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Nitrate Repression of the *Escherichia coli pfl* Operon Is Mediated by the Dual Sensors NarQ and NarX and the Dual Regulators NarL and NarP

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The *pfl* operon is expressed at high levels anaerobically. Growth of *Escherichia coli* in the presence of nitrate or nitrite led to a 45% decrease in expression when cells were cultivated in rich medium. Nitrate repression, however, was significantly enhanced (sevenfold) when the cells were cultured in minimal medium. Regulation of *pfl* expression by nitrate was dependent on the NarL, NarP, NarQ, and NarX proteins but independent of FNR, ArcA, and integration host factor, which are additional regulators of *pfl* expression. Strains unable to synthesize any one of the NarL, NarP, NarQ, or NarX proteins, but retaining the capacity to synthesize the remaining three, exhibited essentially normal nitrate regulation. In contrast, *narL narP* and *narX narQ* double null mutants were devoid of nitrate regulation when cultured in rich medium but they retained some nitrate repression (1.3-fold) when grown in minimal medium. By using *lacZ* fusions, it was possible to localize the DNA sequences required to mediate nitrate repression to the *pfl* promoter-regulatory region. DNase I footprinting studies identified five potential binding sites for the wild-type NarL protein in the *pfl* promoter-regulatory region. Specific footprints were obtained only when NarL was phosphorylated with acetyl phosphate before the binding reaction was performed. Each of the protected regions contained at least one heptamer sequence which has been deduced from mutagenesis studies to be essential for NarL binding (K. Tyson, A. Bell, J. Cole, and S. Busby, Mol. Microbiol. 7:151–157, 1993).

Nitrate respiration is an efficient means of energy generation for *Escherichia coli*. The use of this electron acceptor naturally requires that a particular complement of enzymes is synthesized. Included in this set of enzymes is a respiratory pathway comprising a formate dehydrogenase termed FDH-N (encoded by the *fdnGHI* operon) and a nitrate reductase (encoded by *narGHJI*). The coordinate synthesis of these enzymes occurs only anaerobically and when nitrate is available (33). The presence of nitrate also reduces or prevents the expression of a number of genes, e.g., the *frdABCD* operon encoding fumarate reductase and the *dmsABC* operon encoding dimethylsulfoxide reductase, whose products either are not required during nitrate respiration or are required but in much reduced amounts. A complex system of proteins that is involved in sensing nitrate in the environment and transmitting its presence to the level of gene expression has been discovered (33). The proteins include two sensor-regulator pairs termed NarX-NarL and NarQ-NarP of which NarX and NarQ are membrane-associated histidine kinases and NarL and NarP are transcription regulators (3, 7, 14, 20, 21, 28, 35, 39). The NarQ and NarX proteins are not only capable of sensing nitrate but they are also responsive to nitrite. Mutational studies have demonstrated that either NarX or NarQ is capable of modulating the DNA-binding activity of the NarL and NarP proteins in response to nitrate and nitrite (for a detailed review, see reference 33). The various target operons regulated by nitrate appear to have different specificities for NarL and NarP, and this provides a means of fine-tuning gene expression. Thus, for example, the *narGHJI* operon is regulated exclusively by NarL and is not responsive to NarP, while nitrate induction of the *fdnGHI* operon is controlled mainly by NarL but is also responsive in part to NarP (21).

Recent in vitro studies have demonstrated that a constitutive mutant variant of NarL (NarL V88A) binds to multiple sites in the regulatory regions of several operons, including *narGHJI*, *fdnGHI*, *narK*, and *frdABCD* (14). It was possible to show that NarL (V88A) only bound to specific sites on the DNA in its phosphorylated form. The findings of that study, together with those derived from detailed mutagenesis experiments, have identified a sequence motif (TACYNMT, where Y = C or T, M = A or C, and N = any nucleotide) that is recognized by the NarL and NarP proteins.

Another gene that has been shown to be regulated by nitrate is that encoding the enzyme pyruvate formate-lyase (PFL) (25). PFL is a key enzyme of anaerobic metabolism catalyzing the nonoxidative cleavage of pyruvate to acetyl coenzyme A and formate (11). Formate is the substrate of the FDH-N enzyme and is consequently a major electron donor for nitrate respiration. The *pfl* gene forms part of an operon that is regulated in response to anaerobiosis and is transcribed from seven promoters (24–27, 37). The transcription factors FNR and ArcA modulate anaerobic induction of expression. The regulation of *pfl* by nitrate proved to be somewhat surprising, since nitrate represses expression and consequently reduces the concentration of an electron donor (formate) for nitrate respiration. Examination of the *pfl* regulatory region reveals the presence of several hexamer sequences that conform to the NarL recognition sequence. The aim of this study therefore was to perform a detailed characterization of nitrate regulation of the *pfl* operon and to try to elucidate its physiological relevance with regard to anaerobic nitrate respiration.

MATERIALS AND METHODS

Bacterial strains, vectors, and growth conditions. The bacterial strains, plasmids and phages used in this study are listed in Table 1. Strains were grown either

