JOURNAL OF BACTERIOLOGY, July 1986, p. 362–367
0021-9193/86/070362-06$02.00/0
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Vol. 167, No. 1

Anaerobically Induced Genes of *Escherichia coli*

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Received 13 January 1986/Accepted 15 April 1986

A collection of anaerobically induced gene fusions were isolated, and representative isolates were characterized with respect to their regulatory properties, phenotypes, and approximate map locations. Four fusion strains that had defects in the anaerobic metabolism of asparagine or aspartate were found. These fusions were all repressed by alternate electron acceptors, ammonia, and glucose but were induced by other sugars. Several other fusion strains which demonstrated no observable phenotype showed diverse regulatory responses. The anaerobically induced fusions were scattered around the *Escherichia coli* chromosome more or less at random, suggesting that all the isolates examined were in separate genes.

The natural habitat of *Escherichia coli* is the large intestine of humans and other animals, from which *E. coli* emerged to colonize the scientific laboratory about half a century ago. Although the exact nature of the nutrients used by *E. coli* in the wild remains obscure (20), it is clear that growth normally occurs in the absence of oxygen and that energy must be supplied either by fermentation or by the anaerobic respiration of alternative electron acceptors such as nitrate (15), fumarate (13, 17), trimethylamine oxide (TMAO) (23, 32), or dimethyl sulfoxide (DMSO) (2). Work on *E. coli* and *Salmonella typhimurium* has established a large group of genes which are involved in anaerobic respiration (13, 15, 31). These genes are repressed by oxygen (26, 31) and require the presence of the appropriate electron acceptor as an inducer (30). In addition, the product of the *fnr* gene (*oxrA* in *S. typhimurium*) appears to be an activator protein, necessary for transcription of many anaerobically regulated genes, including those involved in anaerobic respiration (7, 27, 28) and several others such as *pepT* of *S. typhimurium* (16, 31). However, *fnr* mutants grow well under fermentative conditions, and several anaerobically regulated fermentative genes such as *adh* (8), *prd* (5), and *pfl* (25) probably do not require *fnr* activation.

We previously demonstrated by using gene fusions that *E. coli* has approximately 50 anaerobically regulated genes, most of which are unmapped and biochemically uncharacterized (10). Our first collection of anaerobic gene fusions was made with the original Mu d1 phage of Casadaban (3) and proved to be too unstable for convenient further characterization. We therefore made a second collection of anaerobically induced gene fusions by using the modified phage, Mu dX, of Howe (1). Such fusions are much more stable, and we have been able to study the requirements for induction of selected gene fusions and to map several of these. This paper reports our findings.

MATERIALS AND METHODS

Bacterial strains and media. All strains used were *Escherichia coli* K-12 and are listed in Table 1. The minimal medium used was medium E (34), except that M9 (21) was used as indicated when we wished to avoid the presence of citrate (which can be used by anaerobic *E. coli* [19]). Carbon sources were added to a concentration of 0.4% (wt/vol) together with amino acids (50 mg/liter) and vitamins (5 mg/liter) when appropriate. Rich broth contained (per liter) 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract. Superbroth for preparation of Mu lysates contained (per liter) 32 g of tryptone, 20 g of yeast extract, 5 g of NaCl, 2.5 g of $MgSO_4 \cdot 7H_2O$, and 0.2 g of NaOH. PPPS medium contained the nutrients of MacConkey base medium with the dyes and bile salts omitted, i.e., 17 g of Bacto-peptone (Difco Laboratories, Detroit, Mich.), 3 g of Proteose/Peptone, and 5 g of NaCl per liter. Solid media contained Bacto-agar (Difco) (1.5% [wt/vol]). Anaerobic growth was examined by using Oxoid anaerobic jars with a H_2-CO_2 atmosphere generated by using Oxoid Gas Generating Kits. All media used for anaerobic growth were supplemented with the following trace elements: $FeSO_4$ (50 μM), H_2SeO_3 (5 μM), and $(NH_4)_6Mo_7O_{24}$ (5 μM with respect to Mo). Growth was monitored turbidometrically by using a Klett-Summerson colorimeter equipped with a green filter (540 nm). Aerobic liquid cultures (10-ml volume) were incubated in 150-ml culture flasks and shaken rapidly in a New Brunswick G76 Gyrotory water bath (speed setting 6). Anaerobic liquid cultures were grown without agitation in tubes inside an anaerobic jar. Resazurin indicators were used to ensure anaerobic conditions. Cultures were grown for three to four cell doublings before the beta-galactosidase assay. The inocula for beta-galactosidase induction experiments were grown under aerobic conditions and therefore expressed only low basal levels of beta-galactosidase.

