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Transcription of *pfl* Is Regulated by Anaerobiosis, Catabolite Repression, Pyruvate, and *oxrA*: *pfl*::Mu dA Operon Fusions of *Salmonella typhimurium*

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Pyruvate formate-lyase (EC 2.3.1.54), a key enzyme in the anaerobic metabolism of *Salmonella typhimurium*, catalyzes the conversion of pyruvate to acetyl coenzyme A and formate. *pfl*::Mu dA operon fusions were isolated for the study of transcriptional regulation. *pfl* was transcribed both aerobically and anaerobically, but the activity increased about sixfold under anaerobic conditions. The addition of pyruvate, formate, and acetate in nutrient broth did not have any effect on the anaerobic expression of *pfl*. However, the addition of pyruvate to minimal glucose medium increased the anaerobic expression of *pfl*. The expression of *pfl* varied in different growth media. Anaerobic expression of *pfl* was lower when the culture was grown in minimal glucose medium than when it was grown in nutrient broth. When Casamino Acids (Difco Laboratories, Detroit, Mich.) were added to minimal glucose medium, the expression of *pfl* increased proportionally with the amount of Casamino Acids added. The transcription of *pfl* was positively controlled by the *oxrA* gene product and was affected by both the *cya* and *crp* mutations. However, mutations in genes affecting the cyclic AMP-cyclic AMP receptor protein complex or *oxrA* could not completely abolish the anaerobic derepression of *pfl*. In merodiploid strains, *pfl*::Mu dA/F' *pfl*$^+$, the β-galactosidase activities were decreased. The mutations *gyrA*, *oxrC*, and *oxrE*, which affected anaerobic metabolism, did not affect anaerobic expression of *pfl*.

Facultative anaerobic enteric bacteria such as *Salmonella typhimurium* and *Escherichia coli* carry out mixed acid fermentation during anaerobic growth on glucose when electron acceptors such as nitrate and trimethylamine oxide are not available. Pyruvate formate-lyase (PFL; EC 2.3.1.54) is a key enzyme in fermentative growth and is responsible for converting pyruvate to acetyl coenzyme A and formate, which serve as substrates for the production of acetate, ethanol, hydrogen gas, and carbon dioxide (14). The activity of PFL is regulated by a reversible enzymatic interconversion of an active form to an inactive form in response to oxygen (6). Smith and Neidhardt (27) determined that the anaerobic steady-state level of PFL is 10 times higher than the aerobic level. A temporal overproduction of PFL after a shift from aerobic to anaerobic growth has also been demonstrated (27). The expression of the cloned *pfl* gene of *E. coli*, which is the structural gene encoding PFL, is derepressed 5- to 10-fold during anaerobiosis compared with the basal level in aerobically grown *E. coli* (22). However, it is unclear whether the derepression of *pfl* is regulated by oxygen per se or by the accumulation of some inducers. The effects of various global control systems, such as catabolite repression and *fnr* (*oxrA*) regulation (28), on *pfl* transcription are still unclear. We report here the use of *pfl*::Mu dA operon fusions to study the regulation of transcription of *pfl*.

MATERIALS AND METHODS

Bacteria and bacteriophages. The bacterial strains used in this study are listed in Table 1. All *S. typhimurium* strains were derived from strain LT2. Transductions with P22 *int4* phage have been described by Ely et al. (8). The Mu dl derivative Mu dA has been described by Hughes and Roth (12).

Chemicals. Ampicillin, fusaric acid, tetracycline, chlortetracyline, kanamycin sulfate, tryptophan, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and organic acids were from Sigma Chemical Co. (St. Louis, Mo.). MacConkey agar, Bacto-Agar, nutrient broth (NB), nutrient agar, and MacConkey agar base were purchased from Difco Laboratories (Detroit, Mich.).

Culture media. Complex medium consisted of 0.8% NB. Minimal glucose medium (NCE medium with 1% glucose) has been described by Gutnick et al. (10). Medium E (31) with 1% glucose was the minimal medium used for genetic mapping. The MacConkey agar-glucose-trimethylamine oxide media (MGT) MGT and MGT1 have been described previously (15). The concentrations of glucose in MGT1 and MGT were 1 and 0.15%, respectively; and that of trimethylamine oxide was 0.1%. When they were grown aerobically, *pfl* mutants were dark red on MGT1 plates, while the wild-type strains was pink in the center. When grown anaerobically, the *pfl* mutants formed red colonies on MGT, while the wild-type strains formed white colonies. MacConkey nitrate medium has been described by Barrett et al. (3). Under anaerobic conditions on MacConkey nitrate medium, *oxrA* mutants formed tiny dark red colonies, and the wild-type strains formed large white colonies. The fusaric acid medium described by Maloy and Nunn (18) was used to select for tetracycline-sensitive derivatives from tetracycline-resistant parents. Green and X-gal media have been described by Miller (19). When needed, potassium nitrate (20%), glucose (40%), and other carbon sources were autoclaved separately as stock solutions and added to the medium at final concentrations of 1%. Tryptophan was prepared as a 40 mM stock solution, filter sterilized, and added to the autoclaved medium at a final concentration of 1 mM. Ampicillin and tetracycline were added to the autoclaved complex medium at concentrations of 50 and 20 μg/ml, respectively. Ampicillin and tetracycline were added to the

