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Carbon Metabolism Regulates Expression of the *pfl* (Pyruvate Formate-Lyase) Gene in *Escherichia coli*

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The anaerobic expression of *pfl* is reduced both in a strain mutated in the *pgi* gene and in a *pfkA pfkB* double mutant strain when cells are grown in medium supplemented with glucose. When cells are grown in medium supplemented with either fructose or pyruvate, no reduction is observed in these strains. The amount of pyruvate in the cells may be responsible for the reduced expression of *pfl* in the strains mutated in the genes encoding the glycolytic enzymes. Because of the lowered oxygen concentration in the medium, the expression of *pfl* is induced when an exponentially growing culture enters the stationary phase. This induction is increased when the Casamino Acid concentration is raised 10-fold or when the medium is supplemented with NaCl. Superhelicity of DNA is decreased in a *pgi* mutant strain grown in medium supplemented with glucose. The superhelicity is also changed, but the opposite way, in a wild-type strain grown in medium supplemented with Casamino Acids at a high concentration or 0.3 M sodium chloride. Our data show that changes in superhelicity do not affect the aerobic expression of *pfl* but might be important for the anaerobic induction of *pfl*.

During growth under anaerobic conditions, *Escherichia coli* carries out a mixed acid fermentation and energy is obtained by substrate-level phosphorylation. In this mixed acid fermentation, pyruvate is reduced to acetyl coenzyme A and formate by the enzyme pyruvate formate-lyase (Pfl) (see reference 20 for a review). Studies of the enzyme have shown that it is present in an inactive form under aerobic conditions and that it is activated when oxygen no longer is present (see reference 21 for a review). Pfl is among the 18 identified polypeptides that reach their highest level during anaerobic growth (38).

The structural genes coding for Pfl (*pfl* gene) and pyruvate formate-lyase activase (Act; *act* gene) have been cloned (8) and sequenced, and their translation start sites have been determined (30, 33). Seven possible start points for transcription of *pfl* have been found. The abundance of all seven transcripts increases during anaerobic growth, and they are all reduced in an *fnr* mutant strain (33, 34).

Regulatory proteins seem to play a role in the anaerobic regulation of gene expression. All anaerobically induced genes that have been examined are regulated at the level of transcription (10). Studies on *E. coli* and *Salmonella typhimurium* have led to the theory that the proteins fumarate nitrate reductase regulator (Fnr, also known as NirR or OxrA) and OxrC (probably identical to the *pgi* gene product, the glycolytic enzyme phosphoglucose isomerase, Pgi) are responsible for the regulation of two large groups of anaerobically induced genes (18, 23, 28).

Fnr is a positive regulatory protein that is homologous to the cyclic AMP receptor protein Crp (13, 36, 37). Fnr has been shown to be essential for the expression of several anaerobic respiratory genes (7, 23, 28, 37, 40, 41). In contrast, OxrC has been proposed to regulate primarily the synthesis of enzymes with a fermentative or a biosynthetic role (17–19).

DNA supercoiling changes in response to oxygen tension, growth phase, and extracellular osmolarity (4, 14, 16). It has been suggested that changes in DNA supercoiling are responsible for the induction of at least some of the oxygen-regulated genes (29).

Recent experiments suggest that the altered expression of some anaerobically induced genes in cells carrying a *pgi* mutation is due to a change in superhelicity (29). In this report we show that the expression of *pfl* is dependent upon the phosphoglucose isomerase activity as well as the phosphofructokinase activity in the cell. We also show that the expression of the *pfl* operon during aerobic growth is unaffected by increases in osmolarity that have been shown to increase supercoiling.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Strain LJ134 carries a *pfl-lacZ* fusion integrated into the lambda attachment site of LJ24 (Fig. 1B) by the method of Atlung et al. (1a). A P1 lysate grown on strain LJ134 was used to introduce the *pfl-lacZ* fusion into TC3266, TC3340, TC3611, and LJ120 while selecting for kanamycine resistance (Table 1).

Construction of strain TC3264 carrying a defined *lac* deletion. The 12-kb *Pst*I fragment from lambda d*lac*, carrying the intact *lac* operon, the *lacI* gene, and 700 bp upstream, was cloned into the *Pst*I site of pUC8 (42). An *Mlu*I cutback derivative, pTAC3243, with a 3,376-bp deletion encompassing most of the *lacI* and *lacZ* genes, was constructed. The deleted *lac* fragment was recloned with *Hin*dIII and *Sal*I from the pUC8 multiple cloning site into the *Hin*dIII-*Sal*I sites in the *tet* gene of the *rep*(Ts) plasmid pSM491 (obtained from Søren Molin), giving rise to pTAC3258. Plasmid pSM 491 is a Tn*3* derivative of pPM103 (24). Plasmid pTAC3258 was transformed into strain C600 by selecting for ampicillin resistance at 30°C. The transformants were streaked onto plates containing LB medium (26) and 50 μg of ampicillin per ml and incubated at 42°C to select for integration of the plasmid into the chromosome. Four colonies were restreaked at 42°C, inoculated into LB medium containing 300

