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Two Genetically Distinct Pathways for Transcriptional Regulation of Anaerobic Gene Expression in *Salmonella typhimurium*

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Expression of the tripeptide permease gene *tppB* is anaerobically induced. This induction is independent of the *fnr* (*oxrA*) gene product, which is known to be required for the anaerobic induction of several respiratory enzymes. We isolated, characterized, and mapped mutations in two genes, *oxrC* and *tppR*, which prevent the anaerobic induction of *tppB* expression. Mutations in *oxrC* were highly pleiotropic, preventing the anaerobic expression of the formate dehydrogenase component of formate hydrogen lyase (*fhl*), a tripeptidase (*pepT*), and two of the three known hydrogenase isoenzymes (hydrogenases 1 and 3). On the other hand, expression of nitrate reductase, fumarate reductase, and a number of other *fnr* (*oxrA*)-dependent enzymes was not affected by mutations in *oxrC*. Thus, there appeared to be at least two distinct classes of anaerobically induced genes, those which required *fnr* for their expression and those which required *oxrC*. It seems that *fnr*-dependent enzymes perform primarily respiratory functions, whereas *oxrC*-dependent enzymes served fermentative or biosynthetic roles. We found the primary defect of *oxrC* mutants to be a deficiency in phosphoglucose isomerase activity, implying that a product of glycolysis functions as an anaerobic regulatory signal. Mutations in *tppR* were specific for *tppB* and did not affect expression of other *oxrC*-dependent genes. However, *tppR* did exhibit phenotypes other than the regulation of *tppB*. Both *oxrC* and *tppR* mutants were hypersensitive to the toxic NAD analog 6-aminonicotinic acid. This suggests that *oxrC* and *tppR* may play a role in the regulation of NAD biosynthesis or, alternatively, that NAD or a related nucleotide serves as the anaerobic signal for *oxrC*-dependent enzymes.

The enteric bacteria *Escherichia coli* and *Salmonella typhimurium* are facultative anaerobes. When these bacteria are grown under anaerobic conditions, the synthesis of many proteins involved in aerobic respiration is repressed, whereas the synthesis of a specific class of approximately 50 proteins, including a number of respiratory enzymes, is specifically induced (5, 33). The anaerobic induction of many genes encoding respiratory enzymes has been shown to be at the level of transcription (1, 16, 23, 41, 42). In addition, three genes whose products are related in function but are not directly involved in energy generation, the tripeptide permease gene *tppB* and two peptidase genes, *pepT* and *pepN*, are also anaerobically induced (8, 14, 36). The molecular mechanisms by which gene expression is regulated in response to anaerobiosis are poorly understood. In *E. coli*, mutations in the *fnr* gene (also variously called *nirA* or *nirR*) prevent the anaerobic induction of several respiratory enzymes, including nitrate reductase, nitrite reductase, fumarate reductase, dimethyl sulfoxide reductase, and glycerol-3-phosphate dehydrogenase (2, 16, 17, 21, 34). The effect of *fnr* mutations on these genes is at the transcriptional level. The nucleotide sequence of *fnr* shows that the Fnr protein shares considerable homology with the catabolite activator protein CAP. This implies that the Fnr protein may be a DNA-binding protein and suggests the involvement of a nucleotide such as cyclic AMP in the regulation of anaerobic gene expression (31). In *S. typhimurium*, two genes designated *oxrA* and *oxrB* have been shown to be essential for the anaerobic induction of several respiratory enzymes (36). *oxrA* is identical to the *fnr* gene of *E. coli* (14, 36). The *oxrB* gene is, as yet, poorly characterized, although it is required for the anaerobic expression of the same subset of genes as is *oxrA* (*fnr*).

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However, not all anaerobically induced genes are subject to *fnr* control. We recently showed that transcription of *tppB*, which encodes the tripeptide permease, is specifically induced by anaerobiosis and that this induction is *fnr* independent (14). Similarly, the anaerobic induction of a tertiary amine oxidase (*torA*), peptidase N (*pepN*), and certain aspects of formate hydrogen lyase activity have been reported to be independent of *fnr* (8, 23, 25, 28). To investigate this *fnr*-independent pathway, we isolated and characterized mutations which prevent the anaerobic induction of *tppB*. Two distinct and unlinked regulatory genes were defined, designated *oxrC* and *tppR*. The *oxrC* gene was found to play an important regulatory role in anaerobic gene expression. Mutations in the *oxrC* gene were highly pleiotropic and affected the anaerobic synthesis of a number of enzymes whose expression is *fnr* independent. Thus, *oxrC* and *fnr* mutations appeared to define two distinct pathways for the anaerobic induction of gene expression.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derivatives of *S. typhimurium* LT2 unless otherwise indicated. The genotypes and constructions of these strains are described in Table 1.

Media and growth conditions. Cells were grown in LB medium (20) or on LB agar at 37°C with aeration, unless otherwise stated. Mu-containing strains were grown at 30°C to prevent bacteriophage induction. Anaerobic growth was achieved by growing cells in completely filled and sealed vessels or by using Gas-Paks (Oxoid Ltd.). To ensure full aeration of aerobically grown cells, growth was in vigorously shaking conical flasks containing less than 1/20 the flask volume of medium. LC medium is LB to which 2 mM $CaCl_2$, 0.1% glucose, and 0.001% thymidine are added. Minimal medium was based on the E medium of Vogel and Bonner

