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Transcription Initiation at Multiple Promoters of the *pfl* Gene by $E\sigma^{70}$-Dependent Transcription In Vitro and Heterologous Expression in *Pseudomonas putida* In Vivo

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In vitro transcription experiments were used to provide further evidence that the gene encoding pyruvate formate-lyase (EC 2.3.1.54) from *Escherichia coli* is transcribed from seven promoters which cover a region of 1.2 kilobase pairs of DNA (G. Sawers and A. Böck, J. Bacteriol., 171:2485–2498, 1989). The results demonstrated that all promoters were recognized by the major RNA polymerase holoenzyme species $E\sigma^{70}$ in vitro. Further corroboration for multiple functional promoters came from heterologous expression of the *pfl* operon in the obligate aerobe *Pseudomonas putida*. An immunological analysis indicated that the pyruvate formate-lyase protein was synthesized from a multicopy plasmid in *P. putida*, and S1 nuclease protection of RNA transcripts confirmed that all the *pfl* promoters on the plasmid were recognized by the host RNA polymerase. Transcription initiated at the same sites in *P. putida* and in *E. coli* for all the transcripts that were analyzed.

Pyruvate formate-lyase (PFL; EC 2.3.1.54) plays a key role in the anaerobic metabolism of *Escherichia coli* (28); therefore, analysis and characterization of the control mechanisms regulating expression of its gene are of particular interest. The gene encoding PFL, together with that of its activating enzyme (*act*), has been cloned and sequenced (9, 35, 38). More recent analyses have shown that a third gene (*orf*), which encodes a protein of unknown function, lies immediately 5′ to the *pfl* gene (41). The *act* gene is not cotranscribed with *pfl*, but the *orf* and *pfl* genes do form an operon. Studies dealing with the transcriptional regulation of the *pfl* gene have uncovered a remarkably complex, and so far unique, mechanism by which the expression of the gene is controlled (41). The construction of *pfl′-′lacZ* fusions in which the 5′-regulatory sequences of the *pfl* gene were successively deleted, together with analysis of the transcripts produced from these constructs, indicated that transcription initiates from at least six (probably seven) promoters spanning approximately 1,200 base pairs of DNA (41). The data indicate that synthesis of these transcripts is coordinately regulated in response to oxygen, pyruvate, and the presence of the Fnr protein. Three (or four) lie within the *orf* structural gene, one lies in the intergenic region between *orf* and *pfl*, and two more lie upstream of *orf* (Fig. 1). *orf* and *pfl* are cotranscribed from the two uppermost promoters P_6 and P_7, but *pfl* can also be transcribed alone from the operon internal promoters. The consequence of such a mechanism of control is that *pfl* and *orf* are regulated in exactly the same manner; however, there are profound differences in the amounts of the translatable message of each gene. Indeed, it has been estimated that, on a protein basis, PFL is present in a 200-fold excess over ORF (open reading frame) in the cell (41).

Examination of the DNA sequences lying immediately 5′ to the potential start sites of transcription of *pfl* revealed only poor homologies with the canonical −10 and −35 consensus promoter sequences (17, 32), notably in the −35 region (41). One reason for this could be that all are positively regulated by Fnr and perhaps require only sufficient homology to facilitate promoter recognition by RNA polymerase holoenzyme ($E\sigma^{70}$), after which Fnr can function and activate transcription. Poor homology in the −35 region has already been proposed as a characteristic feature of positively regulated genes (37); other Fnr-dependent genes also show poor promoter consensus sequence homologies (10, 25, 31).

Because of the unusual nature of this transcription mechanism, we wished to provide more evidence which would substantiate the existence of multiple promoters in the *pfl* operon. With this aim in mind, we examined transcription of the *pfl* operon in vitro and its heterologous expression in the obligate aerobe *Pseudomonas putida*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The buffered, rich medium (TGYEP [pH 6.5] containing 0.4% [wt/vol] glucose) was used in all growth experiments for RNA preparations (40). For all other growths, LB medium (1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl) was used.

Anaerobic growth was performed as described previously (3). The following antibiotics were used at the indicated final concentrations: chloramphenicol, 20 μg/ml; kanamycin sulfate, 50 μg/ml.

Recombinant DNA technqiues. The procedures of Maniatis et al. (34) were used for plasmid preparations, restriction enzyme digestions, ligations, transformations, 5′-end-labeling of DNA, and agarose gel electrophoresis. Transformation of *P. putida* 2440 was performed as described by Franklin (14). Radioactive DNA size markers were prepared by restriction of pBR322 with either *Hin*fI or *Hpa*II, followed by dephosphorylation with calf intestinal phosphatase (Boehringer GmbH, Mannheim, Federal Republic of Germany) and 5′-end-labeling with [γ-^{32}P]dATP and polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.).