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Characteristics	Reference or source
Strains		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 ptsF25 deoC1 relA1 flbB530 rpsL150* λ$^-$	2
FM420	MC4100 *recA56*	42
RM3132	As MC4100 but *arcA2*zjj::Tn*10*	This work
RM600	As MC4100 but *narL215*::Tn*10*	This work
RM605	As MC4100 but Δ*narX242 zch-2084*::W-Cm *narQ251*::Tn*10d* (Tc)	This work
RM610	As MC4100 but *narP253*::Tn*10d* (Cm)	This work
RM611	As MC4100 but *narP253*::Tn*10d* (Cm) *narL215*::Tn*10*	This work
VJS4322	RK4353 but Δ(*narXL*)*235 narP253*::Tn*10d* (Cm)	V. Stewart
VJS3051	As RK4353 but Δ*narX242 zch-2084*::Ω-Cm *narQ251*::Tn*10d* (Tc) *fdnG108*::MudJ	20
RK5268	*nar205*::Tn*10* (NarG$^-$)	34
RK5278	As RK4353 but *narL215*::Tn*10*	34
ECL618	F′ *arcA2* zjj::Tn*10*	E. C. C. Lin
RM123	FM420, λRM123	25
RM5268	RK5268, λRM123	This work
RM381	FM420, λRM12321	30
RM364	RM2321, λRM123	This work
RM2314	FM420, λRM123, FNR 6	24
RM2315	FM420, λRM123, FNR 7	24
RM2316	FM420, λRM123, FNR 6 + 7	24
BL21	*E. coli* B; F′ *hsdS gal1* DE3	36
Plasmids		
pRS551	Km^r Ap^r *lacZ*$^+$ *lacY*$^+$ *lacA*$^+$	29
pRS552	Km^r Ap^r *lacZ′ lacY*$^+$ *lacA*$^+$	29
pRM123	pRS552 F(*pfl′-′lacZ*) 1397 (Hyb)	25
pRM2321	pRM123 but mutated in the IHF-binding site	30
pRM123/FNR6	pRM123 carrying the FNR-1 mutation in the *pfl* regulatory region	24
pRM123/FNR7	pRM123 carrying the FNR-2 mutation in the *pfl* regulatory region	24
pRM123/FNR6+7	pRM123 carrying the FNR-1 and FNR-2 mutation in the *pfl* regulatory region	24
pSN15	Ap^r *Bam*HI bp −414 to +34 of *pfl* regulatory region in pUC19	30
pSN20	Ap^r *Bam*HI bp −457 to +83 of *pfl* regulatory region in pUC18	30
pSN25	Ap^r *Bam*HI bp −287 to +34 of *pfl* regulatory region in pUC19	This work
pUNarL1	pUC19 *narL*$^+$	This work
pUC19	Ap^r *lacIPOZ*$^+$	41
pTK200	pRS551 Φ(*pfl′-lacZ*) carrying 531 bp from −457 to +83 of *pfl* regulatory region	This work
pRM1001	pRS551 Φ(*pfl′-lacZ*) carrying 161 bp from −457 to −294 of *pfl* regulatory region	N. Drapal
pRM20201	pRS551 Φ(*pfl′-lacZ*) carrying 118 bp from −81 to +37 of *pfl* regulatory region	This work
Phages		
λRS45	*lacZ lacY*$^+$ *imm21 ind*$^+$	29
λRM123	λRS45 Φ(*pfl′-′lacZ*) 1397 (Hyb)	25
λRM12321	λRM123 but mutated in the IHF-binding site	30
λRM123/FNR6	λRM123 carrying the FNR-1 mutation in the *pfl* regulatory region	24
λRM123/FNR7	λRM123 carrying the FNR-2 mutation in the *pfl* regulatory region	24
λRM123/FNR6	λRM123 carrying the FNR-1 and FNR-2 mutation in the *pfl* regulatory region	24
λRM1001	λRS45 Φ(*pfl′-lacZ*) carrying 161 bp from −457 to +83 of *pfl* regulatory region	N. Drapal
λTK200	λRS45 Φ(*pfl′-lacZ*) carrying 531 bp from −457 to −294 of *pfl* regulatory region	This work
λRM20201	λRS45 Φ(*pfl′-lacZ*) carrying 531 bp from −81 to +37 of *pfl* regulatory region	This work

in Luria-Bertani medium (19), buffered rich medium (10 g of Bacto Tryptone, 5 g of yeast extract, 100 mM potassium phosphate [pH 6.5]) according to the methods of Begg et al. (1), or in Werkman minimal medium (which consisted of 50 mM disodium hydrogen phosphate, 100 mM potassium dihydrogen phosphate, 15 mM ammonium sulfate, 1 mM magnesium sulfate, 100 μM calcium chloride, 10 μM sodium selenite, and 10 μM sodium molybdate, pH 6.5). Glucose (20 mM in rich medium and 40 mM in minimal medium) was used as the carbon source in all growth experiments except when cells were grown aerobically in rich medium. Potassium nitrate was added to 40 mM, and sodium nitrite was added to a final concentration of 5 mM. Antibiotics were added at the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 15 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 15 μg/ml. Media and buffers for lambda work were prepared as described previously (26).

Plasmid and strain constructions. Strains RM3132, RM600, RM605, RM610, and RM611 were constructed by P1 kc-mediated transduction by the method of Miller (19), with the appropriate strains used as donors (Table 1).

Plasmid pSN25 was constructed by isolating the *Ase*I-*Alu*I fragment from p29 (22), and after filling in of the protruding ends with the Klenow enzyme (17), *Bam*HI linkers were added and the resulting fragment was cloned into the *Bam*HI site of pUC19 (41).

Plasmid pUNarL1, which was used to overproduce the NarL protein, was constructed as follows. A 694-bp fragment containing the complete *narL* gene (35) was amplified from the chromosome of strain FM420 by PCR with the following oligonucleotides: Nar1, 5′-GGGGATCCAAGGAGATACCCATG AG-3′; and Nar2, 5′-GGGGATCCAAACGACGAACTGCGCTG-3′. After *Bam*HI restriction, the resulting fragment was cloned into the *Bam*HI site of pUC19 to yield plasmid pUNarL1. The authenticity of the *narL* gene was determined by DNA sequencing. The 163- and 118-bp DNA fragments depicted in Fig. 1 which were used to construct λRM1001 and λRM20201, respectively, were amplified from p29 (22) by PCR and appropriately designed primers which incorporated a *Bam*HI restriction site at the 5′ and 3′ ends of the resulting amplified product. Subsequent to amplification, the fragments were restricted with *Bam*HI and cloned into the *Bam*HI site of pRS551 (29). The authenticity of the amplified fragments was determined by DNA sequencing.

β-Galactosidase assays. β-Galactosidase activity was assayed in cultures of exponentially growing cells, and the specific activity was calculated as described by Miller (19). The values reported are the averages of at least three independent experiments performed in triplicate. The standard error of the values reported was not more than 15%.

