JOURNAL OF BACTERIOLOGY, Sept. 1995, p. 5338–5341 Vol. 177, No. 18
0021-9193/95/$04.00+0

Promoter 7 of the *Escherichia coli pfl* Operon Is a Major Determinant in the Anaerobic Regulation of Expression by ArcA

NIKOLA DRAPAL AND GARY SAWERS*

Lehrstuhl für Mikrobiologie der Universität München, 80638 Munich, Germany

Received 27 March 1995/Accepted 3 July 1995

The anaerobically inducible *pfl* operon of *Escherichia coli* has a regulatory sequence comprising 494 bp, which includes two anaerobically regulated promoters, termed P6 and P7. In this study, we show that in its normal context the activity of P7 is constrained and that one important function of the promoter is to mediate controlled ArcA-dependent regulation of the operon.

The *pfl* operon includes two genes, termed *focA* and *pfl* (17). Expression is controlled from seven anaerobically regulated promoters, only two of which, promoter 6 (P6) and promoter 7 (P7), are located within a 494-bp nontranslated regulatory region (10, 11). Anaerobic induction of operon expression is dependent on the ArcA and Fnr transcription factors (10, 12). Recent studies have identified sequences important for regulation by these proteins in the nontranscribed promoter-regulatory region (5, 10–12, 15).

P7 is located most distal to the operon (Fig. 1) and is separated from the next promoter, P6, by approximately 300 bp of DNA. The function of P7 in regulating expression is still unclear. By performing a detailed dissection of the *pfl* promoter-regulatory region, we show that both P6 and P7 have ArcA and Fnr regulatory components and that P7 modulates ArcA-dependent anaerobic regulation.

At least 494 bp of DNA is required for maximal anaerobic regulation of *pfl* expression. To delimit the DNA sequences in the *pfl* promoter-regulatory region that are necessary for maximal anaerobic regulation, defined DNA sequences from the *pfl* promoter-regulatory region (Fig. 1) were amplified by PCR with oligonucleotides derived from the sequence of the *pfl* regulatory region (11) and cloned into the *lacZ* expression vector pRS551 (14). After the various derivatives were transferred to the chromosome of strains MC4100 (wild type [3]), RM102 (*fnr* [2]), RM313 (*arcA* [12]), and RM315 (*arcA fnr* [12]), β-galactosidase activity was measured (9) after aerobic and anaerobic growth in TYEP medium (1). Anaerobic cultures were supplemented with 20 mM glucose. Expression from λRM1031, which includes the complete 494-bp regulatory region of the *pfl* operon, was induced approximately eightfold by anaerobiosis (Table 1). Anaerobic induction was dependent on both ArcA and Fnr. This result is consistent with the findings of previous studies with constructs including promoters 1 to 7 (10–12). Deletion of 43 bp of DNA from the 5′ end of λRM1031 delivered construct λRM811 (Fig. 1). Both aerobic and anaerobic *lacZ* expression from this derivative in strain MC4100 ($arcA^+$ fnr^+) was reduced approximately twofold compared with that from λRM1031 (Table 1). Moreover, anaerobic ArcA regulation of expression was significantly impaired. The 5′ 43 bp of DNA is therefore important for maximal ArcA-dependent anaerobic expression. Removal of DNA sequences from the 3′ end of the regulatory region or separation of the two promoters also resulted in reduced regulation (see below; data not shown). The complete 494 bp of DNA is therefore necessary for maximal anaerobic regulation.

Identification of sequences involved in Fnr regulation of expression. Introduction of a mutation into the Fnr-binding site (Fnr-1) upstream of P6 has been shown to abolish transcription from the P6 promoter (10). The same mutation, when introduced into the λRM1031 derivative, which delivered λRM1033, strongly reduced anaerobic induction of *pfl* expression. This confirms that this DNA sequence is also an important determinant for Fnr-dependent regulation in this shortened derivative. In a *fnr* null mutant, λRM1033 expression was reduced both aerobically and anaerobically, while in an *arcA* mutant, anaerobic regulation was abolished (Table 1). These results indicate first that anaerobic induction in λRM1033 is ArcA dependent and second that some Fnr-dependent regulation is retained, despite the fact that Fnr cannot bind to the Fnr-1 site in vitro (7). The fact that aerobic expression is also reduced in the *arcA* mutant (Table 1) indicates that ArcA is also involved in aerobic expression of *pfl*. Compan and Touati (4) have demonstrated that Fnr regulates *arcA* expression; however, since this regulation is mainly anaerobic, it is unlikely that the effects we observed are secondary to Fnr regulation of *arcA* expression. Rather, it appears that both ArcA and Fnr can regulate *pfl* expression under particular circumstances in aerobically grown cells. Evidence has been presented which also suggests that Fnr regulates the expression of certain genes aerobically (13).

