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A Carbon Starvation Survival Gene of *Pseudomonas putida* Is Regulated by σ^{54}

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By using mini-Tn*5* transposon mutagenesis, two mutants of *Pseudomonas putida* ATCC 12633 were isolated which showed a marked increase in their sensitivity to carbon starvation; these mutants are presumably affected in the Pex type of proteins that *P. putida* induces upon carbon starvation (M. Givskov, L. Eberl, and S. Molin, J. Bacteriol. 176:4816–4824, 1994). The affected genes in our mutants were induced about threefold upon carbon starvation. The promoter region of the starvation gene in the mutant MK107 possessed a strong σ^{54}-type promoter sequence, and deletion analysis suggested that this was the major promoter regulating expression; this was confirmed by transcript mapping in *rpoN*$^+$ and *rpoN* mutant backgrounds. The deletion analysis implicated a sequence upstream of the σ^{54} promoter, as well as a region downstream of the transcription start site, in the functioning of the promoter. Two σ^{70}-type Pribnow boxes were also detected in the promoter region, but their transcriptional activity in the wild type was very weak. However, in a σ^{54}-deficient background, these promoters became stronger. The mechanism and possible physiological role of this phenomenon and the possibility that the sequence upstream of the σ^{54} promoter may have a role in carbon sensing are discussed.

Recent studies have established that, contrary to earlier implicit assumptions, the so-called nondifferentiating bacteria do undergo an elaborate process of molecular realignment upon starvation which leads to the development of a resistant cellular state. Such differentiated cells possess markedly enhanced resistance to a variety of individual stresses (21, 22). These studies have been most advanced in *Escherichia coli* (15, 25, 38), but evidence is accumulating that similar processes operate in marine *Vibrio* species (43) and in *Salmonella typhimurium* (50). This differentiation involves expression of several temporal classes of starvation genes that code for special resistance proteins and depends on alternate σ factors. In *E. coli*, two σ factors, σ^{38} and σ^{32}, have been implicated in the starvation response, with the former playing a specially critical role (20, 27, 40). Evidence has also been presented that these σ factors are regulated primarily at the posttranscriptional level by a mechanism that involves mRNA secondary structure (32, 39) and that carbon starvation in *E. coli* might be sensed through the accumulation of homoserine lactone (18).

Given the ecological niches of *E. coli* and *S. typhimurium*, it is probable that they experience periods of feast and famine. Bacteria in many other habitats probably experience an even greater degree of nutrient scarcity with survival being mostly a matter of coping with prolonged starvation. Members of the genus *Pseudomonas* inhabit soil and groundwater environments which typically are very nutrient poor (11, 16) and thus belong to the latter class of bacteria. Earlier studies established the remarkable starvation resistance of many species of this genus (8, 36). More recently, Molin and his coworkers have shown that, like *E. coli* and other above-mentioned bacteria, *Pseudomonas putida* KT2442 also exhibits a temporal program of gene expression leading to the development of a general resistant state (13). They also showed (12) that, like *E. coli* (35, 47), this bacterium may synthesize two classes of starvation proteins: Pex, concerned with increased cellular resistance, and Cst, concerned with escape from starvation rather than with cellular resistance (35).

Thus, the *P. putida* starvation response qualitatively resembles—at the starvation protein synthesis level—that of *E. coli* and other bacteria. But there are also differences in that the acquired resistance is greater, more proteins appear to be involved, and the major adaptation period lasts longer (12).

We report here the isolation of two mutants of *P. putida* ATCC 12633 which exhibit a marked increase in sensitivity to carbon starvation. According to the terminology used by us (35) and Molin and his coworkers (12), these mutants are therefore likely to be affected in *pex* genes. We have also carried out detailed deletion and primer extension analyses of the starvation promoter region of one of these mutants. Our results show that the major promoter responsible for the regulation of this gene is σ^{54} dependent. This is the first instance implicating σ^{54} in development of cellular resistance to starvation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. All strains and plasmids used in this study are listed in Table 1. *P. putida* ATCC 12633 was purchased from the American Type Culture Collection. Luria-Bertani (LB) and M9 minimal media were prepared as described previously (41). The following antibiotics were used at the indicated concentrations (in micrograms per milliliter) for *P. putida*: tetracycline (50), streptomycin (400), kanamycin (50), and rifampin (150). For *E. coli*, antibiotics were used at the indicated concentrations (micrograms per milliliter): tetracycline (25), ampicillin (100), streptomycin (25), kanamycin (50), and chloramphenicol (34). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 50 μg/ml) was used to detect β-galactosidase production on plates.