TABLE 1. Bacterial strains

Strain[a]	Genotype	Source (reference) and construction
LT2 (A)	Wild type	B. N. Ames
LT2 (Z)	Wild type	B. N. Ames
CH44	Δ*oppBC250*	10
CH602	*pepT7*::Mu dl *oxrA1 zda-893*::Tn*5*	14, 36
CH616	*pepT7*::Mu dl *zda-888*::Tn*10*	14, 36
CH656	Δ*oppBC250 ompR1002*::Mu dl-8[b]	9; Gibson et al., submitted
CH776	Δ*oppBC250 tppB84*::Mu dl-8	This study
CH804	Δ*oppBC250 tppB84*::Mu dl-8 *oxrC101*::Tn*5*	This study
CH805	Δ*oppBC250 tppB84*::Mu dl-8 *oxrC102*::Tn*5*	This study
CH806	Δ*oppBC250 tppB84*::Mu dl-8 *ompR1003*::Tn*5*[b]	This study
CH878	Δ*oppBC250 tppB84*::Mu dl-8 *tppR90*::Tn*5*	This study
CH879	Δ*oppBC250 tppR90*::Tn*5*	Recipient, CH44; donor P22 lysate, CH878
CH881	Δ*oppBC250 oxrC102*::Tn*5*	Recipient, CH44; donor P22 lysate, CH805
CH937	Δ*oppBC250 tppB84*::Mu dl-8 *oxrC102*::Tn*5*/pCH21 (*oxrA*$^+$/*fnr*$^+$)	Recipient, CH805 transformed with pCH21 (14)
CH938	*pepT7*::Mu dl *zda-888*::Tn*10 tppR90*::Tn*5*	Recipient, CH616; donor P22 lysate, CH878
CH940	*pepT7*::Mu dl *zda-888*::Tn*10 oxrC102*::Tn*5*	Recipient, CH616; donor P22 lysate, CH805
CH950*	*fhl*::Mu dl[c] *tppR90*::Tn*5*	Recipient, EB137; donor P22 lysate, CH879
CH951*	*fhl*::Mu dl[c] *oxrC102*::Tn*5*	Recipient, EB137; donor P22 lysate, CH881
CH952*	*hyd*::Mu dl[c] *tppR90*::Tn*5*	Recipient, EB138; donor P22 lysate, CH879
CH953*	*hyd*::Mu dl[c] *oxrC102*::Tn*5*	Recipient, EB138; donor P22 lysate, CH881
CH974*	*fhl*::Mu dl[c] *oxrA1 zda-893*::Tn*5*	Recipient, EB137; donor P22 lysate, CH602
CH975*	*hyd*::Mu dl[c] *oxrA1 zda*-893::Tn*5*	Recipient, EB138; donor P22 lysate, CH602
CH1021*	*oxrC*::Tn*5*	Recipient, LT2 (Z); donor P22 lysate, CH881
CH1298	*zae-1709*::Tn*10*Δ*16*Δ*17* (Cml^r)	This study
EB137*	*fhl*::Mu dl[c]	E. L. Barrett (1)
EB138*	*hyd*::Mu dl[c]	E. L. Barrett (1)
JF165	*pyrD95 pasA5 gal*	J. Foster (6)
SA572	*metA22 trpE2 hisF1009 strA201 xyl-1 ilvA99 pyrE231 malB111*	K. Sanderson
SA2628	*panC2*	K. Sanderson
TN1425	*zja-861*::Tn*5*	C. G. Miller
TN1910	*pepT7*::Mu dl *oxrA1 zda-888*::Tn*10* (15% linked to *oxrA1*)	C. G. Miller (36)
TN2021	*pepT7*::Mu dl *zda-893*::Tn*5* (70% linked to *oxrA1*)	C. G. Miller (36)
TS616	*his-6165 ilv-452 metA22 metE551 trpB2 galE496 xyl-404 rpsL120 flaA66 hsd-26 hsdA29 malE*::Tn*10*[d]	E. T. Palva (22)
TT421	*pan-540*::Tn*10*	J. R. Roth
TT7610	*zeb-609*::Tn*10 supD10*	J. R. Roth (12)
TT7674	*pncA212*::Mu dl-8	J. R. Roth (12)
TT10427	pNK972 (Ap^r)	J. R. Roth (39)
TT10605	*proAB47*/F′128 *pro*$^+$ *lac*$^+$ *zzf-1837*::Tn*10*Δ*16*Δ*17* (Cml^r)	J. R. Roth (39)

[a] All strains are derivatives of LT2 (A) unless indicated (*) to be an LT2 (Z) derivative. This distinction is critical, as the hydrogenase isoenzyme contents of these two wild-type strains differ (29). The wild-type strain in which the *fhl* and *hyd* fusions were constructed is not clear (E. L. Barrett, personal communication), but it is probably LT2 (Z) on the basis of hydrogenase and nitrate reductase activities (15, 29; unpublished results).

[b] Selected as *tppA*; *tppA* is identical to *ompR* (Gibson et al., submitted).

[c] The Mu dl insertion was stabilized.

[d] We found that the Tn*10* in TS616 is not in *malE* but is 20% linked to a *malE* point mutation; the Tn*10* is renamed *zjb-1708*::Tn*10*

(26) and was supplemented with 0.4% glucose, fructose, or glycerol as the carbon source, as indicated. Nutrient broth (NB) was obtained from Difco Laboratories. MacConkey agar plates were prepared from MacConkey agar base (Difco) to which the appropriate sugar was added at 1%. MacConkey agar-nitrate medium is described by Stewart and MacGregor (35), and glycerol-nitrate medium is described by Lambden and Guest (17).

When necessary, minimal medium was supplemented as follows: amino acids, 0.4 mM; ampicillin, 50 μg ml^{-1} or 25 μg ml^{-1} in rich and minimal media, respectively; tetracycline, 20 μg ml^{-1} and 10 μg ml^{-1} in rich and minimal media, respectively; kanamycin, 25 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; streptomycin, 150 μg ml^{-1}; and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 20 μg ml^{-1}. When exogenous electron acceptors were added, they were used at the following concentrations: sodium formate, 0.5 g liter^{-1}; KNO_3, 10 g liter^{-1}; and sodium fumarate, 5 g liter^{-1}.

TABLE 2. Effect of *oxr* mutations on *tppB* expression[a]

Strain	Relevant genotype	β-Galactosidase activity[b] in medium:						
		$+O_2$	$-O_2$	$+O_2$, +leucine	$-O_2$, +leucine	$-O_2$, $-NO_3$	$-O_2$, +formate	$-O_2$, +fumarate
CH776	*tppB84*::Mu d1-8	14	389	289	588	173	183	203
CH805	*tppB84*::Mu d1-8 *oxrC102*::Tn*5*	9	11	313	296	38	33	26
CH878	*tppB84*::Mu d1-8 *tppR90*::Tn*5*	ND	ND	197	228	ND	ND	ND
CH806	*tppB84*::Mu d1-8 *ompR1003*::Tn*5*	3	7	6	8	ND	ND	ND
CH937	*tppB84*::Mu d1-8 *oxrC102*::Tn*5*(pCH21)	23	24	271	289	ND	ND	ND

[a] Cells (except CH878) were grown in minimal glucose medium containing additives as indicated. The concentration of each additive is given in Materials and Methods. Strain CH878 was grown in LB, as it is unable to grow in minimal glucose. We previously showed (14) that *tppB* expression in LB is identical to that in minimal medium to which leucine is added. ND, Not determined.

