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Novel Regulatory Loci Controlling Oxygen- and pH-Regulated Gene Expression in *Salmonella typhimurium*

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Three new loci were discovered, each of which participates in the regulation of anaerobic gene expression. The regulatory gene *earA* negatively regulates the expression of the anaerobiosis-inducible gene *aniG* as well as that of at least three other genes, as determined by two-dimensional polyacrylamide gel electrophoresis. The *earA* locus maps at 86 min. The expression of *aniG* was also shown to be controlled by changes in external pH under aerobic and anaerobic conditions. Maximal expression was observed under anaerobic conditions at an external pH of 6.0. Significant transcriptional activity was also observed under aerobic conditions at pH 6.0. This was in contrast to *hyd*, whose expression was dependent upon anaerobiosis and varied with external pH. The pH dependence disappeared under fully aerobic conditions. Mutations in *earA* had no effect upon *hyd* expression. The two other regulators identified were *oxrF*, which controls *aniH*, and *oxrG*, which, in concert with *oxrA* and *oxrB*, controls *aniC* and *aniI*. The *oxrG* locus was mapped to 88 min and appears to code for a positive regulator. Various *oxr* mutants were subjected to two-dimensional polyacrylamide electrophoretic analysis of anaerobiosis-inducible proteins. Several pathways of anaerobic control were observed by means of these techniques.

There have been several reports identifying genes in *Salmonella typhimurium* and *Escherichia coli* whose expressions are controlled by the presence or absence of molecular oxygen (1, 10, 35, 37). Anaerobiosis-inducible genes with unidentified products have been referred to as *ani* (1) and *oxd* (35). These genes were discovered by generating random *lacZ* operon fusions in the chromosome with Mu d(Ap *lac*) phage. Identified anaerobiosis-inducible genes include those involved with nitrate (*nar*; 4, 14, 34) and fumarate (*frd*; 21, 22) reductases, hydrogen sulfide production (*phs*; 1, 11), cobalamine biosynthesis (*cob*; 13), hydrogenase (*hyd*; 1, 20, 39), some peptidases and peptide permeases (*tppB*, *pepT*; 15, 18, 19, 35), anaerobic L-α-glycerophosphate dehydrogenase (*glpAB*; 23), NAD biosynthesis (*nadAB*; 17), degradative threonine dehydratase (28), formate hydrogen lyase (*fhl*; 31), a tertiary amine oxidase (*torA*; 24, 30), menaquinone synthesis (*men*; 24), and dimethyl sulfoxide reductase (23). Several genes have been implicated in *S. typhimurium* as participating in anaerobic transcriptional control. These include *oxrA*, a positive regulator equivalent to *fnr* in *E. coli* (7, 25, 35); *oxrB*, another positive regulator (35); *oxrC* (*pgi*), not a transcriptional regulator but involved with the generation of an inducing signal (19); and *tppR*, a positive regulator of *tppB* (18).

The most characterized anaerobic regulatory locus is *fnr* of *E. coli*. Mutations in *fnr* prevent the anaerobic induction of several respiratory enzymes, such as the nitrate and fumarate reductases, hydrogenase 2, dimethyl sulfoxide reductase, and glycerol-3-phosphate dehydrogenase (3, 7, 23, 25, 34). The nucleotide sequence of *fnr* has revealed a considerable amount of homology with the cyclic AMP receptor protein (CRP). This suggests that a nucleotide such as cyclic AMP may be involved in the regulation of anaerobic gene expression by binding to Fnr. Not all anaerobically inducible genes are *fnr*$^+$ dependent, however. Various studies have shown that *tppB*, *torA*, *pepN*, certain components of *fhl*, the gene encoding hydrogenase 3, and *phs* do not require Fnr for their expressions (1, 11, 18, 19, 30, 35). The molecular mechanism(s) controlling the expressions of these genes remains obscure.

In a previous study we characterized 16 *ani-lacZ* operon fusions into three classes on the basis of their control by *oxrA* and *oxrB*. Class I loci were regulated by both *oxrA* and *oxrB*, class II loci were controlled by *oxrA* only, and class III loci were independent of both *oxrA* and *oxrB*. We have also noted other regulatory subclasses including (i) genes whose anaerobic expression in minimal medium requires vitamin-free Casamino Acids, (ii) some Casamino Acids-inducible strains whose expression is enhanced by the addition of 20 mM nitrate, (iii) genes whose anaerobic expression is repressed by glucose (catabolite repression), (iv) genes whose anaerobic expression increases in complex medium with the addition of glucose, and (v) *ani* loci, whose expression is repressed by the addition of 20 mM nitrate.

We have utilized three *ani* loci that exhibit different regulatory characteristics to identify three new regulatory loci. The first is *earA*, which acts as a negative regulator of *aniG*, a class III gene whose anaerobic expression requires complex medium and is glucose repressed. The second is *oxrF*, which controls *aniH*, a class III locus whose expression is *oxrAB* independent. The third regulator is *oxrG*, which controls *aniC* and *aniI*, both group I loci whose anaerobic expressions require Casamino Acids and are enhanced by nitrate. It was also determined that the *hyd* and *aniG* loci are regulated by alterations in external pH as well as by anaerobiosis. The *earA* (external acidification regulator) mutations relinquished *aniG* from pH control but not oxygen regulation. To our knowledge this is the first report

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of a gene involved in regulating the transcriptional response of enterobacteria to changes in pH.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The bacterial strains used in this study are listed in Table 1. Cultural conditions, including minimal E medium and complete LB medium, were as described previously (12, 27, 36). No-carbon E medium was supplemented with 10 mM $MgSO_4$ and lactose at 0.2% (NCE-lactose medium) (12). For labeling with $H_2{}^{35}SO_4$, the minimal MOPS (morpholinepropanesulfonic acid) medium of Neidhardt et al. (29) containing 28 μM SO_4 was used. Antibiotic concentrations were 30 μg/ml for ampicillin, 100 μg/ml for kanamycin, and 10 μg/ml (minimal medium) or 20 μg/ml (complex medium) for tetracycline. The pH of liquid media was maintained by including buffers with the appropriate pK_a values as described by Slonczewski et al. (32). The buffers (at 100 mM) included citrate (pH 5.0 to 5.3), MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 5.7 to 6.3), PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.7 to 7.3), or MOPS (pH 7.7 to 8.3). Anaerobiosis was achieved by overlaying a tube of broth with 1 ml of sterile paraffin oil. Plates were incubated in an anaerobic GasPak system (BBL Microbiology Systems). Aerobic conditions were achieved with vigorous shaking (220 rpm) of 5 ml of culture medium in a 50-ml flask. MacConkey nitrate medium was prepared as recommended (2). Transductions employing bacteriophage P22 HT 105/1-*int* were performed as indicated earlier (16). After transductions, nonlysogenic segregants were identified by cross-streaking against phage H5 on the green medium of Chan et al. (6). H5 is a virulent, clear-plaque-forming derivative of P22 which will not infect strains lysogenic for P22.