Transposon mutagenesis. Our strategy for this mutagenesis necessitated a

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TABLE 1. Strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
CC118 λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir* phage lysogen; Rif^r	17
S17-1	*recA thi pro hsdR* mutant M^+ RP4:2-Tc::Mu-Km::Tn*7* Tp^r Sm^r	49
S17-1 λ*pir*	S17-1 λ*pir* phage lysogen	31
HB101	F^- *hsdS20 recA13 arg14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	4
LE392	F^- *hsdS574 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ^-	Promega Corp.
MC1061	*hsdR2 hsdM*$^+$ *hsdS*$^+$ *araD139* Δ(*ara-leu*) Δ*lac galE15 galK16 rpsL mcrA mcrB1*	This laboratory
Pseudomonas putida		
ATCC 12633	Prototroph	American Type Culture Collection
MK1	Derivative of ATCC 12633; Rif^r	This study
MK101	Tn*5* mutant of *P. putida* MK1; Km^r Rif^r	This study
MK104	Tn*5* mutant of *P. putida* MK1; Km^r Rif^r	This study
MK107	Tn*5* mutant of *P. putida* MK1; Km^r Rif^r	This study
MK114	Tn*5* mutant of *P. putida* MK1; Km^r Rif^r	This study
MK201	Tn*5* mutant of *P. putida* MK1; Km^r Rif^r	This study
Plasmids		
pUT mini-Tn*5 lacZ1*	Ap^r Km^r; delivery plasmid for mini-Tn*5 lacZ1*	17
pRK600	Cm^r *ori* ColE1 RK2-Mob$^+$ RK2-Tra$^+$	31
pMMB67EH	Tac expression cloning vector with cloning sites of pUC18; Ap^r	10
pHRP317	Km^r Sm^r Ω streptomycin-spectinomycin cassette	44
pMK101	pMMB67EH containing 6.5-kb *Pst*I fragment from MK107; Ap^r Km^r	This study
pMK103	pMMB67EH containing 14.3-kb *Pst*I fragment from MK107; Ap^r Km^r	This study
pMK1011	pMK101 with 0.65-kb *Eco*RI fragment deleted	This study
pMKU101	pUC19 with 0.65-kb *Eco*RI-*Pst*I inserted; Ap^r	This study
pMKU102, -103	*Bal* 31 deletion derivatives of pMKU101; Ap^r	This study
pMKU104	pMKU103 with *Bst*XI-*Hin*cII fragment deleted; Ap^r	This study
pMKU105	pMKU103 with *Bst*XI-*Bgl*II fragment deleted; Ap^r	This study
pMKU106	pMKU103 with *Apa*I-*Bgl*II fragment deleted; Ap^r	This study
pMKU107	pMKU103 with *Bst*XI-*Apa*I fragment deleted; Ap^r	This study
pMKU108	pMKU103 cut with *Bst*XI and treated with *Bal* 31; Ap^r	This study
pMKU111 to -113	PCR products 1, 2, and 3 cloned into pUC19 cut with *Eco*RI-*Bam*HI; Ap^r	This study
pMKS100	pUC19 with 1.95-kb *Hin*dIII Ω streptomycin-spectinomycin cassette inserted at *Sma*I site; Ap^r Sm^r	This study
pMKB100	1.95-kb *Hin*dIII-*Eco*RI fragment of pMKS100 cloned into the vector pMKB301; Ap^r Sm^r	This study
pMKS101 to -113	pMKU101 to -113 with 1.95-kb Ω streptomycin-spectinomycin cassette inserted at *Hin*dIII site; Ap^r Sm^r	This study
pMKB101 to -113	pMK301 with *Eco*RI-*Hin*dIII fragment from pMKS101 to pMKS113, which contains deleted promoter region and 1.95-kb Ω streptomycin-spectinomycin cassette; Ap^r Sm^r	This study
pMK301	*lacZ1* promoter probe vector; Ap^r	This study

rifampin-resistant strain of *P. putida* (referred to as *P. putida* MK1). This was isolated from the ATCC 12633 strain by successive culture on LB plates containing increasing concentrations of this antibiotic from 25 to 150 μg/ml. The donor strain, *E. coli* S17-1 λ*pir*(pUT mini-Tn*5 lacZ1*), was obtained by mating it with *E. coli* CC118 λ*pir*(pUT mini-Tn*5 lacZ1*), supplied to us by V. de Lorenzo and K. Timmis (17, 31), in the presence of *E. coli* HB101 containing the helper plasmid pRK600.