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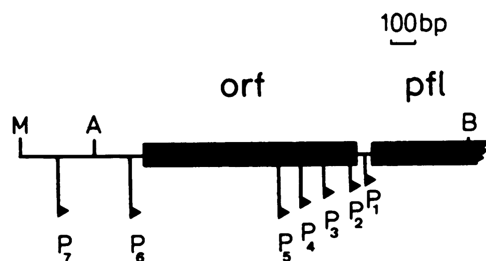

FIG. 1. Transcriptional start sites of the *pfl* operon. The positions of the seven promoters (P) are indicated. The complete gene for the open reading frame (*orf*) and the 5′ end of the pyruvate formate-lyase (*pfl*) gene are shown. Abbreviations: M, *Mlu*I; A, *Asp*700I; B, *Bam*HI.

Construction of plasmid p153E1. The construction of plasmid p153E1 is summarized in Fig. 2. Briefly, the *pfl* and *orf* genes from plasmid p29 were isolated on a 3.5-kilobase (kb) *Asp*700I-*Fsp*I fragment. This fragment then was ligated into the *Eco*RI site of the shuttle vector pBT306.1, after treatment of the cut vector with Klenow enzyme (Boehringer GmbH) and deoxynucleoside triphosphates and dephosphorylation with calf intestinal phosphatase, to generate plasmid p153E1. Plasmid pBT306.1 (a gift from G. Schumacher, Boehringer GmbH) was a construct in which the 1.4-kb *Hae*II fragment containing the kanamycin resistance gene from pACYC177 was inserted in the unique *Pvu*II site of the broad-host-range vector RSF1010.

RNA preparation. RNA was prepared from exponentially growing cells by the hot phenol extraction method described by Aiba et al. (1).

In vitro transcription. In vitro transcription was performed essentially as described by Buttner and Brown (6), with minor modifications. Plasmid p29 (2 μg) was equilibrated with 5 U (10 μg) of RNA polymerase holoenzyme (Boehringer GmbH) in 40 μl of a reaction mixture which included 40 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 0.6 mM EDTA, 15 mM KCl, 1.5 mM dithiothreitol, 0.75 mg of bovine serum albumin per ml, and 10% (vol/vol) glycerol. After 5 min of incubation at 37°C, ATP, CTP, GTP, and UTP were added, each to a final concentration of 0.4 mM, followed by incubation at 37°C for a further 2 min. Further reinitiation of polymerase was prevented by the addition of 5 μg of heparin, after which the reaction was allowed to proceed for a further 15 min at 37°C. The reaction was terminated by phenol extraction followed by ethanol precipitation. In experiments in which purified core enzyme and the σ^{70} subunit were used, the components were mixed in the reaction mixture.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and characteristics	Reference or source
Strains		
E. coli		
MC4100	F^- *araD139* Δ(*argF-lac*) *U169 ptsF25 deoC1 relA1 flbB530 rpsL150* λ^-	7
FM420	MC4100 *recA56*	46
RM202	MC4100 Δ*pfl-25* Ω(*pfl*::*cat* pACYC184) Δ(*srl-recA*) *306*::Tn*10*	40
P. putida mt-2 KT2440	r^- m^+	2
Plasmids		
p29	Cm^r orf^+ pfl^+ act^+	9, 38, 41
pBT306.1	RSF1010 replicon Su^r Sm^r Kan^r (from pACYC177) mob^+	G. Schumacher
p153E1	pBT306.1 pfl^+ orf^+	This study

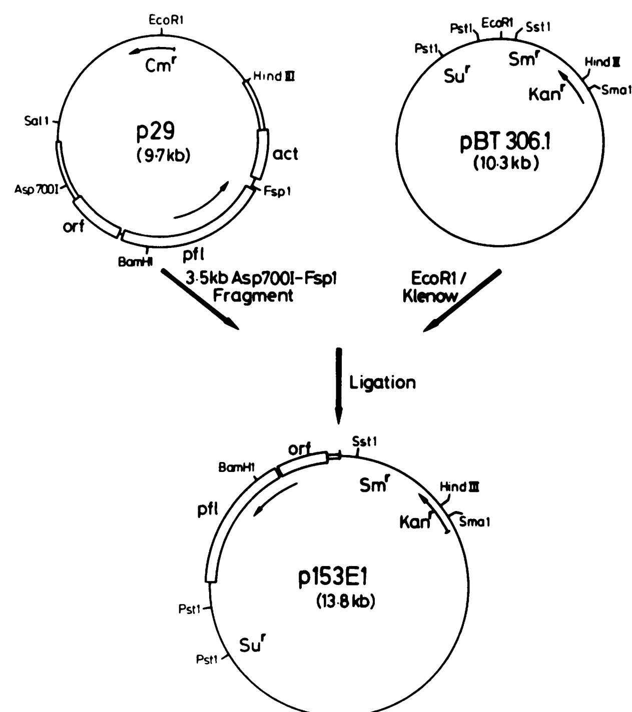

FIG. 2. Construction of plasmid p153E1. Abbreviations: orf, pfl, and act, gene symbols for the open reading frame, pyruvate formate-lyase, and PFL-activating enzyme, respectively; Kan^r, Sm^r, and Su^r indicate kanamycin, streptomycin, and sulfonamide resistance markers, respectively. In the interest of clarity, only the relevant restriction enzyme sites are shown.