Isolation of fusions. The procedure for the isolation of operon fusions was as previously described (3, 10) except for the following modifications. The Mu dX fusion phage of Baker et al. (1) was used instead of Mu d1, and the double lysogen CAG5050 was temperature induced in superbroth. Mu dX lysates were stabilized by the addition of 1 mM $MgSO_4$ and 1 mM $Pb(NO_3)_2$ (final concentrations) before storage. Insertions of Mu dX into the target strain MC4100 were selected aerobically on glucose plus casein hydrolysate (Sigma; 0.1% [wt/vol]) agar containing both ampicillin (50 mg/liter) and chloramphenicol (20 mg/liter). Putative fusions were then picked onto two lactose-MacConkey plates, one of which was incubated aerobically, the other anaerobically. Colonies which were Lac positive anaerobically but Lac negative aerobically were kept for further investigation.

Genetic mapping. A set of Tn*10*-containing Hfr strains with points of origin scattered around the *E. coli* chromo-

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TABLE 1. Bacterial strains

Strain	Relevant markers	Source
MC4100	*araD139* Δ(*argF-lac*)*U169* *rpsL thiA relA*	M. Casadaban
CAG5050	Δ(*proAB lac*) *met his tyr gyrA rpsL* (Mu dX) F' *pro lacZ8305*::Mucts62	C. A. Gross
BW5659	HfrKL98 *zdh*::Tn*10* *mglP1* *xyl-7 lacY1*	B. Wanner
BW6169	HfrAB313 *argA*::Tn*10* *leuB6 lacY thi-1 supE44 gal-6*	B. Wanner
BW6166	HfrJ4(P10) *zhf*::Tn*10* *thi-1 malB16*	B. Wanner
BW6156	HfrP4X *zje*::Tn*10* *relA1* *spoT1 metB1*	B. Wanner
BW7261	HfrC *leu*::Tn*10* *fhuA22* *relA1 spoT1* Δ(*argF-lac*)*U169*, *ompF627*	B. Wanner
CAG5053	HfrBroda7 *zbc*::Tn*10* *relA1*? *spoT1*?	C. A. Gross
CAG5054	HfrKL96 *trp*::Tn*10* *thi-1* *relA1 spoT1*	C. A. Gross
BW6161	HfrKL99 *zed*::Tn*10* *supD* *lac-42 relA1 spoT1 thi-1*	B. Wanner
BW5660	HfrPK19 *srlC300*::Tn*10* Δ(*gpt-lac*)*5*	B. Wanner
BW6159	HfrKL14 *ilv*::Tn*10* *relA1* *spoT1 thi-1*	B. Wanner
BW6175	HfrPK3 *argE*::Tn*10* *thr-1* *thi-1 leuB6 azi-15* *fhuA21 supE44*	B. Wanner
NK6051	HfrH *purE79*::Tn*10* Δ(*gpt-lac*)*5 relA1 spoT1* *thi-1*	N. Kleckner
BW6164	HfrRa-2 *thr*::Tn*10* *supE42* *mal-28 sfa-4*	B. Wanner
BW6165	HfrP801 *argE*::Tn*10* (λ *ind*) *ara-41*	B. Wanner
WNK13 through 34[a]	*aif-13* through aif-34::Mu dX of MC4100	see text

[a] Strains bearing anaerobically induced fusions.

some was used. The Tn*10* insertion in each Hfr strain was close to and behind the origin of transfer and thus acted as a proximal marker which could be positively selected. This set of Hfr strains was constructed in the laboratory of B. Wanner and was made available by B. Bachmann of the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. Each fusion strain was crossed with each Hfr strain by using standard procedures (21), and a 30-min mating period was used. Exconjugants were selected on rich broth agar containing tetracycline (10 mg/liter) (to select for transfer of the Tn*10* into the recipient) and streptomycin (100 mg/liter) (to select against the donor). Colonies obtained were checked for the retention or loss of the Mu dX fusion by testing for sensitivity to ampicillin and chloramphenicol, since Mu dX specifies resistance to both of these. In some cases, more detailed mapping was carried out by cotransduction with P1 *vir* (21).