Many of our Mu d1 and Mu dA lysogens were replaced by the smaller (11.3-kilobase) Mu dJ derivative, which carries kanamycin resistance. Construction and use of Mu dJ (originally referred to as Mu dI1734) was described by Castilho et al. (5).

Isolation of regulatory mutants. Two basic methodologies were employed to isolate regulatory mutations. The first method involved plating 10^7 cells of an appropriate *lacZ* fusion strain per plate onto NCE-lactose medium with incubation under repressive conditions, i.e., aerobically for *ani-lacZ* strains. The method screens for spontaneous regulatory mutations which allow for constitutively high or deregulated expression of the *lacZ* fusion, resulting in β-galactosidase levels suitable to support growth on lactose as a sole carbon source.

TABLE 1. *S. typhimurium* strains used

Strain	Genotype	Source
JF900	*aniC1052*::Mu dA *supD10*	(1)
JF927	*aniA1005*::Mu dA *supD10*	(1)
JF1105	*aniG1072*::Mu dA *supD10*	(1)
JF1114	*aniD1047*::Mu dA *oxrB8* *zxx-895*::Tn*5*	
JF1140	*aniH1069*::Mu dA	(1)
JF1141	*aniI1070*::Mu dA	(1)
JF1294	*aniG1072*::Mu dJ *supD10*	This paper
JF1295	*aniG1072*::Mu dJ	This paper
JF1411	*aniG*$^+$ *earA209 metC1945*::Tn*10*	This paper
JF1493	*aniG1072*::Mu dJ *zig-1935*::Tn*10* (Tn*10* 86% linked to *earA*$^+$)	This paper
SF165	*oxrA1 leu-485 pepT*::Mu d1 *zda-888*::Tn*10*	C. Miller (35)
SF242	*oxrC*::Tn*5*	D. Jamieson (19)

The second method used to isolate regulatory mutants involved screening a random pool of Tn*10* insertions for insertions into regulatory loci (12). P22 HT 105/1-*int* phage propagated on the Tn*10* insertion pool was crossed with the *ani-lacZ* recipient on MacConkey tetracycline medium, and duplicate plates were incubated aerobically (looking for constitutive expression) and anaerobically (looking for a lack of anaerobic induction). Verification of strains containing potential *oxr*::Tn*10* insertions was achieved by showing that subsequent transduction of the Tn*10* to the original *ani-lacZ* strain resulted in a 100% linkage between Tc^r and the deregulated Oxr^- phenotype. The β-galactosidase assay performed on these strains was described by Miller (27). Enzyme activity is expressed in Miller units, i.e., nanomoles of *o*-nitrophenyl-β-D-galactosidase per minute per unit of optical density at 600 nm (A_{600}). For β-galactosidase assays, cells were grown under aerobic or anaerobic conditions to an optical density at 600 nm of 0.4 to 0.5 before the assay. Values represent an average of duplicate measurements.

Genetic manipulations. Tn*10* insertions near regulatory loci were identified by transducing *ani-lacZ oxr* mutants with P22 lysates propagated on a pool of Tn*10* insertion mutants with selection under aerobic conditions on MacConkey tetracycline medium. Light-colored transductants that retained the appropriate Mu d antibiotic resistance marker were purified and backcrossed with the original mutant strain to determine linkage to the *oxr* locus. Deletions extending from a Tn*10* insertion through an *oxr* locus were constructed by using the method of Maloy and Nunn (26). Tn*10*-directed Hfr donors were constructed as originally outlined by Chumley et al. (9) and used in mapping as described previously (1).

Two-dimensional polyacrylamide electrophoresis. The procedure for the two-dimensional analysis of cellular proteins was essentially as described previously (33). Cells were grown in minimal MOPS with vigorous aeration to 2×10^8 cells per ml. The culture was then split; 5 ml was left under aeration, and 5 ml was transferred to a culture tube and overlaid with sterile paraffin oil. Cells were labeled at the point of shift with 50 μCi of $H_2{}^{35}SO_4$ and allowed to grow for one doubling.

Approximately 1.5 ml of labeled cells was suspended in 13 μl of a sodium dodecyl sulfate (SDS) lysing solution, boiled, and run in a pH 5 to 7 isoelectric focusing system followed by 11.5% SDS-polyacrylamide electrophoresis as described earlier (33). Comparisons were made between aerobic and anaerobic samples with equivalent protein (5 to 15 μg) and equivalent disintegrations per minute (1×10^6 to 3×10^6). The coordinates given are those provided in a previously published standard two-dimensional profile of *S. typhimurium* polypeptides (33).

RESULTS

Control of *hyd* and *aniG* loci by external pH. Growth under anaerobic conditions in unbuffered medium such as LB will result in a drop in external pH levels from 7.4 to approximately 6. To determine whether any of our anaerobiosis-inducible fusions, especially those only induced in LB, were regulated by pH, our *ani-lacZ* strains were grown in LB medium buffered as described in Materials and Methods. Two of our fusions, *aniA* (*hyd*) and *aniG*, clearly exhibited a pH effect (Fig. 1). The *hyd-lacZ* strain, JF927, expressed β-galactosidase only under anaerobic conditions, but was