S1 nuclease protection analysis of transcripts. Mapping of transcripts with S1 nuclease was done by the procedure of Berk and Sharp (4), as modified by Wich et al. (45). The 6.1-kb *Bam*HI-*Hin*dIII fragment from plasmid p29 (Fig. 2) was used for mapping the *pfl* transcripts. The fragment was 5′-end-labeled with [γ-^{32}P]dATP at the *Bam*HI site. In a typical experiment, 100,000 cpm of labeled DNA fragment (0.2 to 0.5 pmol) was precipitated together with either 50 μg of in vivo-synthesized RNA or half of the sample produced from the in vitro transcription experiment described above. After gentle drying, the washed pellet was suspended in 30 μl of hybridization buffer that was made up of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4], 1 mM EDTA, 400 mM NaCl, and 80% formamide; heated to 85°C for 15 min; and then immediately placed at the hybridization temperature of 54°C. After 3 h of incubation, 300 μl of S1 nuclease buffer (300 U of S1 nuclease [Boehringer GmbH] in 280 mM NaCl, 50 mM sodium acetate [pH 4.6], 4.5 mM zinc sulfate, and 20 μg of salmon sperm DNA per ml) was added, and the mixture was incubated for 1 h at 30°C. The reaction was stopped by the addition of 10 μl of 0.5 M EDTA, followed by phenol extraction and ethanol precipitation. All precipitates then were suspended in an equivalent volume

(usually 10 μl) of formamide dye (34), and 1 μl from each sample was applied to a denaturing 4% polyacrylamide gel prepared as described by Maniatis et al. (34).

SDS-polyacrylamide gel electrophoresis of proteins. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (29) by using either 8% polyacrylamide gels or gradient gels of 7.5 to 12.5% polyacrylamide. Gels were stained with Coomassie brilliant blue R250. For analysis of crude extracts of *E. coli* or *P. putida* strains, cells were grown to an optical density at 550 nm of between 0.6 and 0.7, and a fraction (equivalent to 1 ml of a culture with an optical density at 550 nm of 1.0) was centrifuged, and the pellet was suspended in 50 μl of disaggregation buffer (6 M urea, 10% [vol/vol] β-mercaptoethanol, 4% [wt/vol] SDS, and 125 mM Tris hydrochloride [pH 6.8]). After the sample was heated to 100°C for 1 min, 15-μl fractions were applied to the gel.

Immunological detection of PFL. After SDS-gel electrophoresis, the proteins were blotted onto nitrocellulose membranes (Amersham Buchler, Braunschweig, Federal Republic of Germany) by the procedure of Towbin et al. (43). The membrane was then washed for 10 min at room temperature with 50 ml of buffer A (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl) containing 1% (wt/vol) gelatin. A total of 25 μl of anti-PFL antiserum (11) then was added to this mixture, and incubation was continued for a further 14 to 16 h. The filter was washed three times with 50 ml of buffer A, after which it was incubated with protein A-horseradish peroxidase conjugate (Bio-Rad, Munich, Federal Republic of Germany) in 50 ml of buffer A containing 1% (vol/vol) Tween 20 for 2 h. After a further wash with buffer A, the filter was developed for 60 min in 50 ml Tris hydrochloride (pH 8.5) buffer containing 9 mg of 4-chloro-1-naphthol (Bio-Rad) and 0.015% (vol/vol) H_2O_2. The reaction was stopped by washing the filter with water, and the filter was air-dried before storage.

RESULTS

In vitro transcription of the *pfl* promoters is mediated by RNA polymerase holoenzyme ($E\sigma^{70}$). In vitro transcription of the *pfl* operon promoters was performed by using purified RNA polymerase holoenzyme ($E\sigma^{70}$) with supercoiled plasmid p29 as the template. The transcripts generated from the 5'-regulatory region were identified by S1 nuclease protection analysis. The use of this technique with a labeled DNA probe ensured that identical conditions could be used to analyze in vivo- and in vitro-generated transcripts, thus facilitating a direct comparison. The $E\sigma^{70}$-dependent in vitro transcription from the *pfl* promoters revealed a transcript pattern very similar to that derived from in vivo-synthesized RNA (Fig. 3; compare lanes 3 and 5); all transcripts found in vivo also were present in in vitro RNA samples. Use of linear templates yielded essentially the same results (data not shown). These results are consistent with the fact that all seven major transcripts identified from in vivo-synthesized RNA (41) were primary transcripts and not the result of processing. Significantly, the RNA polymerase preparation yielded relatively long transcripts (over 1.2 kb in length), a feature which is unusual for *E. coli* RNA polymerase in vitro; the average length of transcript generated is usually about 500 to 600 bases (8). A major difference between in vitro- and in vivo-synthesized transcripts was the lower levels of transcripts from P_1 to P_7 in vitro in comparison with those found in vivo. This is perhaps due to the fact that all of these promoters are positively regulated by Fnr in vivo (40, 41) (see Discussion).