[b] Units are as defined by Miller (20).

Genetic techniques. Transductions were performed by using a high-transducing derivative of phage P22 *int4* as described by Roth (26). Because of its greater transducing capacity, the phage derivative P1 Tn*9* *clr-100* was occasionally used (20, 32). As *S. typhimurium* is normally resistant to P1 infection, *galE* derivatives were used for P1 transduction. Donors and recipients for P1 transduction were grown in LC medium.

Conjugations for HFr matings or F′ transfer were performed as described previously (9, 20). Random chromosomal insertions of Tn*5* were obtained by transduction of the appropriate recipient to Kan^r with a P22 lysate of strain TT3416 as the donor, as described previously (4). Mu d1-8 (Amp^r *lac*) insertions were obtained by transduction of strain TT7610 (*supD*) to Amp^r by using TT7674 as the donor (13). Random chromosomal insertions of the mini-Tn*10*Δ*16*Δ*17* (Cml^r) element were obtained by transduction of strain TT10427 (which carries the transposase helper plasmid pNK972 [39]) to chloramphenicol resistance with a P22 lysate of strain TT10605 (39). After transductions involving either a Tn*5* or Mu phage insertion, the correct location of the insertion and the presence of just a single copy of the transposon in the transductant were ascertained by marker rescue.

Mutants resistant to the toxic peptide alafosfalin were selected by plating washed cells on a minimal glucose plate containing 80 μg of alafosfalin ml^{-1} (9). Screening for sensitivity or resistance to alafosfalin was by radial streaking on an MG plate around a filter disk containing 250 μg of the antibiotic (9). Sensitivity to the toxic NAD analog 6-aminonicotinic acid (6-AMN) was similarly determined by using 10 μg of the analog per disk. Wild-type strains gave a zone of killing with a diameter of about 10 mm, whereas hypersensitive strains showed a 30-mm zone of killing.

Enzyme assays. β-Galactosidase activity was determined as described by Miller, by using the sodium dodecyl sulfate-chloroform permeabilization method (20). Phosphoglucose isomerase (PGI) was assayed as described by Fraenkel and Horecker (7). Nitrate reductase activity was detected by the overlay technique described by Sawers et al. (29).

RESULTS

Isolation of Mu d1-8(Amp^r *lac*) operon fusions to *tppB*. To isolate mutants defective in the anaerobic induction of *tppB*, we took advantage of the relatively simple phenotypic plate screens for reduced expression of β-galactosidase from *tppB-lacZ* fusions. We previously isolated operon fusions between *tppB* and *lacZ* by using the bacteriophage derivative Mu dl(Amp^r *lac*) (14). However, Mu dl-mediated *lacZ* fusions are relatively unstable, the phage transposing to other sites on the chromosome at a significant frequency. We therefore constructed *tppB-lacZ* fusions by using the recently described phage Mu d1-8(Amp^r *lac*) (13). This phage contains an amber mutation in the transposase gene and is consequently stable in strains that do not harbor an appropriate suppressor. A collection of 10,000 random Mu d1-8 insertions into the chromosome of strain TT7610 (*supD*) was made as described in Materials and Methods. From this collection, Mu d1-8 insertions in *tppB* were selected by their resistance to alafosfalin, and the resulting fusions were mapped and characterized as described previously (9, 14). One *tppB*::Mu d1-8 fusion was stabilized by transduction into a wild-type (*supD*$^+$) strain, and this derivative (CH776) was used for all further experiments. CH776 was shown to harbor just a single Mu d1-8 insertion, and this insertion was shown by marker rescue to be responsible for the *TppB* phenotypes. Regulation of β-galactosidase expression from this fusion was similar to that found previously for *tppB*::Mu dl(Amp^r *lac*) fusions (14) (Table 2).

Isolation of anaerobic regulatory mutations. Mutants defective in the anaerobic induction of *tppB* expression were identified by color changes on MacConkey agar-lactose plates. Preliminary experiments showed that color changes on anaerobically incubated MacConkey agar-lactose plates were unreliable due to a general increase in acid production under such conditions. However, when incubated aerobically, colonies of *tppB-lacZ* fusion strains gave a characteristic fish-eye appearance; β-galactosidase was expressed only in the center of a colony which had become anaerobic, whereas the aerobic perimeter of the colony remained white.

To ensure complete inactivation of any regulatory gene and to facilitate its characterization, the transposon Tn*5* was used as a mutagen. A random collection of 12,000 independent Tn*5* insertions in strain CH776 (*tppB84*::Mu d1-8) was pooled, washed twice in minimal medium, and plated on MacConkey agar-lactose plates at a density of about 500 cells per plate. Any colonies which were white or less red than CH776, i.e., having lost the fish-eye appearance, were picked, purified, and characterized further. Those which despite giving an altered colony color on MacConkey agar-lactose plates showed unaltered levels of β-galactosidase activity were discarded as mutations causing a general defect in acid production. Derivatives in which the Tn*5* insertion was essentially 100% linked by cotransduction to the Mu d1-8 insertion were assumed to have insertions in the *lacZ* or *lacY* genes of the Mu derivative and were discarded. It was also anticipated that the screening procedure used would identify Tn*5* insertions in the *tppA* (*ompR*) gene. *tppA* is a positive regulator of *tppB* expression and has recently been shown to be identical with the *ompR* gene (9; M. M. Gibson and C. F. Higgins, submitted for publication). *tppA* (*ompR*)

TABLE 3. Effects of *oxr* mutations on *pepT*, *hyd*, and *fhl* expression[a]

