonies by wild-type, mutant, and recombinant strains of *H. influenzae* on BHI-NAD agar containing heme or PPIX. (Panels 1 and 2) Approximately 100 to 300 CFU of the wild-type Hib in DL302 (panel 1) and the isogenic *oxyR* mutant DL302.22 (panel 2) were spread onto BHI-NAD agar plates containing either heme (20 μg/ml) (A) or PPIX (20 μg/ml) (B). In addition, in panel 2, the recombinant strains DL302.22(pIM130) (C) and DL302.22(pLS88) (D) were plated onto BHI-NAD agar containing PPIX. The mutant strain DL302.22 (E) was also plated onto BHI-NAD agar containing both PPIX and catalase (5 μg/ml). (Panel 3) The transformant strain DL302.22T was plated onto BHI-NAD agar containing heme (A) or PPIX (B).

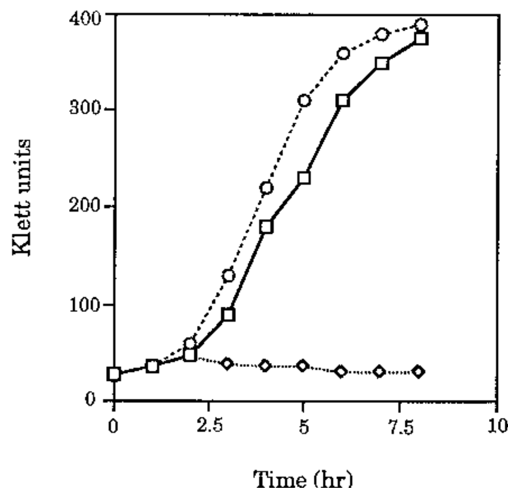

FIG. 10. Growth of wild-type and *oxyR* mutants of *H. influenzae* in broth-based media. The wild-type NTHI TN106 (open circles) and the *oxyR* mutant TN106.13 (open diamonds) were inoculated into BHI-NAD broth containing PPIX (10 μg/ml); the *oxyR* mutant was also inoculated into this broth medium containing catalase (5 μg/ml) (open squares).

H. influenzae strains, a gene probe consisting of the 873-bp *Xba*I fragment from pIM120, which contained most of the *oxyR* structural gene (Fig. 2), was used to probe chromosomal DNA preparations from five NTHI and five Hib strains. In all 10 strains, this probe hybridized to a 10- to 11-kb *Bgl*II fragment of chromosomal DNA (data not shown).

DISCUSSION

All aerobic and facultatively anaerobic organisms must be able to defend against toxic by-products arising as a result of the incomplete reduction of molecular oxygen, the major toxic products from oxygen metabolism being superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radicals (6, 19). Bacteria may be exposed to endogenously generated oxidants as a result of normal respiration but may also be exposed to high levels of environmental oxidants produced by host phagocytic cells as a response to bacterial infection in vivo (30). Consequently, most aerobic organisms constitutively produce basal levels of antioxidant enzymes but can also employ inducible systems which result in the production of high levels of antioxidants in response to sudden oxidative stress (7, 17).

The *oxyR* gene product is a transcriptional activator of hydrogen peroxide-inducible genes in *E. coli* and *S. typhimurium* (7, 61, 63, 69). Both of these organisms become resistant to killing by hydrogen peroxide when pretreated with nonlethal levels of this oxidant (7, 12). During this adaptive response, some 30 new proteins are synthesized, 9 of which are known to be regulated by OxyR (44). The biochemical function of several of these OxyR-regulated gene products is known, including the *katG*-encoded HPI catalase, the glutathione reductase encoded by *gorA*, and the alkyl hydroperoxide reductase activities contributed by the *ahpF* and *ahpC* genes (7, 8, 62). An additional three proteins regulated by OxyR are heat shock proteins, and some proteins induced by glucose and nitrogen starvation also overlap with hydrogen peroxide-inducible proteins (38).

The *H. influenzae katE* gene encoding HPII catalase has been cloned and sequenced (4). A putative OxyR-binding site overlapping the *katE* promoter region has been described, although definitive evidence of OxyR binding to this region of the *H. influenzae* chromosome has not been obtained (4). However, *E. coli* OxyR binds to the promoter region of the *E. coli katG* gene (67). It is of interest that catalase-deficient mutants of Hib Eagan, carrying a mutated *katE* gene, exhibit modestly reduced virulence when compared with the wild-type parent strain in the infant-rat model of infection (3).