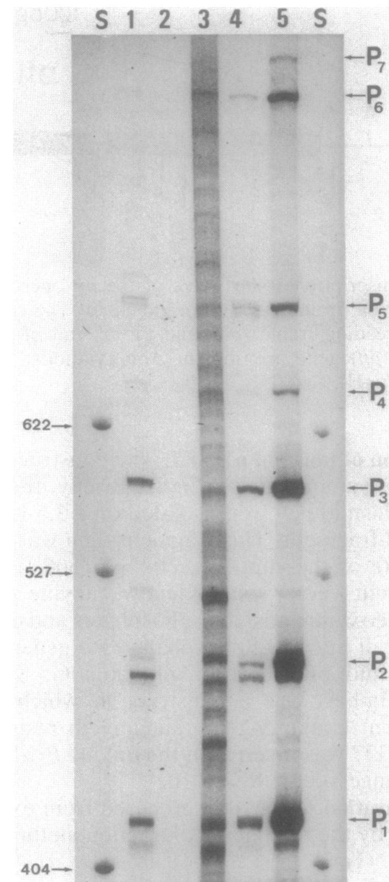

FIG. 3. S1 nuclease protection analysis of in vitro-generated *pfl* transcripts. An autoradiogram is shown of protected DNA fragments generated after treatment of DNA-RNA hybrids with S1 nuclease. The fragments were separated in a denaturing 4% polyacrylamide gel. S, DNA size standards generated by *Hpa*II restriction of pBR322 (numbers to the left of the gel are in bases). Protected fragments generated from RNA transcripts synthesized in vivo from FM420 grown aerobically (lanes 1 and 4) and anaerobically (lane 5) are shown alongside in vitro-synthesized RNA transcripts derived from plasmid p29 in the absence (lane 2) and presence (lane 3) of *E. coli* RNA polymerase holoenzyme ($E\sigma^{70}$). P_1 to P_7 indicate transcripts generated from promoters one to seven, respectively.

In an attempt to substantiate these findings further, transcription was performed with RNA polymerase core enzyme (E) lacking the σ^{70} subunit (Fig. 4). The data show that initiation at the *pfl* promoters was at a very much lower level with core enzyme alone (Fig. 3, lane 5) in comparison with that effected by the holoenzyme (Fig. 3, lane 4). The addition of σ^{70} to the transcription assay (Fig. 3, lane 6) stimulated transcription, yielding the same pattern as that obtained with holoenzyme. Results of this experiment strongly suggest that in vitro transcription of the *pfl* promoters is $E\sigma^{70}$-dependent.

The quality of the enzyme preparations used is shown in Fig. 5. No σ^{70} subunit was visible in the core enzyme preparations (Fig. 5, lane B) in comparison with the holoenzyme (Fig. 5, lane A), which is consistent with the transcription data (Fig. 4). The addition of purified sigma subunit to the core enzyme restored the profile typical for the holoenzyme (Fig. 5, lane C).

Heterologous expression of the *E. coli pfl* gene in *P. putida*. A DNA fragment carrying promoters one to six plus the