Strain	Relevant genotype	β-Galactosidase activity[b] under growth condition(s):			
		$+O_2$	$-O_2$	$+O_2$, +formate	$-O_2$, +formate
TN2021	*pepT7*::Mu dl	45	187	ND	ND
CH940	*pepT7*::Mu dl *oxrC102*::Tn*5*	31	24	ND	ND
TN1910	*pepT7*::Mu dl *oxrA1 (fnr)*	16	28	ND	ND
CH938	*pepT7*::Mu dl *tppR90*::Tn*5*	14	176	ND	ND
EB137	*fhl*::Mu dl	8	209	14	1,332
CH951	*fhl*::Mu dl *oxrC102*::Tn*5*	17	37	ND	144
CH974	*fhl*::Mu dl *oxrA1 (fnr)*	8	112	7	899
CH950	*fhl*::Mu dl *tppR90*::Tn*5*	3	251	ND	1,501
EB138	*hyd*::Mu dl	16	194	ND	268
CH953	*hyd*::Mu dl *oxrC102*::Tn*5*	17	40	ND	ND
CH975	*hyd*::Mu dl *oxrA1 (fnr)*	25	200	ND	218
CH952	*hyd*::Mu dl *tppR90*::Tn*5*	17	220	ND	ND

[a] Cells were grown in NB aerobically or anaerobically with formate added as indicated. ND, Not determined.
[b] Units are as defined by Miller (20).

is not involved in the anaerobic regulation of *tppB* (14). To identify and eliminate *tppA* (*ompR*) mutations, the transductional linkage between each regulatory Tn*5* and an *ompR*::Mu dl insertion (strain CH656) was determined. All strains in which the Tn*5* was closely linked to *ompR* were presumed to be insertions in *ompR* and were not studied further.

Three strains containing putative regulatory Tn*5* insertions remained, CH804, CH805, and CH878. The *tppB*::Mu dl-8 fusion from these strains was transduced into LT2, and regulation of β-galactosidase was shown to be normal (i.e., identical to that of the parental strain CH776). Thus, the Mu dl-8 had not mutated or transposed. Similarly, the Tn*5* insertions were transduced into unmutagenized CH776 to confirm that the strains contained only a single Tn*5* insertion and that reduction in β-galactosidase expression from the *tppB*::Mu dl-8 fusion was due to the Tn*5* insertion and not to an incidental point mutation. β-Galactosidase assays of these strains showed that the three regulatory mutations fell into two classes, designated *oxrC* and *tppR*. Genetic mapping and further phenotypic characterization showed that the Tn*5* insertions in strains CH804 and CH805 were indistinguishable. Thus, all further characterization of the *oxrC* locus was performed by using CH805 (*oxrC102*::Tn*5*).

Mutations in *oxrC* (oxygen regulation) prevented the anaerobic induction of *tppB* expression but had no effect on induction by leucine (Table 2). We previously presented evidence that the anaerobic and leucine-dependent inductions of *tppB* expression are mediated independently (14). The isolation of regulatory mutants which prevent only the anaerobic induction substantiate this view and, in addition, show that anaerobic induction is not simply a consequence of increased intracellular leucine pools.

Mutations in *tppR* (the regulatory locus for *tppB*; as shown below, *tppR* mutations did not have a pleiotropic affect on anaerobic enzymes) were found to confer partial auxotrophy (see below). Thus, *tppR* mutants were unable to grow in minimal medium, and all assays had to be performed on cells grown in LB (which contains leucine). Cells grown aerobically in LB showed a basal level of *tppB* expression due to induction by leucine (Table 2). However, this expression was not increased by growing the cells anaerobically, showing that, like *oxrC* mutations, mutations in *tppR* prevent the anaerobic induction of *tppB* expression.

***oxrC* and *tppR* are not alleles of *fnr*.** The only gene so far which is known to regulate anaerobic gene expression is *fnr* (*oxrA*) (also called *nirA* and *nirR* [17, 21, 31]). We previously obtained evidence that anaerobic expression of *tppB* is independent of *fnr* (14). Although the phenotypes of *oxrC* and *tppR* mutations were very different from those of *fnr* mutations (see below), it remained a possibility that different alleles of *fnr* exhibit different phenotypes. To demonstrate that *oxrC* is not an allele of *fnr*, plasmid pCH21 (containing the *fnr* gene [14]) was introduced into strains CH804 and CH805 and β-galactosidase activity was assayed anaerobically. The cloned *fnr* gene did not complement *oxrC* (Table 2). In addition, we also showed that *oxrC* and *tppR* map to very different chromosomal locations, both from each other and from *fnr* (see below).

***oxrC* is a pleiotropic regulatory gene.** A number of respiratory enzymes are known to be induced by anaerobiosis. Although some of these are *fnr* dependent, others are unaffected by *fnr* mutations. To determine whether *oxrC* mutations are specific to *tppB* or whether *oxrC* defines a pleiotropic anaerobic regulatory locus, the effect of *oxrC* mutations on the expression of a variety of anaerobically induced genes was examined. Operon fusions between *lacZ* and three anaerobically induced genes, *fhl*, *hyd*, and *pepT*, were recently described in *S. typhimurium* (1, 36). An *oxrC*::Tn*5* mutation was introduced into these fusion strains by transduction to Kan^r. The Tn*5* was shown by marker rescue to have remained in *oxrC* and not to have transposed. Mutations in *fnr* (*oxrA*) were also transduced into the *fhl* and *pepT* fusion strains, taking advantage of the Tn*5* insertion in CH602 which is 70% linked to *fnr* (*oxrA*). Derivatives were checked for coinheritance of *fnr* and the Tn*5* insertion by screening for the formation of red colonies on anaerobic MacConkey agar-nitrate plates and by the failure of the derivatives to grow on anaerobic glycerol-nitrate plates.

The *fhl* locus to which the fusion was made probably encodes the formate dehydrogenase component of formate hydrogen lyase (FDH-BV) (1, 15, 24). *fhl* expression is induced anaerobically, and this induction was further enhanced by exogenous formate (Table 3). A mutation in *oxrC* strongly reduced the anaerobic induction of *fhl* but had little effect on induction by formate. In addition, the effects of the *oxrC* mutation could not be suppressed by supplying exogenous formate. Thus, it seems clear that the formate and anaerobic induction of *fhl* expression were mediated by independent processes; *oxrC* mutations affected only the anaerobic induction. In contrast to the effects of *oxrC* mutations, *fnr* mutations only reduced anaerobic *fhl* expres-

TABLE 4. PGI activity[a]

Strain (mutation)	Enzyme activity (μmol of NADPH formed min^{-1} mg^{-1}) in medium:			
	LB		NB	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$
LT2	5.03	10.9	5.94	12.49
CH1021 (*oxrC*)	0.19	0.46	0.13	0.64
JF165 (*pasA*)	ND	ND	0.18	0.25

[a] PGI activity was determined as described in Materials and Methods. Cells were grown aerobically or anaerobically in LB or NB medium. ND, Not determined.