In the present study, we have demonstrated a relationship between the presence of a functional *oxyR* gene and the ability of *H. influenzae* to grow on a medium containing PPIX in place of heme. The growth of *H. influenzae oxyR* mutants on PPIX was completely inhibited (Fig. 9, panel 2B), whereas wild-type strains could form individual colonies on this growth medium (panel 1B). This growth inhibition was reversed by either provision of the *H. influenzae oxyR* gene in *trans* (on the plasmid pIM130) or an exogenous source of catalase (panels 2C and E, respectively). This latter observation indicated that the observed growth inhibition on the PPIX-based medium was probably due to an extracellular accumulation of hydrogen peroxide, derived from either bacterial metabolism or the growth medium itself (34, 48). It is known that many medium components are capable of autoxidation with oxygen to form superoxide. This radical can, in turn, spontaneously undergo dismutation to form hydrogen peroxide. That hydrogen peroxide is involved in the observed growth inhibition of the *oxyR* mutant is supported further by the fact that incorporation of pyruvic acid into the PPIX-based media had the same growth-stimulating effect on an *oxyR* mutant as did the provision of an exogenous source of catalase. Pyruvate and other keto acids detoxify hydrogen peroxide by virtue of their ability to undergo nonenzymatic decarboxylation in the presence of hydrogen peroxide. In this reaction, hydrogen peroxide is broken down, and the products of the reaction include water and carbon dioxide (46, 47).

Hydrogen peroxide can diffuse freely across the outer membrane, and catalase-deficient bacteria cannot catabolize significant amounts of extracellular hydrogen peroxide (15, 42). In fact, an *E. coli oxyR* deletion mutant exhibited a phenotype strikingly similar to that of the *H. influenzae oxyR* mutants,

FIG. 11. Ability of wild-type and mutant Hib strains to grow with heme and hemoglobin. Approximately 10^7 CFU of the wild-type Hib DL302 (panel 1) and the *oxyR* mutant DL302.22 (panel 2) were plated on BHI-NAD agar plates supplemented with PPIX (1 μg/ml) and Desferal (80 μM). Filter paper disks were placed on the agar surface and impregnated with 5 μl of catalase (5 mg/ml) (A), PBS (B), hemoglobin (5 mg/ml) and catalase (5 mg/ml) (C), hemoglobin (5 mg/ml) (D), heme (5 mg/ml) and catalase (5 mg/ml) (E) or heme (5 mg/ml) (F).

being unable to form individual colonies on LB agar plates in the absence of an exogenous source of catalase (60a).

Growth of *H. influenzae oxyR* mutants on PPIX-based media was totally eliminated (Fig. 9, panel 2B); however, the same mutants formed individual colonies when PPIX was replaced by heme (panel 2A). This finding could be interpreted to indicate that hydrogen peroxide production during growth on PPIX is greater than during the utilization of heme, especially since ferrous iron must be reduced for the synthesis of heme from PPIX and iron (23, 40). However, an alternative explanation may be that heme itself can function in a peroxidative manner (10, 20, 68), thereby counteracting the inhibitory effect of hydrogen peroxide, especially at the relatively high heme concentrations (20 μg/ml) supplied in the *H. influenzae* growth medium. This latter hypothesis is supported by the fact that an *E. coli oxyR* deletion mutant, while unable to form individual colonies on LB agar, will do so on LB agar supplemented with free heme (20 μg/ml) (data not shown). The fact that growth of the *H. influenzae oxyR* mutants with heme sources was slightly decreased in the disk-feeding experiments (Fig. 11, panel 2, disks D and F), together with the finding that the provision of catalase restored growth to wild-type levels (panel 2, disks C and E), suggests that even in the presence of exogenous heme, hydrogen peroxide-based toxicity can adversely affect the growth of *H. influenzae* when *oxyR*-regulated gene products are absent.

The lack of expression of OxyR by the NTHI mutant TN106.13 clearly had a deleterious effect on the level of catalase activity expressed by this mutant (Fig. 8), as would have been expected from previous studies involving *E. coli* and *S. typhimurium* (8, 44, 62). Therefore, it is puzzling that the same *oxyR* mutation in Hib DL302.22 did not have a detectable effect on the catalase activity expressed by this Hib mutant (Fig. 8). Clearly, this Hib mutant had the same sensitivity to hydrogen peroxide as its NTHI counterpart did (Fig. 7) and could not grow on BHI-NAD-PPIX medium unless catalase was present (Fig. 9). Testing of the ability of colonies of these wild-type and mutant *H. influenzae* strains to break down hydrogen peroxide, accomplished by adding a drop of 3% hydrogen peroxide to individual colonies and viewing the rate of bubble production, confirmed the catalase activity results obtained in the spectrophotometric assays (data not shown). The molecular basis for this apparent lack of effect of an *oxyR* mutation on catalase activity expressed by the Hib mutant DL302.22 remains unexplained.