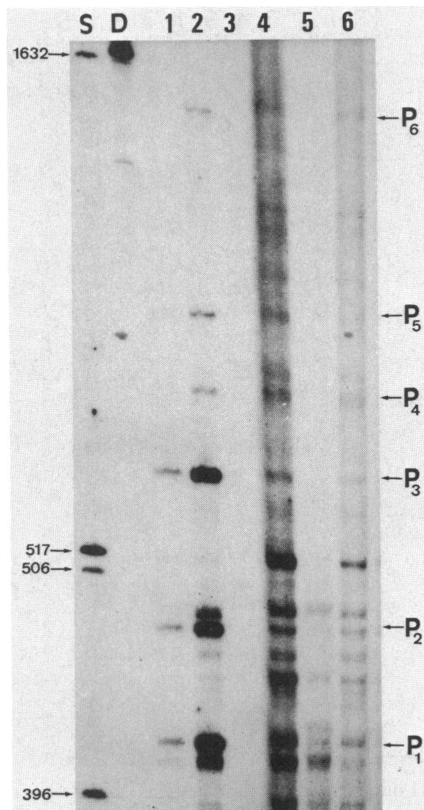

FIG. 4. σ^{70}-dependent transcription of *pfl*. S1 nuclease-protected DNA fragments were separated in a denaturing 4% polyacrylamide gel as described in the legend to Fig. 2. S, DNA size standards generated by *Hin*fI restriction of pBR322 (numbers to the left of the gel are in bases); D, *Bam*HI-*Hin*dIII DNA fragment; lane 1, RNA transcripts from FM420 grown aerobically; lane 2, RNA transcripts from FM420 grown anaerobically; lane 3, RNA transcripts from RM202 (Δ*pfl*) grown anaerobically; lane 4, RNA transcripts generated in vitro from p29 in the presence of RNA polymerase holoenzyme (10 μg); lane 5, RNA transcripts generated in vitro from p29 in the presence of 10 μg of RNA polymerase core enzyme; lane 6, RNA transcripts generated in vitro from p29 in the presence of 10 μg of RNA polymerase core enzyme plus 2.0 μg of the σ^{70} subunit.

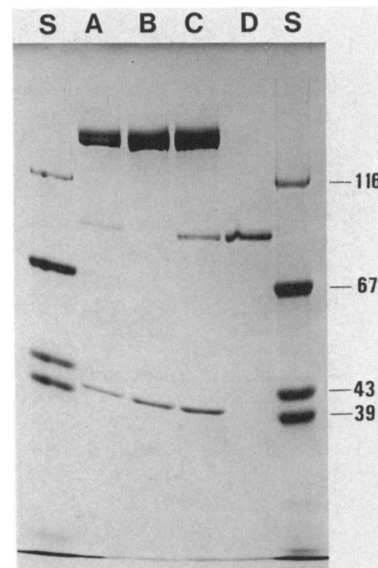

FIG. 5. SDS-polyacrylamide gels of RNA polymerase preparations. Samples were separated on a gradient gel of 7.5 to 12.5% polyacrylamide. S, Protein molecular weight standards (indicated on the right, in thousands) were β-galactosidase (116,000), bovine serum albumin (67,000), aldolase (43,000), and ovalbumin (39,000). Lane A, 10 μg of RNA polymerase holoenzyme (Boehringer GmbH); lane B, 10 μg of RNA polymerase core enzyme; lane C, 10 μg of RNA polymerase core enzyme plus 2 μg of purified σ^{70} subunit; lane D, 5 μg of purified σ^{70} subunit.

complete structural genes encoding ORF and PFL was cloned into the broad-host-range vector pBT306.1 (Fig. 2; see Materials and Methods). The sequences necessary for anaerobic induction of *pfl* expression are retained on this DNA insert (41). *E. coli* MC4100 and *P. putida* 2440 were transformed with the plasmid p153E1, and the protein profiles of the strains were examined by SDS-gel electrophoresis after growth under anaerobic (*E. coli* only) or aerobic (both strains) conditions (Fig. 6A). A strongly staining protein band was present in *P. putida* containing p153E1 (Fig. 6A, lane 6), which showed the same apparent molecular weight as purified PFL from *E. coli* (Fig. 6A, lane 1). No such band was observed for *P. putida*, which did not bear p153E1 (Fig. 6A, lane 7). Significantly more PFL was synthesized in the *E. coli* strain bearing p153E1 compared with that synthesized in the *E. coli* strain without the plasmid (Fig. 6A). These results are consistent with a gene dose effect on PFL synthesis (35, 40). The origin and identity of the protein of approximately 50 kilodaltons which was present in anaerobically grown MC4100 cells, but which was dramatically reduced in plasmid-bearing MC4100 cells, are unclear; however, it was not the product of the *orf* gene, which was not visible on Coomassie brilliant blue R250-stained gels, even when it was expressed from a multicopy plasmid (G. Sawers and A. Böck, unpublished data).

Immunoblotting analysis of the same samples with anti-PFL antibodies confirmed that the Coomassie brilliant blue R250-staining band in *P. putida* containing p153E1 was indeed PFL (Fig. 6B, lane 6) and demonstrated that no protein species, of this or any other size, which showed a cross-reaction with the anti-PFL antibodies was present in *P. putida* (Fig. 6B, lane 7). Overloading of the gel with *P. putida* extract also did not reveal any cross-reacting species (data not shown). These results indicate that (i) the *pfl* gene is expressed in *P. putida* and (ii) there are no translational barriers which may have arisen because of differences in codon usage between *E. coli* and *P. putida*. Significantly, aerobic growth of both *P. putida* and *E. coli* bearing p153E1 resulted in single PFL protein bands (Fig. 6B, lanes 3 and 6) with similar intensities, when the dilution factor of the samples was taken into account, while in *E. coli* strains grown anaerobically, both in the presence and absence of plasmid, a second anti-PFL cross-reacting species was observed (Fig. 6B, lanes 4 and 5). This protein was a degradation product of PFL which resulted from exposure of active, free radical-bearing enzyme to molecular oxygen (A. F. V. Wagner and J. Knappe, manuscript in preparation).