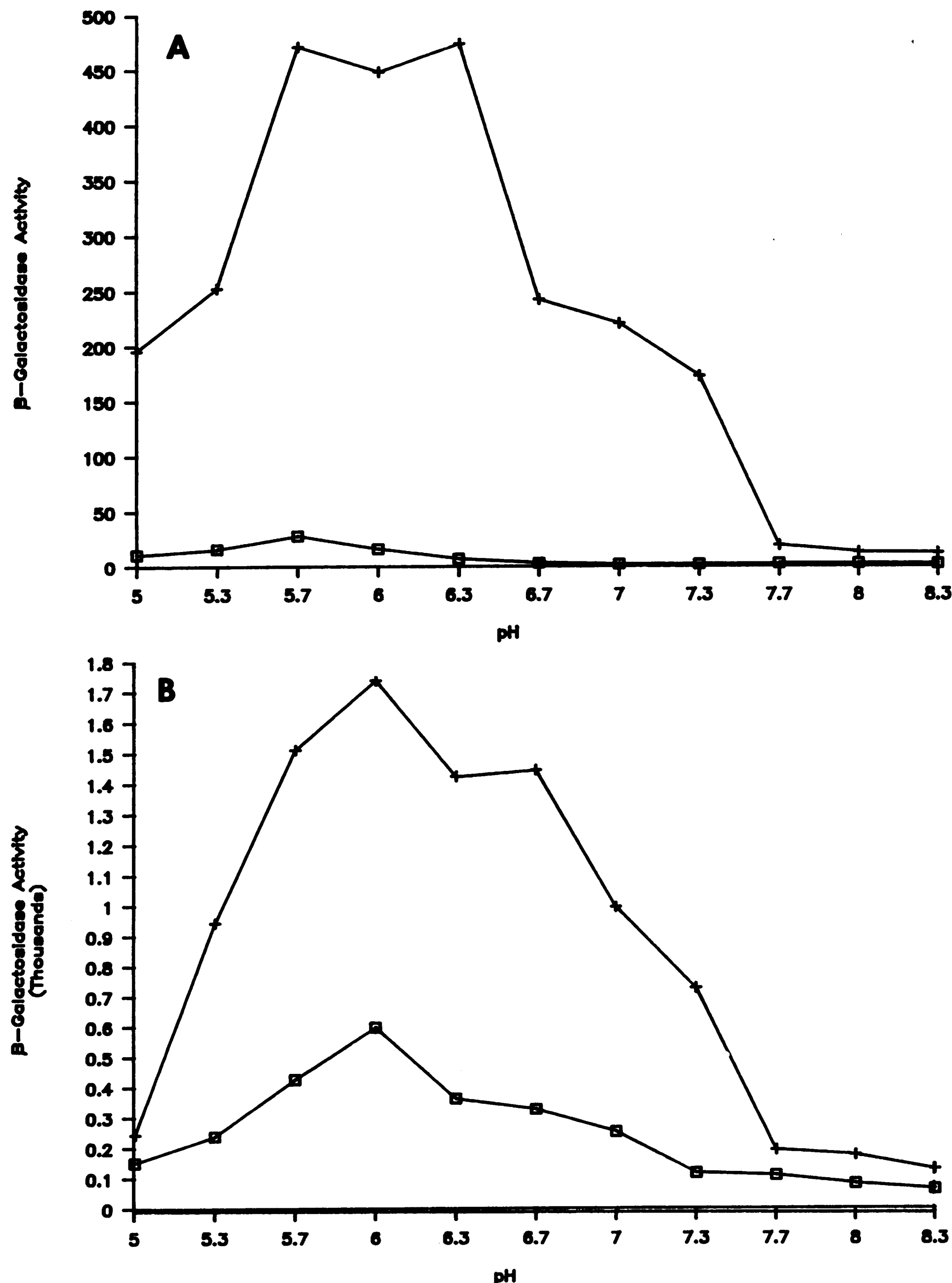

FIG. 1. Transcriptional control of *aniA* (*hyd*) and *aniG* by external pH. Cells were grown to equivalent densities under aerobic (open symbols) or anaerobic (closed symbols) conditions in LB buffered to the pH indicated. Samples were washed and suspended in assay buffer to avoid pH effects on the enzymatic activity. (A) JF927 [*aniA* (*hyd*)-*lacZ*]; (B) JF1295 (*aniG-lacZ*). β-Galactosidase activities are in Miller units as described (8).

strongly influenced by the pH of the growth medium (Fig. 1A). Maximum induction occurred between pH 5.7 and 6.3. Two other independently isolated *hyd*::Mu dJ fusions also exhibited this phenomenon.

Regulation of *aniG* was more complex (Fig. 1B). While induction was best under anaerobic conditions, significant induction was also observed under aerobic conditions when the external pH of the growth medium was adjusted to pH 6.0. Thus, *hyd* and *aniG* are controlled by at least two environmental conditions, oxygen and pH. However, each manifests this control in different ways and via distinct mechanisms. For example, the *earA* mutations described below eliminate pH control for *aniG* but have no effect upon *hyd*.

Isolation of *earA* regulatory mutants. The *aniG* locus is complex in its regulation. It requires a complex medium for induction, is anaerobiosis inducible, and, as described above, is regulated by the external acidification of growth medium. As described in Materials and Methods, regulatory mutations affecting *aniG* were isolated by plating the *lacZ* operon fusion strains under aerobic conditions on NCE medium containing lactose. Mutations resulting in the increased expression of the *ani-lacZ* fusions would allow the mutant cells to grow under the normally repressive condi-

TABLE 2. Regulatory mutations affecting the expression of *aniG*

Strain	Pertinent genotype	β-Galactosidase activity[a]		% Linkage to[b]:	
		Aerobic	Anaerobic	*aniG*	*zig-1935*
JF1294	*aniG*::Mu dJ	29	760		
JF1299	*aniG*::Mu dJ *earA201*	2,910	5,200	0	62
JF1300	*aniG*::Mu dJ *earA202*	3,970	4,960	0	55
JF1301	*aniG*::Mu dJ *earA203*	3,520	6,940	0	69
JF1302	*aniG*::Mu dJ *earA204*	2,530	4,100	0	90
JF1303	*aniG*::Mu dJ *earA205*	2,760	5,913	0	90
JF1304	*aniG*::Mu dJ *earA206*	2,410	6,390	0	72
JF1295	*aniG*::Mu dJ	75	1,170		
JF1355	*aniG*::Mu dJ *earA207*	2,010	4,540	0	73
JF1356	*aniG*::Mu dJ *earA209*	2,370	5,960	0	86
JF1357	*aniG*::Mu dJ $aniG^{PO}210$[c]	1,650	3,140	97	0
JF1358	*aniG*::Mu dJ $aniG^{PO}212$[c]	2,080	4,310	97	0
JF1518	*aniG*::Mu dJ *earA208*	1,950	3,740	0	92
JF1519	*aniG*::Mu dJ $aniG^{PO}211$[c]	1,680	3,270	98	0

[a] β-Galactosidase activity is measured in Miller units (27) (see the text). Cells were grown to equivalent optical densities under aerobic and anaerobic conditions as described in the text.

[b] Cotransduction frequencies were determined using a minimum of 100 scored Tc^r transductants per cross.