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TABLE 1. Bacterial strains used in this study

Strain[a]	Genotype	Origin or source
AZ1516	*trp*::Tn*5*/F'123 *finP trp*$^+$	S. Artz
HSK516	*trp*::Tn*5*	F$^-$ isolate of AZ1516
TT629	*strA1 pyrC7*/F'114 ts *lac zzf-22*::Tn*10*	Chumley et al. (5)
TT7610	*supD501 zeb-609*::Tn*10*	Hughes and Roth (12)
TT8388	*recA1 zeb-609*::Tn*10*/F'128 *zzf-1066*::Mu d1-8	Hughes and Roth (12)
SL5442	*pfl*$^+$ *zbi*::Tn*10*	Hoiseth and Stocker (11)
SCSG378	*pfl*	Hoiseth and Stocker (11)
HSK100	*pfl zbi*::Tn*10*	SGSC37 was transduced with P22(SL5442) to Tet^r and tested for Gas$^-$ and H_2S^-
JT68	*oxrE zda*::Tn*10*	J. Tang
JT73	*oxrA*::Tn*10*	J. Tang
PP1002	*cya-1091*::Tn*10 trpB223*	P. W. Postma
PP1037	*crp-773*::Tn*10 trpB223*	P. W. Postma
HSK1	*trp*::Tn*5 zeb-609*::Tn*10 supD501*	Transduced HSK516 with P22(TT7610) to Tet^r and tested for *supD*
HSK2	*trp*::Tn*5 supD501*	Tet^s isolate from HSK1
HSK3	*pfl zbi*::Tn*10 trp*::Tn*5*	HSK516 was transduced with P22(HSK100) to Tet^r and tested for Gas$^-$ and H_2S^-
HSK4	*pfl*$^+$ *zbi*::Tn*10 trp*::Tn*5*	Isogenic pair with HSK3
HSK14	*pfl-102*::Mu dA *trp*::Tn*5*	This study
HSK15	*pfl-103*::Mu dA *trp*::Tn*5*	This study
HSK16	*pfl-104*::Mu dA *trp*::Tn*5*	This study
HSK21	*pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1101	*cya*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1105	*oxrA*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1109	*gyrA pfl-101*::Mu dA *trp*::Tn*5*	Spontaneous nalidixic acid-resistant isolate of HSK21
HSK1110	*oxrC*$^+$ *zxx*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1111	Δ*oxrC zxx*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1112	*pfl-101*::Mu dA *aroA5330*	This study
HSK1113	*pfl-101*::Mu dA *aroA5330*/F' *pfl*$^+$ *aroA*$^+$	This study
HSK1114	*oxrA*::Tn*10 pfl-101*::Mu dA *aroA5330*/F' *pfl*$^+$ *aroA*$^+$	This study
HSK1116	Δ*oxrA pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1119	*cya*::Tn*10* Δ*oxrA pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1120	*crp*::Tn*10 pfl-101*::dA *trp*::Tn*5*	This study
HSK1121	Δ*cya crp*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1122	Δ*cya pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1123	Δ*cya oxrA*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1125	*pfl-101*::Mu dA *trp*::Tn*5*/F'114 ts *lac*$^+$ *zzf-22*::Tn*10*	This study
HSK1130	*ack*$^+$ *zde*::Tn*10 pfl-101*::Mu dA *aroA5330*/F' *pfl*$^+$ *aroA*$^+$	This study
HSK1131	*ack zde*::Tn*10 pfl-101*::Mu dA *aroA5330*/F' *pfl*$^+$ *aroA*$^+$	This study
HSK1132	*oxrE*$^+$ *zda*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
HSK1133	*oxrE zda*::Tn*10 pfl-101*::Mu dA *trp*::Tn*5*	This study
KL725	*pyrD34 trp-45 his-68 recA1 thi-1 galK35 malA1* (λ^r) *xyl mtl-2 rpsL118*/F' *pfl*$^+$ *aroA*$^+$	B. Low

[a] All strains were derived from wild-type *S. typhimurium* LT2, except strain KL725, which was derived from wild-type *E. coli* K-12.

minimal medium at concentrations of 25 and 10 μg/ml, respectively.