Because of the dilutions of the samples applied to the gels, it was not possible to see any antibody reaction in aerobically grown MC4100 (Fig. 6B, lane 2); however, application of larger amounts of sample resulted in the detection of PFL at a level which was comparable to those found previously (40).

Since we showed that PFL protein was synthesized in *P. putida*, it was of interest to examine the products of *pfl* gene transcription from this strain. This was done by performing an S1 nuclease protection experiment with the RNA isolated

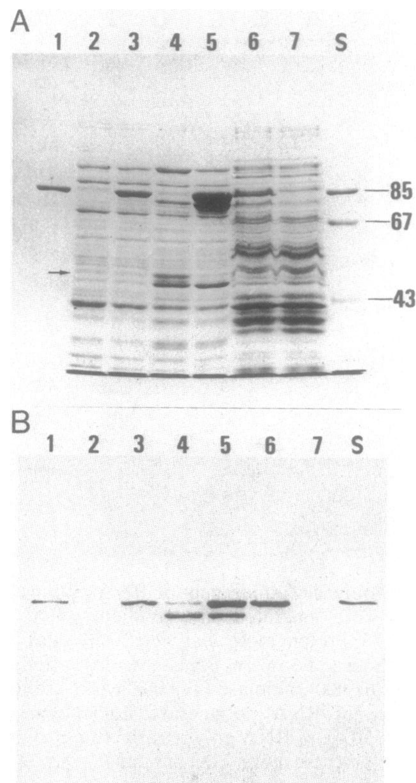

FIG. 6. Immunoblot analysis of *E. coli* and *P. putida* crude cell-free extracts with anti-PFL antibodies. Samples were prepared as described in the text, and proteins were separated on SDS-8% polyacrylamide gels. Shown is a Coomassie brilliant blue R250-stained gel (A) and an immunoblot treated with antibodies directed against PFL (B). Samples included 1.5 μg of purified PFL (11) (lane 1), MC4100 with O_2 (lane 2), MC4100(p153E1) with O_2 (lane 3), MC4100 without O_2 (lane 4), MC4100(p153E1) without O_2 (lane 5), *P. putida*(p153E1) with O_2 (lane 6), *P. putida* with O_2 (lane 7). Protein molecular weight standards (indicated on the right, in thousands) (S) included 3 μg each of PFL (85,000), bovine serum albumin (67,000), and aldolase (43,000). For the immunoblot (B) one-half the amount of material applied in panel A was used for lanes 2, 4, 6, and 7 and one-tenth the amount was used for lanes 1, 3, 5, and S. The arrow indicates the protein of ~50 kilodaltons that was reduced in MC4100 bearing p153E1.

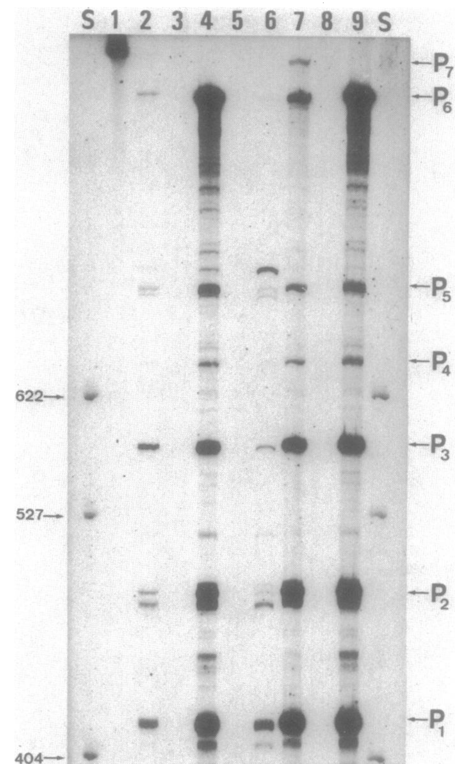

FIG. 7. S1 nuclease protection analysis of *pfl* transcripts from *E. coli* and *P. putida*. Protected DNA fragments were separated in a denaturing 4% polyacrylamide gel, and an autoradiogram is shown. All growths for RNA isolation were performed in TGYEP medium (pH 6.5) plus 0.4% (wt/vol) glucose. S, DNA size standards (indicated on the left, in bases) generated by *Hpa*II restriction of pBR322; lane 1, the *Bam*HI-*Hin*dIII DNA fragment used as hybridization probe; lane 2, FM420 grown aerobically; lane 3, RM202 (Δ*pfl*) grown aerobically; lane 4, RM202 (Δ*pfl*) bearing p153E1 grown aerobically; lane 5, *P. putida* grown aerobically; lane 6, *P. putida* bearing p153E1 grown aerobically; lane 7, FM420 grown anaerobically; lane 8, RM202 (Δ*pfl*) grown anaerobically; lane 9, RM202 (Δ*pfl*) containing p153E1 grown anaerobically. P_1 to P_7 indicate transcripts generated from promoters one to seven, respectively.