It is also apparent that the *H. influenzae oxyR* gene product does not directly regulate the outer membrane proteins involved in transferrin binding by *H. influenzae*. However, the expression of a transferrin-binding phenotype by *E. coli* strains harboring the NTHI *oxyR* gene on a plasmid remains to be explained. It is known that other stress proteins are expressed in *E. coli* and *S. typhimurium* in response to exposure to hydrogen peroxide (7, 44, 62), and it is possible that the transferrin-binding phenotype of the recombinant *E. coli* strain carrying the *H. influenzae oxyR* gene is the result of one of these other proteins, of as yet unidentified function (7, 44), that are induced in *E. coli* by hydrogen peroxide exposure. However, provision of the *E. coli oxyR* gene to *E. coli* RR1 on plasmid pAQ17 (8) did not result in a transferrin-binding phenotype (data not shown), and *E. coli* RR1 cells grown in the presence of increasing levels of hydrogen peroxide did not exhibit a detectable transferrin-binding activity (data not shown). Consequently, the identity of the *E. coli* protein that binds transferrin in the recombinant strain containing the *H. influenzae oxyR* gene remains to be determined.

ACKNOWLEDGMENTS

We thank Gisela T. Storz, John W. Eaton, and Daniel J. Hassett for very helpful discussions and advice.

This study was supported by U.S. Public Health Service grant AI17621 to E.J.H.

REFERENCES

1. **Alexander, H. E.** 1965. The *Haemophilus* group, p. 724–741. *In* R. J. Dubos and J. G. Hirsch (ed.), Bacterial and mycotic infections of man. The J. B. Lippincott Co., Philadelphia.
2. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195:** 133–140.
3. **Bishai, W. R., N. S. Howard, J. A. Winkelstein, and H. O. Smith.** 1994. Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. Infect. Immun. **62:**4855–4860.
4. **Bishai, W. R., H. O. Smith, and G. J. Barcak.** 1994. A peroxide/ascorbate-inducible catalase from *Haemophilus influenzae* is homologous to the *Escherichia coli katE* gene product. J. Bacteriol. **176:**2914–2921.
5. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
6. **Chance, B., H. Sies, and A. Boveris.** 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59:**527–589.
7. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
8. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA **86:**3484–3488.
9. **Cope, L. D., S. E. Thomas, J. L. Latimer, C. A. Slaughter, U. Muller-Eberhard, and E. J. Hansen.** 1994. The 100 kDa heme:hemopexin-binding protein of *Haemophilus influenzae*: structure and localization. Mol. Microbiol. **13:**863–873.
10. **Davies, M. J.** 1988. Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with heme-proteins by electron spin resonance spectroscopy. Biochim. Biophys. Acta **964:**28–35.
11. **de Bruijn, F. J., and J. R. Lupski.** 1984. The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
12. **Demple, B., and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature (London) **304:**466–468.
13. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
14. **Dixon, L. G., W. L. Albritton, and P. J. Willson.** 1994. An analysis of the complete nucleotide sequence of the *Haemophilus ducreyi* broad-host-range plasmid pLS88. Plasmid **32:**228–232.
15. **Eaton, J. W.** 1991. Catalases and peroxidases and glutathione and hydrogen peroxide: mysteries of the bestiary. J. Lab. Clin. Med. **118:**3–4.
16. **Evans, N. M., D. D. Smith, and A. J. Wicken.** 1974. Haemin and nicotinamide adenine dinucleotide requirements of *Haemophilus influenzae* and *Haemophilus parainfluenzae*. J. Med. Microbiol. **7:**359–365.
17. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
18. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, R. C. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Frichman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
19. **Fridovich, I.** 1978. The biology of oxygen radicals. Science **201:**875–880.
20. **George, P., and D. H. Irvine.** 1952. The reaction between metmyoglobin and hydrogen peroxide. Biochem. J. **52:**511–517.
21. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1991. Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**1125–1129.
22. **Gottberg, M., B. Horvath, E. Konderosi, P. Putnoky, F. Rodrigues-Quinones, and D. Kondorosi.** 1986. At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium meliloti*. J. Mol. Biol. **191:**411–420.
23. **Granick, S., and H. Gilder.** 1946. The porphyrin requirements of *Haemophilus influenzae* and some functions of the vinyl and propionic acid side chains of heme. J. Gen. Physiol. **30:**1–13.
24. **Gray-Owen, S. D., S. Loosmore, and A. B. Schryvers.** 1995. Identification and characterization of genes encoding the human transferrin-binding proteins