Enzyme assays. Beta-galactosidase was assayed as previously described (7) except that cultures were grown to mid-exponential phase (~5 × 10^8 cells per ml) before the assay in a variety of media under both aerobic and anaerobic conditions. The units of beta-galactosidase activity were micromoles of *o*-nitrophenylgalactoside hydrolyzed per 10^9 cells per h, at 37°C.

RESULTS

Isolation of anaerobic fusions. The parental strain MC4100 was infected with Mu dX, and insertions resistant to both ampicillin and chloramphenicol were selected as described in the Materials and Methods section. These insertions were screened on MacConkey lactose agar both aerobically and anaerobically under H_2-CO_2 for expression of beta-galactosidase. Those showing induction under anaerobic conditions were examined further. These presumed anaerobic gene fusions have been provisionally designated *aif* (anaerobically induced fusion), since most appear to be in previously undiscovered genes.

Anaerobic induction and effect of sugars. When grown in minimal medium E supplemented with glucose or xylose and casein hydrolysate, all strains grew well both aerobically and anaerobically, but there was no beta-galactosidase induction under anaerobic conditions, except that strains WNK14, WNK15, WNK17, WNK30, and WNK32 were induced approximately twofold. To achieve good anaerobic induction, it was necessary to devise a rich medium (PPPS) which was essentially an artificial MacConkey medium with the dyes, detergents, and sugars omitted (see Materials and Methods for details). Substantial anaerobic induction for most fusions occurred in PPPS supplemented with various fermentable sugars (Table 2). The ratios of induction and the absolute levels of beta-galactosidase varied substantially from strain to strain. The lowest anaerobic induction ratios were usually seen when glucose was the sugar. For example, WNK13, WNK17, and WNK32 were only induced significantly in the absence of glucose. However, certain strains (WNK30 and WNK31) did show greater induction in the presence of glucose than in the presence of other sugars.

In the following sections, the data are presented as the ratio of the amount of beta-galactosidase observed anaerobically to the amount observed aerobically: the anaerobic induction ratio. It is possible to estimate the absolute levels of beta-galactosidase if required by using the appropriate ratios and referring back to the primary beta-galactosidase data in Table 2.

Effect of alternative electron acceptors. Several anaerobic genes were shown to be repressed not only by the presence of oxygen but also by alternative electron acceptors such as nitrate or fumarate (2, 8, 9, 17, 23). Selected anaerobically induced fusions were tested for possible repression by nitrate, TMAO, DMSO, and fumarate. As expected, nitrate, TMAO, and DMSO substantially repressed the majority of our fusion strains (Table 3). Of the fusions, only strain WNK29 was unaffected by nitrate. The effects of TMAO and DMSO were somewhat less severe, both in the extent of repression and in the number of fusion strains affected. Since some of the anaerobic fusion strains such as WNK18 and WNK25 were only induced well in the presence of certain sugars such as lactose or xylose, the effects of electron acceptors were also tested under these conditions (Table 3).

In contrast to the other electron acceptors, fumarate had little significant repressive effect on most anaerobic fusion strains. In fact, strain WNK29 was induced two- to threefold by fumarate, and strain WNK31 showed somewhat greater anaerobic induction in the presence of fumarate (42-fold) than in its absence (26-fold).

Effect of ammonia, citrate, and buffer. The other major difference between PPPS (which showed good induction) and medium E plus glucose (poor anaerobic induction) was the presence of high phosphate, citrate, and ammonia concentrations in medium E. These agents were tested against