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype[a]	Reference or source
C600	*thi-1 thr-1 leu-6 lacY1 supE44 tonA21 rpsL rfbD1*	2
ECL323	*araD139* Δ(*argF-lac*)U169 *flbB5301 deoC1 rpsL150 relA1 fnr-1 zci*::Tn*10*	22
DF40	*pgi-2 tonA22 ompF627 relA1 pit-10 spoT1*	15
DF920	*pfkB20*::Tn*10*	12
AM1	*pfkA1 tonA22 ompF627 relA1 pit-10 spoT1*	27
GC2281	*malB*::Tn*9*	Richard D'Ari
TC3264	*thi-1 thr-1 leu-6 lacY1 lacIZ* Δ(*Mlu*) *supE44 tonA21 rpsL rfbD1*	This work
LJ24	*thi-1 leu-6 lacY1 lacIZ* Δ(*Mlu*) *supE44 tonA21 rpsL rfbD1*	P1 transductant of TC3264[b]
TC3266	*fnr-1 zci*::Tn*10*[c]	P1(ECL323) × TC3264[d]
TC3329	*malB*::Tn*9*[e]	P1(GC2281) × DF40
TC3340	*malB*::Tn*9 pgi-2*[c]	P1(TC3329) × TC3264[f]
TC3319	*argE*::Tn*10*[c]	P1 lysate on S11[g]
TC3342	*pfkA1*[c]	P1(AM1) × TC3319[f]
TC3611	*pfkA1 pfkB20*::Tn*10*[c]	P1(DF920) × TC3342
TC3609	*pfkB20*::Tn*10*[c]	P1(DF920) × TC3264
LJ120	*pgi-2 malB*::Tn*9 fnr-1 zci*::Tn*10*[c]	P1(TC3329) × TC3266
LJ134	*attB*::*pfl-lacZ23*[h]	This work (Fig. 1B)
LJ165	*fnr-1 zci*::Tn*10 attB*::*pfl-lacZ23*[c]	P1(LJ134) × TC3266
LJ166	*pgi-2 malB*::Tn*9 fnr-1 zci*::Tn*10 attB*::*pfl-lacZ23*[c]	P1(LJ134) × LJ120
LJ167	*pfkA1 pfkB*::Tn*10 attB*::*pfl-lacZ23*[c]	P1(LJ134) × TC3611
LJ197	*pgi-2 malB*::Tn*9 attB*::*pfl-lacZ23*[c]	P1(LJ134) × TC3340

[a] Genetic symbols are those of B. Bachmann (3).
[b] Strain TC3264 was transduced to Thr$^+$ with a P1 lysate grown on strain CM1793 (43).
[c] Genotype otherwise like that of TC3264.
[d] The introduction of *fnr-1* was tested by lack of growth on minimal-glycerol-fumarate medium under anaerobic conditions.
[e] Genotype otherwise like that of DF40.
[f] The introduction of *pgi-2* or *pfkA1* was tested as slow growth on glucose-minimal medium.
[g] A P1 lysate grown on strain S11 (1) was used to introduce *argE*::Tn*10*.
[h] Genotype otherwise like that of LJ24.

μg of ampicillin per ml, and grown to saturation at 30°C for excision of the plasmid. The cultures were diluted 1,000-fold in LB medium and then incubated for 6 h at 42°C to shut off replication of the plasmid and to allow the segregation of plasmid-free cells. The cultures were then diluted and plated for single colonies on LB containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Lac$^-$ colonies were tested for ampicillin sensitivity. This procedure reproducibly yielded strains with the desired phenotype at frequencies between 5 and 50%. One of the resultant strains was named TC3264.

Growth media. Cells were grown in either LB medium for all cloning experiments or AB minimal medium (9) supplemented with 1 μg of thiamine per ml, 0.5 or 5% Casamino Acids (Difco), and 2% glucose, 1% glucose 6-phosphate, 2% pyruvate, or 2% fructose as the carbon source. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. The indicator X-gal was added to plates at a concentration of 60 μg/ml.

Aerobic cultures were grown with 50 ml of medium in 250-ml flasks with vigorous shaking at 37°C unless otherwise stated. Anaerobic growth was achieved by bubbling nitrogen into culture flasks or by growing cells in filled capped flasks with gentle stirring in medium prebubbled with nitrogen. For all growth experiments, cultures were pregrown aerobically in the respective media for at least 10 generations, never reaching an A_{450} above 0.5. For anaerobic experiments, the aerobic preculture was diluted at least 10-fold into the nitrogen-prebubbled medium. The cells were grown for about two generations before the experiment was started. For all experiments, growth was monitored by the increase in A_{450} by using a Zeiss PMQ2 spectrophotometer. When the cell density was above 0.5, the samples were diluted appropriately before cell density was measured. Samples were taken at appropriate intervals for the determination of β-galactosidase activity.

DNA technology. Plasmid DNA was prepared as described by Birnboim and Doly (5). Restriction enzymes, T4 DNA ligase, *Bal*31 exonuclease, and the phosphorylated 8-bp *Bam*HI linker were used as recommended by the suppliers (New England BioLabs, Boehringer Mannheim, and Amersham International).

Enzyme assays. β-Galactosidase activity was determined as described by Miller (26) with toluene-permeabilized cells. Units of activity are expressed as described by Miller (26) with one modification: instead of correcting for the A_{550} of cell debris, the debris was removed by high-speed centrifugation.

Determination of changes in the level of supercoiling in the cells. Plasmid DNA was prepared by the method of Birnboim and Doly (5). Changes in the level of supercoiling were determined essentially as described by Ni Bhriain et al. (29). Plasmid topoisomers were separated by agarose gel electrophoresis on 0.7% agarose gels containing 15 or 25 μg of chloroquine per ml. At these chloroquine concentrations, the topoisomers that are more relaxed before electrophoresis migrate faster. Gels were run for at least 20 h at 50 V with TBE buffer (90 mM Tris [pH 8.3], 90 mM borate, 10 mM EDTA). Chloroquine was added to the running buffer at a concentration identical to that in the agarose gel. After electrophoresis, the chloroquine was removed by rinsing for 1 h in distilled water. DNA was transferred to GeneScreen membranes and visualized after blotting (39) with ^{35}S-dATP-labeled pBR322 (6) as a probe.