TABLE 6. Effect of acetate on *tppB* expression[a]

Growth conditions			U of β-galactosidase[b] from strain:	
Leucine	O_2	Acetate	CH776	CH805
−	+	−	80	40
−	−	−	442	60
−	+	+	316	191
−	−	+	527	276
+	+	−	162	ND
+	−	−	495	ND
+	+	+	191	ND
+	−	+	501	ND

[a] Strain CH776 (*tppB84*::Mu d1-8) or CH805 (*tppB84*::Mu d1-8 *oxrC102*::Tn*5*) was grown in minimal glucose medium with leucine, oxygen, and acetate (1%). ND, Not determined.
[b] Units are as described by Miller (20).

sion about twofold and expression was fully restored to wild-type levels by the addition of formate. Thus, the effect of *fnr* mutations on *fhl* expression appears to be indirect, probably the result of decreased formate production in *fnr* strains; it has been suggested that pyruvate-formate lyase activity is *fnr* dependent (28). Like *tppB*, expression of *fhl* is *oxrC* dependent and *fnr* independent. The data from *fhl* operon fusions are fully substantiated by direct assay for enzyme activity (15). Thus, mutations in *oxrC*, but not those in *fnr*, specifically reduced FDH-BV activity.

The *hyd* locus to which the *lacZ* fusion was made has been mapped to 59 min on the chromosome. The lesion is pleiotropic, lacking all three hydrogenase isoenzyme activities (15) and, therefore, seems unlikely to be a hydrogenase structural gene. Several *hyd* genes with pleiotropic phenotypes have been mapped to this 59-min locus, one of which can be phenotypically restored to Hyd^+ by growth in the presence of nickel (38); the *hyd-lacZ* fusion is not nickel suppressible. *oxrC* mutations also prevented the anaerobic induction of expression of this *hyd* locus (Table 3).

pepT encodes an anaerobically inducible tripeptidase whose expression is known to depend on *fnr* function (36). *oxrC* was also required for *pepT* expression from *pepT-lacZ* operon fusions (Table 3). Thus, unlike *tppB*, *hyd*, and *fhl*, *pepT* was both *oxrC* and *fnr* dependent.

Because *lacZ* fusions to other anaerobically induced genes have not been isolated in *S. typhimurium*, the effects of *oxrC* and *fnr* on other respiratory enzymes had to be determined by direct enzyme assay (15). Neither nitrate reductase nor fumarate reductase activities, both of which are *fnr* dependent (17, 21), were affected by *oxrC* mutations (15). Similarly, activity of the respiration-linked hydrogenase (hydrogenase 2) was *fnr* dependent and *oxrC* independent. On the other hand, the formate hydrogenlyase-associated hydrogenase (hydrogenase 3) was *oxrC* dependent and *fnr* independent, whereas hydrogenase 1, which seems to be associated with both hydrogen uptake and formate hydrogenlyase activities, was both *oxrC* and *fnr* dependent. It seems that the effects of *oxrC* were specific to anaerobically induced genes; no effect on the expression of *lacZ* fusions to a variety of oxygen-independent genes was found. It therefore seems clear that *oxrC* mutations defined a pleiotropic regulatory locus required for the expression of several but not all anaerobically induced enzymes. At least for some and probably for all these genes, regulation was at the level of transcription.

TABLE 5. Medium-dependent suppression of *oxrC*[a]

Strain	Relevant genotype	U of β-galactosidase[b] in medium:			
		Minimal glucose	Minimal fructose	NB	LB
CH776	*tppB84*::Mu d1-8	389	375	899	1,292
CH805	*tppB84*::Mu d1-8 *oxrC102*::Tn*5*	11	373	319	1,342
EB137	*fhl*::Mu d1	ND	ND	209	196
CH951	*fhl*::Mu d1 *oxrC102*::Tn*5*	ND	ND	37	190
TN2021	*pepT7*::Mu d1	ND	ND	187	483
CH940	*pepT7*::Mu d1 *oxrC102*::Tn*5*	ND	ND	24	486

[a] Cells were grown anaerobically in the medium indicated, and β-galactosidase was assayed as described in Materials and Methods. ND, Not determined.
[b] Units are as described by Miller (20).

Effects of *tppR* mutations on other anaerobically induced genes. *tppR* was very much more specific than was *oxrC* (Table 3). Thus, *tppR* mutations had no effect on the expression of *lacZ* fusions to *fhl*, *hyd*, or *pepT* and did not affect the activity of the following enzymes: nitrate reductase, fumarate reductase, FDH-BV, respiratory formate dehydrogenase, or any of the three hydrogenase isoenzymes (15, 29).

Effect of *oxrC* and *tppR* mutations on sugar fermentation. During the characterization of *oxrC* mutants, it was noticed that strains harboring an *oxrC* mutation grew as white colonies on green plates. These plates are essentially pH indicators, and this observation therefore implies a defect in fermentation (18). When streaked on MacConkey agar-glucose plates, *oxrC* strains grew as pale pink colonies

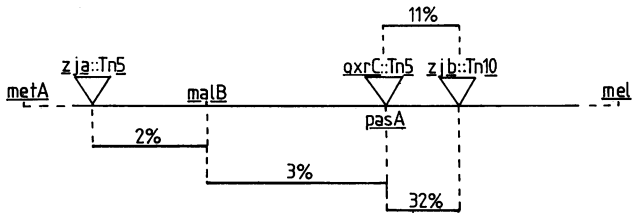

FIG. 1. Transductional mapping of *oxrC*. Cotransduction frequencies, with P22, are indicated. The 2% linkage between the *zja-861*::Tn*5* insertion and *malB* is in agreement with that reported by Strauch et al. (36). The *zjb-1708*::Tn*10* insertion was obtained from strain TS616. It was isolated as a *malE*::Tn*10* insertion (22), but we determined that the strain actually contains a *malE* point mutation and a linked Tn*10* insertion. The *malB* mutation used for mapping was from strain SA572.