Phenotypic characterization. Gas production from pyruvate and glucose was detected by inoculating the bacteria into NB with a durham tube in the presence or absence of 10 mM formate. The tubes were then grown at 37°C as standing cultures for 24 h and scored for the presence of gas in the durham tube. Acid profiles were obtained by high-performance liquid chromatographic analysis of acid products from glucose as described by Guerrant et al. (9). D-Glucose was determined by the *ortho*-toluidine method (7).

Isolation of *pfl*::Mu dA operon fusions. *S. typhimurium* TT8388, which has a Mu dA(Ap^r *lac*) insertion in its F' factor (12), was used as the donor of Mu dA(Ap^r *lac*) to generate operon fusions. P22 lysate prepared from strain TT8388 was used to transduce strain HSK2 to ampicillin resistance on aerobic MGT1 plates containing 50 μg of ampicillin per ml. After 24 h of incubation at 30°C, the plates were examined for red colonies among pink-centered wild-type colonies. Putative *pfl*::Mu dA(Ap^r *lac*) mutants were purified on MGT1 and green plates. Mu dA operon fusions were stabilized by transducing them into strains without a suppressor mutation (12).

Determination of transcription orientation. The temperature-sensitive episome F' ts *lac* Tn*10* (5) was used for the determination of the transcription orientation. In this episome, the orientation of the *lac* operon relative to *oriT* is known. After integration via *lac* homology into the *pfl*::Mu dA fusion strain HSK21 at the nonpermissive temperature, one Hfr strain was obtained. The direction of transcription of *pfl* in this Hfr strain was determined as described before (15).

Bacterial growth conditions. An anaerobic liquid culture was prepared by filling tubes to the top, and the tubes were stoppered with rubber stoppers after inoculation. Growth was started by inoculating a 1% overnight culture grown aerobically in nutrient broth into the tubes. At time intervals, the A_{650} of the cultures was measured directly against a blank of growth medium. Anaerobic incubation of plates was

carried out in anaerobic jars (GasPak; BBL Microbiology Systems, Cockeysville, Md.) by using an atmosphere of 95% H_2–5% CO_2. Aerobic growth of the liquid culture was achieved by growing 20 ml of culture in a 250-ml conical flask with vigorous shaking.

Enzyme assays. The β-galactosidase assay we performed has been described by Miller (19). Toluene-treated cell cultures were used directly for the assay. For the phosphoglucose isomerase assay, the rate of production of D-fructose-6-phosphate from D-glucose-6-phosphate by sonicated crude cell extracts was measured. Quantitative determination of D-fructose-6-phosphate was done by the method of Roe et al. (23). Protein was determined by the method of Bradford (4) by using protein assay reagents (Bio-Rad Laboratories, Richmond, Calif.).

Construction of merodiploid strains. An F′ episome carrying the wild-type *pfl* and *aroA* genes was transferred from *E. coli* KL725 to an *S. typhimurium* strain with the genotype *aroA5330 pfl*::Mu dA. Mating was performed as described previously (19). Transconjugants were purified and checked for F′ and gas production. The F′ episome was maintained by keeping the merodiploid strains in minimal medium and selecting for $AroA^+$ strains. Other strain constructions were done by P22 transduction.

RESULTS

Isolation and characterization of *pfl*::Mu dA fusion. Four *pfl*::Mu dA fusions were isolated as red colonies on MGT1 plates that were incubated aerobically. They were stabilized by transduction into HSK516, which had no suppressor mutation. These mutants had the same phenotype as HSK3 (*pfl*); that is, they all formed red colonies on anaerobic MGT plates, produced gas only in the presence of formate, and produced less H_2S in peptone-iron agar. All insertions were cotransducible with *zbj*::Tn*10*, which was linked to *pfl* (24), with cotransduction frequencies of 70 to 94%. Strain HSK21, which was chosen for further studies, could not produce formate from glucose (data not shown). High-performance liquid chromatographic determination of organic acids in cultures of HSK21 grown anaerobically in minimal glucose medium showed that HSK21 did not accumulate formate. When compared with the parental strain HSK516, PFL mutant HSK21 produced 10-fold less pyruvate, 5-fold less fumarate, slightly less succinate and acetate, but 2-fold more lactate, which constituted about 90% of the total organic acids in the culture of HSK21. The decreased anaerobic growth rates of strains HSK21 and HSK3 were observed in minimal glucose medium (the generation time increased to 8 h). The growth rate, however, could be increased by the addition of trimethylamine oxide or nitrate, indicating that HSK3 and HSK21 are both deficient in fermenting glucose efficiently but have normal anaerobic respiration. The map location and phenotype of HSK21 thus indicate that this strain carries a *pfl*::Mu dA operon fusion. The transcription orientation of *pfl*::Mu dA was determined to be counterclockwise on the genetic map.