from *Haemophilus influenzae*. Infect. Immun. **63:**1201–1210.
25. **Gray-Owen, S. D., and A. B. Schryvers.** 1995. Characterization of transferrin binding proteins 1 and 2 in invasive type b and nontypeable strains of *Haemophilus influenzae*. Infect. Immun. **63:**3809–3815.
26. **Gustafsson, C., and S. R. Warne.** 1992. Physical map of the *oxyR-trmA* region (minute 89.3) of the *Escherichia coli* chromosome. J. Cell Biol. **174:**7878–7879.
27. **Hansen, E. J., C. F. Frisch, and K. H. Johnston.** 1981. Detection of antibody-accessible proteins on the cell surface of *Haemophilus influenzae* type b. Infect. Immun. **33:**950–953.
28. **Hansen, E. J., J. L. Latimer, S. E. Thomas, M. E. Helminen, W. L. Albritton, and J. D. Radolf.** 1992. Use of electroporation to construct isogenic mutants of *Haemophilus ducreyi*. J. Bacteriol. **174:**5442–5449.
29. **Hardie, K. R., R. A. Adams, and K. J. Towner.** 1993. Transferrin-binding ability of invasive and commensal isolates of *Haemophilus* spp. J. Med. Microbiol. **39:**218–224.
30. **Hassett, D. J., and M. S. Cohen.** 1989. Bacterial adaption to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. FASEB J. **3:**2574–2582.
31. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
32. **Herrington, D. A., and P. F. Sparling.** 1985. *Haemophilus influenzae* can use human transferrin as a sole source for required iron. Infect. Immun. **48:**248–251.
33. **Herriott, R. M., E. M. Meyer, and M. J. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101:**517–524.
34. **Hoffman, P. S., L. Pine, and S. Bell.** 1983. Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila*: catalytic decomposition by charcoal. Appl. Environ. Microbiol. **45:**784–791.
35. **Holland, J., P. R. Langford, K. J. Towner, and P. Williams.** 1992. Evidence for in vivo expression of transferrin-binding proteins in *Haemophilus influenzae* type b. Infect. Immun. **60:**2986–2991.
36. **Honore, N., M. H. Nicholas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *E. coli*. EMBO J. **5:**3709–3714.
37. **Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen.** 1994. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type b. Infect. Immun. **62:**2470–2477.
38. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross-protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. **170:**3910–3914.
39. **Kullik, I., M. B. Tolendano, L. A. Tartaglia, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional regulation. J. Bacteriol. **177:**1275–1284.
40. **Loeb, M. R.** 1995. Ferrochelatase activity and protoporphyrin IX utilization in *Haemophilus influenzae*. J. Bacteriol. **177:**3613–3615.
41. **Loosmore, S. M., Y.-P. Yang, D. C. Coleman, J. M. Shortreed, D. M. England, R. E. Harkness, P. S.-C. Chong, and M. H. Klein.** 1996. Cloning and expression of the *Haemophilus influenzae* transferrin receptor genes. Mol. Microbiol. **19:**575–586.
42. **Ma, M., and J. W. Eaton.** 1992. Multicellular oxidant defense in unicellular organisms. Proc. Natl. Acad. Sci. USA **89:**7924–7928.
43. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. N. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83:**8059–8063.
45. **Morton, D. J., and P. Williams.** 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Microbiol. **136:**927–933.
46. **Nath, K. A., H. Enright, L. M. Nutter, M. Fischereder, J.-N. Zou, and R. P. Hebbel.** 1994. Effect of pyruvate on oxidant injury to isolated and cellular DNA. Kidney Int. **45:**166–176.
47. **Nath, K. A., E. O. Ngo, R. P. Hebbel, A. J. Croatt, B. Zhou, and L. M. Nutter.** 1995. alpha-Ketoacids scavenge H_2O_2 in vitro and in vivo and reduce menadione-induced DNA injury and cytotoxicity. Am. J. Physiol. **268:**C227–C236.
48. **Norrod, E. P., and S. A. Morse.** 1982. Presence of hydrogen peroxide in media used for cultivation of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **15:**103–108.