The mating experiment to transfer pUT mini-Tn*5 lacZ1* from *E. coli* S17-1 λ*pir* (pUT mini-Tn*5 lacZ1*) to *P. putida* MK1 was performed essentially as described by Herrero, de Lorenzo, and Timmis (17, 31). The *E. coli* strain was grown overnight with shaking at 37°C in 2 ml of LB medium containing 100 μg of ampicillin per ml and 50 μg of kanamycin per ml. *P. putida* MK1 was cultured similarly except that the incubation temperature was 30°C and no antibiotics were added to the LB medium. Ten to fifty microliters of the donor and recipient cultures was mixed in 5 ml of 10 mM $MgSO_4$. The mixture was filtered (Millipore membrane, 25 mm, 0.45 μm pore size), and the filter was placed on an LB plate which was incubated at 30°C for 8 to 18 h. After mating, each filter was suspended in 5 ml of 10 mM $MgSO_4$ and aliquots (100 to 500 μl) were plated on the selection medium (LB agar with 150 and 50 μg of rifampin and kanamycin, respectively, per ml). Only *P. putida* MK1 exconjugants that had a mini-Tn*5 lacZ1* insert in their chromosome could grow on such plates. These were visible within 24 to 48 h of incubation. When inserted in proper orientation, the *lacZ* gene in such exconjugants serves as a reporter for the expression of individual promoters.

Screening of starvation response gene mutants. Screening of starvation response gene mutants was done by a modified version of our previously described protocol (14). Each kanamycin- and rifampin-resistant exconjugant was transferred by a toothpick onto M9 agar plus X-Gal plates containing either 0.025 or 0.3% glucose and grown at 30°C. During the 10- to 15-h incubation period, starvation conditions for glucose were established only in the low-glucose plates, and selectants that turned blue (or more intensely blue) only on these plates were isolated.

Starvation in liquid media. Late-exponential-phase cells in 0.1% glucose-M9 medium or LB broth were subcultured into the homologous medium. In the ensuing exponential phase, another subculture was made by 10-fold dilution into prewarmed media as specified in the Results section. Incubation was at 30°C with shaking at 150 rpm. Samples were removed at appropriate intervals and analyzed for A_{660}, β-galactosidase activity, and viability. β-Galactosidase activity was measured as described by Clark and Switzer (7), and viability was measured by spreading serial dilutions on LB plates (14). β-Galactosidase activity is given in Miller units.

Cloning of Tn*5*-flanking DNA. The plasmid pMMB67EH (10) was used to clone the Tn*5*-flanking DNA from mutant MK107 (Table 1 and the Results section). This plasmid has a broad host range, can replicate in both *P. putida* and

E. coli, and can be readily transferred from *E. coli* into *P. putida* by conjugation in the presence of a helper plasmid (pRK600).

Total genomic DNA was prepared as described by Ausubel et al. (2). Purified DNA was partially digested with *Pst*I, and the 6- to 15-kb DNA fragments were ligated to *Pst*I-digested, dephosphorylated pMMB67EH. The ligated mixture was electroporated into *E. coli* MC1061 (33), and the transformants were selected on LB plates in the presence of ampicillin and kanamycin.

Transfer of plasmids from *E. coli* MC1061 to *P. putida* was carried out by triparental mating (1) in the presence of pRK600 (borne by *E. coli* HB101). Loopfuls of parental cells, which were grown overnight on appropriate plates, were mixed in LB medium in a 1:1:1 ratio and incubated for 3 to 5 h at 30°C. Transconjugants were selected by plating dilutions of the mating mixture on appropriate selective media.

DNA sequencing. Double-stranded DNA was sequenced by the dideoxy chain termination method (45) with the Sequenase version 2.0 kit (U.S. Biochemical Co.). The universal forward and reverse oligonucleotide primers provided with the kit were used for annealing the complementary strands at both ends. ^{35}S-dATP-labeled mixtures were run in an 8% polyacrylamide gel containing 50% urea.