TABLE 7. Genes or enzymes affected by *oxr* mutations[a]

Gene or enzyme function	Effect of mutation:		
	oxrA (fnr)	*oxrC*	*tppR*
tppB (tripeptide permease)	+	−	−
NAD pool levels[b]	+	−	−
fhl (93 min; FDH-BV)	+	−	+
Hydrogenase 3	+	−	+
Nitrate reductase	−	+	+
Hydrogenase 2	−	+	+
Fumarate reductase	−	+	+
Respiratory formate dehydrogenase	−	+	+
pepT (tripeptidase)	−	−	+
Hydrogenase 1	−	−	+

[a] Data concerning the regulation of hydrogenase isoenzyme activity and the activities of a number of respiratory enzymes by *oxrC*, *tppR*, and *fnr* are described by Jamieson et al. (15). +, Function is present in strains carrying the mutation; −, function is reduced or absent in strains carrying the mutation. When a gene is indicated, we demonstrated that the effect of *oxr* mutations is at the transcriptional level. When only the enzyme is listed, we assayed activity and did not assay transcription directly (see text).

[b] As indicated by 6-AMN hypersensitivity.

compared with the dark red color of $oxrC^+$ strains. It therefore seemed likely that *oxrC* mutants were defective in either glucose transport or metabolism. As the major route for glucose uptake is via the phosphotransferase system (PTS), the activities of the PTS enzymes were assayed. No significant differences in enzyme I activity or in vitro phosphorylation were found between $OxrC^+$ and $OxrC^-$ strains (P. W. Postma, personal communication). In addition, *oxrC* mutants were found to ferment mannitol on MacConkey agar-mannitol plates. As mannitol is transported only via the PTS, it seemed unlikely that the effects of *oxrC* on carbohydrate fermentation were at the level of transport; it seems instead that there was a defect in the production of acetate, formate, or both from glucose. To identify the defect in glycolysis, *oxrC* mutants were tested for their ability to ferment various sugars. *oxrC* mutants were found to ferment fructose, galactose, and arabinose normally and to grow on glycerol as the sole carbon source, yet they were defective in both glucose and maltose fermentation. This suggests a defect in PGI activity. On assay, it was found that PGI activity increased twofold in response to anaerobiosis. This effect, while not major, is in agreement with data presented previously (30). However, crude extracts of *oxrC* mutants were shown to have a 37-fold reduction in PGI activity, although activity was not totally abolished (Table 4). This finding was somewhat surprising, as PGI is not thought to be a major regulatory enzyme in glycolysis. In contrast to *oxrC* mutants, *tppR* mutants ferment glucose normally on MacConkey agar-glucose plates and are not defective in PGI activity (data not shown).

***tppR* mutations result in auxotrophy.** Although *tppR* mutants fermented glucose normally, they were found to grow extremely slowly on minimal glucose medium, suggesting an auxotrophic requirement. *tppR* mutants grew normally in LB, and growth on minimal medium could be restored by the addition of 0.25% LB or 0.25% Casamino Acids (Difco). The addition of aspartate or methionine, but not of any other amino acid, also stimulated growth of *oxrD* mutants, although it did not completely restore growth to wild-type levels. The precise nature of the requirement for multiple amino acids remains unclear.

Suppression of the *oxrC* phenotype. During characterization of the *oxrC* mutation, we noticed that the effects of *oxrC* on the anaerobic induction of *tppB* observed in minimal medium were completely suppressed by growth in LB. Thus, when grown in LB, *oxrC* derivatives of *pepT*, *tppB*, or *fhl* operon fusions showed normal anaerobic induction of β-galactosidase expression (Table 5). NB, on the other hand, did not suppress the *oxrC* lesion. This implies that a component present in LB but not in NB is responsible for the phenotypic suppression. However, although growth in LB suppressed the effect of *oxrC* mutants on *tppB*, *fhl*, and *pepT* expression, the defect in PGI activity remained (Table 5). This implies that either the *oxrC* effects on *tppB* and PGI are mediated by different routes or, alternatively, that *oxrC* causes a defect in PGI synthesis and, as a consequence of this defect, the expression of other genes is altered. This latter view was shown to be correct. Thus, fructose or any other sugar which entered the glycolytic pathway below PGI suppressed all the OxrC phenotypes except the loss of PGI activity (Table 6; unpublished data). Presumably, fructose or other sugars present in LB but not in NB also mediate suppression by this medium. Further evidence that the primary defect in *oxrC* mutants is loss of PGI activity comes from the map location of this gene (see below).

The observation that the primary defect in *oxrC* mutants is the absence of PGI activity implies that a product of glycolysis which is synthesized in altered amounts during anaerobic growth plays a role in the anaerobic induction of *oxrC*-dependent enzymes. Two possible candidates for such a signaling molecule are acetate and formate. Formate cannot play this role, as exogenous formate did not suppress the effects of *oxrC* mutations on *tppB* or *fhl* expression (Table 2). Acetate induced *tppB* expression aerobically and stimulated anaerobic induction in an *oxrC* strain (Table 6). However, as *tppB* could be induced independently by either leucine or anaerobiosis, it seems possible that acetate simply mimics the effects of exogenous leucine and does not affect anaerobic expression directly. The data in Table 6 support this view. Thus, neither acetate nor formate appear to be the mediators of *oxrC*-dependent anaerobic gene expression.

Effect of nucleotide analogs. During the mapping of *oxrC* and *tppR* mutations (see below), both were found to confer hypersensitivity to the toxic nucleotide analog 6-AMN. This implies that the mutations cause a defect in nucleotide biosynthesis. 6-AMN hypersensitivity is a result of reduced NAD pools (6, 12). The hypersensitivity of *oxrC* mutants to 6-AMN was suppressed by fructose, showing it to be a direct consequence of the defect in PGI, whereas fructose had no effect on the hypersensitivity of *tppR* mutations. As the *nadA* and *nadB* genes are anaerobically inducible (3, 11), it may be that the *oxrC* and *tppR* mutations interfere directly with the regulation of NAD synthesis.

Effect of electron acceptors on *tppB* expression. Expression of *tppB* is induced anaerobically, and this induction was prevented by the *oxrC* mutation. The addition of potential electron acceptors other than oxygen (e.g., nitrate and fumarate) to anaerobic cultures reduced the anaerobic induction to some extent but by no means completely repressed *oxrC* function (Table 2).

