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Oxygen Regulation in *Salmonella typhimurium*

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Regulation by oxygen of the peptidase T (*pepT*) locus of *Salmonella typhimurium* was studied by measuring β-galactosidase levels in strains containing a *pepT*::Mu d1(Ap^r *lac*) operon fusion. β-Galactosidase was induced in anaerobic cultures and late-exponential and stationary-phase aerated cultures. Peptidase T activity also was induced under these growth conditions. *pepT*$^+$ but not *pepT* strains will utilize as amino acid sources the tripeptides Leu-Leu-Leu and Leu-Gly-Gly only when grown anaerobically. Mutations at two loci, *oxrA* and *oxrB* (oxygen regulation) prevent induction of the *pepT* locus. The *oxrA* locus is homologous to the *fnr* locus of *Escherichia coli*. We have isolated 12 independent Mu d1 insertions (*oxd*::Mu d1, oxygen dependent) that show induction of β-galactosidase in anaerobic cultures and stationary-phase aerated cultures. These insertions fall into nine classes based on map location. All of the *oxd*::Mu d1 insertions are regulated by *oxrA* and *oxrB* and therefore define a global regulon that responds to oxygen limitation.

Peptidase T of *Salmonella typhimurium* is an aminotripeptidase that removes the N-terminal amino acid from a variety of tripeptides (28). The enzyme was first identified as a band of hydrolytic activity towards tripeptides seen after native polyacrylamide gel electrophoresis of cell extracts and staining for peptidase activity (18). Mutants lacking peptidase T (*pepT*) were obtained by screening extracts of mutagenized colonies for those unable to hydrolyze the peptides Met-Ala-Ser and Met-Gly-Gly (28). This screen was carried out with a strain of *S. typhimurium* lacking several aminopeptidases (peptidases N, A, and B) which also are capable of hydrolyzing tripeptides. Peptidase T cannot be specifically assayed in extracts of wild-type strains because of the tripeptidase activity of these other enzymes (18).

The ability of peptidase T to allow growth of *S. typhimurium* on peptides as amino acid sources was tested in strains lacking peptidases N, A, and B. Compared with *pepT*$^+$ strains, the *pepT* mutants are not deficient in using as amino acid sources any of the peptides which the enzyme hydrolyzes in vitro (28). Peptidase T is the only *S. typhimurium* peptidase studied which does not permit growth on at least one peptide substrate (17, 18, 29, 30). The inability of peptidase T to allow growth on tripeptides results from insufficient peptidase activity in the wild type. Mutants (*pto*, peptidase T overproducer) that overproduce peptidase T are able to use peptidase T substrates as amino acid sources (27). The *pto* mutations are tightly linked to *pepT*, are *cis* dominant, and increase β-galactosidase expression from a *pepT7*::Mu d1(Ap^r *lac*) operon fusion (*pepT*::*lac*) (2, 27).

The occurrence of mutants that overproduce peptidase T suggested that the enzyme might be regulated. To identify a regulatory pattern for peptidase T we have used the *pepT*::*lac* fusion to search for conditions that affect β-galactosidase levels. In this communication we report that peptidase T levels are regulated by oxygen. In addition, we describe the identification of other genes regulated by oxygen and two regulatory loci which control all of these genes.

MATERIALS AND METHODS

Bacterial strains. Strains used in this work are derivatives of *S. typhimurium* LT2 or *Escherichia coli* K-12. Strains used repeatedly are listed in Table 1.

Media and growth conditions. Media and growth conditions have been described previously (28). Growth on peptides, electron acceptors, and nitrogen sources was tested by plating 0.1 ml of an overnight culture in 2.5 ml of soft agar on a minimal plate and placing crystals of the test substrate on the soft agar. Growth was scored after overnight incubation. Chlorate resistance was tested by placing a filter paper disk saturated with 3% $KClO_3$ on a lawn of the strain to be tested growing on nutrient agar supplemented with 0.2% glucose. Sensitive strains showed a zone of growth inhibition after overnight anaerobic incubation. Culture growth was measured by determining the optical density at 600 nm (OD_{600}) on a Gilford spectrophotometer. For anaerobic growth, liquid cultures either were grown in filled volumetric flasks with the medium overlaid with mineral oil or were sparged with 5% CO_2–95% N_2, and plates were incubated in Brewer jars under an atmosphere of 5% CO_2–95% N_2.

Cultures for β-galactosidase assays were grown in 50 ml of E medium plus glucose (0.4%) in a 125-ml screw cap Erlenmeyer flask incubated in a model G-76 Gyrotory shaker (New Brunswick Scientific Co., Inc.) at a speed setting of 5.

Genetic techniques. Mutagenesis with diethyl sulfate (Sigma Chemical Co.) was performed as described by Roth (24). Mutagenesis with Mu d1(Ap^r *lac* cts62) was performed as described previously (6, 27). Transduction with P22 (HT 12/4 *int*-3) was performed by the method of Roth (24). To transduce *pepT7*::Mu d1(Ap^r *lac* cts62 *X*) into recipient strains, ca. 10^8 cells were infected at a multiplicity of infection of 10 PFU per cell and plated to give ca. 100 Amp^r colonies. The *X* mutation in the Mu d1 prophage immobilizes it and allows it to be transduced into other strains without zygotic induction (1, 28). For transduction to antibiotic resistance, the phage and bacteria were mixed and preincubated for at least 30 min before plating on selective medium. Hfr crosses were performed by broth mating as described by Sanderson et al. (25). F′ episomes were transferred from strain to strain by plate matings as described by Sanderson et al. (25).