TABLE 2. Anaerobic induction of beta-galactosidase

Strain	Beta-galactosidase activity (μmol of ONPG[a]/h per 10^9 cells) for the following additives[b] to PPPS:								
	None			Glucose			Xylose		
	Air	Ana	Ratio	Air	Ana	Ratio	Air	Ana	Ratio
WNK13	489	1,622	3.3	381	533	1.5	710	2,200	3.1
WNK14	2,622	4,633	1.8	3,357	5,978	1.7	3,056	4,000	1.3
WNK15	1,422	1,544	1.1	754	1,733	2.3	1,027	1,611	1.6
WNK16	2,867	1,922	0.7	2,178	102	0.04	1,167	1,922	1.7
WNK17	852	2,703	3.2	322	604	1.9	889	6,667	7.5
WNK18	570	1,082	2.0	460	968	2.1	762	5,767	7.6
WNK25	1,744	1,378	0.8	1,333	2,889	2.2	121	1,478	12.2
WNK29	314	3,633	11.6	—[c]	—	—	532	632	1.2
WNK30	711	2,111	3.0	33	738	22.0	670	2,411	3.6
WNK31	648	16,555	26.0	173	11,333	65.0	293	10,622	36.0
WNK32	706	4,589	6.5	44	58	1.3	254	2,356	9.3
WNK33	286	288	1.0	22	0	0.0	1,356	399	0.3
WNK34	221	889	4.0	89	524	6.0	150	1,367	9.1
MC4100	0	0	—	0	0	—	0	0	—

[a] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[b] Cultures were grown in PPPS with the additives indicated. Air, Aerobic; Ara, anaerobic. Ratio = anaerobic activity/aerobic activity.
[c] Too low for accurate measurement.

the anaerobic fusion strains (Table 4). HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer was also tested in place of phosphate or citrate.

Citrate, which is not used by aerobic *E. coli* but which may be used anaerobically (19), resembled fumarate in its effects on the anaerobic gene fusion strains WNK29 and WNK31. These both showed increased expression in the presence of either fumarate or citrate, whereas most other fusion strains were not greatly affected. Since citrate is probably metabolized via oxalacetate to fumarate, this is not surprising.

Citrate also repressed the expression of beta-galactosidase by strains WNK13, WNK18, WNK25, and WNK34. However, in these cases, similar effects were shown by both HEPES buffer and (except for strain WNK34) phosphate. This repression was thus probably a nonspecific effect of buffering. In contrast, phosphate appeared to affect these fusion strains specifically. Strain WNK31 appeared to be two- to threefold repressed, whereas strains WNK17 and WNK34 showed induction by phosphate, particularly when compared with samples buffered with either citrate or HEPES instead of phosphate.

The presence of ammonia strongly repressed beta-galactosidase production by fusion strains WNK13, WNK18, WNK25, and WNK34, with milder effects on WNK17 and WNK32. In contrast, WNK31 was little affected, and the fusion in WNK29 appeared to be induced by ammonia. The poor induction in the presence of glucose and the repression of most fusions by ammonia were probably responsible for the poor expression of beta-galactosidase which we found for most of these fusion strains in medium E plus glucose.

Phenotypes of anaerobically induced gene fusions. Since the insertion of Mu into a gene will typically eliminate gene function, we attempted to find phenotypic alterations in our collection of anaerobic gene fusion strains. The ability to grow in minimal medium with acetate, lactate, succinate,

TABLE 3. Regulation by alternate electron acceptors

Additive and strain[a]	Anaerobic induction ratio[b] for the following additives:				
	None	Nitrate	TMAO	DMSO	Fumarate
None					
WNK13	3.3	1.7	0.4	1.1	2.8
WNK17	3.2	2.0	4.0	4.8	4.4
WNK18	2.0	1.0	0.7	0.7	1.2
WNK25	0.8	0.7	1.2	1.1	0.8
WNK29	11.6	9.4	11.0	11.1	28.0
WNK31	26.0	2.3	5.3	7.6	42.0
WNK32	6.5	1.4	7.5	4.0	2.6
WNK34	4.0	1.0	1.7	0.8	4.1
Lactose					
WNK13	1.2	1.1	0.9	2.8	1.1
WNK17	5.0	1.5	6.6	2.6	2.9
WNK18	0.8	0.7	0.8	0.6	1.1
WNK25	0.8	0.8	0.9	0.6	1.0
WNK29	9.0	4.4	7.5	5.1	12.9
WNK31	29.5	5.3	29.7	13.4	37.0
WNK32	4.0	1.9	3.8	9.1	8.4
WNK34	5.0	1.4	2.0	2.1	2.2

[a] Cultures were grown in PPPS medium with no addition or with 0.8% lactose. Similar results were obtained with xylose (data not shown).
[b] Anaerobic induction ratio is anaerobic beta-galactosidase activity (μmol of ONPG per h per 10^9 cells)/aerobic beta-galactosidase activity. ONPG, *o*-Nitrophenyl-β-D-galactopyranoside. Cultures were grown in PPPS medium with the additive indicated. Final concentration for nitrate, TMAO, and DMSO was 0.1% (wt/vol) and for fumarate, 0.2%.