from aerobically grown *P. putida* and *P. putida* bearing p153E1 and comparing the derived transcription products with those from *E. coli* wild-type and Δ*pfl* strains, with and without p153E1 (Fig. 7). As expected, no protected DNA fragments were found when RNA from *P. putida* was examined (Fig. 7, lane 5). However, in the strain bearing p153E1, all six promoters present on the plasmid (the insert in p153E1 did not have the DNA sequence of promoter 7) were recognized by the host RNA polymerase (Fig. 7, lane 6); and significantly, the 5' ends of all transcripts were very similar to those found both in wild-type *E. coli* grown aerobically (Fig. 7, lane 2) and in strain RM202 (Δ*pfl*) bearing p153E1 (Fig. 7, lane 4). Under anaerobic growth conditions, the transcript from promoter 7 was induced in strain FM420 (Fig. 7, lane 7), as were the other transcripts; and as expected, under these optimal expression conditions, no transcript from promoter 7 was synthesized from p153E1 in strain RM202. These results provide further evidence that support the idea that the *pfl* transcripts originate from multiple promoters. The strengths of the signals generated from protected DNA fragments were the same for RNA from aerobically and anaerobically grown RM202 carrying p153E1 (Fig. 7, lanes 4 and 9). This was due to a limitation in the amount of the radioactive DNA fragment used for hybridization with the RNA. In these two cases the RNA was in excess (data not shown).

Although the patterns of the signals found in aerobically grown *E. coli* and *P. putida* bearing plasmids were similar (Fig. 7, lanes 2 and 6), the relative intensities of some of the transcripts were different. In particular, the upper band of the cluster of three bands generated from P_5 was stronger in *P. putida* than in *E. coli*, and the intensity of the transcript from P_6 showed the opposite tendency. The reason for this is unclear, but it is possible that it was a plasmid effect or that it perhaps resulted from the absence of the appropriate transcription factors in *P. putida*.

DISCUSSION

Recent analysis of the *pfl* operon by S1 nuclease protection, primer extension, and construction of various *lacZ* gene fusions provided evidence which indicated that the *pfl*

gene is transcribed from seven promoters (41). In this report we have provided two further lines of evidence in support of this conclusion. First, through in vitro transcription experiments with RNA polymerase holoenzymes ($E\sigma^{70}$), we showed that a similar transcript pattern was observed from $E\sigma^{70}$ RNA compared with that generated from RNA isolated from growing cells. Second, expression of the *pfl* gene in the obligate aerobe *P. putida* revealed the same transcripts that were seen for the wild-type *E. coli pfl* gene. Taken together these data are entirely consistent with the fact that the 5′ ends of the transcripts arise through distinct initiation events and are not the result of the processing of a single, long transcript.

Sequence analysis of the DNA regions 5′ to the transcription start sites of the *pfl* gene (41) indicated that only a low level of similarity exists between the promoters and the canonical −10 and −35 promoter consensus sequences (17). The promoters termed P_1 to P_5 had two of the three conserved bases, which are deemed to be essential for recognition by $E\sigma^{70}$ (19), in the −10 and −35 regions, with the −10 region exhibiting variable spacing with respect to the start site of transcription (41). In vitro transcription from all five of these promoters was found to be stimulated by the addition of purified σ^{70} to a core enzyme preparation of RNA polymerase (Fig. 4). It was observed that core enzyme alone initiated specific transcription to a small extent. This could have been the result of very small amounts of σ^{70} in the preparation; however, on examination of the enzyme preparations on an SDS-polyacrylamide gel, no traces of the σ^{70} subunit could be observed (Fig. 5). The other possible explanation for this phenomenon perhaps lies in the fact that when supercoiled plasmids are used as templates in in vitro transcription experiments, supercoiling can partially suppress nonspecific binding of core enzyme to DNA (27).

$E\sigma^{70}$-dependent transcription also was found for promoters six and seven, which showed good homology with the −10 consensus sequence but very poor homology with the −35 consensus sequence (41). That only the −10 region of these promoters was sufficient for recognition by $E\sigma^{70}$ in vitro is not an unprecedented finding, since Ponnambalam et al. (36) have demonstrated recently that the −35 sequence of the *galP2* promoter of *E. coli* can be removed without having any deleterious effect on the extent of transcription in vitro.