Data from former studies have demonstrated that Fnr also regulates P7 activity (10, 12). There is a sequence (termed Fnr-2 in Fig. 1) upstream of P7 which exhibits similarity to the consensus Fnr-binding site (TTGAT-N_4-ATCAA [16]) but only in the right half of the sequence. This sequence was mutagenized from ATCAA to ATGAG (10) to create λRM1032 (Fig. 1). The mutation reduced anaerobic expression by approximately 2.5-fold in an MC4100 genetic background compared with expression from the wild-type λRM1031 construct (Table 1). This result verifies the importance of this sequence to the regulation of *pfl* expression. When λRM1032 was analyzed in an *arcA* mutant, both anaerobic expression and aerobic expression were elevated compared with those of the λRM1031 (wild-type sequence) derivative in the *arcA* mutant. One possible explanation for this result is that as a result of the Fnr-2 mutation, Fnr no longer can bind to this sequence, which consequently affects the interaction of ArcA with the P7 regulatory sequences. That an Fnr half-site is recognized by Fnr in vitro has been shown recently for the *ndh* promoter (6). In this

* Corresponding author. Present address: Laboratory of Nitrogen Fixation at the University of Sussex, Brighton BN1 9RQ, England. Phone: 1273 678250. Fax: 1273 678133. Electronic mail address: gary.sawers@bbsrc.ac.uk.