Overproduction and purification of NarL. For production of NarL protein,

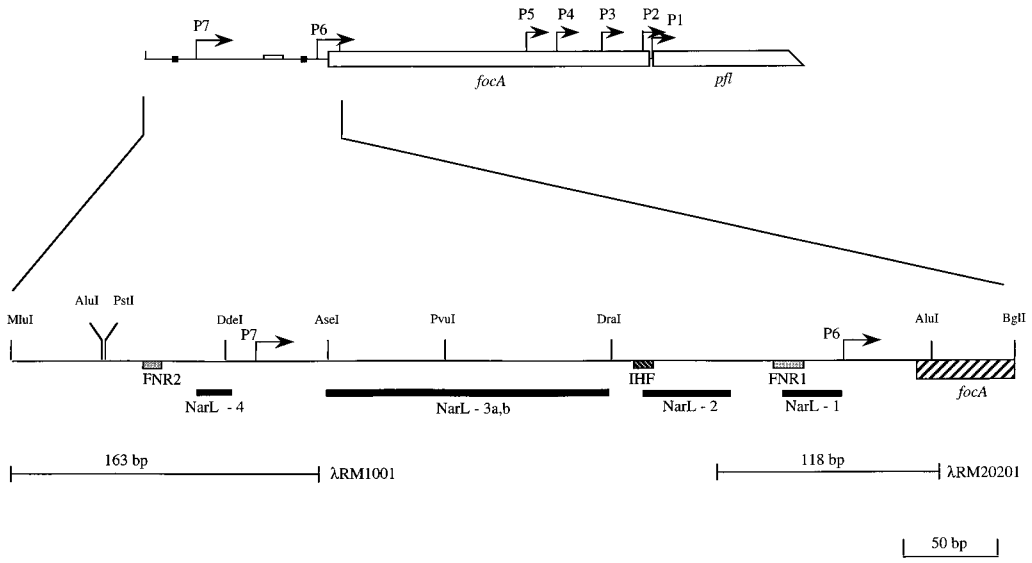

FIG. 1. Schematic representation of the regulatory region of the *pfl* operon. The locations of the 5′ ends of the transcripts generated by promoters 1 to 7 are designated by P1 to P7, respectively. The lower portion of the figure depicts the location of the binding sites for IHF, the FNR protein (FNR1 and FNR2), and DNA sequences protected by NarL-phosphate from DNase I attack (NarL-1 through NarL-4). The small black boxes signify FNR-binding sites, and the small raised box depicts the IHF-binding site. The protected regions NarL-3a and -3b are depicted as a single site because it was not possible to delineate clearly the boundaries of the protected segments on the noncoding strand. It is likely, however, that they represent two or more distinct sites (see Discussion). The thin lines in the map represent the DNA sequences fused with the *lacZ* gene to create derivatives λRM1001 and λRM2021.

250- to 500-ml cultures containing strain BL21 transformed with pUNarL were grown aerobically in LB medium to late logarithmic phase (optical density at 600 nm, 1.0 to 1.5) at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.4 mM, and cells were grown for an additional 3 h at 37°C. Bacteria were harvested by centrifugation at 4°C and stored at −20°C. Cells from a 500-ml induced BL21/pUNarL1 culture were resuspended in 2.5 ml of 50 mM potassium phosphate (pH 7.5)–2 mM dithiothreitol (DTT)–5 mM benzamidine and passed through a French press cell twice at a cell pressure of 1.03×10^2 MPa (16,000 lb/in^2). To shear chromosomal DNA, the cell lysate was sonicated three times for 1 min at 4°C. The resulting cell lysate was clarified by centrifugation at 15,000 × *g* for 15 min. The pellet was discarded, and the centrifugation step was repeated. The crude extract was then centrifuged at 100,000 × *g* for 2 h. The ammonium sulfate concentration of the S100 supernatant (2 ml) was brought to 50%, the supernatant was stirred slowly for 45 min, and the precipitate was collected by centrifuging at 15,000 × *g* for 30 min. The precipitate was resuspended in 2 ml of buffer A (50 mM Tris-HCl [pH 8.0], 2 mM DTT) and desalted by passage over a G-25 Sephadex column (5 ml) equilibrated in the same buffer. The desalted material was loaded in paired 2-ml portions onto a 5-ml MonoQ anion exchange column equilibrated with buffer A, and the column was developed with a 20-ml gradient from 0 to 0.5 M NaCl in the same buffer. Fractions containing NarL were identified by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis and, after the peak tubes were pooled, concentrated with a Centriprep-10 filter (Amicon) and applied to a 120-ml Superdex G-75 gel filtration column equilibrated with buffer A. Fractions containing NarL were identified, pooled, and concentrated as described above. Glycerol was added to the purified NarL protein to yield a final concentration of 10% (vol/vol), and the protein was stored at −20°C.

Phosphorylation of NarL with acetyl phosphate. NarL phosphorylation by acetyl phosphate was carried out at 25°C. Purified NarL protein was incubated in TEGD buffer (50 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 1 mM DTT, 10% glycerol) containing 5 mM magnesium chloride and 50 mM acetyl phosphate for 30 min to 3 h.

Preparation of DNA probes for DNase I footprinting. DNA fragments carrying different portions of the *pfl* operon promoter-regulatory region were asymmetrically labeled by filling in one restriction end with the Klenow enzyme in the presence of [α-^{32}P]dATP. Fragment I, comprising *pfl* operon regulatory sequences from bp, −414 to +34, was isolated from plasmid pSN15 by digestion with *Eco*RI and *Hin*dIII and labeled on the coding strand at the vector-derived *Eco*RI site downstream of promoter 6 (Fig. 1). Fragment II, comprising *pfl* operon regulatory sequences from bp, −287 to +34, was isolated from plasmid pSN25 by digestion with *Eco*RI and *Hin*dIII and labeled on the noncoding strand at the vector-derived *Hin*dIII site. Fragment III carrying bp −457 to +83 of the *pfl* operon regulatory region was isolated from plasmid pSN20 by digestion with *Eco*RI and *Hin*dIII and was labeled on the coding strand upstream of promoter 7 (Fig. 1) at the vector-derived *Eco*RI site.