[c] Superscript PO indicates the mutation probably resides in the promoter-operator region of *aniG*.

tions if these mutations eliminated the complex medium requirement as well as either pH or oxygen control. Regulatory mutations affecting *aniG* were readily obtained and are listed in Table 2 along with the β-galactosidase levels obtained under aerobic and anaerobic conditions. The assay conditions actually combined all components of *aniG* expression, since under anaerobiosis the pH of LB during growth drops to 6.3 to 6.4. To determine whether the mutations were linked to *aniG*, possibly representing promoter or operator lesions, the *aniG*::Mu dJ from each regulatory mutant was transduced into *S. typhimurium* LT2, and Km^r transductants were subsequently scored for oxygen regulation. Three strains containing mutations linked to *aniG* (presumably promoter or operator mutations) were saved (JF1357, JF1358, and JF1519). The remaining mutations were not linked to *aniG* and possibly represent lesions in one or more *trans*-acting regulatory loci. These mutations, while allowing a large increase in *aniG* expression, did not eliminate oxygen control. For reasons discussed below, this class of mutations affects the external acidification regulation of *aniG* and is referred to as *ear* mutations. The Mu dJ insertion into *aniG* was removed from an *ear* mutant via cotransduction with a Tn*10* insertion near $aniG^+$ (JF1494) selecting for Km^s Tc^r colonies. Reintroduction of *aniG*::Mu dJ as well as other *ani-lacZ* fusions into one such $aniG^+$ *earA* strain (JF1411) was used to determine the effect of *earA* on other *ani* loci in our collection. Only *aniG* proved to be dependent upon *earA* for its regulation.

The *earA* product: negative regulator of *aniG* affecting pH control. Strains containing Tn*10* insertions located near *earA* were isolated as described in Materials and Methods. One *aniG*::Mu dJ strain (JF1493) containing a Tn*10* linked 86% to $earA^+$ was used to generate deletions through this regulator. After selection for fusaric acid resistance (FA^r) and Tc^s, deletion mutants were screened on MacConkey medium under aerobic and anaerobic conditions. Many of the FA^r Tc^s mutants constitutively expressed *aniG-lacZ* at derepressed levels, indicating *earA* is a negative regulator. The Tn*10* insertion mutant was also used to map the *earA* gene on the *S. typhimurium* chromosome by constructing Hfr donors with origins of transfer at the point of insertion. These studies place *earA* at 86 min. The *earA* mutations allowed almost constitutive expression of *aniG* in minimal medium, where *aniG* is not normally expressed due to the lack of a complex medium requirement, glucose repression, and alkaline pH repression. We found that *aniG-lac* expression in *earA* mutants, including *earA* deletions, consistently remained oxygen repressible by two- to threefold in minimal

TABLE 3. Effects of an *earA* deletion on the pH, oxygen, and complex medium dependence of *aniG-lacZ* expression

Strain	Genotype	Medium[a]	β-Galactosidase[b]		Anaerobic fold increase
			Aerobic	Anaerobic	
JF1295	*aniG-lacZ*	1. LB	53	926	17
		2. Minimal glucose	10	13	1
		3. Minimal glycerol-NO_3	21	52	2.5
		4. Minimal glycerol-NO_3 + LB	17	603	35
		5. LB, pH 7.9	39	109	2.8
		6. LB, pH 6.3	102	691	6.8
JF1506	*aniG-lacZ* Δ*earA*	7. LB	1,702	5,203	3.0
		8. Minimal glucose	1,784	3,616	2.0
		9. Minimal glycerol-NO_3	3,350	7,100	2.1
		10. LB, pH 7.9	4,666	8,630	1.9
		11. LB, pH 6.3	2,615	6,814	2.6

[a] Glycerol was added to 40 mM and potassium nitrate was added to 20 mM where indicated.

[b] Miller units (see the text).

TABLE 4. Effects of *oxrG* and *oxrH* mutations on *ani-lacZ* expression

Strain	Genotype	β-Galactosidase activity[a]	
		Aerobic	Anaerobic
JF1140	*aniH*::Mu dA	46	327
JF1410	*aniH*::Mu dA *oxrF405*	927	836
JF900	*aniC*::Mu dA	16	250
JF1417	*aniC*::Mu dA *oxrG*::Tn*10*	13	5
JF1141	*aniI*::Mu dA	7	48
JF1509	*aniI*::Mu dA *oxrG*::Tn*10*	6	12

[a] *aniH*::Mu dA strains were grown in minimal medium, and *aniC*::Mu dA and *aniI*::Mu dA strains were grown in LB. Units and growth are as described in Table 2, footnote *a*.

medium or in complex medium buffered to either pH 6.3 or 7.8. However, pH control was eliminated (Table 3). The β-galactosidase values provided in Table 3 illustrate several points. First, the anaerobiosis-inducible level for an *earA*$^+$ strain (JF1295) in LB reflects both oxygen and pH controls (line 1). The values agree nicely with those found in the LB, pH 7.9 aerobic culture (line 5) and the LB, pH 6.3 anaerobic culture (line 6). Second, the Δ*earA* strain (JF1506) lost external acidification control relative to *earA*$^+$ cells (Table 3, lines 5 and 6, *earA*$^+$; lines 10 and 11, Δ*earA*), but still maintained a two- to threefold anaerobiosis inducibility in all media including minimal glucose medium. Third, the data illustrate the complex medium dependence of *aniG* expression (Table 3, lines 3 and 4) and the loss of this dependence in *earA* mutants (lines 8 and 9).

Isolation of *oxrF* and *oxrG* regulatory mutants. A procedure similar to that employed for *aniG* was used to isolate regulatory mutations affecting *aniH*, a locus anaerobically induced in minimal medium but unaffected by *oxrA* or *oxrB*. One mutation, *oxrF405*, proved to be unlinked to *aniH* (Table 4). This mutation caused a complete deregulation of *aniH* but did not affect any of the other *ani-lacZ* operon fusions. This was accomplished by methods similar to those noted above for *earA* mutations. We have been unable to map *oxrF* as yet, but it clearly appears to be a unique regulator since it does not exhibit the characteristics of other known regulators.