Mapping of Mu d1 insertions was performed by transduction to Tet^r with phage lysates grown on various strains, each containing a Tn*10* insertion at a known site on the chromosome as described by Maurer et al. (16). The donor strains were a collection of ca. 50 strains, each carrying a Tn*10* insertion at a different locus. Approximately 5×10^8

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TABLE 1. Bacterial strains[a]

Strain	Genotype
TN1304	*leuBCD485 pepN90 pepA16 pepB11 pepP1 pepQ1*
TN1379	*leuBCD485*
TN1668	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*)
TN1672	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *pto-2 zce-850*::Tn*10*
TN1686	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *zce-850*::Tn*10*
TN1893	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *pto-13*
TN1895	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *oxrA1*
TN1909	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *oxrA*$^+$ *zda-888*::Tn*10*
TN1910	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *oxrA1 zda-888*::Tn*10*
TN1989	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *leuBCD485*
TN2062	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *leuBCD485 oxrA1 zda-888*::Tn*10*
TN2063	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *leuBCD485 oxrA2*::Tn*10*
TN2064	*pepT7*::Mu d1(Ap^r *lac* cts62 *X*) *leuBCD485 oxrB8 zxx-895*::Tn*5*

[a] The insertion *zce-850*::Tn*10* is 75% cotransducible with *pepT* (28). The insertion *zda-888*::Tn*10* is 15% cotransducible with *oxrA* (see the text). The insertion *zxx-895*::Tn*5* is 45% cotransducible with *oxrB8*.

recipient cells were spread on a nutrient agar tetracycline plate, and drops of phage lysates were spotted on this lawn. The plates were incubated at 30°C for 4 h and then at 42°C overnight. Transductants which lost the Mu d1 prophage became temperature resistant and formed colonies on the selective plates. The results of these crosses were confirmed by single transductional crosses, performed by selecting Tet^r and scoring for the Lac and Amp phenotypes conferred by the Mu d1 prophage. Insertions of a Tn*10* derivative Tn*10*Δ16Δ17 (7) near Mu d1 fusions were identified in a similar way by using P22 transducing lysates grown on a set of ca. 300 strains carrying random insertions of this transposable element (A. Kukral and R. Maurer, manuscript in preparation).

Enzyme assays. β-Galactosidase was measured by the method of Miller (20). Hydrolysis of peptides by crude soluble extracts (28) was measured by high-pressure liquid chromatography of trinitrophenyl derivatives to analyze reaction products (T. H. Carter, Ph.D. thesis, Case Western Reserve University, Cleveland, Ohio, 1982). Met-Gly-Gly was obtained from Bachem. *N*-Acetyl-Ala$_3$ was from Sigma Chemical Co. The protein concentration was determined by the method of Lowry et al. (15), with bovine serum albumin as the standard.

RESULTS

Regulation of peptidase T. An operon fusion of the *lac* structural genes to the *pepT* locus (*pepT*::*lac*) was used to search for conditions that regulate synthesis of peptidase T. Strain TN1668 (*pepT*::*lac*) was grown in various media, and β-galactosidase activity was measured. We found no evidence that β-galactosidase levels were significantly affected by growth on alternative carbon sources or nitrogen sources, by starvation for carbon or nitrogen, or by changes in growth rate (data not shown). However, β-galactosidase levels were dependent on the growth phase of the cultures from which cells were harvested. Stationary-phase cells showed significantly higher enzyme levels than did exponential cells. The levels of β-galactosidase throughout the growth cycle therefore were measured (Fig. 1). During exponential growth in minimal glucose medium, the β-galactosidase activity remained constant at ca. 25 U. During the last few doublings before the culture entered stationary phase, the specific activity increased sixfold to ca. 150 U (Fig. 1). In strain TN1672 which contains the *pto-2* mutation, β-galactosidase remained constant throughout the growth cycle at ca. 300 U (Fig. 1). Strain TN1668 (*pepT*::*lac*) showed increased expression of β-galactosidase in stationary phase when grown in minimal medium containing either glucose or glycerol as the carbon source.

To determine whether peptidase T production is regulated in this fashion as well, extracts of strain TN1304 (*leu pepN pepA pepB pepP pepQ*) were prepared from cultures at several optical densities, and the specific activity of peptidase T was assayed. The specific activity of peptidase T increased 15-fold between the exponential phase and the stationary phase (Table 2). The specific activity of another peptidase, dipeptidyl, carboxypeptidase (30), was assayed in the same extracts. The levels of dipeptidyl carboxypeptidase did not differ significantly in exponential- and stationary-phase cells (Table 2).

Effect of nutrient and O_2 limitation on *pepT* expression. It seemed possible that limitation for some nutrient might be the signal for increasing peptidase T levels late in exponential phase. Therefore, *pepT* expression was measured in cultures containing limiting concentrations of nutrients. Cultures with limiting glucose, ammonia, or leucine did not show elevated levels of β-galactosidase expressed from the *pepT* promoter before or after cessation of growth (Fig. 2; data not shown). Because the O_2 supply is frequently cited as an important factor limiting growth, we tested the effect of anaerobiosis on expression of β-galactosidase from the *pepT*::*lac* fusion. Exponentially growing cells from anaerobic cultures contained β-galactosidase levels comparable to those of stationary-phase cells from aerobic cultures (ca. 400 U). It therefore is likely that oxygen limitation

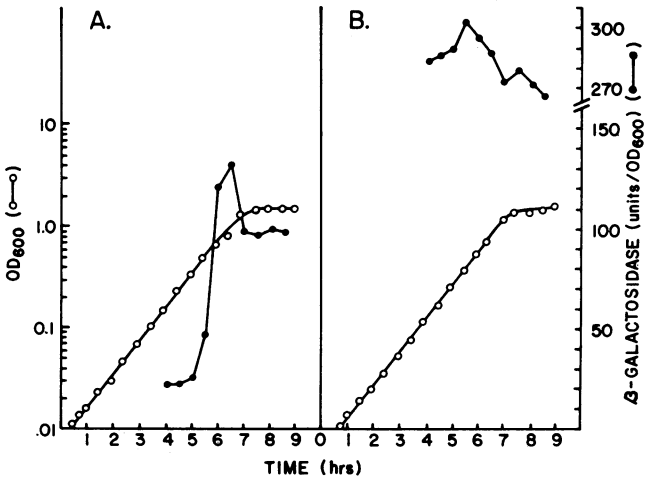

FIG. 1. β-Galactosidase levels during the bacterial growth cycle. Strains were grown in E medium plus 0.4% glucose. Samples for β-galactosidase assay were removed at 0.5-h intervals and kept on ice until assayed by the method of Miller (20). (A) TN1668, *pto*$^+$ *pepT*::*lac*; (B) TN1672, *pto-2 pepT*::*lac*.

during late-exponential phase causes increased *pepT* expression in aerobic cultures.