Chromosomal locations of the *oxrC* and *tppR* genes. The approximate location of the *oxrC* gene on the *S. typhimurium* chromosome was determined by introducing the mutation into various HFr strains. These derivatives were used as conjugation donors with a series of auxotrophic

strains as recipients, selecting for prototrophic transconjugants. The prototrophic colonies were screened for kanamycin resistance to determine the percentage of coinheritance of *oxrC* with each auxotrophic marker (data not shown). These data showed *oxrC* to be located in the 89.5- to 96-min region of the *S. typhimurium* chromosome flanked by *metA* and *purA*. To more precisely locate *oxrC* on the chromosome, the P22-mediated cotransductional linkage of *oxrC102*::Tn*5* with markers in this region of the chromosome was determined (Fig. 1). *oxrC* was found to be 3% linked to a *malB* point mutation and 11% linked to a Tn*10* insertion (*zjb-1708*::Tn*10*) in this region of the chromosome. The well-characterized *zja-861*::Tn*5* insertion is located between *metA* and *malB*, 2 to 5% linked to *malB*. However, no linkage (less than 1%) between *zja-861*::Tn*5* and *oxrC* or *zjb-1708*::Tn*10* could be detected. It therefore seems clear that *oxrC* must lie on the *mel* side of *malB*. Because the size of the Tn*10* and Tn*5* transposons is relatively large compared with the transducing capacity of P22, the precise location of *oxrC* with respect to *zjb-1708*::Tn*10* was determined by P1-mediated transduction. Three-point crosses were performed with strain CH1027 (*mal*$^+$ *oxrC102*::Tn*5*) as the recipient and strain CH1080 (*mal zjb-1708*::Tn*10*) as the donor. Transductions were carried out selecting for Tet^r recombinants. A total of 100 recombinants were subsequently screened for coinheritance of *oxrC*::Tn*5* (Kan^r) and the Mal$^-$ phenotype. The recombinant phenotypes were as follows: Kan^r Mal$^+$, 7%; Kan^r Mal$^-$, 0%; Kan^s Mal$^+$, 83%; and Kan^s Mal$^-$, 10%. The three-point crosses indicated that *oxrC* is located between *malB* and *zjb-1708*::Tn*10*.

The presumed structural gene for PGI has been mapped approximately to this region of the chromosome (27). As *oxrC* mutants are deficient in PGI activity and because suppression by fructose indicates that the PGI defect is the primary cause of all *oxrC* phenotypes, it seems probable that the two mutations are at the same locus. A mutation which confers 6-AMN hypersensitivity (*pasA* [6]) is also located in this region of the chromosome. We showed that the *pasA* mutation is located between *malB* and *zjb*::Tn*10* and is linked to these markers to about the same extent as is *oxrC* (Fig. 1), taking into account the differences in cotransduction frequencies, which result when mapping point (*pasA*) and insertion (*oxrC*::Tn*5*) mutations. As *oxrC* is also hypersensitive to 6-AMN, it seems likely that the two mutations are in the same gene. We therefore tested a *pasA* mutant and showed it to be defective in glucose but not fructose fermentation on MacConkey agar plates, indicating a defect in PGI activity. Subsequent assays for PGI activity confirmed this defect (Table 5). Thus, *oxrC*, *pgi*, and *pasA* are almost certainly alleles of the same locus, the primary defect being a deficiency in PGI. It seems likely that this locus encodes the structural gene for PGI, although it remains a possibility that it encodes a positive regulator of *pgi* expression.

Because *tppR* mutations confer a general auxotrophy, mapping was facilitated by isolating a mini-Tn*10* insertion closely linked to the *tppR*::Tn*5* mutation. This was achieved by transducing strain CH878 to Cml^r with a P22 lysate grown on a collection of random mini-Tn*10* (Cml^r) insertions in the *S. typhimurium* chromosome. This collection of insertions was prepared as described in Materials and Methods. The Cml^r derivatives were screened for those which had simultaneously become Kan^s. The cotransductional linkage between one such mini-Tn*10* insertion (*zae-1709*::Tn*10*Δ*16*Δ*17*) and the *tppR*::Tn*10* insertion was found to be greater than 95%. This mini-Tn*10* insertion did not confer the *tppR* phenotypes. The approximate map location of the *tppR*-linked mini-Tn*10* insertion was determined to be between *leu* (2 map units) and *pro* (7 map units) by HFr mapping, as described for *oxrC* above. P22 cotransduction showed both the *tppR*::Tn*5* and the *tppR*-linked mini-Tn*10* insertions to be 66% linked to a *panC*::Tn*10* insertion (TT421) and 78% linked to a *panC* point mutation (SA2628), both at 3 min on the *S. typhimurium* chromosome. *tppR* is not, as far as we have been able to determine, an allele of any of the known genes in this region of the chromosome.

DISCUSSION

The transcription of a number of bacterial genes is coordinately induced by anaerobiosis. This global regulatory system has been the subject of much interest, yet little is known of the mechanisms by which the anaerobiosis is sensed or how the anaerobic switch is controlled. The only gene known to play a key role in the control of anaerobic gene expression is *fnr* (*oxrA*), a pleiotropic regulatory gene required for the anaerobic induction of a number of genes including *nar*, *frd*, *glp*, and *pepT* (17, 21, 36). However, not all anaerobically induced genes are *fnr* dependent. We previously showed that the anaerobic induction of the tripeptide permease gene *tppB* is independent of the *fnr* gene product (14). In this study, we identified two genetic loci, *oxrC* and *tppR*, which were required for the anaerobic induction of *tppB*. Both were highly pleiotropic but exhibited different phenotypes. Our results imply the existence of at least two distinct classes of anaerobically induced genes. These two classes of genes seemed to respond to different regulatory signals, and regulation was apparently mediated by entirely different mechanisms. The first class of anaerobically induced genes were *fnr* dependent and *oxrC* independent. In contrast, the second class of genes, which included *tppB* and *fhl* (the structural gene for FDH-BV), were *fnr* independent and were defined by their dependence on the normal function of the pleiotropic regulatory locus *oxrC*. All the anaerobically induced genes we examined fell into these two classes, with two exceptions, *pepT* and hydrogenase 1, whose expression required the function of both *oxrC* and *fnr*. Those genes identified as belonging to each regulatory pathway are shown in Table 7. Interestingly, there seemed to be a functional distinction between the two classes of anaerobically induced genes; *fnr*-dependent enzymes served primarily respiratory roles, whereas *oxrC*-dependent enzymes served fermentative or biosynthetic roles. This observation is substantiated (15) in an accompanying paper in which the effects of *fnr* and *oxrC* on respiratory and fermentative hydrogen metabolism are examined. Particularly significant is the finding that hydrogenase 1 was linked to both respiratory and fermentative hydrogen metabolism, compatible with the dual regulation of this isoenzyme by both *fnr* and *oxrC*.