DNase I protection. DNase I footprinting assays were performed as follows. ^{32}P-labeled DNA probes (2 pmol) were mixed together with different amounts of the phosphorylated NarL in a buffer that contained 50 mM Tris-HCl (pH 7.5), 12.5 mM magnesium chloride, 1 mM EDTA, 1 mM DTT, 20% glycerol (vol/vol), and 0.1 M potassium chloride. The total reaction volume was 50 μl. After incubation on ice for 15 min, the samples were kept at room temperature for 1 min. Then, 50 μl of a solution containing 10 mM magnesium chloride and 5 mM calcium chloride was added and incubation was allowed to proceed for 1 min more. Subsequently, 2 μl of DNase I (0.00125 μg/ml; Boehringer Mannheim) was added and digestion was stopped after 30 s by the addition of 90 μl of stop solution (20 mM EDTA, 1% SDS, 0.2 M NaCl, and 250 μg of carrier DNA per ml). The reaction products were then purified by phenol-chloroform extraction, precipitated with ethanol, dissolved in formamide dye, and separated on 6.6% sequencing gels. Sequencing reactions were performed as described by Maxam and Gilbert (18). Occasionally, products of sequence reactions with plasmid DNA obtained by the dideoxy method (23) were used as size standards on sequencing gels.

Gel mobility shift assay with purified NarL. Mobility shift assays were performed exactly as described previously, using the 531-bp *Bam*HI fragment from pSN20 which includes the complete *pfl* promoter-regulatory sequence (30). The DNA fragment was radioactively labeled with [α-^{32}P]dATP with the Klenow fragment of DNA polymerase I. NarL or NarL-phosphate was used at concentrations ranging from 5 to 50 μM. Reaction products were analyzed in 4% nondenaturating polyacrylamide gels prepared in Tris-borate-EDTA buffer (17).

Other methods. Operon and protein fusions to the *lacZ* gene were transferred to λRS45 exactly as described previously (25). SDS-polyacrylamide gel electrophoresis was carried out in 12.5% (wt/vol) polyacrylamide gels by following the procedure of Laemmli (13). Protein concentration was determined by the method of Lowry et al. (15), with bovine serum albumin as the standard. N-terminal amino acid sequencing was performed with an Applied Biosystems gas phase sequencer.

RESULTS

Maximal nitrate repression of *pfl* expression is dependent on functional NarL, NarP, NarX, and NarQ proteins. A *lacZ* fusion including the complete *pfl* regulatory region plus the *focA* gene (37) was used to study the effect of nitrate on *pfl* gene expression (Table 2). Nitrate, when used as a supplement to buffered rich medium, reduced anaerobic *pfl* gene expression by approximately 45%. This result is in accord with previous observations (25). Surprisingly, nitrate repression was much stronger (about sevenfold) when the bacteria were grown in minimal medium than in rich medium (Table 2). It

TABLE 2. Influence of nitrate and various mutant *nar* alleles on anaerobic *pfl-lacZ* expression

Strain[a]	β-Galactosidase enzyme sp act[b]				
	Rich medium			Minimal medium	
	−Nitrate	+Nitrate	+Nitrite	−Nitrate	+Nitrate
FM420 (wild type)	4,180	2,320	2,670	1,650	230
RM600 (*narL*)	2,960	2,820	2,740	1,820	580
RM610 (*narP*)	4,540	2,710	2,780	1,660	220
RM611 (*narL narP*)	4,980	5,550	3,880	1,710	1,270
RM605 (*narX narQ*)	4,700	5,220	3,630	1,615	1,200
RK5268 (*narG*)	2,160	2,680	ND[c]	1,615	890

[a] All strains carried a chromosomal copy of λRM123.
[b] Specific activity is expressed in Miller units (19).
[c] ND, not determined.

TABLE 3. Effects of different regulatory mutations on anaerobic *pfl-lacZ* expression

Strain[a]	β-Galactosidase enzyme sp act[b]			
	Rich medium		Minimal medium	
	−Nitrate	+Nitrate	−Nitrate	+Nitrate
RM123 (wild type)	4,180	2,320	1,650	230
RM364 (*arcA*)	3,680	2,660	1,600	200
RM381 (IHF bind$^-$)	2,480	1,350	1,310	240
RM2314 (FNR1 bind$^-$)	370	210	250	35
RM2315 (FNR2 bind$^-$)	4,700	1,920	1,870	190
RM2316 (FNR1+2 bind$^-$)	310	160	350	50

[a] Strains bear a chromosomal copy of λRM123 or a mutant allele therof.
[b] Specific activity is expressed in Miller units (19).

should be noted that basal anaerobic *pfl* expression in cells grown in minimal medium attained levels that were only 40% that of cultures grown in rich medium but the regulation pattern is identical to that observed in rich medium (data not shown). The reason for this reduced expression is currently unclear.

To determine whether nitrate repression is indirect and due to changes in metabolism caused by growth in the presence of the electron acceptor or whether it results from a direct interaction of components of the nitrate-dependent sensor-regulator machinery with the *pfl* operon regulatory region, the *pfl-lacZ* expression in various *narX*, *narL*, *narQ*, and *narP* mutants after growth of the strains in the presence or absence of nitrate was monitored. Nitrate repression could not be relieved in strains with genetic backgrounds in which each gene was mutated singly, regardless of the growth medium used (Table 2 and data not shown). It was noted, however, that in *narL* mutants the level of expression in nitrate-supplemented minimal medium was twofold higher than that of the *narL*$^+$ strain. A further salient feature of *narL* mutants was that they reproducibly exhibited 30% lower β-galactosidase activity levels than the wild type after growth in rich medium in the absence of nitrate (Table 2). This difference indicates that *narL*$^+$ influences *pfl* expression in the absence of added nitrate.

Nitrate repression could be relieved completely only in strains grown in rich medium and unable to synthesize either functional NarL and NarP proteins or NarX and NarQ proteins (Table 2). This result indicates that these two nitrate-sensing systems are redundant with regard to the nitrate repression of the *pfl* operon. Analysis of *pfl* expression in the double null mutants after growth in minimal medium revealed that nitrate repression could not be relieved completely. Both the *narL narP* double mutant RM611 and the *narX narQ* strain RM605 exhibited a residual 1.3-fold nitrate repression (Table 2). This finding suggests that either a further nitrate-sensing system exists in *E. coli* or the direct nitrate repression of the *pfl* operon mediated by the NarLPQX system is augmented by metabolic modulation of expression in the presence of nitrate.