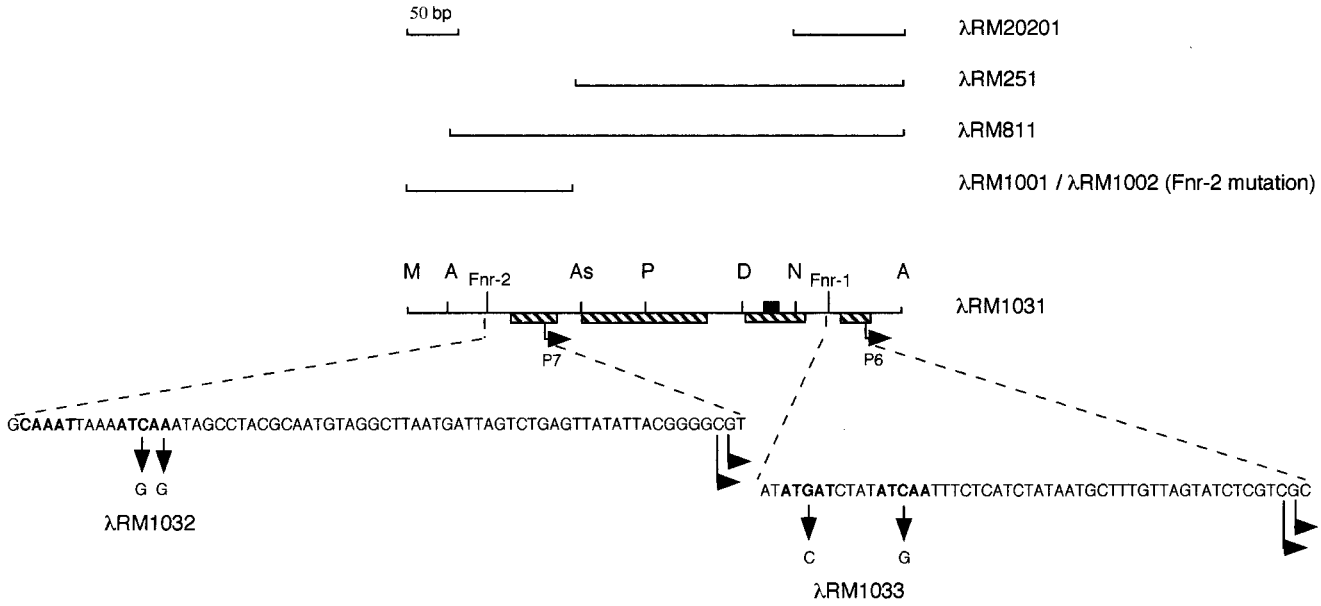

FIG. 1. Schematic representation of *lacZ* fusion constructs with different portions of the *pfl* regulatory region. The DNA fragments in each construct are presented as horizontal bars above the insert of λRM1031. The corresponding names of the respective derivatives are shown on the right of the diagram. Constructs λRM1031, λRM1001, λRM811, and λRM20201 have inserts bearing wild-type DNA sequences. Constructs λRM1032 and λRM1002 carry a mutation in the Fnr-2-binding sequence (10); construct λRM1033 is mutated in the Fnr-1-binding site. Putative Fnr-binding sites are shown, and the regions of DNA protected by ArcA from DNase I cleavage (5) are depicted as hatched boxes, while the location of the integration host factor-binding site (15) is shown as a black box above the sequence. The DNA sequences below the insert of λRM1031 show expanded portions of P6 and P7 and their regulatory sequences. Only the noncoding strand of the sequence is shown. The putative Fnr-binding sites are shown in boldface type. The vertical arrows show the mutations introduced and the names of the resultant mutant derivatives of λRM1031. The locations of the transcription initiation sites of P6 and P7 determined with RNA isolated from wild-type strain MC4100 (10, 11) are shown by angled arrows. In both cases, initiation occurs at two adjacent residues. For P6, both residues are used with equal frequency, while for P7, the guanosine is preferred over the cytosine residue. Abbreviations: A, *Alu*I; As, *Ase*I; D, *Dra*I; N, *Nla*III; M, *Mlu*I; P, *Pvu*I.

case, Fnr bound at that half-site has been proposed to function conjointly with a Fnr dimer bound at a complete Fnr-binding site to repress transcription. Fnr bound at the half-site upstream of P7 may function together with bound ArcA (5) to activate expression from the P7 promoter. This also has been suggested to occur at the *arcA* regulatory region (4).

TABLE 1. Expression of *pfl* promoter-regulatory region derivatives

lacZ derivative	Host genotype	β-Galactosidase activity[a]		
		Aerobic ($+O_2$)	Anaerobic ($-O_2$)	Induction ratio ($-O_2/+O_2$)
λRM1031 (wild-type sequence)	*arcA*$^+$ *fnr*$^+$	450	3,705	8.2
	arcA$^+$ *fnr*	340	1,060	3.1
	arcA fnr$^+$	240	490	2.0
	arcA fnr	320	320	1.0
λRM811	*arcA*$^+$ *fnr*$^+$	220	1,890	8.6
	arcA$^+$ *fnr*	220	650	3.0
	arcA fnr$^+$	145	1,370	9.5
	arcA fnr	245	505	2.0
λRM1033 (Fnr-1 site mutation)	*arcA*$^+$ *fnr*$^+$	220	720	3.3
	arcA$^+$ *fnr*	70	300	4.2
	arcA fnr$^+$	80	60	0.75
	arcA fnr	80	80	1.0
λRM1032 (Fnr-2 site mutation)	*arcA*$^+$ *fnr*$^+$	210	1,395	6.6
	arcA$^+$ *fnr*	185	720	3.9
	arcA fnr$^+$	480	2,510	5.2
	arcA fnr	180	295	1.6

[a] Enzyme activity was measured by the method of Miller (9).

P7 is required to fine-tune anaerobic ArcA-dependent regulation. The results presented so far indicate that two DNA sequences upstream of P7 are important in ArcA-dependent regulation of anaerobic expression (compare λRM811 and λRM1032 in Table 1). To confirm the importance of P7 for anaerobic ArcA regulation of *pfl* expression, a construct (λRM251) completely lacking P7 but retaining all DNA sequences below the *Ase*I restriction site (Fig. 1), including the high-affinity ArcA-binding site (5), was constructed. Expression in the wild-type host (*arcA*$^+$ *fnr*$^+$) was induced over 20-fold, in contrast to the approximately 8-fold anaerobic induction observed with λRM1031 (Table 2). Significantly, anaerobic regulation was only partially dependent on ArcA; β-galactosidase activity was reduced less than 2-fold in an *arcA* mutant, although it was reduced 7.5-fold when all the P7 sequences were included on the DNA insert (compare λRM1031 in the *arcA fnr*$^+$ host). Removal of P7 therefore increases the dependence on Fnr of P6 despite ArcA-binding sites being retained on the operon fusion (Fig. 1) (5). These data indicate that ArcA regulation requires P7 and that P7 or DNA sequences in the vicinity of P7 are involved in modulating anaerobic induction of *pfl* expression.