RESULTS

Effects of changes in the glycolytic activity on the expression of *pfl*. Strain LJ134 carries a *pfl-lacZ* fusion containing the

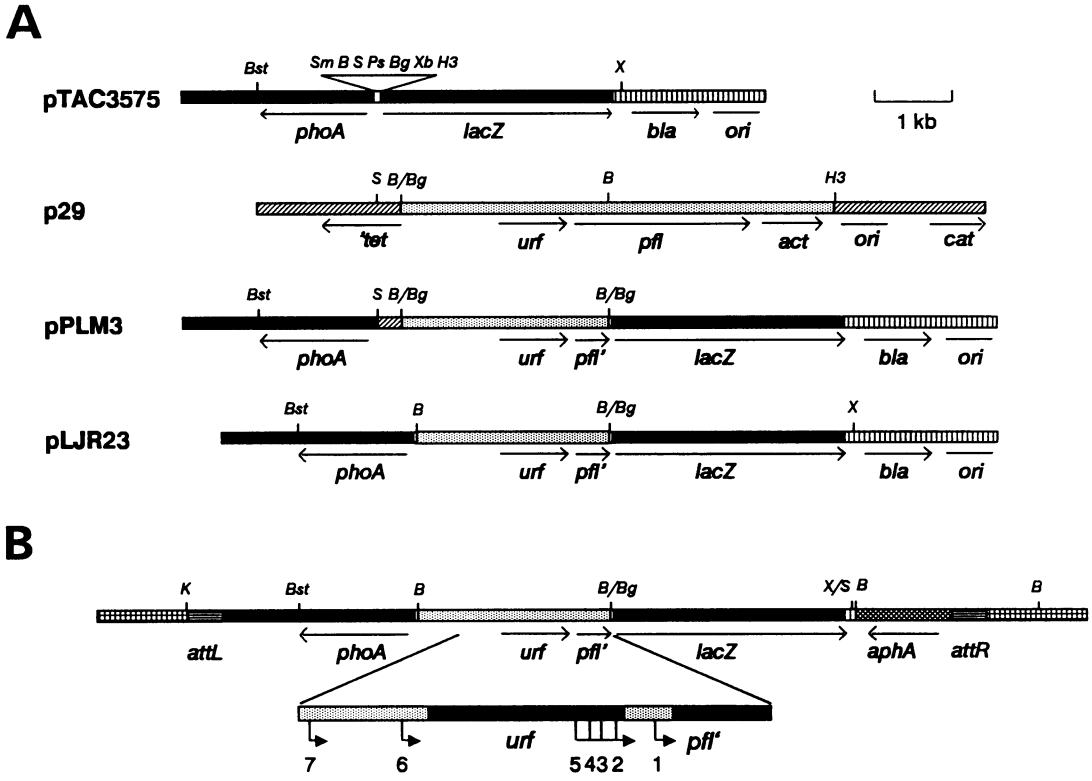

FIG. 1. Structure of plasmids and integrated *pfl-lacZ* fusion. (A) Plasmids pCB267 (35) and pTAC3575 (derivative of pCB267 with an *Xho*I linker inserted in the *Eco*O109 site between *lacZ* and *bla*) were used for construction of the *pfl-lacZ* transcriptional fusions. Plasmid pPLM3 was constructed by digesting plasmid p29 (8) with *Sal*I and *Bam*HI. The 2,600-bp *Bam*HI-*Sal*I fragment was cloned into *Sal*I-*Bgl*II-restricted pCB267. Plasmid pLJR23 was constructed by treating *Bam*HI-restricted pPLM3 with *Bal*31 exonuclease. A *Bam*HI linker was inserted, and inserts were recloned into *Bam*HI-*Hin*dIII-restricted pTAC3575. The insert (2,254 bp) from pLJR23 was cloned into the M13mp19 vector (25). The exact size of the deletion was determined by using the Sanger et al. sequencing technique (31). Symbols: ■, *phoA* and *lacZ* DNA; □, synthetic DNA; ▥, pBR322-derived DNA; ▨, pACYC184 DNA; ▩, *pfl* DNA; ▩, Tn*5*-derived DNA; ▦, DNA flanking *attB*. The location and extent of the various genetic loci are indicated by lines below the plasmid DNA and identified by the genetic symbol. The arrows indicate the directions of transcription. Restriction enzyme symbols: *B*, *Bam*HI; *Bg*, *Bgl*II; *Bst*, *Bst*EII; *H3*, *Hin*dIII; *Ps*, *Pst*I; *S*, *Sal*I; *Sm*, *Sma*I; *X*, *Xho*I; *Xb*, *Xba*I. (B) Integration of promoter fusion from pLJR23 into the *attB* site. This strain is denoted LJ134 (*attB*::*pfl-lacZ23*). The enlargement of the *pfl* promoter region shows the positions of the seven transcript start points according to Sawers and Böck (33).

entire regulatory region upstream of *pfl* (Fig. 1B). Strain LJ134 was used to search for growth conditions that affect synthesis of pyruvate formate-lyase. We wanted especially to identify the signals for anaerobic induction of *pfl*. We found a slight reduction in the expression of *pfl* in cells grown in medium supplemented with glucose when compared with that in cells grown in medium supplemented with fructose (Tables 2 and 3). The anaerobic induction of the *pfl* gene was of the same magnitude in both media. Expression of the *pfl-lacZ* fusion was induced 10- to 20-fold by growth under anaerobic conditions.

Introduction of an *fnr* mutation only reduced this induction by a factor of 3 (Table 2). Expression of *pfl* was also significantly reduced in the *pgi* mutant strain when it was grown anaerobically in minimal medium supplemented with glucose. The reduction was in the same range as that observed in the *fnr* mutant (Table 2). The expression of *pfl* in a *pgi fnr* double mutant strain was similar to the expression of the *fnr* mutant.