Nitrite has been shown to affect expression of several anaerobic respiratory genes (21, 33). It was observed that addition of nitrite to rich medium also reduced expression of the *pfl-lacZ* construct (Table 2). As with nitrate-dependent repression, nitrite repression was lifted only when the strain carried lesions in both the *narL* and *narP* genes or in both the *narX* and *narQ* genes.

The metabolism of nitrate influences *pfl* expression. A result which supports the contention that the metabolism of nitrate may contribute to nitrate repression of *pfl* expression in minimal medium was obtained by examining *pfl-lacZ* expression in a *narG* mutant, which lacks a functional nitrate reductase (Table 2). The *narG* mutation reduced the degree of nitrate repression after growth in minimal medium to only twofold, compared with sevenfold repression in the wild type. It is noteworthy that fermentative growth of the *narG* mutant derivative in rich medium resulted in a reduction of *pfl* expression similar to that seen in a *narL* mutant (Table 2). This similarity suggests that the reduction in expression may be a metabolic effect that is dependent on a functional nitrate reductase enzyme rather than a direct interaction between NarL and the regulatory region.

Nitrate repression is independent of IHF, FNR, and ArcA. To test whether any of the other characterized transcriptional regulators that modulate *pfl* expression are required for nitrate repression, we examined the effects of nitrate supplementation on various mutant derivatives of the *pfl* promoter-regulatory region in a wild-type genetic background or the wild-type promoter-regulatory region in different genetic backgrounds (Table 3). Nitrate repression was still apparent after growth of an *arcA* mutant in rich and minimal media. Strain RM381, which carries a mutation that prevents binding of integration host factor (IHF) at the *pfl* promoter-regulatory region (30), was also unaffected in nitrate repression. Furthermore, promoter derivatives that were mutated in the putative FNR-binding sites at promoter 7 (FNR2 bind$^-$) and promoter 6 (FNR1 bind$^-$) (24) still exhibited nitrate repression (Table 4). This result is surprising, in particular for strain RM2314, since the mutation in the FNR-binding site reduces anaerobic expression dramatically; however, despite this reduction nitrate still caused a sevenfold repression in promoter activity after growth in minimal medium. These results indicate that repression by nitrate is independent of the three transcription factors ArcA, IHF, and FNR.

TABLE 4. Identification of DNA sequences in the *pfl* operon upstream regulatory region required for nitrate-dependent repression of anaerobic *pfl-lacZ* expression

Strain	β-Galactosidase enzyme sp act[a]			
	Rich medium		Minimal medium	
	−Nitrate	+Nitrate	−Nitrate	+Nitrate
FM420 λTK200	5,210	3,310	1,770	690
FM420 λRM20201	1,460	1,340	950	600
FM420 λRM1001	1,850	2,225	570	360

[a] Specific activity is expressed in Miller units (19).

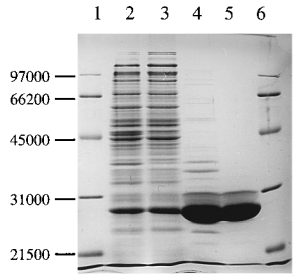

FIG. 2. Purification of NarL protein. Purification was performed as described in Materials and Methods. The protein composition at each purification step was analyzed on an SDS–12.5% polyacrylamide gel. The samples and amounts of protein applied to each lane are as follows: lanes 1 and 6, molecular weight standards; lane 2, the S100 supernatant of BL21/pUNarL1 grown in the presence of IPTG (25 μg); lane 3, redissolved and desalted 50% ammonium sulfate pellet (25 μg); lane 4, the pooled fractions from MonoQ anion exchange chromatography (25 μg); lane 5, the pooled and concentrated fractions after Superdex G-75 gel filtration (25 μg). The molecular weight markers used (with the molecular weights shown from top to bottom at the left of the gel) were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

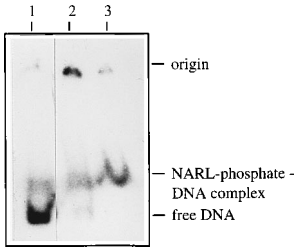

FIG. 3. Specific binding of NarL-phosphate to the *pfl* promoter-regulatory region. The autoradiogram shows the result of a mobility shift assay in which 0.13 pmol of a radioactively labeled *Bam*HI fragment from pSN20 was incubated without protein (lane 1), with 1 nmol of NarL (lane 2), and with 1 nmol of NarL-phosphate (lane 3).

Localization to the *pfl* regulatory region of sequences required for nitrate repression. The studies presented so far employed *pfl-lacZ* fusions that included the complete regulatory region plus the DNA sequences encompassing promoters 1 to 5 (24–26). We constructed various fusion derivatives to delimit the sequences necessary for mediating nitrate regulation (Fig. 1). A construct that contained only the DNA sequences including promoters 1 to 5 fused with the *lacZ* gene exhibited neither anaerobic induction of expression (26) nor nitrate repression (data not shown). In contrast, a derivative (λTK200) that included the complete nontranslated regulatory region (the *Mlu*I-*Bgl*I fragment in Fig. 1) was still regulated by nitrate in both rich and minimal media (Table 4). Strain FM420λRM20201 contains a *pfl′-lacZ* transcriptional fusion that includes only 118 bp of DNA sequence from promoter 6 (Fig. 1). Expression from this derivative exhibited nitrate repression, but only after growth in minimal medium (Table 4). Similarly, a derivative (λRM1001) that included only promoter 7 DNA sequences was partially repressed by nitrate, again only after growth in minimal medium. These results therefore indicate that there is more than one DNA sequence involved in the mediation of nitrate regulation of the *pfl* operon.