TABLE 4. Effect of medium additives on anaerobic induction

Strain	Anaerobic induction ratio[a] for the following additives:				
	None	Ammonia (10 mM)	Phosphate (50 mM)	Citrate (20 mM)	HEPES (50 mM)
WNK13	3.1	1.1	0.9	0.7	1.0
WNK17	7.5	4.6	15.0	8.8	3.0
WNK18	7.6	1.1	1.0	1.2	0.9
WNK25	12.0	1.3	1.9	1.5	1.1
WNK29	1.2	13.5	9.5	38.3	30.0
WNK31	36.0	40.1	16.0	50.1	36.8
WNK32	9.3	5.6	4.8	4.0	6.1
WNK34	9.1	1.0	10.1	1.8	2.9

[a] Anaerobic induction ratio is anaerobic beta-galactosidase activity (μmol of ONPG per h per 10^9 cells/aerobic beta-galactosidase activity. ONPG, *o*-Nitrophenyl-β-D-galactopyranoside. Cultures were grown in PPPS plus xylose with the additives at the final concentrations indicated. These concentrations are approximately equivalent to those in medium E. Phosphate, citrate, and HEPES were sodium salts adjusted to pH 8.0 before addition.

TABLE 5. Anaerobic use of amino acids

Strain	Growth[a] on medium E plus:		
	Asparagine	Aspartic acid	Serine
WNK13	+	++	++
WNK17	−	++	++
WNK18	−	−	++
WNK25	−	+	++
WNK29	+	+	++
WNK31	+/−	++	++
WNK32	+	++	++
WNK34	+	+/−	++
MC4100	+	+	++

[a] Growth was tested anaerobically on minimal medium E, glycerol, and the indicated amino acid for 3 days at 37°C. −, +/−, +, and ++ respresent levels of growth, with ++ being the best. All strains grew with glycerol plus fumarate. No strains were able to grow on fumarate alone or on glycerol alone under these conditions.

glycerol, fructose, glucose, gluconate, or several other sugars as a sole carbon source was tested aerobically, and no differences were found between the gene fusion strains and the parent strain, MC4100. The ability to grow by fermenting a selection of sugars (glucose, fructose, xylose, gluconate, mannose, mannitol) anaerobically was also unchanged. All strains yielded positive reactions when tested for formate dehydrogenase, pyruvate formate lyase, and nitrate reductase by colony staining methods (6, 12), and all were able to grow with TMAO as the anaerobic electron acceptor.

E. coli cannot ferment glycerol anaerobically and requires the presence of a suitable electron acceptor such as fumarate for growth on this substrate (18). Asparagine can be converted to aspartate by asparaginase (4) and thence to the alternative electron acceptor fumarate by aspartase (14). Fumarate could be replaced by either of these amino acids for the anaerobic growth of wild-type *E. coli* on glycerol (Table 5). However, several fusion strains could not use asparagine (WNK17, WNK25, WNK34), and one, WNK18, used neither asparagine nor aspartic acid to replace fumarate (Table 5). However, the parent strain, MC4100, and all the fusion strains could still use these two amino acids as the sole source of nitrogen (data not shown). All strains grew anaerobically on glycerol plus fumarate or on glycerol plus serine, which can be deaminated by L-serine deaminase (22) to pyruvate, a fermentable substrate.

Genetic mapping of anaerobic fusions. Preliminary experiments indicated that our Mu dX insertions were unstable when moved from one strain to another by P1 cotransduction with ampicillin or chloramphenicol to select transductants. Typically, 10 to 50% of the transductants showed altered expression of the *lac* genes carried by the Mu dX fusion vector. In addition, most of our anaerobic gene fusions had no phenotype that was useful for genetic manipulations. We therefore mapped the Mu dX insertions with a set of Hfr strains with different origins of transfer which possessed insertions of Tn*10* within 10 to 20 min of the point of origin. The Tn*10* insertions are transferred as proximal markers during conjugation and obviated the need for a selectable phenotype in the recipient.