49. **Patrick, C. C., A. Kimura, M. A. Jackson, L. Hermanstorfer, A. Hood, G. H. McCracken, Jr., and E. J. Hansen.** 1987. Antigenic characterization of the oligosaccharide portion of the lipooligosaccharide of nontypeable *Haemophilus influenzae*. Infect. Immun. **55:**2902–2911.
50. **Patrick, C. C., S. E. Pelzel, P. A. Gulig, C. J. McCracken, J. D. Radolf, and E. J. Hansen.** 1989. Antigenic evidence for the synthesis of two different lipooligosaccharides by some strains of *Haemophilus influenzae* type b. Infect. Immun. **57:**1971–1978.
51. **Pidcock, K. A., J. A. Wooten, B. A. Daley, and T. L. Stull.** 1988. Iron acquisition by *Haemophilus influenzae*. Infect. Immun. **56:**721–725.
52. **Russell, D. W., and N. D. Zinder.** 1987. Hemimethylation prevents DNA replication in *E. coli*. Cell **50:**1071–1079.
53. **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.
54. **Sanders, J. D., L. D. Cope, and E. J. Hansen.** 1994. Identification of a locus involved in the utilization of iron by *Haemophilus influenzae*. Infect. Immun. **62:**4515–4525.
55. **Sanders, J. D., L. D. Cope, G. P. Jarosik, I. Maciver, J. L. Latimer, G. B. Toews, and E. J. Hansen.** 1993. Reconstitution of a porin-deficient mutant of *Haemophilus influenzae* type b with a porin gene from nontypeable *H. influenzae*. Infect. Immun. **61:**3966–3975.
56. **Saris, P. E. J., L. G. Paulin, and M. Uhlen.** 1990. Direct amplification of DNA from colonies of *Bacillus subtilis* and *Escherichia coli* by the polymerase chain reaction. J. Microbiol. Methods **11:**121–126.
57. **Schryvers, A. B.** 1988. Characterization of the human transferrin and lactoferrin receptors in *Haemophilus influenzae*. Mol. Microbiol. **2:**467–472.
58. **Schryvers, A. B.** 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121–130.
59. **Schryvers, A. B., and B. C. Lee.** 1989. Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*. Can. J. Microbiol. **35:**409–415.
60. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254:**34–38.
60a. **Storz, G.** Personal communication.
61. **Storz, G., and S. Altuvia.** 1994. OxyR regulon. Methods Enzymol. **234:**217–223.
62. **Storz, G., and L. A. Tartaglia.** 1992. OxyR: a regulator of antioxidant genes. J. Nutr. **122:**627–630.
63. **Storz, G., and M. B. Tolendano.** 1994. Regulation of bacterial gene expression in response to oxidative stress. Methods Enzymol. **236:**196–207.
64. **Stragier, P., and J. C. Patte.** 1983. Regulation of diaminopimelate decarboxylase synthesis in *Escherichia coli*. I. Identification of a *lysR* gene encoding an activator of the *lysA* gene. J. Mol. Biol. **168:**333–350.
65. **Stull, T. L.** 1987. Protein sources of heme for *Haemophilus influenzae*. Infect. Immun. **55:**148–153.
66. **Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa.** 1991. Purification and characterization of the *Escherichia coli* OxyR protein, the positive regulator for a hydrogen peroxide-inducible regulon. J. Biochem. **109:**262–266.
67. **Tartaglia, L. A., C. J. Gimeno, G. Storz, and B. N. Ames.** 1992. Multigenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. **267:**2038–2045.
68. **Timmins, G. S., M. J. Davies, and U. Muller-Eberhard.** 1995. A study of the effects of complexation of heme by hemopexin upon its reactions with organic peroxides. Biochem. Soc. Trans. **23:**244S.
69. **Tolendano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell **78:**897–909.
70. **Tomb, J. F., G. J. Barcak, and M. S. Chandler.** 1989. Transposon mutagenesis, characterization, and cloning of transformation genes of *Haemophilus influenzae*. J. Bacteriol. **171:**3796–3802.
71. **Wek, R. C., and G. W. Hatfield.** 1986. Nucleotide sequence and *in vivo* expression of the *ilvY* and *ilvC* genes in *Escherichia coli* K12. J. Biol. Chem. **261:**2441–2450.
72. **White, D. C., and S. Granick.** 1963. Hemin biosynthesis in *Haemophilus*. J. Bacteriol. **85:**842–850.
73. **Willson, P. J., W. L. Albritton, L. Slaney, and J. K. Setlow.** 1989. Characterization of a multiple antibiotic resistance plasmid from *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **33:**1627–1630.

Editor: B. I. Eisenstein