The *oxrG* locus was identified after the transduction of strain JF900 (*aniC*::Mu dA) with a P22 lysate propagated on a pool of Tn*10* insertion mutants. One Tc^r transductant (JF1417) contained an insertion which proved to prevent the induction of *aniC* (Table 4). The OxrG phenotype was 100% cotransducible with Tc^r. The *oxrG*::Tn*10* insertion is 57% linked to *oxiE* at 88 min. Since the *oxyR* locus resides in that region, we wondered whether our *oxrG* locus was actually *oxyR*. The *oxyR* gene is a positive regulator that controls the hydrogen peroxide adaptive response (8). An insertion into *oxyR* would lead to extreme sensitivity to H_2O_2. This did not occur with the *oxrG*::Tn*10* strain. The *oxrG*$^+$ and *oxrG*::Tn*10* strains gave equivalent zones of inhibition surrounding filter paper disks soaked in 3% H_2O_2.

Transfer of the *oxrG*::Tn*10* insertion to other *ani* strains revealed that *aniI* is also controlled by OxrG (Table 4). It is significant that *aniC* and *aniI*, which map at 93 and 30 to 35 min, respectively, are both class I anaerobiosis-inducible genes. Their expressions in minimal media require Casamino Acids, are enhanced by nitrate, and are also controlled by the *oxrA* and *oxrB* products. The fact that the insertion will not permit anaerobic induction suggests that OxrG is a positive regulator of *aniC* and *I*.

Two-dimensional electrophoretic analysis of anaerobiosis-inducible proteins. We have published a compilation of environmental stress-inducible proteins including those that are anaerobiosis inducible (ANI proteins; 33). The resulting protein index was used in this study to compare the protein patterns obtained with various *oxr* mutants. The aim was to begin mapping the control circuitry involved with anaerobic gene expression. Figure 2 is representative of normally observed ANI proteins as presented earlier (33). Next to each ANI protein is the protein number used in reference 33. The gels obtained from *oxr* mutants (Fig. 3 and 4) and the tabular presentation of these results (Table 5) may be easily compared with the reference photograph in Fig. 2. When available, two-dimensional gel electrophoresis was performed with several different mutants from each class, all with identical results.

Analysis of the *oxrA* gels (Fig. 3A and B) reveals that 15 of the 33 ANI proteins are under OxrA control. Most of the affected proteins, 14, were uninducible, reflecting the lack of positive control by OxrA. However, one protein (no. 13, ANI-3) was expressed constitutively, suggesting that OxrA may negatively control some *ani* genes. The *oxrB* gels (Fig. 3C and D) suggest that OxrB positively controls the production of at least six ANI proteins, all of which were also controlled by OxrA. In addition, three previously unidentified proteins were observed, of which two were constitutively expressed (25 × 62; 55 × 62) and the other was now inducible (94 × 37). This new inducible protein was also observed with *oxrA*.

A similar phenomenon was observed for an *oxrC* mutant (Fig. 3E and F), although it should be noted that *oxrC* (*pgi*)

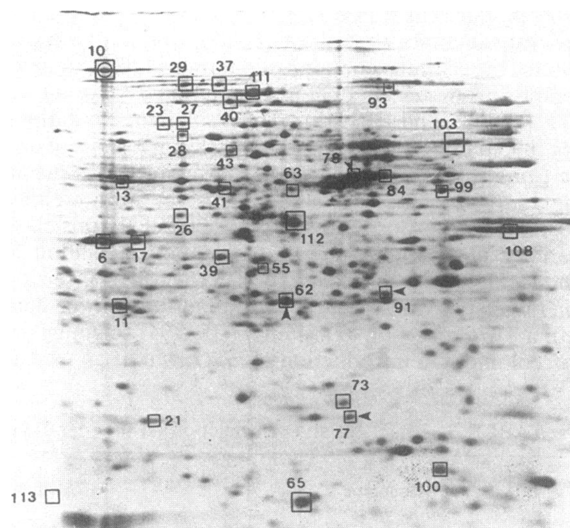

FIG. 2. Standard two-dimensional profile of anaerobiosis-inducible proteins. Growth conditions are described in the text. The first dimension was an isoelectric focusing gel (negative pole to left, positive pole toward the right) containing 1.6% ampholytes (pH 5 to 7) and 0.4% ampholytes (pH 3 to 10). The second dimension was an 11.5% polyacrylamide gel. The boxed proteins are those whose productions increase in response to anaerobiosis (ANI proteins) as indicated in reference 33. The ANI protein indicated with a circle was also temperature inducible; arrows indicate ANI proteins which increase in response to one or more starvation stresses. Each ANI protein is numbered in a manner corresponding to the published catalog (33).