The growth properties of *pepT*$^+$ and *pepT* strains also reflect regulation of peptidase T by oxygen levels. When grown anaerobically, *pepT*$^+$ strains are able to utilize peptide substrates of peptidase T as amino acid sources. These tripeptides did not serve as amino acid sources during aerobic growth (Table 3). Only growth on tripeptides, not dipeptides or tetrapeptides, was affected by anaerobiosis. This suggests that the cell does not contain a group of anaerobic peptidases induced by O_2 limitation. An *E. coli* peptidase mutant, CM89 (19), shows the same effect of anaerobiosis on peptide utilization, indicating that *E. coli* contains a tripeptidase regulated in the same manner as peptidase T of *S. typhimurium*.

Regulatory loci for *pepT*. We have sought mutations that alter regulation of *pepT*. We expected that such mutants might be detectable by screening mutagenized cultures of the *pepT*::*lac* fusion strain for colonies with an altered appearance on lactose indicator plates. When grown on MacConkey-lactose agar, colonies of the *pepT*::*lac* strain TN1668 had dark-red centers and light peripheries (fisheye colonies). Strains carrying a *pto* mutation formed uniformly red colonies. This suggested that cells in the center of the wild-type colony are expressing relatively high β-galactosidase levels (as are cells from a stationary-phase liquid culture or an anaerobic culture), whereas cells on the periphery of the colony contain relatively low β-galactosidase levels (as do cells from aerated, exponentially-growing liquid cultures). We expected that mutants with altered regulation of the *pepT* locus could be detected on MacConkey agar as colonies that are uniformly red (indicating high β-galactosidase levels in all cells) or that lack the red center (indicating low β-galactosidase levels in all cells). Such strains might contain mutations linked to the *pepT* locus (e.g., *pto* or *lac* mutations) or mutations unlinked to *pepT*. Unlinked mutations might represent alterations in *trans*-acting factors that control transcription of *pepT*.

To isolate mutations affecting *pepT*::*lac* expression, strain TN1668 was mutagenized with diethyl sulfate and plated on MacConkey agar. Colonies that differed in appearance from the parent were picked and purified. Mutant phenotypes included uniformly red colonies, colonies with darker, lighter, or smaller red centers, pink colonies, and white colonies. Few completely white colonies were picked and characterized because they were expected to be *lacZ* or *lacY* mutants. From ca. 30,000 colonies screened, 125 mutants were tested for the presence of mutations that were unlinked to *pepT*. This was done by transducing each of these strains to Tet^r on MacConkey-lactose-tetracycline agar with phage grown on a strain (TN1686) containing a Tn*10* insertion 75% linked to *pepT*::*lac*. If a recipient strain carries a mutation linked to *pepT*, some of the transductants from this cross should regain the wild-type (fisheye) colony appearance. For strains with mutations unlinked to *pepT*, all of the transductants should retain the mutant phenotype. To verify that these strains contained mutations unlinked to *pepT*, each strain was used as a donor in a transduction cross with LT2 (wild type, no *lac* fusion) as recipient, and Amp^r transductants were selected. This cross (mutant donor × wild-type recipient) should move the *pepT*::*lac* fusion into a clean genetic background and separate it from any unlinked mutations that affect expression of β-galactosidase. Mutations linked to *pepT* should give a mixture of wild-type and mutant phenotypes reflecting coinheritance of *pepT*::*lac* and the linked mutation. Unlinked mutations should give only wild-type (fisheye) colonies because the *pepT*::*lac* fusion and the mutation altering expression of β-galactosidase cannot be cotransduced.

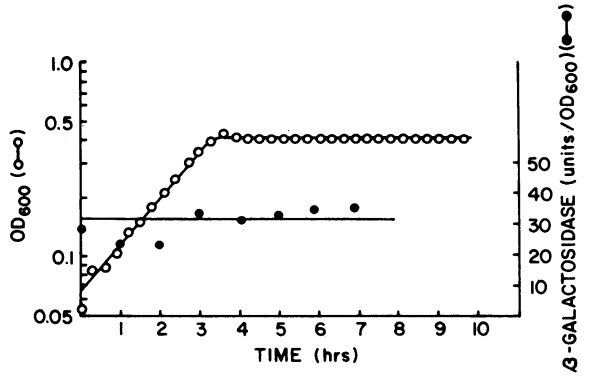

FIG. 2. β-Galactosidase levels in glucose-limited culture. Strain TN1668 (*pto*$^+$ *pepT*::*lac*) was grown in NCE medium plus 0.04% glucose. Samples were removed at 1-h intervals and kept on ice until assayed for β-galactosidase by the method of Miller (20).

TABLE 2. Peptidase T and dipeptidyl carboxypeptidase levels[a]

OD_{600} of culture	Peptidase T Met-Gly-Gly hydrolysis (μmol of methionine per min/mg)	Dipeptidyl carboxypeptidase *N*-acetyl-Ala_3 hydrolysis[b] (μmol of alanine per min/mg)
0.12	0.048 (1.0)	0.082 (1.0)
0.44	0.117 (2.4)	0.103 (1.25)
1.02	0.743 (15.5)	0.094 (1.15)

[a] Extracts were prepared from 1-liter cultures grown in E medium plus 0.4% glucose. The extract for testing cells in exponential growth was made from two pooled 1-liter cultures with optical densities of 0.089 and 0.15 (averaged to 0.12). Met-Gly-Gly hydrolysis was assayed in 50 mM Tris-hydrochloride (pH 7.5) containing 10 mM Met-Gly-Gly and 1 mM $MnCl_2$. *N*-Acetyl-Ala_3 hydrolysis was assayed in 0.1 M sodium barbital (pH 8.1) containing 10 mM *N*-acetyl-Ala_3 and 0.1 mM $CoCl_2$. The values within parentheses measure relative activity.