Each anaerobic gene fusion strain was crossed with each Hfr strain. The exconjugants were selected on streptomycin and tetracycline and checked for loss of the chloramphenicol and ampicillin resistances associated with the Mu dX insertions. This mapping procedure yielded two types of information (Table 6). First, cotransfer of the tetracycline resistance with the Mu dX insertion indicated linkage of the fusion to the Tn*10* insertion of the donor. Second, failure of certain Hfr strains to transfer in the wild-type equivalent of the gene fusion indicated the position of the Mu dX insertion relative to the point of origin. For example, the *aif-17* fusion of WNK17 was linked to Tn*10* insertions in the 55- to 65-min region of the genetic map. In addition, neither strain BW6159 (KL14 origin at 65 min, transferring clockwise) nor strain BW6163 (KL16 origin, 60 min, anticlockwise) could transfer in the wild-type allele. Thus, *aif-17* must have lain anticlockwise of the KL14 origin and clockwise of the KL16 origin, i.e., between 60 and 65 min, Table 6 summarizes the mapping data for eight fusion strains. No linkage data were obtained for WNK31, which presumably lies in one of the regions poorly covered by the set of Hfr::Tn*10* strains used. The eight fusions selected for mapping were scattered around the chromosome in various locations, indicating that all of these fusions were probably in separate genes (Table 6).

DISCUSSION

Discrete map positions and varied regulatory properties were observed for these fusion strains. This supports our previous contention that *E. coli* contains many anaerobic genes, most of which remain to be identified (10). Our selection procedure for anaerobically induced fusions was carried out in rich medium under conditions designed to favor genes involved in fermentative growth rather than anaerobic respiration. We found that most of the anaerobic fusions were repressed by the presence of electron acceptors such as nitrate, DMSO, and TMAO. This is expected for genes involved in fermentative growth (8); the only exception was strain WNK29, which was induced approximately 10-fold anaerobically irrespective of the presence of nitrate or TMAO. Although nitrate, TMAO, and DMSO acted to

TABLE 6. Map location of Mu dX fusions

Fusion strain	Approximate location (min)[a]	Hfr origins nearby[b]		Map limits[c]
		Transfer	No transfer	
WNK13	50–65	PK19 (44,C), AB313 (82,A), KL16 (58,A)	KL14 (65,C), KL96 (41,A)	50–58
WNK17	55–65	PK19 (44,C), AB313 (82,A)	KL14 (65,C), KL16 (60,A)	60–65
WNK18	10–30	Broda7 (30,A), Broda8 (18,C)	HfrC (12,A), KL96 (42,A)	18–28
WNK25	10–20	HfrC (12,A), Broda7 (30,A)	P4X (5,A), Broda8 (18,C)	5–12
WNK29	10–30	Broda7 (30,A)	Broda8 (18,C), HfrC (12,A)	12–18
WNK32	55–60	AB313 (82,A)	PK3 (80,C), KL16 (60,A)	60–70
WNK34	0–20	HfrC (12,A)	P4X (5,A), Broda8 (18,C)	8–12

[a] Derived from cotransfer with Tn*10* insertion.
[b] Hfr origins are designated by point of origin (minutes) and direction of transfer (C, clockwise; A, anticlockwise).
[b] Derived from consideration of both cotransfer with Tn*10* insertions and which Hfr origins effect early transfer.

TABLE 7. Regulatory properties of anaerobic fusions

Fusion strain	Property[a]							
	Map limits (min)	Phenotype[b]	Ammonia	Electron[c] acceptors	Fum/Cit[d]	Buffer[e]	Sugars[f]	Glucose
WNK17	60–65	Asn	R	R	NC	NC	I	R
WNK18	18–28	Asp, Asn	R	R	NC?	R	I	R
WNK25	5–12	Asn	R	R	NC?	R	I	R
WNK34	8–12	Asn	R	R	NC	R	I	R
WNK13	50–58	+	R	R	NC	R	NC	R
WNK29	12–18	+	I	NC	I	I	R	R
WNK31	?	+	NC	R	I	NC	NC	I
WNK32	60–70	+	NC	R	R	NC	NC	R

[a] R, Repression; I, induction; NC, no significant change. These terms refer to anaerobic conditions.
[b] Inability to use asparagine (Asn) or aspartate (Asp) as an electron acceptor; +, no growth defect yet observed.
[c] Electron acceptors were general effect of nitrate, TMAO, or DMSO, but excluding fumarate.
[d] Fumarate or citrate.
[e] Buffers were general effect of HEPES, citrate, or phosphate when these are similar.
[f] Sugars such as xylose, lactose, or maltose but excluding glucose.

repress most of the anaerobic fusions, fumarate had little repressive effect in most cases and even induced strain WNK29. Although it is generally accepted that anaerobic respiration is preferable to fermentation, it should be remembered that whereas two ATP molecules are produced per molecule of nitrate reduced, only one ATP molecule per fumarate molecule is obtained (15). In addition, fumarate reduction results in the loss, as the waste product succinate, of four potentially useful carbon atoms each time an ATP molecule is synthesized. Overall, fumarate respiration may offer little advantage over sugar fermentation.