β-Galactosidase activities. *pfl*::Mu dA fusion strains expressed β-galactosidase aerobically in NB, and the activity was derepressed about sixfold when they were grown anaerobically in NB (Table 2). Supplementation of pyruvate, formate, and acetate in NB did not affect the anaerobic expression of *pfl* in strain HSK21. When HSK21 was grown aerobically, it had a differential rate of 320 U/A_{650} unit. A shift of the aerobic culture to anaerobiosis increased the differential rate of β-galactosidase expression 10-fold to 3,130 U/A_{650} unit. When HSK21 was grown in minimal glucose medium, the anaerobic β-galactosidase expression was lower than that in NB. However, the addition of pyruvate to the minimal glucose medium increased *pfl* expression. Moreover, the addition of Casamino Acids (Difco) to the minimal glucose medium increased both the growth rate and the expression of *pfl*::Mu dA. Supplementation of 1% Casamino Acids to minimal glucose medium increased the anaerobic β-galactosidase expression to the anaerobic expression level in NB. When HSK21 was grown in minimal glycerol nitrate medium, expression of the *pfl*::Mu dA fusion was almost as low as that at the aerobic level.

TABLE 2. Effects of oxygen and growth media on the expression of *pfl*::Mu dA (*lac*) in HSK21

Basal medium and growth condition[a]	Supplement	β-Galactosidase sp act[b]
NB		
Aerobic	None	270
Anaerobic	None	1,900
	Pyruvate, 0.1%	1,630
	Formate, 0.05%	1,780
	Acetate, 0.1%	1,690
Minimal glucose		
Aerobic	None	227
	Pyruvate, 0.05%	223
	Pyruvate, 0.1%	287
	Pyruvate, 0.5%	199
Anaerobic	None	606
	Casamino acids, 0.1%	1,490
	Casamino acids, 1.0%	2,290
	Pyruvate, 0.05%	1,240
	Pyruvate, 0.1%	1,500
	Pyruvate, 0.5%	1,090
Minimal glycerol (anaerobic)	Nitrate, 0.1%	396

[a] Aerobic growth was achieved by growing 20 ml of culture in a 250-ml conical flask with vigorous shaking. Anaerobic growth was achieved by incubating tubes that were filled to the top with medium and stoppered.

[b] Specific activity is expressed as nanomoles of *o*-nitrophenol per minute per A_{650} unit.

Regulation of *pfl* by *oxrA*. The *oxrA* mutation affects the expression of many genes encoding components of anaerobic respiration systems, such as nitrate reductase (16), but it does not seem to affect genes of the fermentative pathway (13). Since the *pfl* gene product is involved in both fermentation and anaerobic respiration, it was necessary to investigate whether it is controlled by *oxrA*. To answer this question, we moved an *oxrA*::Tn*10* insertion into the *pfl*::Mu dA fusion and assayed for the β-galactosidase activity of the anaerobically grown culture. In the *oxrA* background, anaerobic transcription of the fusion was lowered by about twofold, but the aerobic transcription was unaffected and the anaerobic transcription was still threefold higher than aerobic transcription. Thus, *pfl* is under the global regulation of *oxrA*, but the *oxrA* gene product is not absolutely essential for the anaerobic induction of *pfl*.

Catabolite repression. The effect of catabolic repression on the regulation of *pfl* was investigated in *cya pfl*::Mu dA and *crp pfl*::Mu dA double mutants. Isogenic pairs were studied. Both *cya* and *crp* mutations lowered the expression of *pfl*::Mu dA by about twofold (Table 3). The effect of *cya* could be reversed by the addition of 5 mM cyclic AMP (cAMP) to the growth medium, indicating that the absence of cAMP affects *pfl* transcription. The regulation of *pfl* by

TABLE 3. Effects of different gene mutations on the expression of the *pfl*::Mu dA (*lac*) operon fusion[a]

Strain	Relevant mutation, in addition to *pfl*::Mu dA (*lac*)	β-Galactosidase sp act under the following growth conditions[b]:	
		Aerobic	Anaerobic
HSK21	None	270	1,900
HSK1101	*cya*::Tn*10*	192	693
HSK1120	*crp*::Tn*10*	167	710
HSK1121	Δ*cya crp*::Tn*10*	192	791
HSK1105	*oxrA*::Tn*10*	313	577
HSK1116	Δ*oxrA*	201	751
HSK1119	Δ*oxrA cya*::Tn*10*	161	273
HSK1122	Δ*cya*	176	704
HSK1123	Δ*cya oxrA*::Tn*10*	191	282
HSK1109	*gyrA*	279	1,790
HSK1110	*oxrC*$^+$ *zxx*::Tn*10*	275	1,630
HSK1111	Δ*oxrC zxx*::Tn*10*	266	1,660
HSK1132	*oxrE*$^+$ *zda*::Tn*10*	194	1,570
HSK1133	*oxrE zda*::Tn*10*	169	1,240

[a] Cultures were grown to the stationary phase in N Broth and harvested for β-galactosidase assays. Aerobic growth was achieved by growing 20 ml of culture in a 250-ml conical flask with vigorous shaking. Anaerobic growth was achieved by incubating tubes that were filled to the top with medium and stoppered.