Primer extension analysis. Transcript mapping to locate the transcriptional start site in the 655-bp genomic fragment of pMK101 (Table 1; see also Fig. 4) was performed by a modified version of the previously described method (29). Total RNA from *P. putida* MK107 or MK1 containing pMK101 or KT2440 (see Results) was prepared by using the RNeasy Total RNA Kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's recommendations. Three primers (101, 5′-AAATCGCCTAACTTGCATTATCCA; 102, 5′-CATGCAAATTCG GATCAGCCAGGC; and 103, 5′-GGGCCGCTTCGATCAGCGAACGTT, which are complementary to nucleotides +264 to +287, +164 to +187, and +63 to +86 downstream of S1 [see Fig. 5], respectively) were end labeled by T4 polynucleotide kinase (BRL Life Sciences) with [γ-^{32}P]dATP and used for primer extension and sequencing reactions. Two picomoles of the primers was incubated with 5 to 20 μg of RNA in 10 μl of annealing buffer (2 mM Tris HCl [pH 7.8], 0.2 mM EDTA, 0.25 M KCl) at 58°C for 30 min and allowed to cool. The nucleic acid was mixed with 0.33 mM (each) four deoxynucleoside triphosphates–20 mM Tris HCl (pH 8.7)–10 mM $MgCl_2$–100 μg of actinomycin D per ml–5 mM dithiothreitol in a final volume of 25 μl. After 20 U of Moloney murine leukemia virus reverse transcriptase (BRL Life Sciences) was added, the mixture was incubated at 37°C for 30 min and precipitated with ethanol. The pellets were dissolved in 5 μl of Tris-EDTA buffer, and an aliquot, mixed with formamide loading buffer, was analyzed in an 8% sequencing gel. Denatured pMKU101 DNA (Table 1 and below) served as the template for the sequencing reactions.

Construction of a promoter-probe vector and deletion analysis. Deletion analysis to localize the promoter sequences necessitates subcloning of small DNA fragments into vectors with minimal transcriptional read-through from plasmid sequences. Parales and Harwood (44) have constructed an IncQ plasmid RSF1010-based vector and have utilized a two-step procedure which permits directional cloning of small DNA fragments downstream of an Ω streptomycin-spectinomycin resistance cassette and upstream of a reporter gene. As this cassette is flanked by short inverted repeats carrying transcription and translational signals in both orientations, read-through from plasmid sequences is minimized. Thus, the promoter activity of the cloned DNA can be reliably measured.

We used the above principle to construct the transcriptional promoter probe vector pMK301, which has the same multicloning sites as pUC19. Since we used the latter plasmid to subclone the 0.66-kb insert from pMK101 and its various deletion derivatives (Table 1 and the Results section), the homology of restriction sites between pUC19 and pMK301 facilitated the transfer of the DNA fragments into this probe vector.

pMK301 was constructed from pMMB67EH and the promoterless *trp′-′lacZ* fusion in pUT mini-Tn*5* (Fig. 1). pMMB67EH is a P*tac* expression vector also based on the IncQ replicon RSF1010 (10). A 1.5-kb *Eco*RI-*Pvu*II fragment from pMMB67EH containing P*tac* and *lacI*^{q} was replaced with a 3.3-kb *Eco*RI-*Hin*dIII digest from the pUT-mini-Tn*5*::*lacZ1* plasmid (17). The *Hin*dIII site was end filled to make it compatible with the *Pvu*II-generated site.

DNA fragments cloned into pUC19 were transferred to this vector as is illustrated in Fig. 1 for the approximately 0.66-kb chromosomal DNA fragment contained in pMKU101. A 1.95-kb *Hin*dIII fragment containing the Ω streptomycin-spectinomycin cassette, excised from pHRP317 supplied by C. Harwood, was cloned into pMKU101, generating pMKS101 (Fig. 1); selection for the recombinant clones was on ampicillin-streptomycin plates. In the second step, the DNA fragment containing the promoter and the Ω streptomycin-spectinomycin cassette was transferred to pMK301. pMKS101 was cut with *Eco*RI and partially digested with *Hin*dIII. The resulting fragment was cloned into *Eco*RI-*Hin*dIII-treated pMK301, generating pMKB101 (Fig. 1). The *E. coli* pMKB101 transformants were selected and screened on plates containing ampicillin-streptomycin and X-Gal. Transfer of the plasmid from *E. coli* to *P. putida* was done as already described.