In addition to electron acceptor repression, we observed several other regulatory phenomena, including catabolite repression, nitrogen regulation, and effects apparently unique to anaerobic conditions (Table 7). Four strains, WNK17, WNK18, WNK25, and WNK34, fell into a reasonably coherent group. All four were defective in using asparagine or aspartate to replace fumarate as the source of an alternative electron acceptor. All four were induced by the provision of xylose or lactose (poorly catabolite-repressing sugars) but were repressed by glucose and also by ammonia. There are three roles that the cleavage of asparagine or aspartate to ammonia plus fumarate might play: provision of fumarate, provision of ammonia as a nitrogen source, or neutralization of acid fermentation products by released ammonia. The repression of these four fusions by electron acceptors superior to fumarate and their repression by ammonia suggest that the first two roles may both occur under appropriate conditions. The induction by non-catabolite-repressing sugars and the repression of most, although not all, of this group of fusions by the addition of buffers suggests that the release of ammonia may also make a substantial contribution to deacidification. The catabolite repression of these four fusions by glucose is less easy to explain. Perhaps, as suggested above, fermentation of glucose is actually superior to fumarate reduction since, although somewhat less energy efficient, it is simple and rapid, an important consideration in the wild where there is competition between organisms and where rapid uptake of growth substrates can be as important as efficient use. The whole question of catabolite repression under anaerobic conditions is problematical. It has been suggested that catabolite repression is relieved anaerobically, at least in the case of the lactose operon (11). Other experiments, however, have shown severe catabolite repression of the anaerobic formate dehydrogenases of *E. coli* (24). Most recently, Guest and co-workers (28, 33) have suggested that cyclic AMP regulation under anaerobic conditions may involve binding of cyclic AMP by the Fnr regulatory protein rather than, or in addition to, the usual cyclic AMP receptor protein.

The other fusions form a less homogeneous group. Strains WNK13, WNK31, and WNK32 are unaffected by nonrepressing sugars and are repressed by nitrate but show considerable variation in their responses to ammonia, catabolite repression, fumarate, and buffers. Strain WNK31 shows little regulation except nitrate-TMAO repression and induction by fumarate or citrate. Since citrate can be used anaerobically (19) and is converted to acetate plus oxalacetate (hence fumarate) by citrate lyase, it is possible that the *aif-31* gene is involved in some aspect of fumarate respiration. However, strain WNK31 shows no growth defect with fumarate as the electron acceptor.

The fusion in strain WNK29 stands out from all the rest in being unaffected by electron acceptors, repressed by sugars (both xylose and glucose), and induced by all buffers. WNK29 also lacks any phenotype under conditions so far tested, and its unique regulatory properties remain an enigma.

The eight fusion strains discussed above were selected from a larger collection after preliminary investigation as being reasonably representative in their properties. Strauch et al. (31) also isolated anaerobically induced fusions in *S. typhimurium*; however, their gene fusions were subjected mostly to *fnr* regulation and were thus probably not involved in fermentative growth. In contrast, Smith and Neidhardt (29) showed the anaerobic induction of several proteins in *E. coli* by using two-dimensional gels. Since these authors used minimal glucose medium, in which most of our fusions show no anaerobic induction, it seems unlikely that any of our gene fusions correspond to Smith and Neidhardt's anaerobic proteins. It is clear that many anaerobic genes remain to be characterized. We are presently investigating anaerobic nitrogen regulation and catabolite repression in more detail, since there is a substantial body of information concerning the aerobic operation of these regulatory phenomena.

ACKNOWLEDGMENT

This research was supported by a grant to D.C. from the U.S. Department of Energy, Office of Basic Energy Sciences (contract DE-AC02-82ER12095).

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