We investigated whether the Pgi protein is acting directly on the expression of *pfl*, thereby acting as an activator of transcription. We also investigated whether the reduction of *pfl* gene expression is caused by a lack of glycolytic activity or by an accumulation of glucose 6-phosphate, the substrate of the Pgi enzyme, in the cell (Fig. 2). Parallel cultures were grown in minimal medium containing either 2% glucose or 2% fructose as the carbon source (Table 2). The reduced *pfl* expression could be due to the absence of the Pgi enzyme per se. One would then expect a reduced *pfl* expression in a *pgi* mutant strain, independent of the carbon source. Alter-

TABLE 2. Effect of different regulatory mutations on *pfl-lacZ* expression[a]

Strain	Genotype	Sp act of β-galactosidase (U/ml × A_{450})			
		Aerobic		Anaerobic	
		Fructose	Glucose	Fructose	Glucose
LJ134	Wild type	0.4	0.2	7.0	5.7
LJ165	*fnr*	0.4	0.3	3.0	1.8
LJ166	*pgi fnr*	0.4	0.4	3.8	1.3
LJ167	*pfkA pfkB*	0.4	0.4	5.9	1.8
LJ197	*pgi*	0.3	0.4	7.2	1.9

[a] Cells were grown exponentially in minimal medium supplemented with 0.5% Casamino Acids, 50 μg of kanamycin per ml, and either 2% glucose or 2% fructose. The values are averages of at least two different experiments, each with at least five independent measurements.

TABLE 3. Anaerobic expression of the *pfl-lacZ* fusion as a function of the carbon source[a]

Strain	Genotype	Sp act of β-galactosidase (U/ml × A_{450})		
		Glucose	Glucose + pyruvate	Glucose 6-phosphate
LJ134	Wild type	5.0	6.5	7.5
LJ197	*pgi*	1.7	5.5	1.0

[a] Cells were grown exponentially under anaerobic conditions in minimal medium supplemented with 0.5% Casamino Acids, 50 μg of kanamycin per ml, and either 2% glucose, 2% glucose plus 2% pyruvate, or 1% glucose 6-phosphate. The values in the table are averages of at least two different experiments, each with at least five independent measurements.

natively, an accumulation of glucose 6-phosphate or a lack of glycolytic activity in the cell could be responsible for the drastic reduction in *pfl* gene expression. In this case one would expect a reduction of expression in the *pgi* mutant strain only in the glucose medium and an expression similar to that of the wild-type strain in the fructose medium. Expression of *pfl* in the *pgi* mutant strain, LJ197, was only reduced in medium supplemented with glucose (Table 2). This conclusion was confirmed by a similar experiment with strain LJ167, which is mutated in both of the genes (*pfkA* and *pfkB*) encoding the glycolytic enzyme phosphofructokinase, which catalyzes the phosphorylation of fructose 6-phosphate to fructose 1,6-diphosphate (Fig. 2). In this double mutant we observed a similar reduction of the expression of *pfl* in medium supplemented with glucose and no reduction in medium supplemented with fructose. We therefore conclude that it is not the Pgi protein that acts directly on *pfl* gene expression.

When the *pgi* mutant strain is grown in medium supplemented with both glucose and pyruvate, there is no reduction in the expression of *pfl* compared with that in the wild-type strain (Table 3). Presumably, the mutant strain accumulates glucose 6-phosphate intracellularly when grown

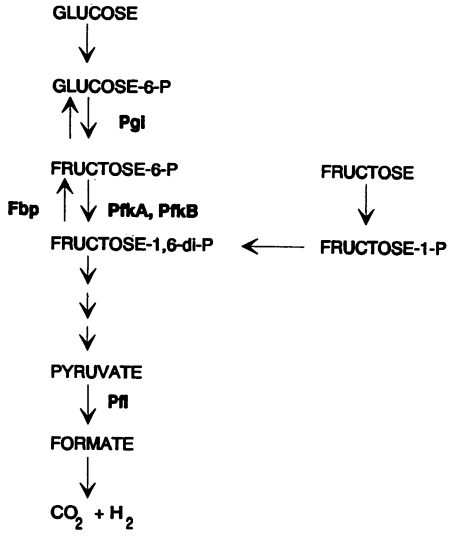

FIG. 2. Glycolysis and fermentation of pyruvate to yield formate and the further metabolization of formate to dihydrogen and carbon dioxide during anaerobic growth. The pathways are schematized. Pgi, phosphoglucose isomerase; PfkA and PfkB, phosphofructokinase; Fbp, fructose-1,6-bisphosphatase; Pfl, pyruvate formate-lyase.

on glucose as a carbon source. Therefore the reduced expression of *pfl* cannot be due to repression by glucose 6-phosphate but may be due to a decreased amount of pyruvate in the cells.

Effect of amino acids and sodium chloride on the transcription of *pfl*. We found that β-galactosidase synthesis was induced when an aerobically grown culture entered the stationary phase (Fig. 3). We were able to decrease the stationary-phase induction by increasing the shaking (Fig. 4A and B) and to increase it by decreasing the aeration (Fig. 4C and D). We therefore conclude that the stationary-phase induction of *pfl* is due to a lowered concentration of oxygen in the medium.

Under normal aeration conditions, high concentrations of Casamino Acids (5%) stimulated stationary-phase β-galactosidase synthesis five-fold compared with that in a culture with 0.5% Casamino Acids (Fig. 3). The induction is in the same range as that observed for the induction of *pfl* expression by shifting to anaerobic growth (Table 2) (32). The Casamino Acids used in these experiments contain a high concentration of NaCl. Medium supplemented with 5% Casamino Acids has a concentration of NaCl of 0.3 M, and it is therefore possible that the induction of *pfl* was stimulated by the increased concentration of NaCl. To test this possibility, we added NaCl to a final concentration of 0.25 M to a culture supplemented with 0.5% Casamino Acids. The final concentration of NaCl in this medium was 0.28 M. Expression of *pfl* in the presence of 0.25 M NaCl was as high as that in the presence of 5% Casamino Acids (Fig. 3). The data indicate that an increased concentration of NaCl stimulates the stationary-phase induction of *pfl*.

We wanted to test whether the addition of NaCl to the medium only affects the stationary-phase induction of *pfl* or whether it enhances the expression of *pfl* in general. To test this possibility we added NaCl to final concentrations of 0.2, 0.3, and 0.4 M to cultures growing in minimal medium supplemented with 0.5% Casamino Acids. The increased NaCl concentration had no effect on the expression of *pfl* either aerobically or anaerobically (Table 4). We conclude that the anaerobic induction of *pfl* is affected by changes in sodium chloride concentration only under the semianaerobic conditions obtained upon entering the stationary phase.