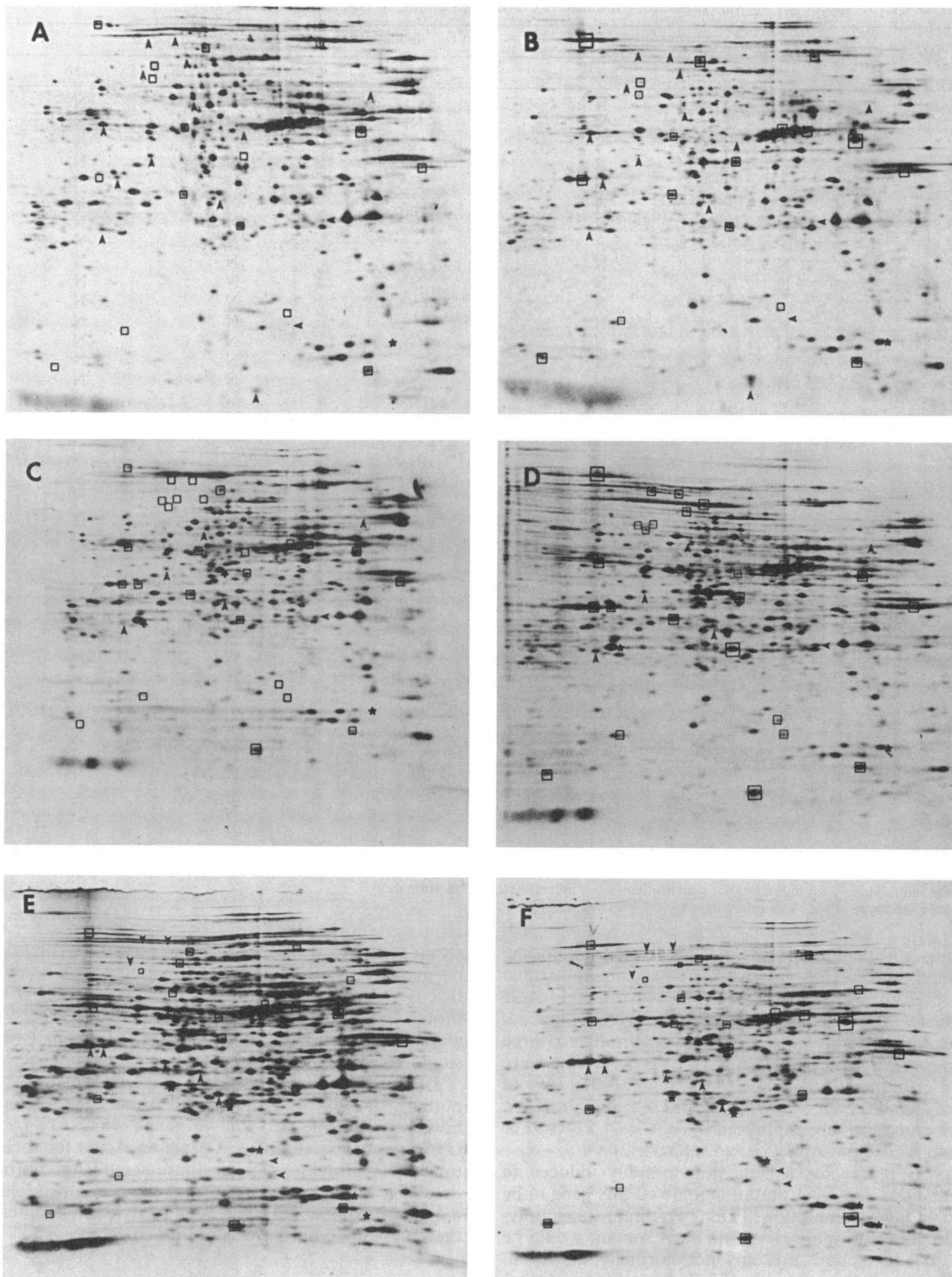

FIG. 3. Two-dimensional profiles of anaerobiosis-inducible proteins from *oxrA*, *oxrB*, and *oxrC* mutants. (A, C, and E) Profiles from aerobically grown cells; (B, D, and F) anaerobically grown cells. (A and B) Strain SF165 (*oxrA1*); (C and D) JF1114 (*oxrB8*); (E and F) SF242 (*oxrC*::Tn*5*). SDS-boiled lysates were prepared as described (33) and run in a two-dimensional system including a pH 5 to 7 (right to left) isoelectric focusing gel and 11.5% SDS-polyacrylamide gel electrophoresis. Acidic proteins are situated to the right of each gel. Boxes indicate normally regulated ANI proteins; arrowheads indicate abnormally regulated ANI proteins; stars designate new proteins. Multiple labelings were done for each strain, and a minimum of two gels were run for each labeling. Each run included a control parent sample for comparative purposes. Only consistently induced proteins are indicated. Tabular results from this figure are presented in Table 5.

TABLE 5. Effects of various regulatory mutations on the two-dimensional profile of anaerobiosis-inducible proteins

Protein no. and designation[a]	Induction[b]					
	oxrA	*oxrB*	*oxrC*	*earA*	*oxrF*	*oxrG*
6. ANI-1	N	N	C	N	N	N
10. SIN-27	N	N	N	N	N	N
11. ANI-2	–	–	N	N	N	N
13. ANI-3	C	N	+/–	N	N	N
17. ANI-4	–	N	C	N	N	N
21. ANI-5	N	N	+/–	N	N	N
23. ANI-6	N	N	–	N	N	N
26. ANI-7	–	–	–	N	N	N
27. ANI-8	N	N	N	N	N	N
28. ANI-9	N	N	N	N	N	N
29. ANI-10	–	N	+/–	N	N	N
37. ANI-11	–	N	+/–	N	N	N
39. ANI-12	N	N	C	N	N	N
40. ANI-13	–	N	N	N	N	N
41. ANI-14	N	N	N	N	N	N
43. ANI-15	–	–	N	N	N	N
55. ANI-16	–	–	N	N	N	N
62. SIN-10	–	N	C	N	N	N
63. ANI-17	–	N	N	N	N	N
65. ANI-18	+/–	N	C	N	N	N
73. ANI-19	N	N	–	N	N	N
77. SIN-17	–	N	–	N	N	N
78. SIN-18	N	N	N	N	N	N
84. ANI-20	N	N	N	N	N	N
91. SIN-21	–	+/–	N	N	N	N
93. ANI-21	N	N	N	N	N	N
99. ANI-22	N	N	+/–	N	N	N
100. ANI-23	N	N	N	N	N	N
103. ANI-24	–	–	N	N	N	N
108. ANI-25	N	N	N	N	N	N
110. ANI-26	N	N	N	N	N	N
112. ANI-27	N	N	N	C	N	N
113. ANI-28	N	N	N	N	N	N
New proteins[c]	94 × 37 (I)	25 × 62 (C) 55 × 67 (C) 94 × 37 (I)	43 × 64 (C) 64 × 44 (C) 57 × 59 (I) 93 × 27 (C) 88 × 32 (I)	49 × 83 (C) 51 × 83 (C)		

[a] From reference 33.
[b] N, Normal induction; –, no induction; C, constitutive; +/–, induction less than normal.
[c] Coordinates are those in Fig. 1 of reference 33.

is not a transcriptional regulator but is involved in generating an undefined signal from glucose necessary for anaerobic induction of some genes. With *oxrC*, the levels of 14 ANI proteins were altered and 5 new proteins were observed. Five of the ANI proteins were constitutively produced (three of these were also negatively regulated by *oxrA*). Nine were uninduced or induced to a lesser extent than normal (five of these were also affected by *oxrA*). Of the five new proteins, three were constitutively expressed (43 × 63; 64 × 44; 93 × 27) and two were now ANI (57 × 57; 83 × 32).

While *aniG* represents a gene not normally induced in minimal medium, the *earA* mutation allowed this gene to be expressed under this condition. Thus, two-dimensional polyacrylamide gel electrophoresis of an *earA* mutant would be expected to reveal new proteins not normally observed. Representative gels presented in Fig. 4 show this to be true. Figures 4A and B show an $earA^{+}$ pattern under aerobic and anaerobic conditions, while Fig. 4C and D show polypeptides from an *earA* mutant. Two new proteins were observed, both of which were constitutively expressed. Surprisingly, one of the normal ANI proteins was also constitutively produced.