[b] The extracts used for these assays contain a dipeptidase (peptidase D) which converts Ala-Ala (the product of hydrolysis of *N*-acetyl-Ala-Ala-Ala by dipeptidyl carboxypeptidase) to Ala. Small amounts of Ala-Ala (less than 5% of the Ala levels) were detected in the reaction products.

Four of the strains tested formed uniformly red colonies on MacConkey-lactose medium. All of these strains had mutations linked to the *pepT* locus. Mapping data for one of these mutants, TN1893, is shown in Table 4. These strains probably carry *pto* mutations. No unlinked mutation was found that elevates β-galactosidase production. One mutant (TN1895) which had decreased β-galactosidase levels (pink colony) carried a mutation unlinked to *pepT* (Table 4). We have called the mutation in this strain *oxrA1* (oxygen regulation). Tn*10* and Tn*5* insertions linked to *oxrA* were isolated. In transduction crosses with these insertions the *oxrA1* allele behaves as a mutation at a single locus (Table 4). A Tn*10* insertion into the *oxrA* locus also has been isolated.

In another attempt to isolate regulatory mutants, a strain with a chromosomal duplication of *pepT7*::Mu d1 (unpublished data) was used to reduce the occurrence of *lacZ* and *lacY* mutations. The strain was mutagenized and screened on MacConkey agar for pink or white colonies. Mutants were tested by transduction to determine whether they contained mutations linked to *pepT* or *oxrA*. In this experi-

TABLE 3. Growth on peptides

Strain[a]	O_2	Growth on				
		Leu	Leu-Leu	Leu-Gly-Gly	Leu-Leu-Leu	Leu-Leu-Leu-Leu
TN1298 (*pepT*$^+$)	+	+	−	−	−	−
	−	+	−	+	+	−
TN1420 (*pepT*)	+	+	−	−	−	−
	−	+	−	−	−	−
CM89 (*pepT*$^+$)	+	+	−	−	−	−
	−	+	−	+	+	−

[a] TN1298, *leu485 pepN90 pepA16 pepB11 pepP1 pepQ1 supQ309Δ(proAB pepD)*; TN1420, *leu485 pepN90 pepA16 pepB11 pepP1 pepQ1 supQ302Δ(proAB pepD)*; CM89, *E. coli* K-12 *leu-9 Δpro-lac met thyA pepN102 pepA11 pepB1 pepQ10*.

ment another *oxrA* allele and a mutation at another locus, *oxrB*, were isolated. Tn*5* insertions linked to *oxrB* have been isolated, and in crosses using this Tn*5* insertion the *oxrB8* allele also behaves as a mutation at a single locus.

The effect of *oxr* mutations on β-galactosidase expressed from the *pepT* promoter was measured. The *oxrA* and *oxrB* mutants failed to induce β-galactosidase as the cultures entered stationary phase or during anaerobic growth (Table 5).

Mapping of *oxrA*. To map the *oxrA* locus we used a Tn*10* insertion (*zda-888*::Tn*10*) 15% linked to *oxrA*. Hfr strains with origins of transfer near *oxrA* were constructed with F′ *lac zzf*::Tn*10* episomes as described by Chumley et al. (5). These Hfr's have an origin of transfer at the site of the chromosomal Tn*10* insertion near *oxrA*. With Hfr's constructed at the Tn*10* near *oxrA*, the only region of the map at which a discontinuity in the gradient of transfer (determined by the number of recombinants formed with particular auxotrophs) occurred was the interval between *pyrC* (22 map units) and *trp* (34 map units) (Table 6). This indicates that the origin of the Hfr's lies between *pyrC* and *trp*. To further define the position of *oxrA*, the Tn*10* insertion near *oxrA* was introduced into an Hfr with its origin at 0 map units and a clockwise direction of transfer. This strain TN2015 (HfrK4 *serA13 rfa-3058 zda-888*::Tn*10*) was mated to auxotrophic mutants. Prototrophic recombinants were selected and scored for Tet^r. The linkage of the Tn*10* insertion to auxotrophic markers was: *proAB25*, 7 map units, 6% (3/50); *pyrD13*, 21 map units, 14% (7/50); *trp-43*, 34 map units, 84% (42/50); *his-644*, 42 map units, 54% (27/50); and *argH88*, 88 map units, 2% (1/50). The high linkage to *trp* suggests that *oxrA* maps near the *trp* end of this interval of the *Salmonella* map. The *oxrA1* mutation was not cotransducible by P22 with *purB13*, *pyrF696*::Tn*10*, *trp-2451*::Tn*10*, or the following transposon insertions: *zce-862*::Tn*10*, *zce-864*::Tn*10*, and *zce-859*::Tn*5* (linked to *purB*; 27); *zcf-845*::Tn*10* and *zcf-847*::Tn*5* (linked to *dcp*; 30); and *zcd-6*::Tn*10*, *zcd-7*::Tn*10*, *zcd-8*::Tn*10*, *zcd-10*::Tn*10*, and *zcd-2*::Tn*10* (linked to *pncA*, *gdhA*, or *nit*; 23).

Identification of other genes regulated by anaerobiosis. We wondered whether other genes might show a pattern of regulation similar to that of *pepT*. In an attempt to identify such genes we screened independent Mu d1(Ap^r *lac*) insertion strains for those which formed fisheye colonies on MacConkey-lactose medium. As noted above, the colony appearance of the *pepT*::*lac* strain (red center, light edge) and of mutants with altered *pepT*::*lac* expression (*pto*, uniformly red; *oxrA*, uniformly pink) suggests that colony appearance on MacConkey-lactose agar is a reflection of the *pepT* regulatory pattern. If this is correct, strains containing *lac* fusions to other genes regulated in the same way might form similar colonies on MacConkey-lactose agar.

In one experiment ca. 800 Amp^r insertion mutants of TN1379 (*leuBCD485*) were selected on MacConkey lactose-ampicillin agar. Most of the colonies showing any red color seemed to be darker in the center of the colony. Consequently, the choice of colonies that most closely resembled the *pepT*::*lac* strain was somewhat subjective. Colonies were chosen for study that had a light edge and a red center, a sharp boundary between these two areas of the colony, and no precipitation of bile salts around isolated colonies. These strains were assayed for β-galactosidase during exponential and stationary phase (Table 7). Of 12 strains with the fisheye appearance, 4 showed an induction ratio (stationary phase/exponential phase) of at least 6. All four isolates that showed a high induction ratio were unable to grow on minimal lactose medium (Table 7). Of the strains that did not show significant induction of β-galactosidase, only one was unable to use lactose as the sole carbon source. The Lac phenotype presumably reflects the low levels of β-galactosidase during exponential growth. All of the Lac$^-$ strains had less than 15 U of activity in exponential phase.