Superhelical density of plasmid DNA in a wild-type strain and a *pgi* mutant strain under different growth conditions. Plasmid topoisomers were separated by agarose gel electrophoresis on an 0.7% agarose gel containing 25 μg of chloroquine per ml. At this chloroquine concentration, the topoisomers that are more relaxed before electrophoresis migrate faster. When the Casamino Acids concentration was raised from 0.5% to 5%, an increase in the level of supercoiling was observed (Fig. 5, lanes A and D). The level of supercoiling was increased to the same extent as that in medium supplemented with 0.5% Casamino Acids and either 0.3 M NaCl or 2% pyruvate (lanes B and C). Anaerobiosis increased the level of supercoiling (lanes A and I), although to a lesser extent than did increased osmolarity (lane B) and the presence of pyruvate (lanes C and J). However, an increase in the level of supercoiling is not sufficient for induction of *pfl* when the environment is aerobic (Table 4).

The lack of induction in glycolytic mutants could be due to an altered level of supercoiling. We therefore determined the level of supercoiling of plasmid pBR322 in the *pgi* mutant strain. Under aerobic conditions, we observed no difference in the level of supercoiling compared with that of the wild-type strain (Fig. 5, lanes A and F). The level of supercoiling is decreased anaerobically in a *pgi* mutant strain

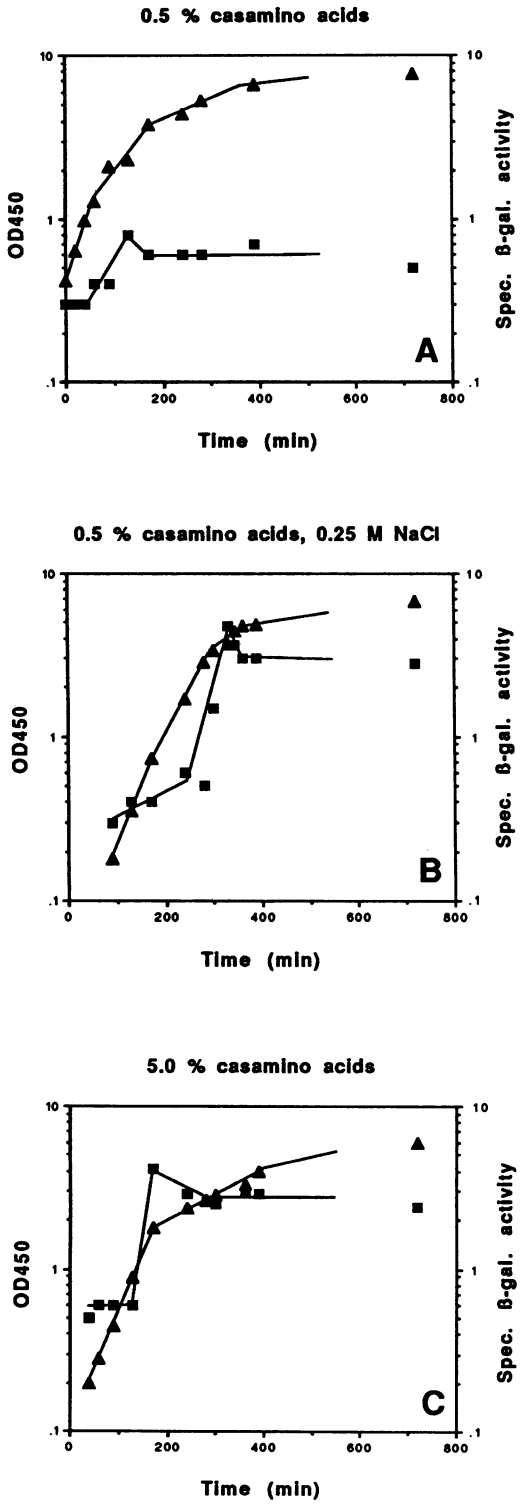

FIG. 3. Induction of *pfl* expression in high-osmolarity medium. Strain LJ134 was grown in minimal medium supplemented with 2% glucose, 50 μg of kanamycin per ml, and either (A) 0.5% Casamino Acids, (B) 0.5% Casamino Acids and 0.25 M NaCl, or (C) 5% Casamino Acids. Parallel 50-ml cultures were grown in 250-ml flasks with shaking at 130 rpm. Cultures were pregrown as described in Materials and Methods. Samples were taken at intervals for determination of β-galactosidase activity. Symbols: ▲, cell density; ■, β-galactosidase specific activity. The last value in each panel indicates a measurement after incubation overnight.

when cells are grown in medium supplemented with glucose. No difference in the level of supercoiling is observed when plasmid is isolated from cultures grown in minimal medium supplemented with fructose (lanes K and N). Under both aerobic and anaerobic conditions, an increase in the level of supercoiling was observed when cells were grown in medium supplemented with pyruvate (lanes A, C, I, and J).

The experiment shown in Fig. 5 was repeated with a lower chloroquine concentration (15 μg/ml). We found exactly the same pattern of the distribution of the topoisomers as on the 25-μg/ml chloroquine gel shown in Fig. 5, but the decreased chloroquine concentration gave a decreased mobility of the topoisomers (data not shown). This decrease in the mobility of the topoisomers in the presence of a decreased level of the unwinding ligand is due to the introduction of fewer positive supercoils into the DNA.

We suggest that the reduced anaerobic expression of *pfl* in the *pgi* mutant strain might be due to the decrease in supercoiling of DNA caused by a lack of glycolytic activity.

DISCUSSION

In this study we examined the regulation of the *pfl* operon by focusing on the signals that are important for anaerobic induction.