Expression from P7 in isolation is not oxygen regulated. P7 was isolated on a discrete 163-bp DNA fragment and fused with the *lacZ* gene (λRM1001 [Fig. 1]). Expression from this construct was strong, constitutive, and no longer influenced by the oxygen status (Table 2). This finding suggests, therefore, that removal of downstream DNA sequences perturbs the action of these two transcription factors at this promoter. In strains defective in ArcA and Fnr synthesis, expression was reduced both aerobically and anaerobically. The expression from λRM1002 (mutated in the Fnr-2 sequence) in an *arcA*$^+$

TABLE 2. Expression from deletion derivatives of the *pfl* promoter-regulatory region derivatives

lacZ derivative	Host genotype	β-Galactosidase activity[a]		
		Aerobic ($+O_2$)	Anaerobic ($-O_2$)	Induction ratio ($-O_2/+O_2$)
λRM251	*arcA*$^+$ *fnr*$^+$	220	4,550	20.6
	arcA$^+$ *fnr*	290	910	3.1
	arcA fnr$^+$	380	2,500	6.6
	arcA fnr	340	580	1.7
λRM1001	*arcA*$^+$ *fnr*$^+$	1,590	1,835	1.1
	arcA$^+$ *fnr*	1,190	1,080	0.9
	arcA fnr$^+$	830	1,080	1.3
	arcA fnr	910	690	0.8
λRM1002	*arcA*$^+$ *fnr*$^+$	770	690	0.9
	arcA$^+$ *fnr*	640	520	0.8
	arcA fnr$^+$	530	500	0.9
	arcA fnr	570	400	0.8
λRM20201	*arcA*$^+$ *fnr*$^+$	450	1,900	4.2
	arcA$^+$ *fnr*	380	600	1.6
	arcA fnr$^+$	1,170	1,270	1.1
	arcA fnr	400	620	1.55

[a] The standard error of the reported values was not more than 15%.

fnr$^+$ genetic background was reduced to the same level as that from λRM1001 in an *arcA fnr* double null mutant (Table 2). This result provides further evidence consistent with the notion that the Fnr-2 sequence is important for P7 activity.

Using an oligonucleotide that hybridizes specifically within the *lacZ* gene, we determined the transcription initiation site of P7 on λRM1001 (Fig. 2A). Approximately 0.2 pmol of ^{32}P-labelled oligonucleotide was incubated with 15 μg of total RNA, and primer extension reactions were performed exactly as described previously (10, 11). Transcription initiated 2 residues upstream from that identified for the chromosomally encoded promoter (Fig. 1) (10, 11). The slight shift in the initiation site may indicate that the constraint normally imposed on transcription in the wild-type context is relieved somewhat when the downstream control sequences are removed. It should be noted that the 5′ end of the transcript derived from P7 on λRM1031 was the same as the wild-type start site and that transcription was induced anaerobically (data not shown).

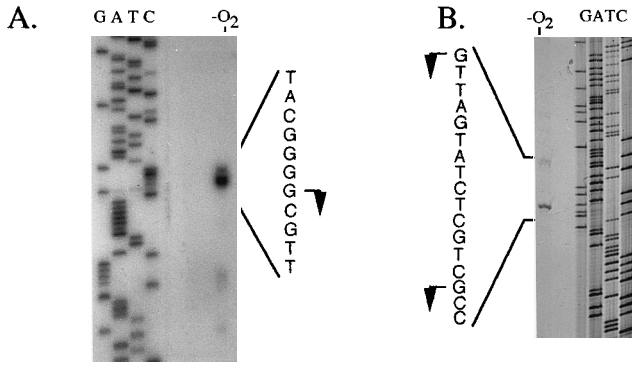

FIG. 2. Determination of the transcription start site in the *pfl-lacZ* fusions. Total RNA (15 μg) isolated from MC4100 containing λRM1001 (A) or λRM 20201 (B) grown anaerobically (lanes $-O_2$) was analyzed by primer extension. Reaction products were separated in denaturing 8% polyacrylamide gels (8).