The gel electrophoresis performed with the *oxrF* mutant strain did not reveal any newly expressed proteins or any normal ANI proteins with abnormal control. The reason for this is not apparent. One possible explanation is that the protein(s) controlled by *oxrF* is expressed poorly or degraded rapidly, preventing its observation on two-dimensional gels.

Two-dimensional gels of the *oxrG* mutant also revealed nothing out of the ordinary. It should be remembered, however, that the two known genes which are targets for OxrG require vitamin-free Casamino Acids for their induction. ANI proteins such as these would not normally be detected by our method of labeling in unsupplemented minimal medium. Thus, the lack of detectable difference in the polypeptide profile was not unexpected.

DISCUSSION

Investigations into the transcriptional regulation of anaerobic gene expression have revealed a complex system of controls. Three independent pathways for anaerobic induction were previously described. Studies have shown *oxrA* and *oxrB* to be pleiotropic positive control loci which are associated primarily with regulating respiratory functions.

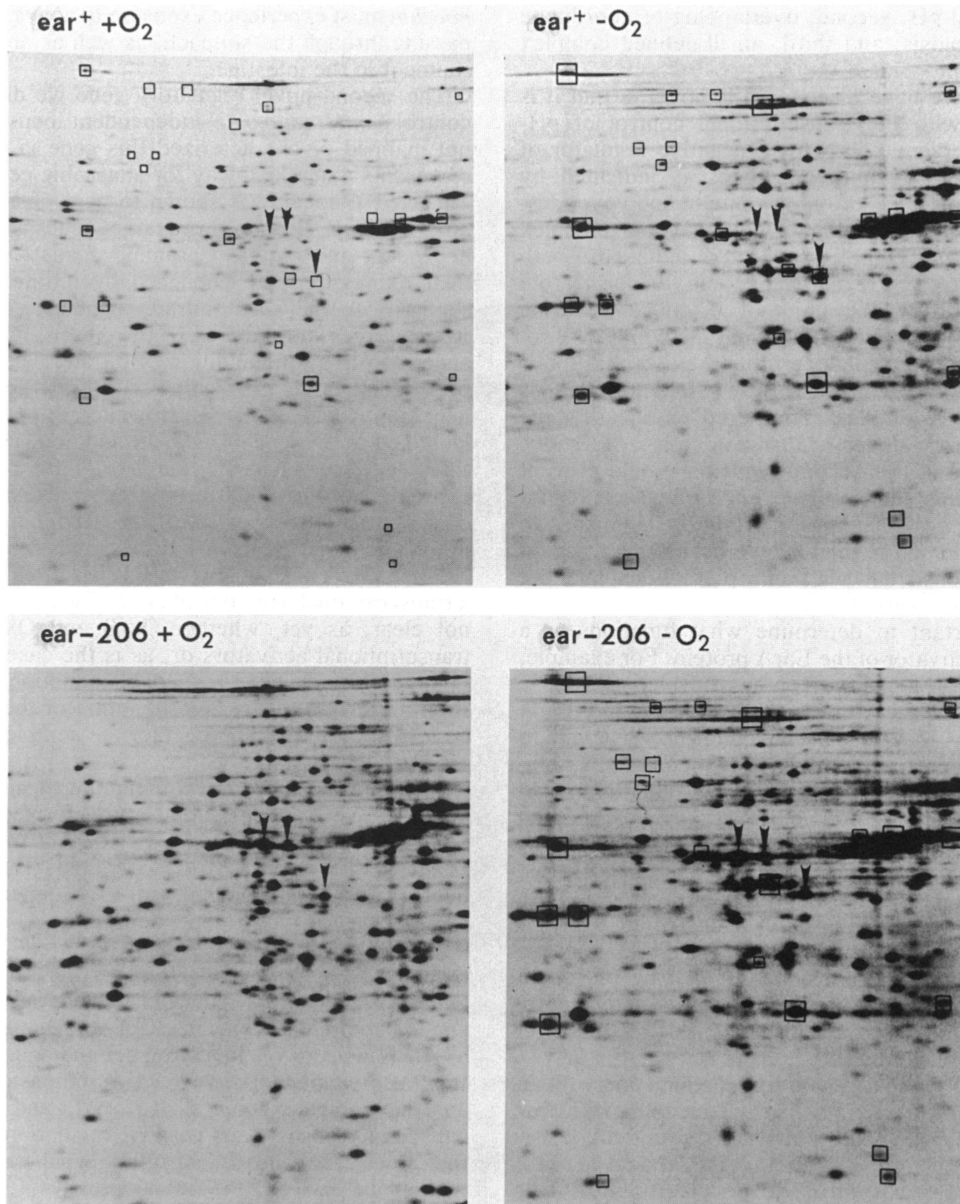

FIG. 4. Two-dimensional profiles of ANI proteins from *earA*$^+$ and *earA* mutants. Top panels are two-dimensional gels of SDS lysates of strain JF1294 (*earA*$^+$). The two lower panels are from JF1304 (*earA206*). Boxes indicate normal ANI proteins. Arrowheads indicate constitutively produced proteins found in the *earA* mutant. Each panel is only a section of a complete gel.

The *oxrC* (*pgi*) gene is involved in the generation of an inducing signal associated primarily with fermentative activities, and *tppR* is a specific regulator of *tppB* (18, 19, 35). The present study has uncovered several new anaerobic regulatory loci and has attempted to outline overlapping controls through two-dimensional polyacrylamide gel electrophoretic analysis of anaerobiosis-inducible proteins.

Two of the anaerobiosis-inducible loci identified in an earlier report, *hyd* and *aniG*, were also found to respond to changes in external pH, with maximal expression occurring under acidic conditions. None of the other *ani-lacZ* fusions responded to these acidification conditions. It is interesting that *hyd* only responded to a pH stimulus if it was placed in an anaerobic situation, whereas *aniG* responded under both aerobic and anaerobic environments. Maximal expression of *aniG*, however, required both anaerobiosis and an acid pH. It was observed that growth in complex unbuffered LB under anaerobic conditions resulted in a progressive drop in external pH to approximately 6. This would explain in part why the locus was induced so well under our assay conditions. However, it seems clear that *aniG* is not normally expressed in minimal medium because of some requirement present in complex medium. After growth, the minimal medium used consistently measured pH 7.2, a hydrogen-ion concentration which should be satisfactory for showing some anaerobic induction. Yet *aniG* expression was barely detectable in minimal medium. The *earA* mutations, therefore, not only relinquish *aniG* from pH control but also eliminate the complex medium requirement, since these mutants showed largely deregulated *aniG* expression in minimal medium. These results indicate, first, a novel control mechanism involving transcriptional responses to

changes in external pH; second, overlapping or coordinate control by anaerobiosis; and third, an ill-defined complex medium requirement.