TABLE 4. Transductional mapping of mutations affecting β-galactosidase expression from *pepT7*::Mu d1 (Ap^r *lac*)[a]

Donor (genotype, phenotype)	Recipient	Selection	Colony types on MacConkey agar		
			Fisheye	Red	Pink
TN1668 (*pepT*::*lac*, fisheye)	LT2	Amp^r	37/37	0	0
TN1893 (*pepT*::*lac pto-13*, red)	LT2	Amp^r	9/40	31/40	0
TN1895 (*pepT*::*lac oxrA1*, pink)	LT2	Amp^r	40/40	0	0
TN1686 (*pepT*::*lac zce-850*::Tn*10*, fisheye)	TN1895	Tet^r	0	0	100/100
TN1909 (*pepT*::*lac zda-888*::Tn*10*, fisheye)	TN1895	Tet^r	7/60	0	53/60
TN1910 (*pepT*::*lac oxrA1 zda-888*::Tn*10*, pink)	TN1668	Tet^r	207/238	0	31/238

[a] Selection for Amp^r requires inheritance of *pepT*::*lac*. The insertion *zce-850*::Tn*10* encodes Tet^r and is 75% cotransducible with *pepT*. The insertion *zda-888*::Tn*10* was isolated on the basis of its linkage to *oxrA1*.

The *pepT*::*lac* strain (ca. 30 U in exponential phase) shows only weak growth on minimal lactose medium (27).

Effect of the *oxrA1* mutation. To test whether these fusions are regulated by *oxrA*, the *oxrA1* mutation was introduced by cotransduction with a linked Tn*10* insertion. Phage grown on TN1910 (*oxrA1 zda-888*::Tn*10*; 15% linked) was used to transduce each of the *lac* fusion strains to Tet^r on MacConkey lactose-tetracycline agar, and the transductants were scored for colony type. Some strains showed only the fisheye colony type, similar to the recipient strains in the cross. Other strains showed two types: fisheye colonies resembling the recipient strain and pink colonies virtually indistinguishable from *pepT*::*lac oxrA1* strains. All four strains with high induction ratios (>6) for β-galactosidase gave pink colonies after introduction of the *oxrA1* mutation (Table 7).

One strain (TN1932) showed no induction of β-galactosidase but did give pink transductants after introduction of the *oxrA1* mutation (Table 7). Because induction ratios were measured in minimal glucose medium but colony appearance was scored on MacConkey agar, a rich medium, it seemed possible that the gene carrying the Mu d1 insertion in TN1932 might show a high induction ratio only when grown in a rich medium. To test this, β-galactosidase levels were measured in cells grown in nutrient broth. TN1932 grown in this medium had 3.6 U of β-galactosidase during exponential phase and 221 U during stationary phase. Therefore, all strains showing high induction ratios (>6) respond to the *oxrA* mutation, and no strain with a low induction ratio showed any *oxrA* effect. All strains with high induction ratios also gave pink transductants after introduction of the *oxrB8* mutation.

Because the criteria for choosing strains with colony appearances similar to *pepT*::*lac* strains were somewhat subjective, the screening process may have missed some *lac* fusion strains that induce β-galactosidase in stationary phase. In a second experiment all Mu d1 insertion mutants of TN1379 that showed any red color (from pink to deep red) on MacConkey agar were transduced to Tet^r with phage on the *oxrA1 zda-888*::Tn*10* strain (TN1910). Of 887 Amp^r colonies, 53 (ca. 5%) expressed enough β-galactosidase to be detected on MacConkey plates. All 53 strains that expressed β-galactosidase were tested for growth on minimal lactose medium and for the effect of introducing the *oxrA1* mutation (formation of pink colonies). Four strains (TN1942, TN1943, TN1944, and TN1945) were found that expressed less β-galactosidase when the *oxrA1* mutation was introduced into the strain. All of these strains had the fisheye phenotype and showed no growth or only marginal growth on minimal lactose medium. The β-galactosidase levels of these strains in exponential and stationary phase were measured (Table 8). All four strains showed high induction ratios. These four strains also gave pink colonies on MacConkey-lactose agar after introduction of the *oxrB8* mutation.

TABLE 5. Effect of regulatory mutations on *pepT*::*lac* expression during stationary-phase and anaerobic growth

Strain	Culture condition	OD_{600}	β-Galactosidase (U)
TN1989 *oxrA*$^+$	Aeration	0.187	23
	Aeration	2.39	156
	CO_2/N_2	0.099	381
TN2062 *oxrA1*	Aeration	0.180	24
	Aeration	2.98	39
	CO_2/N_2	0.153	30
TN2063 *oxrA2*::Tn*10*	Aeration	0.292	25
	Aeration	3.59	23
	CO_2/N_2	0.148	30
TN2064 *oxrB8*	Aeration	0.276	25
	Aeration	3.49	26
	CO_2/N_2	0.099	50

TABLE 6. Mapping of *oxrA* by conjugation crosses with Hfr's with transfer origins near *oxrA*

Donor	No. of colonies when mated with:[a]	
	TT1333 (*trp*::Tn*10*)	TT459 (*pyrC*::Tn*10*)
TN1999 (*leuBCD485 zda-888*::Tn*10*/ F'ts114 *lac*$^+$ *zzf-20*::Tn*10*)	340	20
TN2000 (*leuBCD485 zxx-888*::Tn*10*/ F'ts114 *lac*$^+$ *zzf-21*::Tn*10*)	86	296
TN2001 (*leuBCD485 zxx-888*::Tn*10*)/ F'ts114 *lac*$^+$ *zzf-22*::Tn*10*)	256	39

[a] TT1333 and TT459 were obtained from J. Roth.