Expression from P6 in isolation is regulated by oxygen. Expression from P6 (λRM20201) was induced approximately 4-fold in the *arcA*$^+$ *fnr*$^+$ host strain by anaerobiosis, but the degree of induction was reduced to only 1.6-fold in a *fnr* null mutant (Table 2). Remarkably, in an *arcA* mutant, expression was derepressed aerobically, indicating that ArcA and Fnr are both involved in regulating this promoter, even when upstream regulatory sequences are missing. These findings suggest that P6 activity is repressed by ArcA in aerobically grown cells and that there is a complex interplay between ArcA and Fnr at P6. That ArcA regulates expression of P6 is in accord with DNase I footprinting studies which have identified an ArcA-binding site that overlaps P6 (5) (Fig. 1). A primer extension experiment verified that in λRM20201 the site of transcription initiation was identical to that of the wild-type promoter (Fig. 1 and 2B).

Concluding remarks. The main conclusions that can be drawn from this work are as follows: (i) P7 activity is important in modulating ArcA-dependent anaerobic regulation of *pfl* expression. (ii) A DNA sequence located upstream of P7 and exhibiting similarity to a Fnr half-site (ATCAA) is an important determinant in the ArcA and Fnr regulation of *pfl* expression. (iii) Both the P6 and P7 promoters are regulated by ArcA and Fnr. (iv) Separation of the two promoters reveals that they have a nonadditive effect on *pfl* expression. (v) P7 possesses a strong intrinsic activity that is not responsive to oxygen when the promoter is isolated but that is regulated by oxygen in conjunction with P6 (see also reference 10). (vi) The complete 494-bp promoter-regulatory region is necessary for maximal anaerobic regulation of *pfl* expression. Taken together, these data are consistent with a functional interaction occurring between the ArcA and Fnr proteins (4, 12).

We are extremely grateful to Manuela Kaiser, Mike Merrick, and Barry Smith for their constructive criticism of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Begg, Y. A., J. N. Whyte, and B. A. Haddock.** 1977. The identification of mutants of *Escherichia coli* deficient in formate dehydrogenase and nitrate reductase activities using dye indicator plates. FEMS Microbiol. Lett. **2:**47–50.
2. **Birkmann, A., R. G. Sawers, and A. Böck.** 1987. Involvement of the *ntrA* gene product in the anaerobic metabolism of *Escherichia coli*. Mol. Gen. Genet. **210:**535–542.
3. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76:**4530–4533.
4. **Compan, I., and D. Touati.** 1994. Anaerobic activation of *arcA* transcription in *Escherichia coli*: roles of Fnr and ArcA. Mol. Microbiol. **11:**955–964.
5. **Drapal, N., and G. Sawers.** 1995. Purification of ArcA and analysis of its specific interaction with the *pfl* promoter-regulatory region. Mol. Microbiol. **16:**597–607.
6. **Green, J., and J. R. Guest.** 1994. Regulation of transcription at the *ndh* promoter of *Escherichia coli* by Fnr and novel factors. Mol. Microbiol. **12:**433–444.
7. **Kaiser, M., and G. Sawers.** Unpublished data.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
9. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
10. **Sawers, G.** 1993. Specific transcriptional requirements for positive regulation of the anaerobically inducible *pfl* operon by ArcA and Fnr. Mol. Microbiol. **10:**737–747.
11. **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.
12. **Sawers, G., and B. Suppmann.** 1992. Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and FNR proteins. J. Bacteriol. **174:**3474–3478.
13. **Sawers, R. G., E. Zehelein, and A. Böck.** 1988. Two-dimensional gel electro-

phoretic analysis of *Escherichia coli* proteins: influence of various anaerobic growth conditions and the *fnr* gene product on cellular protein composition. Arch. Microbiol. **149:**240–244.
14. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based protein and operon fusion cloning tools. Gene **53:**85–96.
15. **Sirko, A., E. Zehelein, M. Freundlich, and G. Sawers.** 1993. Integration host factor is required for anaerobic pyruvate induction of *pfl* operon expression in *Escherichia coli*. J. Bacteriol. **175:**5769–5777.
16. **Spiro, S., and J. R. Guest.** 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Rev. **75:**399–428.
17. **Suppmann, B., and G. Sawers.** 1994. Isolation and characterization of hypophosphite-resistant mutants of *Escherichia coli*: identification of the FocA protein, encoded by the *pfl* operon, as a putative formate transporter. Mol. Microbiol. **11:**965–982.