[b] Specific activity is expressed as nanomoles of *o*-nitrophenol per minute per A_{650} unit.

cAMP seemed to be via the cAMP receptor protein (CRP) because the *pfl*::Mu dA strain which carried both *cya* and *crp* mutations did not have β-galactosidase activities different from those of fusions with either mutation alone (Table 3). On the other hand, cAMP did not appear to exert its effect on *pfl* transcription via the *oxrA* gene product, although the OxrA (Fnr) protein has homology with CRP (26). The results given in Table 3 indicate that the effects of *cya* and *oxrA* are independent of each other. However, the transcription of *pfl*::Mu dA in the *cya oxrA* double mutation background was still derepressed by anaerobiosis, albeit to a lesser extent. Therefore, in addition to *oxrA* and catabolite repression, other factors must be involved in the aerobic-anaerobic control of *pfl* transcription.

Regulation in merodiploids. We constructed merodiploids to study the regulation of *pfl*::Mu dA expression in a Pfl$^+$ background. The F'114 episome, which carries a fragment of the *E. coli* chromosome containing the wild-type *pfl* structural gene, was conjugated into *aroA* derivatives of *pfl*::Mu dA. The F' episome was maintained by complementation of the *aroA* mutation in the chromosome. The exoconjugant became Gas$^+$, indicating that the Pfl function is complemented in the merodiploid. Other mutations were then transduced into the merodiploid to study their effects on *pfl* expression. In the merodiploid with the Pfl$^+$ background, anaerobic expression of *pfl*::Mu dA was decreased (Table 4). The *oxrA* mutation further decreased *pfl*::Mu dA expression in the merodiploid, but *pfl* expression was still higher under anaerobic conditions. It was previously observed that Ack$^-$ mutants had the same phenotype as Pfl$^-$ mutants (20), suggesting that in *ack* mutants *pfl* is not expressed. Comparison of the isogenic merodiploid pair, which differed only in that one was Ack$^-$ and the other was Ack$^+$, revealed that the *ack* mutation did not affect *pfl* expression. On the contrary, the merodiploid with the *ack* mutation had an increased *pfl* expression, reaching a level similar to that in the haploid. Perhaps both *pfl* and *ack* mutations caused an accumulation of an inducer which induced *pfl* expression. Pyruvate seemed to be the inducer, as exogenous pyruvate added to the minimal glucose medium stimulated *pfl* expression (Table 2).

Regulation by *gyrA*, *oxrC*, and *oxrE*. *gyrA* is suggested to be a regulatory gene for the expression of anaerobic genes because *gyrA* mutants cannot grow anaerobically on nutrient agar plates (34) and anaerobic induction of *E. coli* formate dehydrogenase (hydrogenase-linked) is enhanced by gyrase inhibition (2). We tested the effect of the *gyrA* mutation on *pfl* expression. A *gyrA* derivative of *pfl*::Mu dA was isolated by its resistance to nalidixic acid (20 μg/ml). *pfl* expression was not affected in this strain (Table 3). Jamieson and Higgins (13) have described a mutation, *oxrC*, which decreases phosphoglucose isomerase activity and affects the transcription of *fhl* and *hyd*. We have isolated an *oxrC* mutant strain (H. S. Kwan and K. K. Wong, unpublished data) which has the same phenotype as that of the *oxrC* mutant strain isolated by Jamieson and Higgins (13). Neither strain fermented glucose, but both strains fermented fructose and both strains had only about 1/10 of the wild-type phosphoglucose isomerase activity. Our *oxrC* mutation did not affect *pfl* transcription (Table 3). We moved another mutation, *oxrE*, which causes a deficiency in formate-nitrate reduction (J. S. Tang and E. L. Barrett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K120, p. 213), into *pfl*::Mu dA and determined the β-galactosidase activity. *oxrE* did not affect *pfl* expression.

DISCUSSION

Our results indicate that the transcription of *pfl* is regulated at the following three levels: (i) derepression by an-

TABLE 4. β-Galactosidase activities of merodiploid strains[a]

Strain	Relevant genotype	β-Galactosidase sp act under the following growth conditions[b]:							
		NB		MG		MGPyr (0.1%)		MGPyr (1%)	
		$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$
HSK1112	*pfl*::Mu dA	238	1,080	172	485	229	1,050	186	1,040
HSK1113	*pfl*::Mu dA/F' *pfl*$^+$	192	630	69	422	90	550	98	901
HSK1130	*pfl*::Mu dA *ack*$^+$/F' *pfl*$^+$	222	632	117	451	111	530	130	919
HSK1131	*pfl*::Mu dA *ack*/F' *pfl*$^+$	189	1,040	149	669	167	639	181	877
HSK1114	*pfl*::Mu dA *oxrA*::Tn*10*/F' *pfl*$^+$	219	393	76	129	92	162	97	159

[a] All strains had a *aroA5330* background.