Effect of oxygen. The β-galactosidase levels of strains with *oxrA* controlled *lac* fusions were measured during anaerobic growth (Table 9). All strains tested showed β-galactosidase levels comparable to or higher than those of stationary-phase aerobic cultures. The loci to which *lac* is fused in these strains therefore have been designated *oxd* (oxygen dependent).

Mapping of *oxd* loci. The *oxd*::Mu d1(Ap^r *lac*) insertions were tested for cotransduction with a set of ca. 50 different Tn*10* insertions at known sites on the chromosome. The map positions of two *oxd*::Mu d1 insertions were identified (Table

TABLE 7. Characterization of Mu d1(Ap^r *lac*) insertion mutants with the fisheye appearance on MacConkey agar

Isolate	β-Galactosidase (U)[a]			NCE-lactose growth[b]	*oxrA1* effect[c]
	Exponential	Stationary	Fold increase		
4 (TN1932)	5.5	5.0	1	−	+
8 (TN1933)	6.0	35	6	−	+
9	53	102	2	+	−
10	50	140	3	+	−
11	250	255	1	+	−
12	69	25	<1	+	−
13 (TN1934)	6.5	63	10	−	+
14	110	100	1	+	−
15	170	130	<1	+	−
16 (TN1935)	13	180	14	−	+
17	320	175	<1	+	−
18 (TN1936)	7.0	235	34	−	+
19[d]	470	900	2	+	−
TN1668 (*pepT*)	33	717	22	+/−	+

[a] A stationary-phase culture was diluted at least 10^4 into 100 ml of E medium plus glucose plus leucine late in the afternoon and incubated at 30°C overnight in a shaking water bath. The next morning the OD_{600} of the culture was ≤0.2. The culture was assayed for β-galactosidase when the optical density was ≤0.2 (exponential) and assayed for β-galactosidase again on the next day (stationary).

[b] Growth was tested by streaking strains on NCE agar plus 0.2% lactose + 0.4 mM leucine.

[c] Each strain was transduced to Tet^r on MacConkey-lactose agar with phage grown on strain TN1910 (*zda-888*::Tn*10 oxrA1*). +, Transductants were of two colony types, one fisheye and one pink; − transductants resembled the recipient strain (fisheye).

[d] This strain was uniformly red on MacConkey plates.

TABLE 8. β-Galactosidase levels in strains with *oxrA1* controlled *lac* fusions

Strain[a]	β-Galactosidase (U)[b]		
	Exponential	Stationary	Fold increase
TN1942	1.4	8.4	6
TN1943	79	278	3.5
TN1944	8.7	139	16
TN1945	8.4	104	12
*TN1950	4.6	55	12
TN1989 (*pepT*)	37	371	10

[a] TN1942 through TN1945 were derived from TN1379. TN1950 was derived from TN1304.

[b] A stationary-phase culture was diluted at least 10^4 into 100 ml of E medium plus glucose plus leucine late in the afternoon and incubated at 30°C overnight in a shaking water bath. The next morning the OD_{600} of the culture was ≤0.2. The culture was assayed for β-galactosidase when the optical density was ≤0.2 (exponential) and again on the next day (stationary).

10). Strain TN1935 contains the mutation *oxdA5*::Mu dl, which is linked to *metC* (64 map units), and strain TN1942 contains the mutation *oxdB7*::Mu dl, which is linked to *mel* (93 map units). All other *oxd*::Mu dl insertions are not cotransducible with *pepT*, *mel*, or *metC*.

To gain better information about how many different loci are represented among our fusions, we tested all of them for linkage to a group of ca. 300 random insertions of Tn*10*Δ16Δ17 isolated by Kukral and Maurer (manuscript in preparation). Tn*10*Δ16Δ17 insertions linked to all but one oxd fusion (*oxd-7*) were found. The *oxd-3*, *oxd-9*, and *oxd-10* fusions were linked to the same Tn*10*Δ16Δ17 insertions with approximately the same linkage frequency. All other *oxd* fusions were linked to unique Tn*10*Δ16Δ17 insertions.

Growth on anaerobic respiratory substrates. When grown anaerobically, *E. coli* induces several proteins that allow growth on nonfermentative carbon sources (e.g., glycerol) with electron acceptors other than oxygen (e.g., nitrate, fumarate, or trimethylamine *N*-oxide). A regulatory locus, *fnr* (also called *nirA* or *nirR*), which prevents induction of these proteins has been described previously (3, 13, 21). The *fnr* locus maps near *trp* (3), in approximately the same region as *oxrA*. We therefore tested the *oxr* and *oxd* mutants for anaerobic growth on these electron acceptors. The *oxrA* mutants were unable to use nitrate as an electron acceptor or a nitrogen source but could use fumarate and trimethylamine *N*-oxide as electron acceptors and nitrite as a nitrogen source. The *oxrB8* mutant utilized all these compounds.

One *oxd* mutant, TN1943 (*oxd*-8::Mu dl) was unable to use nitrate or trimethylamine *N*-oxide as electron acceptors. This phenotype suggested that the *oxd-8* mutation might affect one of the *chl* loci. Mutations at these loci confer chlorate resistance because they are pleiotropically deficient in several molybdo-enzymes which reduce chlorate to the toxic compound chlorite. All of the *oxd* fusions therefore were tested for chlorate resistance. Only *oxd-8*::Mu dl was chlorate resistant. In a transduction cross with a Tn*10*Δ16Δ17 linked to *oxd-8* as donor and *oxd-8*::Mu dl, all of the Lac$^-$ ampicillin-sensitive recombinants regained chlorate sensitivity. The chlorate-resistant phenotype therefore results from the *oxd* mutation and not from a secondary mutation.

TABLE 9. β-Galactosidase levels during anaerobic growth[a]

Strain	β-Galactosidase (U)
TN1668	773
TN1933	185
TN1934	53
TN1935	213
TN1936	408
TN1942	270
TN1943	318
TN1944	233
TN1945	85

[a] Cells were harvested from cultures growing exponentially (OD_{600} < 0.38) in E medium plus 0.4% glucose plus 0.4 mM leucine, overlaid with mineral oil.