This strategy was used to construct pMKB101 through -113 (and the corresponding intermediate pMKU and pMKS series of plasmids [Table 1; see also Fig. 7), carrying different deletion fragments derived from the 0.66-kb chromosomal DNA insert of pMK101. pMKB100 was constructed as a control and carried only the Ω streptomycin-spectinomycin cassette.

The various deletion fragments of the 0.66-kb chromosomal DNA (of pMK101) were prepared in several ways. pMKU102 and -103 (Table 1; see also Fig. 7) were constructed from pMKU101. The latter was cut with *Hin*dIII and digested with *Bal* 31 for different durations. The resulting fragments were end filled with Klenow fragment, treated with *Eco*RI, and ligated into *Eco*RI-*Sma*I-cut pUC19. pMKU104 through -109 were derived from pMKU103 by either using further *Bal* 31 digestion or removing fragments by making use of appropriate restriction sites. For example, pMKU105 was constructed by deletion of the region between the *Bst*XI and *Bgl*II sites of pMKU103 and religation after making blunt ends with Klenow fragment, and pMKU108 and -109 were made by digestion of *Bst*XI-cut pMKU103 with *Bal* 31 for different time periods, followed by ligation.

Three deletions were generated by PCR, after the start sites had been determined by transcript mapping (see Fig. 5 and 6). Amplitaq DNA polymerase (Perkin-Elmer Cetus) was used according to the manufacturer's recommendations. Reaction conditions involved denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min. Four oligonucleotide primers were used: PCR1, 5′-GAGGGATCCCTGGAGCCAAGGTTGGAA-3′; PCR2, 5′-GAGGGATCCAATAGTCTGCAAAGGGCC-3′; PCR3, 5′-GAGGGATCC GGGCAGGGTCAGGAAGAT-3′; and PCR4, 5′-GAGGAATTCGGCCTAG GCGGC-3′. The *Bam*HI site (underlined) was added to the 5′ ends of PCR1, -2, and -3. PCR1 through -3 are complementary to nucleotides −42 to −23, −10 to +8, and +7 to +26 relative to S1, respectively (see Fig. 5). PCR4 served as the reverse primer complementary to sequences in pMKU101 to which the *Eco*RI site was added. pMKU101 was used as the template. PCR products were purified (QIAEX Gel Extraction Kit; Qiagen Inc.) and ligated into pUC19, generating pMKU111 through pMKU113. These plasmids were used to generate the corresponding pMKS and pMKB series plasmids as described above. The PCR-generated products were sequenced; no sequence alterations were found.

Rapid isolation of plasmid DNA was done by the modified method of Birnboim and Doly (3).

RESULTS

Transposon mutagenesis and isolation of starvation gene mutants of *P. putida*. We used the pUT plasmid, bearing the mini-Tn*5*::*lacZ1* transcriptional fusion vector (17), for transposon mutagenesis of *P. putida*. This plasmid can replicate only in strains that produce the R6K-specified π protein (17), such as the λ*pir* phage-lysogenized *E. coli* strains. It therefore acts as a suicide plasmid in organisms like *P. putida* that do not produce this protein. To facilitate counterselection against the donor *E. coli* strain, we isolated a Rif^r strain of *P. putida*, ATCC 12633 (Materials and Methods), and used Rif^s *E. coli* S17-1 λ*pir* as the donor strain. Since the pUT-bearing *E. coli* (CC118 λ*pir*) strain supplied to us is Rif^r, we first transferred the plasmid to *E. coli* S17-1 λ*pir*.

Selection for the *P. putida* exconjugants was carried out on LB plates containing kanamycin (the antibiotic marker of Tn*5*) and rifampin. The presence of the latter antibiotic eliminated the donor *E. coli* cells, and that of the former ensured selection only of *P. putida* cells containing a chromosomal Tn*5* insertion. Over 3,000 Km^r Rif^r exconjugants were obtained. Screening on M9 plates with 0.3 or 0.025% glucose permitted isolation of 60 mutants presumptively affected in starvation genes (Materials and Methods). When tested in liquid glucose minimal medium, these expressed enhanced β-galactosidase activity upon starvation, while showing constant lower levels during exponential phase, as is illustrated for selected mutants in Fig. 2. MK201 was included as a control; this mutant did not exhibit increased blue color on low-glucose plates and presumably represented mutation in a growth-related gene. As expected, it did not exhibit increased β-galactosidase activity during starvation in the liquid minimal medium, in contrast to the other mutants.