[b] Abbreviations: NB, nutrient broth; MG, minimal glucose medium; MGPyr (0.1%), minimal glucose-pyruvate (0.1%) medium; MGPyr (1%), minimal glucose-pyruvate (1%) medium; $+O_2$, aerobic conditions; $-O_2$, anaerobic conditions. Aerobic growth was achieved by growing 20 ml of culture in a 250-ml conical flask with vigorous shaking. Anaerobic growth was achieved by incubating tubes that were filled to the top with medium and stoppered. Specific activity is expressed as nanomoles of *o*-nitrophenol per minute per A_{650} unit.

aerobiosis, (ii) catabolite repression, and (iii) derepression by pyruvate. The response of the transcription of *pfl* to anaerobiosis appeared to be partially mediated by the *oxrA* (*fnr*) gene product, because the expression of *pfl* could not reach the fully derepressed level in the *oxrA* background. However, there was still a threefold increase when the growth was shifted from aerobic to anaerobic conditions.

Full anaerobic expression of *pfl* also required cAMP and CRP, indicating that *pfl* expression is controlled by catabolite repression. The low expression of *pfl* when the *pfl*::Mu dA strain (HSK21) was grown in the presence of glucose also indicated catabolite repression. The action of cAMP did not require the *oxrA* gene product but required CRP. This observation is similar to that reported on the role of cAMP and the Fnr protein on anaerobic fumarate reduction (29, 30). The *cya* mutation affects anaerobic growth of *E. coli* in glycerol-fumarate medium (30), and cAMP is apparently required for anaerobic growth in minimal glucose medium (21). Catabolite repression is thus involved in both anaerobic respiration and fermentation. In this study we showed that catabolite repression is also involved in the regulation of the anaerobic expression of the *pfl* gene. However, the *cya* and *crp* mutations might have affected *pfl* expression indirectly by decreasing the internal level of pyruvate (the effect of pyruvate is discussed below). When *E. coli* is grown in minimal glucose medium enriched with NB, the concentration of cAMP is about three times higher under anaerobic growth conditions than it is under aerobic growth conditions (29). It is thus possible that catabolite repression is one of the signals of anaerobiosis which induces the expression of anaerobic genes. If this is the case, however, cAMP is not a specific signal of the availability of molecular oxygen but remains a general signal that responds to the energy state of the culture. It is possible that a specific genetic regulatory network responding to molecular oxygen does not exist. Alternatively, it is possible that the regulatory network is one that responds to the presence of different terminal electron acceptors, including oxygen (aerobic or anaerobic respiration), or the absence of terminal electron acceptors (fermentation). Jamieson and Higgins (13) have proposed two distinct anaerobic regulatory systems: anaerobic respiration and fermentation. Because they are energy-producing pathways, aerobic and anaerobic respirations do not seem to be fundamentally different. We can consider aerobic and anaerobic respirations as one kind of energy-producing pathway, just respiration, because in both aerobic and anaerobic respirations, electron transport chains are involved and ATP appears to be generated by the chemiosmotic mechanism (17). On the other hand, the catabolic pathways and components involved in respiration and fermentation are quite different. We propose an extension of the proposal that there are two distinct systems for the transcriptional regulation of anaerobic gene expression, namely, anaerobic respiration and fermentation, to include aerobic respiration, combining aerobic and anaerobic respirations into a single category, respiration. A global regulatory network may exist to control the shift from respiration to fermentation rather than from aerobiosis to anaerobiosis. We are trying to isolate regulatory mutations which affect both aerobic and anaerobic respiration systems.