DISCUSSION

The overall response of cells to several major physiological stresses or changes has been studied genetically with the Mu dl(Ap^r lac) vector to identify genes induced by DNA damage (10) or phosphate starvation (31) and biochemically by two-dimension gel electrophoresis to identify polypeptides whose synthesis is regulated by heat shock (8, 14) or growth rate (22). These phenomena have been termed global regulation. We believe that the group of genes (*oxd*) defined by our Mu dl(Ap^r *lac*) insertions comprise another global regulon characterized by high levels of gene products in anaerobic cultures or in stationary-phase aerobic cultures Expression of these genes is reduced by mutations at the *oxrA* and *oxrB* loci. Mu dl insertions into these genes occur at a frequency of 1 in 200 among all Mu dl lysogens. Based on map position, 10 different loci are represented among the 12 Mu dl fusions. Only one class contains more than one member, so it is likely that several other anaerobically induced loci remain undiscovered. By using two-dimensional gels, Smitha and Neidhardt (26) observed in *E. coli* 18 polypeptides that are present at higher steady-state levels in anaerobically grown cells. Most of these polypeptides showed 2- to 5-fold increases in anaerobiosis, whereas our anaerobic/aerobic ratios were considerably higher (5- to 60-fold). It is possible that the products of the *oxd* loci defined by our Mu dl insertions are not resolved by the two-dimensional gel system used by Smith and Neidhardt. However, a more likely explanation is that their aerobic reference culture, grown to an OD_{420} of 1.0, was already partially induced for these gene products. We find that an OD_{420} of 1.0 corresponds to an OD_{600} of 0.53. At this cell density peptidase T levels have reached 50% of the maximum under our culture conditions.

The induction ratio for β-galactosidase expressed from the *pepT* promotor shows some variability in different experiments (Fig. 1 and Tables 5, 7, and 8). Most of this variability is probably due to variations in the rates of aeration as cultures enter stationary phase. We have found that the most reproducible measure of the induction ratio is anaerobic exponential phase/aerobic exponential phase. This can be

TABLE 10. Mapping of *oxd*::Mu dl mutations by transduction[a]

Donor	Recipient	% Linkage (Lac$^-$/Tet^r)
TT14 (*metC*::Tn*10*)	TN1935 (*oxdA5*::Mu dl)	64 (32/50)
TN1214 (*zce-850*::Tn*10*)	TN1935	0 (0/200)
TT1662 (*mel*::Tn*10*)	TN1942 (*oxdB7*::Mu dl)	28 (14/50)
TN1214 (*zce-850*::Tn*10*)	TN1942	0 (0/200)

[a] Transductants were selected on medium containing tetracycline and tested on MacConkey agar for lactose fermentation. The insertion *zce-850*::Tn*10* is 75% linked to *pepT*. TT14 and TT1662 were obtained from J. Roth.

calculated as 381 U/23 U = 16.7 for *pepT* (Table 5). This proves to be the best method for determining induction ratios of the *oxd* loci as well.

The *pepT* gene is the only one of this group of genes for which a gene product is known. The physiological significance of this mode of regulation for an aminotripeptidase is not clear. Perhaps tripeptides are present at high levels in some natural anaerobic environment, and peptidase T is induced to degrade them. Clearly the levels of peptidase T are sufficiently high in cells growing anaerobically to allow the use of some tripeptides that cannot be used by aerobically growing peptidase-deficient cells. An alternative possibility is that peptidase T plays some role in regulating the response to anaerobiosis, perhaps by processing a peptide signal molecule. The observation that *pepT* mutants show normal regulation of the *oxd*::Mu dl(Amp^r *lac*) fusions (K. Strauch, unpublished data) argues against this possibility. Because a multiply-peptidase-deficient *E. coli* K-12 strain uses certain tripeptides only when grown anaerobically, this organism seems to contain a similarly regulated tripeptidase, presumably tripeptidase TP (9). Regulation of tripeptidase activity by oxygen levels therefore is conserved in *E. coli* and *S. typhimurium*.

Some proteins have been observed previously to be regulated much like peptidase T. Under conditions of oxygen-limited growth and in stationary phase, cytochromes b_{558}, a_1, and *d* are present in higher levels than in well-aerated, exponentially growing cells (10). Other respiratory proteins involved in electron transport to acceptors other than O_2 also are expressed during anaerobiosis. We have considered the possibility that our regulatory locus, *oxrA*, corresponds to an *E. coli* locus that pleiotropically affects several anaerobic respiratory pathways. Mutations at this locus (variously called *fnr*, *nirA*, or *nirR*) prevent the expression of several anaerobic respiratory proteins (fumarate reductase, nitrate reductase, nitrite reductase, trimethylamine *N*-oxide reductase, and formate hydrogenlyase). These *E. coli* mutants have lost the ability to use nitrate or fumarate as electron acceptors or nitrite as a nitrogen source (13, 21). The *fnr* mutations map at 29.5 min on the *E. coli* map, in approximately the same region as *oxrA* in *S. typhimurium*. Strains carrying *oxrA* mutations cannot use nitrate as an electron acceptor or as a nitrogen source, but they do use fumarate as an electron acceptor and nitrite as a nitrogen source. (The *oxrB* mutant is not deficient in utilizing any of these substrates.) Although these phenotypes are clearly different from that of *E. coli fnr* mutants, it is possible that *S. typhimurium* contains furmarate and nitrite reductase activities that are not repressed by oxygen. Multiple trimethylamine *N*-oxidase reductases, some of which are constitutive, are present in *S. typhimurium* (12). Higgins and co-workers (9a) have shown that the *E. coli fnr*$^+$ gene can complement *oxrA1* in *S. typhimurium* for expression of β-galactosidase from the *pepT*::*lac* fusion, indicating that *oxrA* and *fnr* are homologous loci. This locus must have a much broader regulatory significance than previously recognized, including the regulation of at least one gene (*pepT*) having no obvious role in respiration.