Starvation survival of starvation gene mutants. The carbon starvation survival capacity of two mutants, MK107 and MK114, was compared with that of the wild type, MK1 (Fig. 3). There was some twofold increase in cell number of MK1 during the first 3 days of starvation. This increase in cell number in early starvation is consistent with previous findings with *P. putida* (13) and other bacteria (25). Over the next 17 days, there was only a slight decrease in culture viability, followed by

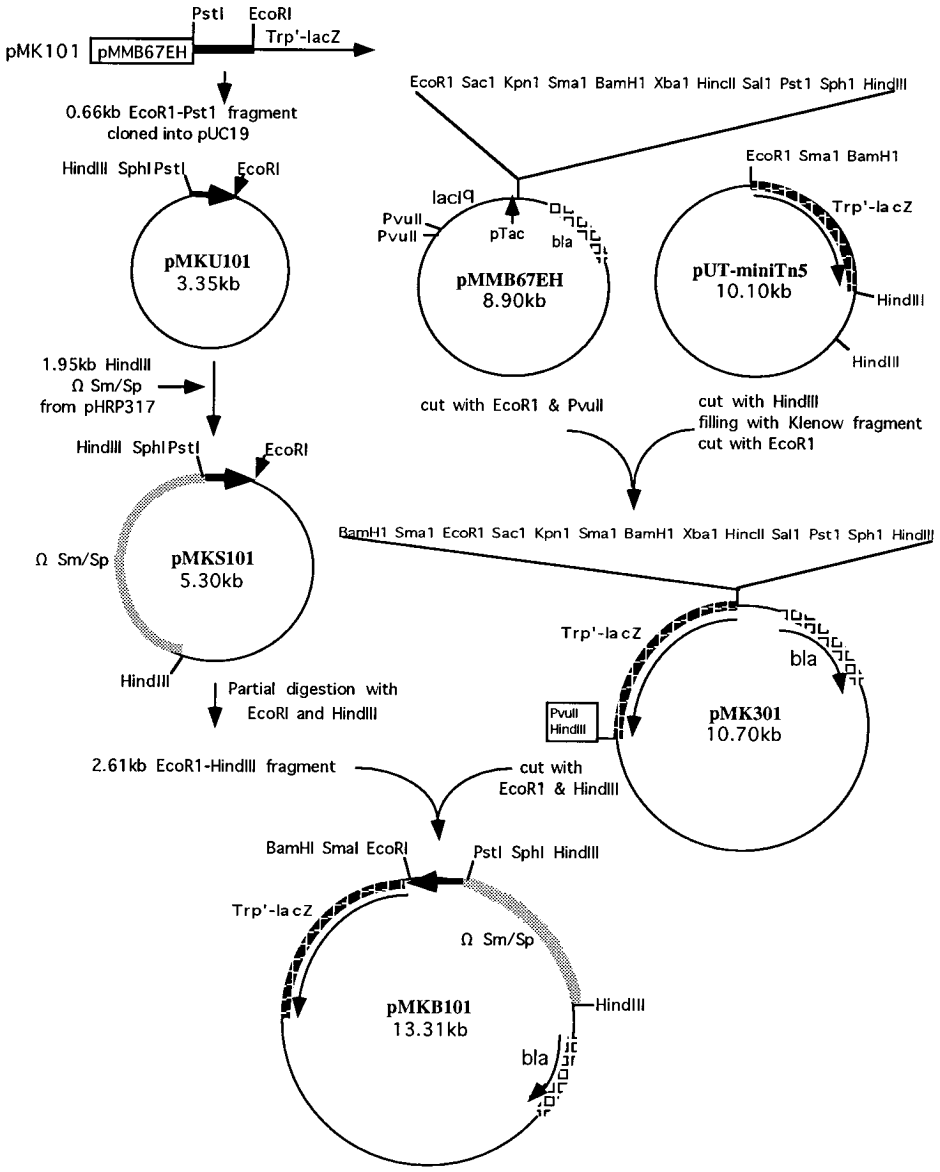

FIG. 1. Construction of promoter probe vector pMK301 and the cloning of the DNA fragment from pMKS101 into it to obtain pMKB101. See text for details.

a somewhat higher rate of viability loss. Nonetheless, even after 30 days of starvation, which was the duration of the experiment, the culture remained >30% viable.