The normal 10- to 20-fold anaerobic induction was reduced 3-fold in *fnr*, *pgi*, and *pfkA pfkB* mutants (Table 2). The reduction in the *fnr* mutant was independent of the carbon source, whereas the reduction in the two mutants blocked in glycolysis was only observed with glucose as the carbon source. Anaerobic induction was normal with fructose, which both mutants can utilize as a carbon source, indicating that the reduction is due to a block in glycolysis. The anaerobic induction in glucose medium could be fully restored by the addition of pyruvate, which has been shown to induce *pfl* expression (32).

We have shown that the level of supercoiling of a plasmid is decreased in a *pgi* mutant strain. This is probably due to carbon starvation of the cells, which has been shown to decrease DNA supercoiling (4). The level of supercoiling is also decreased in the *pfkA pfkB* double mutant strain. Plasmid DNA isolated from *pfkA* or *pfkB* single mutant strains showed no change in the superhelicity of the DNA, and the expression of *pfl* was unaffected in these strains (29a). The reduced expression of *pfl* in the *pgi* and *pfkA pfkB* mutant strains might be due to the decreased superhelicity of the DNA.

The superhelicities of the plasmid and the chromosome could be different. All of the experiments shown in Table 2 have also been carried out with the *pfl-lacZ* fusion plasmid pPLM3 (Fig. 1A). We found no difference in the regulation of *pfl* expression compaired with that of the integrated *pfl-lacZ* fusion (29a). We investigated whether the regulation of the *pfl* expression was affected by the location of the promoter fusion by repeating all of the experiments shown in Tables 2 and 4 with a strain containing the same *pfl-lacZ* fusion as LJ134 integrated into the *pfl* locus at 20 min. We found a higher expression of the *lacZ* gene, but the regulation of expression was unaffected (29a).

An increase in the level of supercoiling has been proposed to be a signal for anaerobic induction (29). In this report we have shown that the expression of *pfl* under aerobic and fully anaerobic conditions is unaffected by an increase in the superhelicity of the DNA caused by altering the osmolarity. An increase in osmolarity, however, increases the anaerobic induction of *pfl* under the semianaerobic conditions that

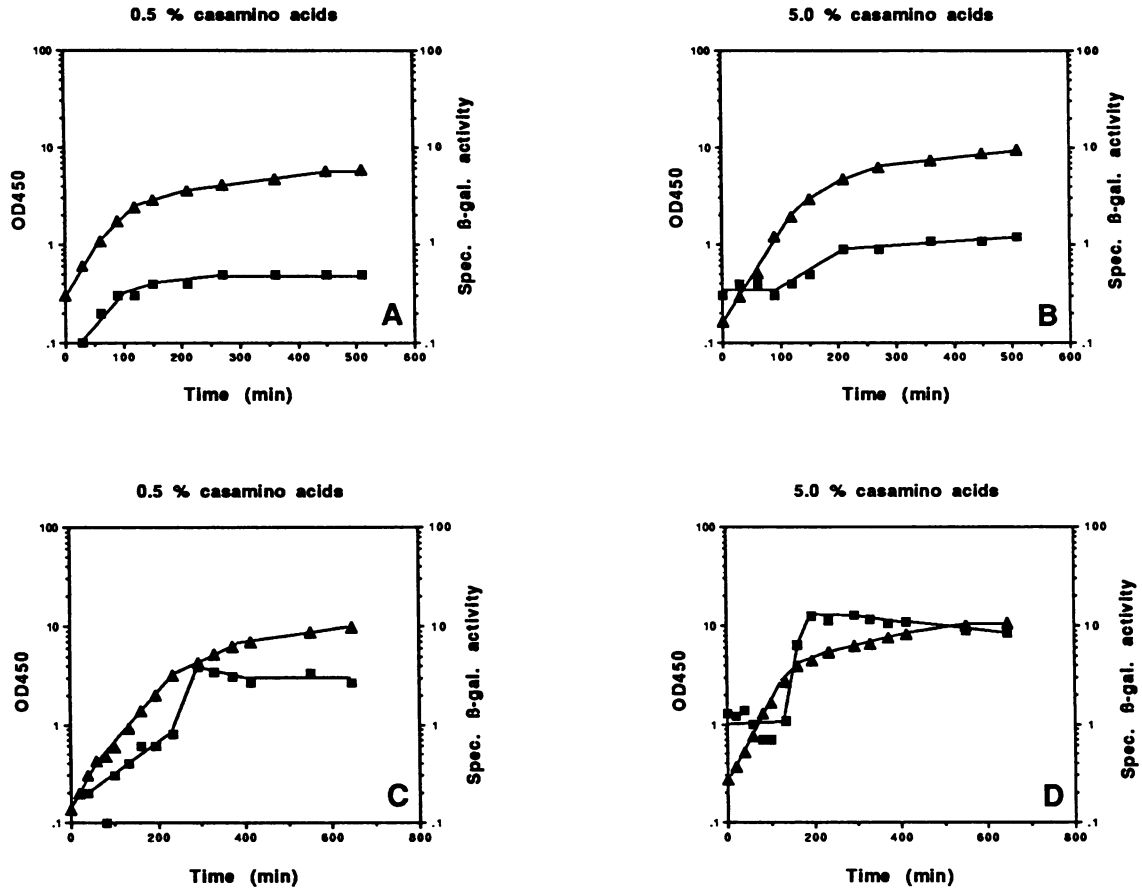

FIG. 4. Stationary-phase induction of *pfl* expression. Strain LJ134 was grown to the stationary phase in minimal medium supplemented with 2% glucose, 50 μg of kanamycin per ml, and either 0.5% (A and C) or 5% (B and D) Casamino Acids. (A and B) Parallel cultures of 50 ml were grown in 250-ml flasks with shaking at 160 rpm. (C and D) Parallel cultures of 75 ml were grown in 250-ml flasks with shaking at 120 rpm. Cultures were pregrown as described in Materials and Methods. Samples were taken at intervals for determination of β-galactosidase activity. Symbols: ▲, cell density; ■, β-galactosidase specific activity.

occur upon entry into the stationary phase. The addition of NaCl to the medium might increase the intracellular pyruvate concentration by increasing the glutamate concentration (see reference 11 for a review). We therefore cannot exclude the possibility that the increased anaerobic induction of *pfl* in high-osmolarity medium is due to an increased intracellular pyruvate concentration, since we found that a high external concentration of pyruvate increases the supercoiling per se.