The third level of regulation of *pfl* expression is derepression by the substrate pyruvate. The addition of pyruvate to the culture grown in rich medium (NB) did not affect *pfl* expression, presumably because of the ample supply of pyruvate as the catabolic product of alanine and cysteine degradation. The role of pyruvate could only be observed when the *pfl*::Mu dA mutant was grown in minimal glucose medium in which the anaerobic expression of *pfl* reached the level of *pfl* expression in rich medium. The addition of Casamino Acids had a similar effect, presumably because Casamino Acids contain amino acids which can be converted to pyruvate during growth of the bacteria. Expression of *pfl* in merodiploids was also derepressed by pyruvate, but a higher pyruvate (1%) concentration was required (Table 4). The F′ *pfl*$^+$ episome probably lowered *pfl* expression by providing PFL, which converted pyruvate to formate and acetyl coenzyme A. An additional *ack* mutation could change the expression of *pfl* in the merodiploid to the full level, probably because the *ack* mutation somehow inhibits PFL activity, causing pyruvate to accumulate and thus derepressing *pfl*. It is still not clear, however, whether pyruvate acts directly as an inducer or acts indirectly by releasing catabolite repression. The catabolic reduction charge, defined as NADH/(NADH + NAD$^+$), has been suggested to affect anaerobic metabolism (1, 32). The conversion of pyruvate to lactate can regenerate NAD$^+$ from NADH and can lower the catabolic reduction charge. The effect of pyruvate may be related to the catabolic reduction charge. We have isolated a mutation, *pflR* (33), which eliminates the anaerobic induction of *pfl*. The primary defect caused by the *pflR* mutation is a deficiency in lactate production. A deficiency in lactate production would probably affect the regeneration of NAD$^+$. It seems that a high catabolic reduction charge affects the anaerobic expression of *pfl*. Alternatively, it is possible that lactate is a signal for anaerobic induction of *pfl*. We have observed that when the wild type is grown anaerobically in minimal glucose medium with nitrate, the final concentration of lactate is negligible (Kwan and Wong, unpublished data), and the anaerobic level of expression of *pfl* in nitrate medium is comparable to the aerobic level. Furthermore, Pecher et al. (22) have shown that the aerobic level of expression of the cloned *pfl* structural gene on growth at the expense of D-lactate is twice as high as that at the expense of D-glucose. It is possible that lactate somehow titrates some repressors and causes the derepression of *pfl*.

The pleiotropic mutations *oxrC*, *gyrA*, and *oxrE*, which are known to affect anaerobic metabolism, did not affect the expression of *pfl*. Since the *oxrC* mutant did not grow in minimal medium, we tested its effect on *pfl* expression in NB. Thus, if an inducing signal is generated during glycolysis, the inducing signal may be counteracted by catabolites that are produced during growth in NB. As pyruvate can be generated from the degradative products of amino acids, our data do not necessarily conflict with the fact that *oxrC* (*pgi*) is required for the generation of an inducing signal. Yamamoto and Droffner (34) have suggested that DNA supercoiling plays a significant role in anaerobic gene expression. Their conclusion was based on the isolation of strict aerobic mutants containing mutations in *gyrA* and *gyrB* which lead to little or no DNA gyrase activity. Our *gyrA* mutant may not have completely lost gyrase activity, and thus, the effect was not obvious. The effect of supercoiling on the expression of *pfl* should be tested with gyrase inhibitors such as coumermycin. None of the mutations, when tested alone, could completely eliminate the anaerobic derepression of *pfl*, indicating that multiple signals are involved.

Since this paper was submitted for publication, it was described in another report (25) that (i) there is anaerobic induction (12-fold) and pyruvate stimulation (2-fold) of the *pfl* gene from *E. coli* K-12; (ii) complete anaerobic induction requires a functional *fnr* gene product, but the dependence is

not absolute; and (iii) nitrate partially represses *pfl* expression. These results agree with our findings presented above on the regulation of expression of the *pfl* gene from *S. typhimurium* LT2.