The *earA* locus we have discovered is novel in that it is directly involved with the transcriptional control of pH-regulated loci. The gene codes for a negative regulator of *aniG* and at least two additional genes as indicated by two-dimensional analysis of *earA* mutant polypeptides. Since mutations in *earA* eliminate both pH and complex medium control of *aniG*, the EarA protein may sense both conditions or may itself be regulated by them. Its normal role would be to repress the transcription of certain genes during alkaline growth conditions or in minimal medium. We propose that an additional regulatory protein exists that interacts with some component of LB (e.g., peptide). This regulator-peptide (?) complex is required to release *aniG* from EarA repression under acidic conditions. Mutations resulting in the loss of this second regulator would prevent the induction of *aniG*. Furthermore, *earA* mutations resulting in constitutive *aniG* expression would be dominant to mutations in this second regulator. We have recently identified mutations consistent with our predictions and are characterizing the responsible locus.

It will be important to determine what functions as a corepressor or inactivator of the EarA protein. For example, what role does proton motive force, of which ΔpH is a component, play in activating or inactivating the EarA repressor? The *earA* locus has been cloned and was found to code for a 33-kilodalton protein which is membrane associated. This raises interesting questions concerning the mechanism by which EarA senses external acidification and subsequently releases *aniG* from negative control (J. Foster, manuscript in preparation). The other pH-regulated locus, *aniA* (*hyd*), which exhibited strict requirements for both acidic and anaerobic conditions, was unaffected by *earA* mutations. Thus, it would seem there are also multiple pathways for pH regulation. Control of *hyd* by pH could very likely be *oxrA* independent since *hyd* is still significantly inducible (26-fold) in an *oxrA* background as compared to an *oxrA*$^+$ (80-fold; 1) background.

Pecher et al. (31) noted a similar pH phenomenon with an *hyd*::*lac* operon fusion in *E. coli* using medium with low buffering capacity. Their study could not differentiate between higher rates of formate accumulation at acid pH or a pH effect on the system controlling *hyd* transcription. Our data were obtained by using a heavily buffered medium and suggest that pH rather than formate accumulation was responsible for the effect. The data presented for *aniA* (*hyd*) and *aniG* lend support to the theory that the transmembrane potential is sensed by one or more effector molecules (EarA?).

A recent report by Slonczewski et al. described pH-regulated gene expression in *E. coli* (32). Both of the *exa* (external acidification) loci described also exhibited an anaerobic control component similar to what we describe for *aniG*. The selection method used in our study, MacConkey medium, was much different from that used by Slonczewski et al. (32) for isolating external acid-inducible fusions. Their method involved altering the medium pH through buffering. MacConkey medium relies upon acid production by the organisms to incorporate dye into a Lac$^+$ colony. Thus, our method screens both for genes which respond to anaerobiosis as well as for genes which are regulated by fluctuations in external pH. Further analysis of these and similar pH-controlled loci could lead to a greater understanding of the mechanism(s) of pH homeostasis. It is not insignificant that in the course of producing gastrointestinal disease *S. typhimurium* must experience exposure to acidic conditions while passing through the stomach, as well as an anaerobic environment in the intestine.

The second novel regulatory gene we discovered, *oxrF*, controls *aniH*, an *oxrAB*-independent locus. While we have not mapped or characterized this gene in detail, it clearly represents a new pathway for anaerobic gene expression.

The *oxrG* locus was shown to be a positive regulator of *aniC* and *aniI*. Both of these target genes are *oxrA* and *oxrB* dependent and require Casamino Acids for induction, and their expressions are enhanced by nitrate. There is some similarity to the *narC* (nitrate reductase) locus in *E. coli*, whose expression is repressed by oxygen and enhanced under anaerobiosis by the addition of nitrate. The *narL* locus has been identified as controlling the nitrate-dependent component of *narC* induction (34). However, this locus has not been identified in *S. typhimurium*. In addition, it appears that all three loci, *oxrA*, *oxrB*, and *oxrG*, are required for any expression of the *aniCI* regulon, whereas *narC* requires only *oxrA* and *narL*. It is possible that the *oxrG* product is involved in the Casamino Acids or nitrate signals rather than oxygen.

Since OxrA is analogous to Fnr in *E. coli*, it appears to be a transcriptional activator of anaerobic gene expression. It is not clear, as yet, whether OxrB and OxrG are actually transcriptional activators or, as is the case with OxrC, are involved with the generation of an induction signal molecule. In either instance, the determination of their functions will be important in piecing together the complexities of anaerobic gene expression.

In summary, there are a minimum of nine pathways for anaerobic gene expression as characterized by genetic and biochemical techniques: (i) *oxrA* dependent, (ii) *oxrAB* dependent, (iii) signal *oxrABC* dependent, (iv) *oxrABG* dependent, (v) *oxrAC* dependent, (vi) *oxrC* dependent, (vii) *earA* dependent, (viii) *oxrF* dependent, and (ix) *tppR* dependent. Of the 33 ANI proteins normally detected, 22 are controlled by one of these pathways. However, 11 were not affected by any of the regulatory mutations tested, suggesting the presence of additional pathways. Our studies with *earA* indicate overlapping controls involving pH and anaerobiosis as well. It is also significant that the two-dimensional polypeptide analysis indicates that *oxrA*, *oxrB*, and *oxrC* have the capacity for positive and negative regulation depending upon the target gene. This is not unlike what has been observed with cyclic AMP-CRP.

Yamamoto and Droffner (38) have presented data suggesting that DNA supercoiling plays a significant role in anaerobic gene expression. Their theory centers around the isolation of strict aerobic mutants containing mutations in *gyrA* and *gyrB* which lead to little or no DNA gyrase activity. Our data do not necessarily conflict with theirs, since the alteration of supercoiling under anaerobic conditions could be the signal that triggers the transcription of various *oxr* regulatory loci. However, evidence from *E. coli* indicates *fnr* (*oxrA*) is transcribed under both aerobic and anaerobic conditions (22). Thus, it would appear that at least one anaerobic regulator does not respond itself to supercoiling. Altered chromosome structure could, however, facilitate Fnr access to target promoter sequences. Undeniably the phenomena of anaerobic gene expression as well as pH regulation are intricate processes whose mechanistic complexities will require a major effort to understand.

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