NarL-phosphate binds specifically to the *pfl* promoter-regulatory region. The *narL* gene was placed under the control of the *lac* promoter of pUC19, which facilitated overproduction of the NarL protein in response to addition of IPTG to the growing cultures. NarL was purified (Fig. 2) essentially by the procedure of Walker and DeMoss (40). The purification scheme was slightly modified by introducing an ammonium sulfate fraction step subsequent to preparation of the S100 extract. NarL eluted from the MonoQ column between concentrations of 100 and 200 mM NaCl and migrated in the Sephadex G-75 column as a 26-kDa monomer. N-terminal amino acid sequence analysis identified the sequence Ser-Asn-Gln, which confirms that the purified protein was NarL (35). Yields from 1.8 g of cell paste were reproducibly in the range of 3.0 to 4.0 mg.

To examine whether NarL binds specifically to the *pfl* regulatory region, the purified protein was tested in a mobility shift experiment. The results in Fig. 3 show that, when incubated with NarL, the radioactively labeled DNA fragment remained at the origin of the gel (lane 2) despite a greater-than-1,000-fold molar excess of nonspecific competitor DNA. A single retarded DNA-protein complex could be resolved in the polyacrylamide gel only by incubating the NarL protein with acetyl phosphate before the mobility shift assay was performed (Fig. 3, lane 3). It has been demonstrated that incubation of the NarL protein with acetyl phosphate results in the transfer of the phosphate group to the protein (28).

DNase I footprinting analysis was employed to define the location of the NarL-phosphate-binding sites more precisely. The results in Fig. 4A show five areas of the regulatory region that are protected by NarL-phosphate from attack by DNase I. No specific footprint was observed with nonphosphorylated NarL protein. The protected regions span almost the complete regulatory sequence (see Fig. 5). Binding site 1 (NarL-1 in Fig. 4 and 5) extends over the −10 region of promoter 6, while the NarL-4 binding site spans the −35 sequence of promoter 7 (Fig. 5). The locations of both sites are compatible with NarL-phosphate repressing transcription from both promoters.

The NarL-2 binding site partially overlaps the recognition sequence for IHF, while sites NarL-3a and NarL-3b are located between promoter 7 and the IHF-binding site (Fig. 5). The last two binding sites were readily distinguishable by footprinting the coding strand (Fig. 4A) but less so by analysis of the noncoding strand (Fig. 4B and C). The binding constants for NarL-phosphate on the coding and the noncoding strands were in the range of 1.2 to 1.66 μM.

DISCUSSION

The data presented in this report demonstrate that the expression of the *pfl* operon is negatively regulated by nitrate and that when cells are cultured in a mineral salts medium this regulation is markedly stronger than when they are cultured in rich medium. Nitrate regulation is mediated exclusively by the dual sensors (NarX and NarQ) and the dual regulators (NarL and NarP) in rich medium. In minimal medium, these four factors are also the principal means by which nitrate is sensed and transmitted to the level of *pfl* gene expression; however, there remains a significant degree of nitrate repression when either both sensors or both regulators are inactivated. This finding suggests either that another nitrate-sensing system which regulates the expression of the *pfl* operon exists or that the metabolism of nitrate in some way influences expression. Although we cannot exclude the former possibility (see reference 21), we favor the latter argument, mainly because nitrate repression could be substantially relieved in a mutant incapable of synthesizing a functional respiratory nitrate reductase. This result strongly suggests communication between the metabolic (redox) status, possibly transmitted through other transcriptional regulators, and *pfl* expression.