TABLE 4. Effects of medium supplements on anaerobic induction of *pfl-lacZ* fusion[a]

Supplement(s)		Sp act of β-galactosidase (U/ml × A_{450})	
Casamino Acids (%)	NaCl (M)	Aerobic	Anaerobic
0.5	None	0.3	5.2
5	None	0.5	6.7
0.5	0.2	0.5	5.0
0.5	0.3	0.5	4.3
0.5	0.4	0.4	3.0

[a] Strain LJ134 was grown exponentially in minimal medium supplemented with 2% glucose, 50 μg of kanamycin per ml, and Casamino Acids and/or NaCl at the concentrations indicated. The values of β-galactosidase specific activity in the table are averages of at least two different experiments, each with at least five independent measurements.

The expression of *pfl* is the same in the *pgi* and the *fnr* mutant strains when cells are grown anaerobically in medium supplemented with glucose. When the expression of *pfl* was determined in a *pgi fnr* double mutant strain, no further reduction was observed (Table 2). The data presented in this report show that carbon metabolism plays a key role in the anaerobic expression of *pfl*. This regulation might be mediated by the ability of the Fnr protein to bind the DNA. When DNA supercoiling is reduced, the Fnr protein may no longer be able to bind and activate transcription of the *pfl* operon. This interpretation is further confirmed by the increased stationary-phase induction of *pfl* expression when sodium chloride is added to the growth medium. Furthermore, Sawers and Böck (33) showed that the addition of pyruvate to a culture growing in glucose medium induced the transcription of *pfl* slightly. In this report we showed that the supercoiling of DNA is increased in the presence of pyruvate at a high concentration. This increase in the level of DNA supercoiling might favor the binding of the Fnr protein.

The regulatory region upstream of *pfl* has been described as extremely unusual in that it might contain several promoters, all anaerobically induced (33, 34). A deletion analysis of the *pfl* regulatory region indicates a high degree of cooper-

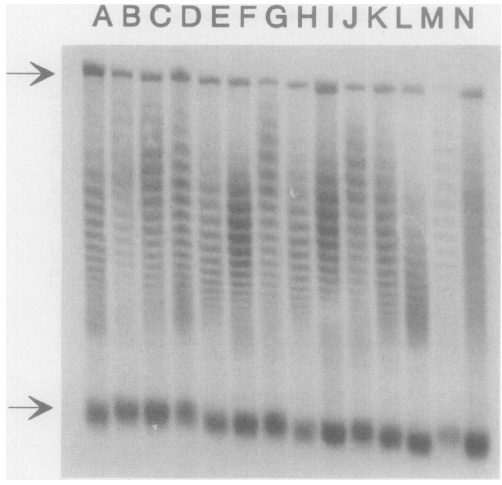

FIG. 5. Effect of *pgi* mutation, carbon source, and NaCl on DNA supercoiling. LJ24 (wild type) and TC3340 (*pgi*) were transformed with pBR322. Plasmid DNA was isolated from cells grown exponentially in minimal medium supplemented with 100 μg of ampicillin per ml and 0.5% Casamino Acids except in the experiment shown in lane D. Lanes: A, LJ24 grown aerobically with glucose; B, LJ24 grown aerobically with glucose and 0.3 M NaCl; C, LJ24 grown aerobically with pyruvate; D, LJ24 grown aerobically with glucose and 5% Casamino Acids; E, LJ24 grown aerobically with fructose; F, TC3340 grown aerobically with glucose; G, TC3340 grown aerobically with pyruvate; H, TC3340 grown aerobically with fructose; I, LJ24 grown anaerobically with glucose; J, LJ24 grown anaerobically with pyruvate; K, LJ24 grown anaerobically with fructose; L, TC3340 grown anaerobically with glucose; M, TC3340 grown anaerobically with pyruvate; N, TC3340 grown anaerobically with fructose. The gel contains chloroquine at a concentration of 25 μg/ml. The direction of migration is from top to bottom. The upper arrow indicates the more supercoiled DNA, and the lower arrow indicates the more relaxed DNA.

ativity of the promoters. Removal of the area containing transcript start sites 7, 6, and 5 or the area containing transcript start sites 4, 3, 2, and 1 resulted in reduced activity of the remaining transcripts (29a). Furthermore, investigations of the DNA in the upstream promoter region have shown that it contains a region exhibiting conformational changes, also known as a bent DNA region (29a). This suggests that long-distance regulation is involved in the regulation of *pfl*. Changing the superhelicity of the DNA in a certain way could disrupt such regulation.

Our results strongly suggest that there is more than one signal for regulation of the anaerobic induction of *pfl*, since there is a residual significant induction (two- to threefold) in *pgi*, *fnr*, and *pgi fnr* mutants.