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LITERATURE CITED

1. **Andersen, K. B., and K. V. Meyenburg.** 1977. Charges of nicotinamide adenine nucleotides and adenylate energy charge as regulatory parameters of the metabolism in *Escherichia coli*. J. Biol. Chem. **252**:4151–4156.
2. **Axley, M. J., and T. C. Stadtman.** 1988. Anaerobic induction of *Escherichia coli* formate dehydrogenase (hydrogenase-linked) is enhanced by gyrase inactivation. Proc. Natl. Acad. Sci. USA **85**:1023–1027.
3. **Barrett, E. L., C. E. Jackson, H. T. Fukumoto, and G. W. Chang.** 1979. Formate dehydrogenase mutants of *Salmonella typhimurium*: a new medium for their isolation and new mutant classes. Mol. Gen. Genet. **177**:95–101.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Chumley, F. G., R. Menzel, and J. R. Roth.** 1979. Hfr formation directed by Tn*10*. Genetics **91**:639–655.
6. **Conradt, H., M. Hohmann-Berger, H. P. Hohmann, H. P. Blaschkowski, and J. Knappe.** 1984. Pyruvate formate-lyase (inactive form) and pyruvate formate-lyase activating enzyme of *Escherichia coli*: isolation and structural properties. Arch. Biochem. Biophys. **228**:133–142.
7. **Dubowski, K. M.** 1962. An o-toluidine method for body-fluid glucose determination. Clin. Chem. **8**:215–235.
8. **Ely, B., R. M. Weppleman, H. C. Massey, Jr., and P. E. Hartman.** 1974. Some improved methods in P22 transduction. Genetics **76**:625–631.
9. **Guerrant, G., M. A. Lambert, and C. W. Moss.** 1982. Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. J. Clin. Microbiol. **16**:355–360.
10. **Gutnick, D., J. M. Calvo, T. Klopotowski, and B. N. Ames.** 1969. Compounds which serve as the sole source of carbon or nitrogen for *Salmonella typhimurium* LT-2. J. Bacteriol. **100**: 215–219.
11. **Hoiseth, S. K., and B. A. D. Stocker.** 1985. Genes *aroA* and *serC* of *Salmonella typhimurium* constitute an operon. J. Bacteriol. **163**:355–361.
12. **Hughes, K. T., and J. R. Roth.** 1984. Conditional transposition defective derivative of Mu d1(Amp *lac*). J. Bacteriol. **159**: 130–137.
13. **Jamieson, D. J., and C. F. Higgins.** 1986. Two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in *Salmonella typhimurium*. J. Bacteriol. **168**: 389–397.
14. **Knappe, J., H. P. Blaschkowski, P. Gröbner, and T. Schmitt.** 1974. Pyruvate formate-lyase of *Escherichia coli*: the acetyl-enzyme intermediate. Eur. J. Biochem. **50**:253–263.
15. **Kwan, H. S., H. W. Chui, and K. K. Wong.** 1988. *ack*:: Mud1–8(Ap^r *lac*) operon fusions of *Salmonella typhimurium* LT2. Mol. Gen. Genet. **211**:183–185.
16. **Kwan, H. S., and K. K. Wong.** 1986. A general method for isolation of Mu *d*1–8(Ap^r *lac*) operon fusions in *Salmonella typhimurium* LT2 from Tn*10* insertion strains: *chlC*::Mu *d*1–8. Mol. Gen. Genet. **205**:221–224.
17. **Maloney, P. C.** 1987. Coupling to an energized membrane: role of ion-motive gradients in the transduction of metabolic energy, p. 222–243. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
18. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**: 1110–1112.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Pascal, M.-C., M. Chippaux, A. Abou-Jaoude, H. P. Blaschkowski, and J. Knappe.** 1981. Mutants of *Escherichia coli* K12 with defects in anaerobic pyruvate metabolism. J. Gen. Microbiol. **124**:35–42.
21. **Patrick, J. M., and W. J. Dobrogosz.** 1973. The effect of cyclic AMP on anaerobic growth in *Escherichia coli*. Biochim. Biophys. Res. Commun. **54**:555–561.
22. **Pecher, A., H. P. Blaschkowski, J. Knappe, and A. Böck.** 1982. Expression of pyruvate formate-lyase of *Escherichia coli* from the cloned structural gene. Arch. Microbiol. **132**:365–371.
23. **Roe, J. H., J. H. Epstein, and N. P. Goldstein.** 1949. A photometric method for the determination of inulin in plasma and urine. J. Biol. Chem. **178**:839–845.
24. **Sanderson, K. E., and J. R. Roth.** 1983. Linkage map of *Salmonella typhimurium*, edition VI. Microbiol. Rev. **47**:410–453.
25. **Sawers, G., and A. Böck.** 1988. Anaerobic regulation of pyruvate formate-lyase from *Escherichia coli* K-12. J. Bacteriol. **170**:5330–5336.
26. **Shaw, D. J., D. W. Rice, and J. R. Guest.** 1983. Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli*. J. Mol. Biol. **166**:241–247.
27. **Smith, M. W., and F. C. Neidhardt.** 1983. Proteins induced by anaerobiosis in *Escherichia coli*. J. Bacteriol. **154**:336–343.
28. **Strauch, K. L., J. B. Lenk, B. L. Gamble, and C. G. Miller.** 1985. Oxygen regulation in *Salmonella typhimurium*. J. Bacteriol. **161**:673–680.
29. **Unden, G., and A. Duchene.** 1987. On the role of cyclic AMP and the Fnr protein in *Escherichia coli* growing anaerobically. Arch. Microbiol. **147**:195–200.
30. **Unden, G., and J. R. Guest.** 1984. Cyclic AMP and anaerobic gene expression in *Escherichia coli*. FEBS Lett. **170**:321–325.
31. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **93**:237–244.
32. **Wimpenny, J. W. T., and A. Firth.** 1972. Levels of nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide in facultative bacteria and effect of oxygen. J. Bacteriol. **111**:24–32.
33. **Wong, K. K., and H. S. Kwan.** 1988. A novel mutation causing the deficiency in lactate production of *Salmonella typhimurium* during mixed acid fermentation, p. 293–300. *In* S. T. Chang, K. Y. Chan, and N. Y. S. Woo (ed.), Recent advances in applied biology and biotechnology. The Chinese University Press, Hong Kong.
34. **Yamamoto, N., and M. L. Droffner.** 1985. Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **83**: 2077–2081.