We did not observe a measurable difference in the degrees

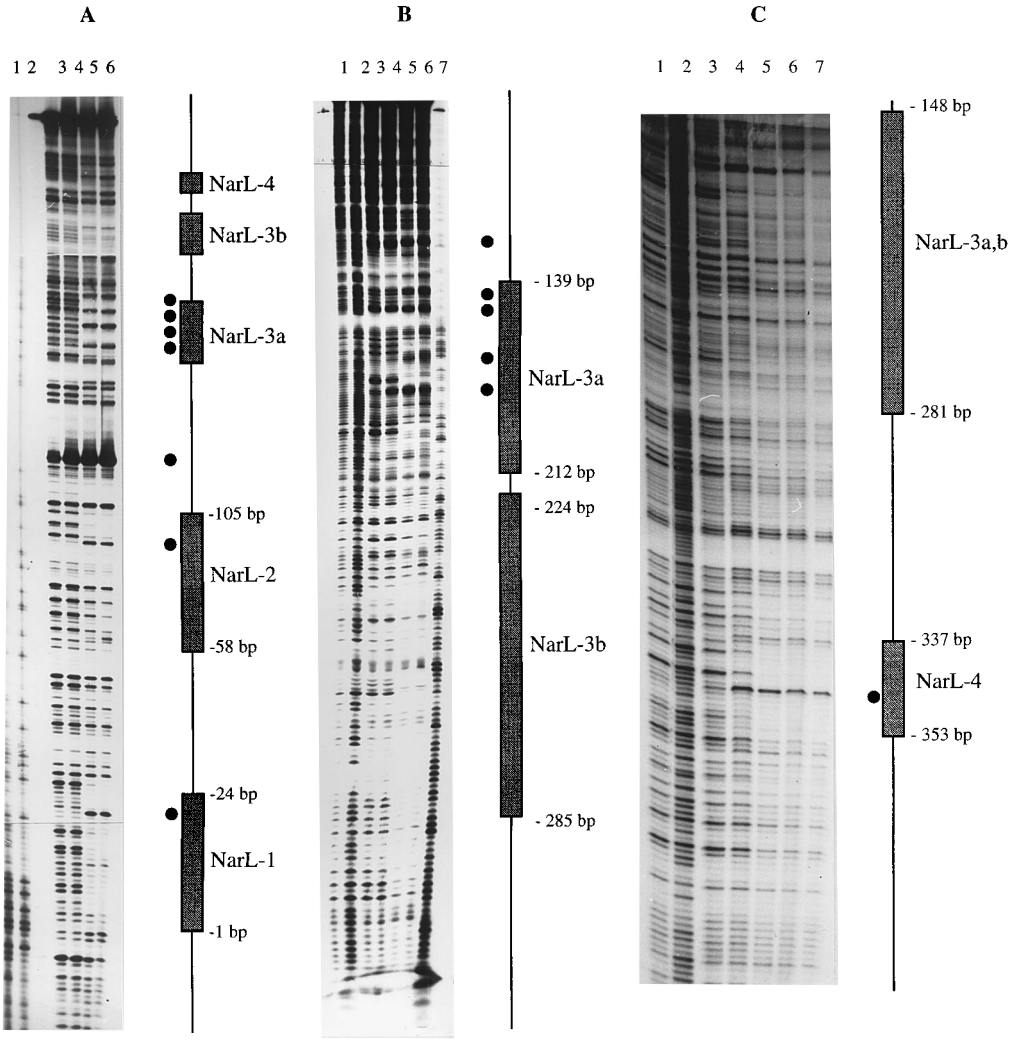

FIG. 4. DNase I footprinting of NarL binding to the *pfl* operon upstream regulatory region. Three different 3′-end-labeled DNA fragments were incubated with progressively larger amounts of NarL protein, and the reaction products were resolved on sequencing gels as described in Materials and Methods. (A) Fragment I derived from plasmid pSN15 (bp −414 to +34 of the *pfl* operon regulatory region) and labeled on the coding strand. Lanes 1 and 2, T+C and C from the Maxam and Gilbert sequencing reactions of the corresponding fragment; lane 3, no protein; lane 4, 1.25 nmol of NarL protein; lane 5, 0.83 nmol of phosphorylated NarL; lane 6, 1.25 nmol of phosphorylated NarL protein. (B) Fragment II derived from plasmid pSN25 (bp −287 to +34 of the *pfl* operon regulatory region) and labeled on the noncoding strand. Lane 1, no protein; lane 2, 1.25 nmol of NarL protein; lane 3, 0.15 nmol of phosphorylated NarL; lane 4, 0.3 nmol of phosphorylated NarL; lane 5, 0.6 nmol of phosphorylated NarL; lane 6, 1.25 nmol of phosphorylated NarL protein; lane 7, T+C from the Maxam and Gilbert reaction of the corresponding fragment. (C) Fragment III derived from plasmid pSN20 (bp −457 to +83 of the *pfl* operon regulatory region) and labeled on the noncoding strand. Lane 1, no protein; lane 2, 1.25 nmol of NarL protein; lane 3, 0.15 nmol of phosphorylated NarL; lane 4, 0.3 nmol of phosphorylated NarL; lane 5, 0.6 nmol of phosphorylated NarL; lane 6, 1.25 nmol of phosphorylated NarL protein; lane 7, 2.0 nmol of phosphorylated NarL protein. The binding sites, together with their boundaries, are diagrammed on the right of each panel. The black dots signify hypersensitive sites.

of nitrate repression of *pfl* operon expression in single *narX* and *narQ* mutants. Therefore, NarX and NarQ are equally effective in sensing the presence of nitrate and relaying this signal to the regulator components of the pathway, a finding which has been extensively studied for other nitrate-responsive operons (21, 28, 33). Either of the response regulators, NarL or NarP, was sufficient to confer maximal nitrate and nitrite repression in rich medium, while the NarL protein appeared to be marginally more effective when cells were grown in minimal medium. The two regulator components are therefore essentially redundant with regards to the regulation of the *pfl* operon. For many of the other nitrate-regulated genes studied to date, the distinction between a preference for one regulator is more clear-cut than for *pfl*. For example, nitrate induction of the *narGHJI* operon (21) and the *narK* gene (12) is controlled exclusively by NarL, while induction of the *fdnGHI* operon is controlled principally by NarL, but NarP also effects a degree of control (observed with a *narL* mutant) (21). Expression of the *dmsABC* and the *frdABCD* operons is repressed in the presence of nitrate, and this repression is dependent on the NarL protein (8, 10, 21, 28). A more complex scenario is presented by the regulation of two more recently identified genes, termed *aeg*-46.5 and *aeg*-93 (4). Nitrate induction of *aeg*-46.5 is enhanced in a *narL* mutant (4) and is mediated by NarP (21). Regulation of *aeg*-93 is more complex than that of *aeg*-46.5, since nitrite is an effective activator and it appears that NarL represses expression in the presence of nitrate but activates it in the presence of nitrite, while NarP is an activator with both nitrate and nitrite. Clearly, there is a complex inter-

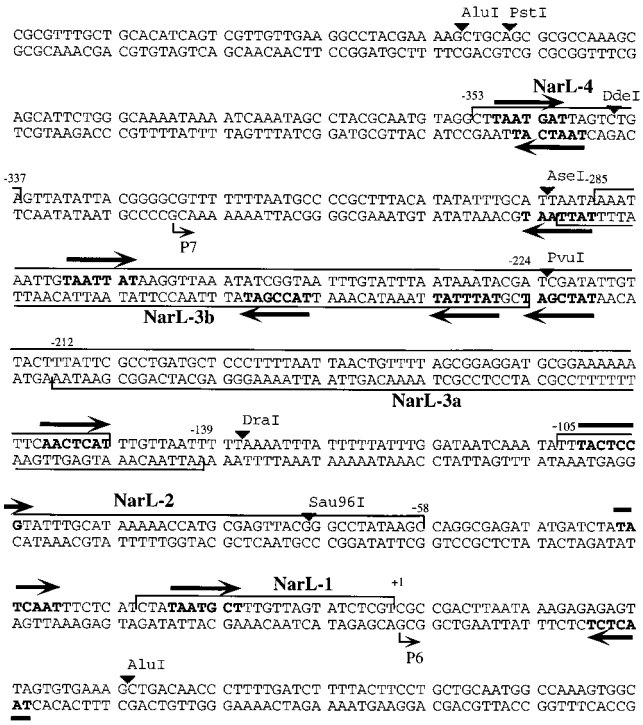

FIG. 5. DNA sequence of the *pfl* operon upstream regulatory region, showing NarL-phosphate-binding sites. The transcription initiation sites of promoters 6 and 7 (angled arrows) and the sequences protected by NarL-phosphate binding from DNase I digestion (brackets) are indicated. The numbering is with respect to the major transcription initiation site from promoter 6, which is taken as +1 (24). Sequences that exhibit a high degree of similarity to the NarL heptamer consensus of TACYNMT (where Y = C or T and M = A or C) are in boldface letters and, depending on the orientation, are overlined or underlined with a thick arrow. Restriction sites are indicated by the arrowheads above the sequence.

play between these four proteins at different target operons (see reference 23).