The mutants exhibited much greater starvation sensitivity. The increase in cell number was smaller and was complete within about a day. The viability was then lost at a roughly constant rate, and by the end of the experiment (30 days), less than 1 to 2% of the cells remained viable; the difference on the 19th day of starvation was even more dramatic: 100% viability for the wild type versus 5 to 20% for the mutants (Fig. 3).

Both the starved mutants produced high levels of β-galactosidase during starvation (Fig. 2), and this raises the possibility that their impaired starvation survival resulted from this extra energy drain and not from the affected genes having a direct role in starvation survival. As described below, we have cloned the starvation gene-*lacZ* fusion from strain MK107 on plasmid pMKB101 (Table 1). This plasmid was transferred into strain KT2440 (resulting in strain MK1071); as a control, the same plasmid minus the fusion (pMKB100) was transferred into another culture of KT2440, generating strain MK1072. Unlike the control MK1072, MK1071 produced the same high levels of β-galactosidase during starvation as strain MK1 containing pMKB101. Both MK1071 and -1072 lost viability at the same rate (data not shown), indicating that β-galactosidase synthesis was not the determining factor in the increased starvation sensitivity of MK107.

Neither strain MK107 nor MK114 exhibited any difference in growth rate or yield compared with the wild type. Thus, the effect of their mutations is specific to starvation survival.

Cloning of the starvation promoter region. Chromosomal DNA from strain MK107 was digested into 6- to 15-kb fragments as described in Materials and Methods. These were ligated into the plasmid pMMB67EH, which was electropo-

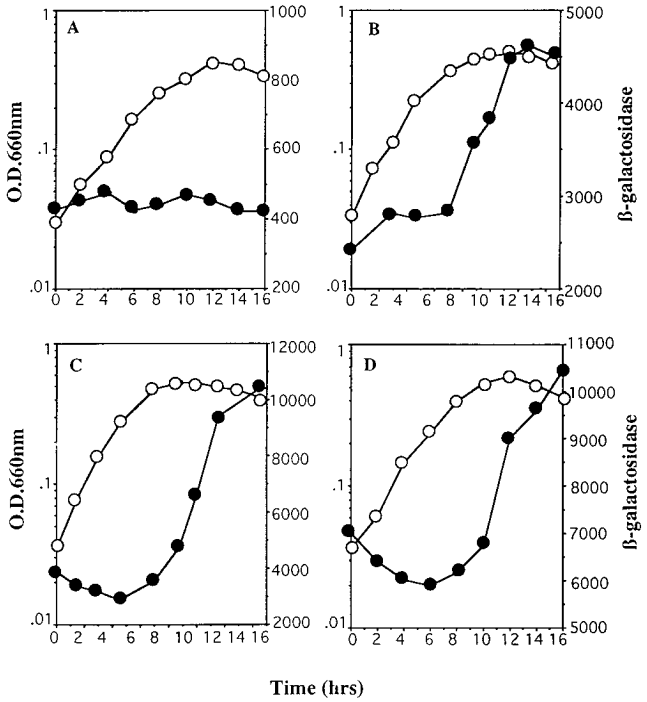

FIG. 2. β-Galactosidase synthesis during growth and stationary phases in the mini-Tn*5*::*lacZ1* fusion mutants of *P. putida* MK1. (A to D) MK201, MK104, MK107, and MK114, respectively. Cultures were grown on M9 medium plus 0.1% glucose; other methods are described in the text. Open symbols, growth; solid symbols, β-galactosidase activity.