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LITERATURE CITED

1. **Bukhari, A. I.** 1975. Reversal of mutator phage Mu integration. J. Mol. Biol. **96**:87–99.
2. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533.
3. **Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M.-C. Pascal.** 1981. Operon fusions in the nitrate reductase operon and study of the control gene *nirR* in *Escherichia coli*. Mol. Gen. Genet. **182**:477–479.
4. **Chippaux, M., D. Giudici, A. Abou-Jaoude, F. Casse, and M. C. Pascal.** 1978. A mutation leading to the total lack of nitrite reductase activity in *Escherichia coli* K12. Mol. Gen. Genet. **160**:225–229.
5. **Chumley, F. G., R. Menzel, and J. R. Roth.** 1979. Hfr formation directed by Tn*10*. Genetics **91**:639–655.
6. **Csonka, L. N., M. M. Howe, J. L. Ingraham, L. S. Pierson III, and C. L. Turnbough, Jr.** 1981. Infection of *Salmonella typhimurium* with coliphage Mu dl (Ap^r *lac*): construction of *pyr*::*lac* gene fusions. J. Bacteriol. **145**:299–305.
7. **Foster, T. J., M. A. Davis, D. E. Roberts, K. Takeshita, and N. Kleckner.** 1981. Genetic organization of transposon Tn10. Cell **23**:201–213.
8. **Herendeen, S. L., R. A. VanBogelen, and F. C. Neidhardt.** 1979. Levels of major proteins of *Escherichia coli* during growth at different temperatures. J. Bacteriology **139**:185–194.
9. **Hermsdorf, C. L.** 1978. Tripeptide-specific aminopeptidase from *Escherichia coli* AJ005. Biochemistry **17**:3370–3376.
9a. **Jamieson, D. J., and C. F. Higgins.** 1984. Anaerobic and leucine-dependent expression of a peptide transport gene in *Salmonella typhimurium*. J. Bacteriol. **160**:131–136.
10. **Kenyon, C. J., and G. C. Walker.** 1981. Expression of the *E. coli uvrA* gene is inducible. Nature (London) **289**:808–810.
11. **Kranz, R. G., and R. G. Gennis.** 1982. Isoelectric focusing and crossed immunoelectrophoresis of heme proteins in the *Escherichia coli* cytoplasmic membrane. J. Bacteriol. **150**:36–45.
12. **Kwan, H. S., and E. L. Barrett.** 1983. Roles for menaquinone and the two trimethylamine oxide (TMAO) reductases in TMAO respiration in *Salmonella typhimurium*: Mu *d*(Ap^r *lac*) insertion mutations in *men* and *tor*. J. Bacteriol. **155**:1147–1155.
13. **Lambden, P. R., and J. R. Guest.** 1976. Mutants of *Escherichia coli* K12 unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. **97**:145–160.
14. **Lemaux, P. G., S. L. Herendeen, P. L. Bloch, and F. C. Neidhardt.** 1978. Transient rates of synthesis of individual polypeptides in *E. coli* following temperature shifts. Cell **13**:427–434.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Maurer, R., B. C. Osmond, E. Shekhtman, A. Wong, and D. Botstein.** 1984. Functional interchangeability of DNA replication genes in *Salmonella typhimurium* and *Escherichia coli* demonstrated by a general complementation procedure. Genetics **108**:1–23.
17. **McHugh, G. L., and C. G. Miller.** 1974. Isolation and characterization of proline peptidase mutants of *Salmonella typhimurium*. J. Bacteriol. **120**:364–371.
18. **Miller, C. G., and K. Mackinnon.** 1974. Peptidase mutants of *Salmonella typhimurium*. J. Bacteriol. **120**:355–363.
19. **Miller, C. G., and G. Schwartz.** 1978. Peptidase-deficient mutants of *Escherichia coli*. J. Bacteriol. **135**:603–611.
20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Newman, M., and J. A. Cole.** 1978. The chromosomal location and pleiotropic effects of mutations of the *nirA*$^+$ gene of *Escherichia coli* K12: the essential role of *nirA*$^+$ in nitrite reduction and in other anaerobic redox reactions. J. Gen. Microbiol. **106**:1–12.
22. **Pedersen, S., P. L. Bloch, S. Reeh, and F. C. Neidhardt.** 1978. Patterns of protein synthesis in *E. coli*: a catalog of the amount of 140 individual proteins at different growth rates. Cell **14**:179–190.
23. **Rosenfeld, S. A., S. M. Dendinger, C. H. Murphy, and J. E. Brenchley.** 1982. Genetic characterization of the glutamate

dehydrogenase gene (*gdhA*) of *Salmonella typhimurium*. J. Bacteriol. **150**:795–803.
24. **Roth, J. R.** 1970. Genetic techniques in studies of bacterial metabolism. Methods Enzymol. **17**:3–35.
25. **Sanderson, K. E., H. Ross, L. Ziegler, and P. H. Mäkelä.** 1972. F^+, Hfr, and F′ strains of *Salmonella typhimurium* and *Salmonella abony*. Bacteriol. Rev. **36**:608–637.
26. **Smith, M. W., and F. C. Neidhardt.** 1983. Proteins induced by anaerobiosis in *Escherichia coli*. J. Bacteriol. **154**:336–343.
27. **Strauch, K. L., T. H. Carter, and C. G. Miller.** 1983. Overproduction of *Salmonella typhimurium* peptidase T. J. Bacteriol. **156**:743–751.
28. **Strauch, K. L., and C. G. Miller.** 1983. Isolation and characterization of *Salmonella typhimurium* mutants lacking a tripeptidase (peptidase T). J. Bacteriol. **154**:763–771.
29. **Vimr, E. R., L. Green, and C. G. Miller.** 1983. Oligopeptidase-deficient mutants of *Salmonella typhimurium*. J. Bacteriol. **153**:1259–1265.
30. **Vimr, E. R., and C. G. Miller.** 1983. Dipeptidyl carboxypeptidase-deficient mutants of *Salmonella typhimurium*. J. Bacteriol. **153**:1252–1258.
31. **Wanner, B. L., and R. McSharry.** 1982. Phosphate-controlled gene expression in *Escherichia coli* K12 using Mu*d*1-directed *lacZ* fusions. J. Mol. Biol. **158**:347–363.