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REFERENCES

1. **Atlung, T.** 1984. Allele-specific suppression of *dnaA* (Ts) mutations by *rpoB* mutations in *Escherichia coli*. Mol. Gen. Genet. **197**:125–128.
1a. **Atlung, T., A. Nielsen, L. J. Rasmussen, L. J. Nellemann, and F. Holm.** A versatile method for integration of genes and gene fusions into the λ attachment site of *Escherichia coli*. Gene, in press.
2. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
3. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12. Microbiol. Rev. **54**:130–197.
4. **Balke, V. L., and J. D. Gralla.** 1987. Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli*. J. Bacteriol. **169**:4499–4506.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Cloning and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
7. **Chippaux, M., D. Giudici, A. Abou-Jaoude, F. Casse, and M. C. Pascal.** 1978. A mutation leading to total lack of nitrite reductase activity in *Escherichia coli* K-12. Mol. Gen. Genet. **160**:225–229.
8. **Christiansen, L., and S. Pedersen.** 1981. Cloning, restriction endonuclease mapping and post-transcriptional regulation of *rpsA*, the structural gene for ribosomal protein S1. Mol. Gen. Genet. **181**:548–551.
9. **Clark, D. J., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23**:99–112.
10. **Clark, D. P.** 1984. The number of anaerobically regulated genes in *Escherichia coli*. FEMS Microbiol. Lett. **24**:251–254.
11. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**:121–147.
12. **Daldal, F., and D. G. Fraenkel.** 1981. Tn*10* insertions in the *pfkB* region of *Escherichia coli*. J. Bacteriol. **147**:935–943.
13. **De Crombrugghe, B., S. Busby, and H. Buc.** 1984. Cyclic AMP receptor protein: role in transcription activation. Science **224**: 831–838.
14. **Dorman, C. J., G. C. Barr, N. Ni Bhriain, and C. F. Higgins.** 1988. DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. J. Bacteriol. **170**:2816–2826.
15. **Fraenkel, D. G., and S. R. Levisohn.** 1967. Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase. J. Bacteriol. **93**:1571–1578.
16. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddel, I. R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. Cell **52**:569–584.
17. **Jamieson, D. J., and C. F. Higgins.** 1984. Anaerobic regulation of a peptide transport gene in *Salmonella typhimurium*. J. Bacteriol. **160**:131–136.
18. **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.
19. **Jamieson, D. J., R. G. Sawers, P. A. Rugman, D. H. Boxer, and C. F. Higgins.** 1986. Effects of anaerobic regulatory mutations and catabolite repression on regulation of hydrogen metabolism and hydrogenase isoenzyme composition in *Salmonella typhimurium*. J. Bacteriol. **168**:405–411.
20. **Knappe, J.** 1987. Anaerobic dissimilation of pyruvate, p. 151–155. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella thyphimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
21. **Knappe, J., and G. Sawers.** 1990. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. FEMS Microbiol. Rev. **75**:383–398.
22. **Kuritzkes, D. R., X.-Y. Zhang, and E. C. C. Lin.** 1984. Use of ϕ(*glp-lac*) in studies of respiratory regulation of the *Escherichia coli* anaerobic *sn*-glycerol-3-phosphate dehydrogenase genes (*glpAB*). J. Bacteriol. **157**:591–598.
23. **Lambden, P. R., and J. R. Guest.** 1976. Mutants of *Escherichia coli* K-12 unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. **97**:145–160.

24. **Meacock, P. A., and S. N. Cohen.** 1979. Genetic analysis of the interrelationship between plasmid replication and incompatibility. Mol. Gen. Genet. **174:**135–147.
25. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19:**269–276.
26. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Morrissey, A. T. E., and D. G. Fraenkel.** 1969. Chromosomal location of a gene for fructose-6-phosphate kinase in *Escherichia coli*. J. Bacteriol. **100:**1108–1109.
28. **Newman, B. M., and J. A. Cole.** 1978. The chromosomal location and pleiotropic effects of mutation of the *nirA*$^+$ gene of *Escherichia coli* K-12: the essential role of *nirA*$^+$ in nitrite reduction and in other anaerobic redox reactions. J. Gen. Microbiol. **106:**1–12.
29. **Ni Bhriain, N., C. J. Dorman, and C. F. Higgins.** 1989. An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. **3:**933–942.
29a. **Rasmussen, L. J.** 1991. Ph.D. thesis. The Technical University of Denmark, Lyngby, Copenhagen.
30. **Rödel, W., W. Plaga, R. Frank, and J. Knappe.** 1988. Primary structures of *Escherichia coli* pyruvate formate-lyase and pyruvate formate-lyase activating enzyme deduced from the DNA nucleotide sequences. Eur. J. Biochem. **177:**153–158.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Sawers, G., and A. Böck.** 1988. Anaerobic regulation of pyruvate formate-lyase from *Escherichia coli* K-12. J. Bacteriol. **170:**5330–5336.
33. **Sawers, G., and A. Böck.** 1989. Novel transcriptional control of the pyruvate formate-lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. J. Bacteriol. **171:**2485–2498.
34. **Sawers, G., A. F. V. Wagner, and A. Böck.** 1989. Transcription initiation at multiple promoters of the *pfl* gene by Eσ70-dependent transcription in vitro and heterologous expression in *Pseudomonas putida* in vivo. J. Bacteriol. **171:**4930–4937.
35. **Schneider, K., and C. F. Beck.** 1986. Promoter probe vectors for the analysis of divergently arranged promoters. Gene **42:**37–48.
36. **Shaw, D. J., and J. R. Guest.** 1982. Nucleotide sequence of the *fnr* gene and primary structure of the Fnr protein of *Escherichia coli*. Nucleic Acids Res. **10:**6119–6130.
37. **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.
38. **Smith, M. W., and F. C. Neidhardt.** 1983. Proteins induced by anaerobiosis in *Escherichia coli*. J. Bacteriol. **154:**336–343.
39. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
40. **Spiro, S., and J. R. Guest.** 1987. Activation of the *lac* operon of *Escherichia coli* by a mutant Fnr protein. Mol. Microbiol. **1:**53–58.
41. **Unden, G., and J. R. Guest.** 1985. Isolation and characterization of the Fnr protein, the transcriptional regulator of anaerobic electron transport in *Escherichia coli*. Eur. J. Biochem. **146:** 193–199.
42. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
43. **von Meyenburg, K., and F. G. Hansen.** 1980. The origin of replication, *oriC*, of the *Escherichia coli* chromosome: genes near *oriC* and construction of *oriC* deletion mutations. ICN-UCLA Symp. Mol. Cell. Biol. **19:**137–159.