Although a similar pattern was observed when in vitro- and in vivo-generated transcripts were compared, the relative amounts of the specific transcripts in vitro were much lower than those found in the in vivo RNA samples, indicating that transcription in vitro is inefficient. Also noticeable was the higher degree of nonspecific initiation in vitro compared with what was observed with RNA in vivo, although on close examination, the majority of these signals also were observed in in vivo-generated RNA samples (Fig. 3) (41), albeit to a much lesser extent. These phenomena could be the consequence of inappropriate DNA conformation in vitro. A further explanation for this discrepancy could be due to a requirement for a specific transcription factor(s), e.g., Fnr, which was absent in the transcription assay. Positively regulated promoters, like those of the *pfl* operon (41), generally require the addition of the appropriate factor in vitro to stimulate efficient transcription from the promoter. This has been demonstrated for the *cat* and *lac* genes (30, 33), which are dependent on the catabolite activator protein. The Fnr protein is known to be one of the factors required for high-level expression of the *pfl* promoters in vivo (40, 41), since transcription is reduced approximately five- to sixfold in a strain that is unable to synthesize the Fnr protein. More recent experiments, however, indicate that Fnr is not the only factor necessary for *pfl* gene transcription (41; Sawers and Böck, unpublished data). The isolation of such factors in a pure and active form should allow us to answer some of these questions.

Although the Fnr protein has been purified, progress toward elucidation of its mechanism of action has been hampered by the extreme susceptibility of the N-terminal region of the protein to proteolysis (42, 44). Interestingly, this region contains several cysteine residues and has been proposed by Spiro and Guest (42) to play a role in sensing anaerobiosis. Perhaps through the use of in vitro transcription an assay system could be developed to permit the study of this phenomenon in more detail.

Heterologous expression of the *pfl* operon was achieved in *P. putida*, and remarkably, all six of the promoters included on the plasmid construct were transcribed by the *Pseudomonas* RNA polymerase and all initiation sites were the same as those in *E. coli*. These data taken together with the in vitro transcription data and the analysis of deletion clones of the *pfl* promoter region (41) lend further support to the idea of the existence of multiple promoters in the *pfl* operon. Although an alternative explanation could be that a specific transcript processing system which recognizes and cleaves the same sequences exists in *E. coli* and *P. putida*, it is more plausible to assume that the promoters are recognized by an RNA polymerase holoenzyme with similar sequence recognition properties. It has been shown that *P. putida* has an enzyme which recognizes contact points on promoters similar to those recognized by $E\sigma^{70}$ (15). More recent evidence indicates that *Pseudomonas* species also contain forms of RNA polymerase which have minor factor species (12, 39), analagous to NtrA of *E. coli* (16, 18, 20). In view of the fact that transcription of *pfl* in *E. coli* is known to be independent of NtrA (5, 41) and in consideration of the available data, the evidence supports the contention that the *pfl* promoters, like those of other Fnr-dependent genes, are recognized and transcribed in vivo by $E\sigma^{70}$, as has been suggested by the work of Spiro and Guest (42) and Cole and colleagues (24).

The initiation sites of several *Pseudomonas* genes transcribed in *E. coli* have been shown to be the same as those in the natural host (13, 21–23); however, we have not been able to find any reports of the transcriptional analysis of an *E. coli* gene in *Pseudomonas* species. Little is known about the expression of *E. coli* genes in *Pseudomonas* species, but the general conclusion that could be drawn from the studies performed is that *Pseudomonas* species are much more permissive hosts for expression of *E. coli* genes than is *E. coli* for expression of genes from *Pseudomonas* species (26). Nonregulated *E. coli* genes are well expressed in *P. putida*, while regulated genes are less well expressed. Although *pfl* falls into the latter category of genes, its expression nevertheless was good in *P. putida*; the degree of transcription from the multicopy plasmid in *P. putida* was qualitatively comparable to that of aerobically grown *E. coli* (Fig. 7).

Examination of the protein profile of *P. putida* containing the plasmid-borne *pfl* gene revealed that the *pfl* transcripts are efficiently translated, indicating that there is no translational barrier for this gene in *P. putida*. Previous analyses have determined that when *Pseudomonas* genes are transcribed from inducible *E. coli* promoters, there is also only limited restriction to translation in *E. coli* (26). Synthesis of PFL in *P. putida* yields a suitable substrate for biochemical analysis since the enzyme cannot be activated in the heterologous host because of the absence of the gene encoding the PFL-activating enzyme. Therefore, only the complete poly-

peptide is produced without contaminating amounts of the degraded form of the enzyme (see Fig. 6), which results from the oxygen-dependent cleavage of the activated enzyme species (Wagner and Knappe, manuscript in preparation).

As discussed above, the amounts of *pfl* transcripts produced in *P. putida* containing p153E1 were comparable to those produced in wild-type *E. coli*; however, there was considerably more PFL protein synthesized in *P. putida* (Fig. 6). We do not know whether this is due to increased transcript stability, more efficient translation, or a longer half-life of the PFL protein; but such heterologous expression studies open up a whole new avenue of research which can be applied to our understanding of the regulation of this protein, both at the molecular biological and biochemical levels.

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