It was anticipated that nitrate repression would not show a dependence on the presence of ArcA, FNR, or IHF, which proved to be the case. An initially perplexing finding, however, was that it was not possible, by using *lacZ* operon fusions, to localize a single region of DNA as being sufficient to confer nitrate repression; expression from promoter 6 and promoter 7 derivatives in isolation proved to be susceptible to repression by nitrate. This apparent paradox was clarified by performing in vitro DNase I footprinting studies with purified NarL protein. Five regions of the *pfl* regulatory region were protected by NarL from DNase I cleavage. The locations of the sequences in the *pfl* promoter-regulatory region occupied by NarL are summarized in Fig. 5. Sites 1 and 4 overlap promoters P6 and P7, respectively, and may account for the nitrate repression seen in vivo with *lacZ* fusion derivatives including these regions. The NarL-2 site is located between the FNR-binding site of P6 and partially overlaps the IHF recognition sequence. An extremely large region of DNA that extends from downstream of P7 and adjoins the IHF recognition sequence was also protected (NarL-3a and -3b). Footprinting studies using the coding strand separated this region into two well-defined sites (Fig. 4a and Fig. 5); however, analysis of the noncoding strand produced a less clear-cut result. Similar problems were encountered by Li et al. (14) for NarL binding to the *frdA* and *narK* control regions. It is perhaps significant that the DNA sequence encompassed by NarL-3a and NarL-3b is extremely AT rich, and it is feasible that the combined effects of extensive DNA bending and protein-protein interaction between DNA-bound NarL monomers hinders DNase I access to the DNA. Hydroxyl-radical footprinting and mutagenesis studies will aid in defining these sites more precisely. A recent study by Li et al. (14) identified multiple NarL-binding sites in the *fdnG*, *narG*, *narK*, and *frdA* regulatory regions; therefore, our results are in excellent agreement with their findings.

All five sites of NarL-DNA interaction contain at least one sequence that conforms to the NarL heptamer consensus (5, 38) TAC C/T N A/C T in minimally six positions (Fig. 5). Of a total of 12 putative heptamer sequences, only 2 lie outside the boundaries of a site protected by NarL. The sites are arranged on both strands and exhibit diverse spacing, suggesting that they are recognized by NarL monomers.

In order to obtain specific binding of the NarL protein to the DNA, it was necessary to incubate it with acetyl phosphate prior to the binding experiments, indicating that phosphorylation of the protein is a prerequisite for specificity (14, 16). Binding constants ranged from 1.2 to 1.7 μM, which was of the same order of magnitude as the range found for the constitutive mutant form of the protein NarL (V88A) used in a previous study (14). Clearly, the true binding constant will be significantly lower than the estimated value, since it is unlikely that all of the NarL monomers were in a form capable of binding DNA. No significant difference in DNA-binding activity was noted when the phosphorylation reaction was allowed to proceed for up to 4 h at room temperature, and maximal activity was attained after 10 min of incubation. We have succeeded in purifying the NarP protein, and like NarL, it shows no specific interaction with DNA in the (presumably) dephosphorylated state. Unfortunately, attempts to activate specific DNA-binding properties of the protein by incubation with acetyl phosphate or carbamoyl phosphate did not yield reproducible footprints (data not shown).

Two important questions are raised by the findings presented. First, what is the physiological significance of the nitrate repression of the *pfl* operon? Second, what mechanisms are involved in the enhancement of nitrate repression upon growth in minimal medium? In a redox environment which supports only fermentative growth, it is crucial that the cell generates a minimum of reducing equivalents, and the nonoxidative cleavage of pyruvate by PFL serves this purpose ideally. The availability of nitrate as an oxidant presents the cell with an alternative means of maintaining redox balance and generating ATP. If pyruvate is cleaved by PFL, then the product formate is metabolized by the energy-conserving FDH-N–nitrate reductase pathway (32). The pyruvate dehydrogenase (PDH) complex provides an alternative route for pyruvate metabolism, assuming that the NADH generated can be channeled to the quinone pool and hence to nitrate reductase. Indeed, it has been noted that nitrate enhances PDH complex synthesis and activity two- to threefold in anaerobically growing cells (6). Batch culture studies with phenotypically PFL^- and PDH^- mutants have shown that, when cultured anaerobically in minimal medium with glucose and nitrate, the wild type and both single mutants grow equally well (9). Therefore, either PFL or PDH can be used to catabolize pyruvate in nitrate-respiring cells. This provides a rationale for nitrate repression of PFL enzyme synthesis in favor of the PDH complex. The degree of nitrate repression is limited in rich medium, perhaps because the cell has little problem generating ATP and maintaining high growth rates. In minimal medium, the contribution of NADH as an electron donor for nitrate respiration is probably significantly higher than in rich medium (32) and the cell will thus tend to catabolize more pyruvate

through the PDH complex. Studies with *Enterococcus faecalis* have demonstrated that by modulating the pH of the culture medium, and hence the intracellular NADH/NAD^+ ratio, it is possible to influence whether PDH or PFL is used to catabolize pyruvate (31). By analogy, the increased synthesis and use of the PDH complex in nitrate-respiring cells may provide an explanation for the enhanced repression of the *pfl* operon by nitrate in minimal medium.

The mechanism which the cell employs to repress *pfl* expression with nitrate clearly involves NarL and NarP, but a further factor(s) which dictates whether repression is strong or weak, perhaps in response to the intracellular redox status, must be involved. A feasible scenario would involve influencing the extent of occupancy of the NarL-binding sites. Recent in vitro studies have demonstrated that purified ArcA binds to several distinct sites in the *pfl* regulatory region and these overlap substantially those regions protected by NarL in footprinting studies (5a). Thus, competition between NarL and ArcA for their corresponding binding sites in the *pfl* promoter-regulatory region would provide a simple explanation for the variable level of nitrate repression. However, if this argument holds, then a strain incapable of synthesizing ArcA should show stronger nitrate repression in rich medium than in minimal medium, which is not what is observed. Thus, the situation is clearly highly complex and much experimentation will be required to elucidate the details of this regulation.

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