Surprisingly, *oxrC* mutants were found to be defective in sugar fermentation, and this was shown to be due to a defect in PGI activity. We showed that the absence of this enzyme was the primary defect of *oxrC* mutants and that all other phenotypes, including the defects in anaerobic induction of gene expression, were a direct consequence of the loss of PGI activity. Thus, none of the phenotypes of *oxrC* mutants, except the deficiency in PGI, were observed when cells were grown on fructose or on other sugars which enter the glycolytic pathway below PGI. Evidence that *oxrC* maps to approximately the same chromosomal location as does *pgi* implies that the two genes are identical (i.e., that *oxrC* is the structural gene for PGI). However, we cannot rule out the

possibility that *oxrC* encodes a positive regulator of *pgi*. Indeed, this latter possibility is suggested by the observation that, despite the *oxrC* lesion being due to a Tn*5* insertion, residual PGI activity is still detectable and this residual activity shows two- to threefold anaerobic induction. Thus, if *oxrC* is the structural gene for PGI, there must be at least one additional isoenzyme. In addition, the *oxrC* gene in *S. typhimurium* is located on the opposite side of *malB* than *pgi* is located in *E. coli*. Either the *pgi* structural gene is located at somewhat different chromosomal positions in the two species or, alternatively, *oxrC* encodes a positive regulator of *pgi*. In this context it is worth noting that *S. typhimurium* lacks an XylE function (P. J. Henderson, personal communication); *xylE* in *E. coli* has been mapped close to *pgi*. Interestingly, a mutation with the properties of a *pgi* lesion that maps to a similar chromosomal location has been shown to prevent the anaerobic induction of threonine dehydratase in *E. coli* (19). A mutation which confers 6-AMN hypersensitivity (*pasA*) is also located in this region of the chromosome (6). As *oxrC* mutants were also supersensitive to this analog, we tested the *pasA* mutation and found it to be deficient in PGI activity and to be genetically inseparable from *oxrC*. Thus, it seems that mutations at a single locus, whose primary defect was the loss of PGI activity and which was probably the structural gene for the enzyme, could give rise to each of these phenotypes.

How does loss of PGI activity prevent anaerobic enzyme induction? Because *oxrC* mutations had no effect on anaerobic metabolism when fructose was used as a carbon source, it seems clear that it is not the PGI protein itself which plays a regulatory role but that the anaerobic induction of *oxrC*-dependent enzymes must require a normal flow of carbon down the glycolytic pathway. Presumably, the defect in glycolysis prevents the synthesis of a metabolic intermediate which functions as a regulatory signal. The nature of this compound remains a matter for speculation. Two possible candidates are acetate or formate, which are produced during fermentation and which accumulate during the switch from respiratory to fermentative energy generation. However, we showed that neither of these compounds is the anaerobic signaling molecule, although each does play a role in the regulation of specific genes (e.g., formate increases the anaerobic induction of *fhl*, and acetate affects *tppB* expression indirectly, possibly by altering intracellular leucine pools). An alternative possibility is that *oxrC* mutations lead to an imbalance in NAD biosynthesis and that an NAD-related nucleotide or, alternatively, the NAD/NADH ratio serves as an indicator of anaerobiosis. This hypothesis is suggested by the observed hypersensitivity of *oxrC* mutants to 6-AMN, which implies a decreased intracellular pool of NAD (6, 12). Under anaerobic conditions, the NAD/NADH ratios are known to alter (40). It is easy to envision a mechanism by which a defect in glycolysis (the *oxrC* mutants) could mimic this effect as dihydroxyacetone phosphate (a glycolytic intermediate) is required for NAD biosynthesis (12). An alternative explanation for the 6-AMN hypersensitivity, that *oxrC* and *tppR* prevent the anaerobic induction of the *nadA* and *nadB* genes, has been shown to be incorrect (E. Ellis and C.F.H., unpublished data). This question requires further analysis.

The *tppR* mutation defined a second locus which affected the expression of *tppB*. *tppR* was located at 3 min on the *S. typhimurium* chromosome. Unlike *oxrC*, this mutation did not prevent the anaerobic induction of *fhl*, *pepT*, or any other known anaerobically induced gene which we tested. However, *tppR* was pleiotropic in that it caused a deficiency in NAD biosynthesis and resulted in a complex auxotrophic requirement. The exact nature of the defect was unclear. It is noteworthy that the NAD and amino acid biosynthetic defects of *tppR* mutants were apparent during both aerobic and anaerobic growth. In addition, the poor growth (auxotrophic requirement) of *tppR* mutants in minimal media could be suppressed by growth in rich media (LB), whereas the anaerobic induction of *tppB* is not restored by growth in LB. Thus, unlike *oxrC*, the failure of *tppR* mutants to induce *tppB* expression anaerobically did not seem to be a secondary consequence of a defect in metabolism. *tppR* specifically regulated *tppB* expression, rather than defining a component of a global anaerobic regulatory pathway.

We have shown here that the anaerobic induction of several genes encoding products involved in carbohydrate and amino acid metabolism (*oxrC* dependent) was regulated by a more or less distinct mechanism from that controlling the induction of respiration-linked enzymes (*fnr* dependent). *oxrC*-dependent genes required the normal function of glycolysis, whereas the *fnr*-dependent enzymes are apparently unaffected by altered patterns of fermentation. Clearly the mechanisms by which genes are regulated by anaerobiosis are varied and complex. We defined two apparently independent pathways by which anaerobic gene expression is controlled. To learn whether additional pathways exist and to identify the various components of each pathway will require further genetic analysis.

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