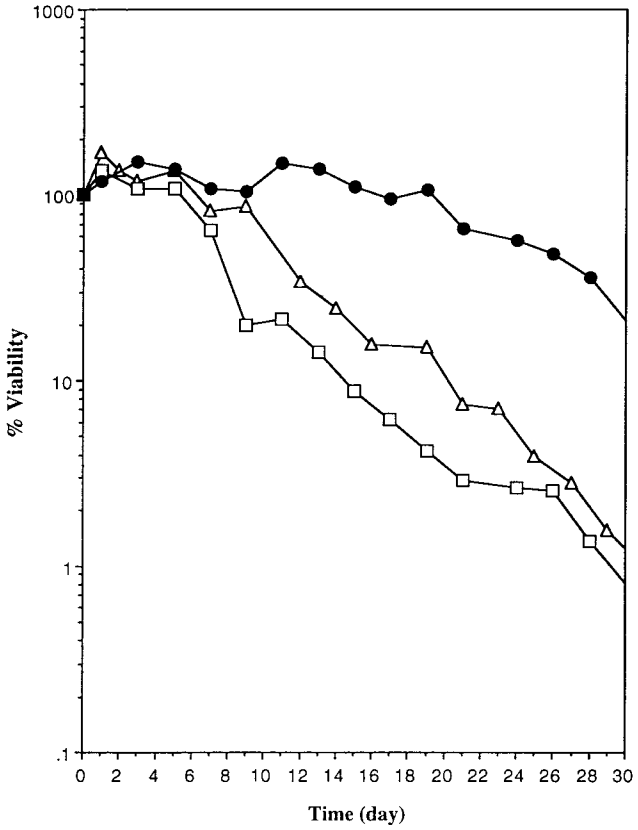

FIG. 3. Starvation survival of *P. putida* MK1 and its mutants. Cells were cultured in 125-ml flasks containing 50 ml of M9 medium plus 0.05% glucose. One hundred percent viability corresponded to 4×10^8 to 6×10^8 cells per ml. Symbols: ●, *P. putida* MK1; △, MK107; □, MK114.

rated into *E. coli* MC1061, and the transformants were plated on kanamycin (the antibiotic marker of mini-Tn*5*) plates. Fifteen Km^r transformants were subjected to restriction analysis; all possessed a common fragment carrying the *lacZ* and Km^r genes, confirming insertion of the mini-Tn*5*::*lacZ* transposon into their chromosome.

Two of the recombinant plasmids containing the largest and smallest chromosomal DNA inserts (pMK101, with 6.5 kb, and pMK103, with 14.3 kb [Fig. 4]) were transferred from *E. coli* into *P. putida* MK1 by triparental mating as described in Materials and Methods and were tested for the starvation phenotype, i.e., β-galactosidase induction upon starvation. The recombinant *P. putida* strains were grown in liquid M9 medium, and β-galactosidase activity was monitored during growth and after the exhaustion of glucose. Both the strains exhibited similar levels of enzyme activity during growth and similar degrees of induction upon the onset of starvation (Fig. 4). This pattern of β-galactosidase synthesis is very similar to that shown by the fusion strain MK107 (Fig. 2), and the results indicated that the 0.66-kb chromosomal DNA insert upstream of the reporter gene in pMK101 contained all the information required for starvation promoter activity. A *P. putida* strain bearing plasmid pMK1011, which is pMK101 without the 0.66-kb chromosomal DNA insert (Table 1), was used as a control; it showed no β-galactosidase induction upon starvation (Fig. 4), confirming that the regulatory sequences contained in the 0.66-kb chromosomal DNA fragment were necessary for starvation-mediated induction.

Localization of the starvation promoter regulatory region in the pMK101 insert DNA. The 655-bp chromosomal insert of pMK101 was sequenced, and a putative open reading frame and Shine-Dalgarno sequence were identified by computer analysis, with the Genetics Computer Group sequence analysis software package (Fig. 5). This information enabled us to construct primers for transcript mapping in order to determine the transcription start site of this gene. Three primers were used, and transcript mapping was conducted as described in Materials and Methods. Three DNA bands were observed, regardless of the primer used, as reverse transcription products of the RNA isolated from starved *P. putida* MK1 containing pMK101 (as well as from *P. putida* MK107, without the plasmid [Fig. 6]). The smallest-sized product was by far the most abundant. This product corresponded to transcriptional start site 1 (S1 in Fig. 5) and was 270 bp upstream of the putative translational start site. Thirteen nucleotides upstream of this site was a GG-N_{10}-GC sequence. This is a perfect match to the canonical sequence recognized by σ^{54}-RNA polymerase (P1 [Fig. 5]) (42). The much weaker start sites (S2 and S3 [Fig. 6]) exhibited upstream promoter sequences, P2 and P3, with resemblance to those recognized by $E\sigma^{70}$ (Pribnow boxes [Fig. 5]).

The RNA obtained from the exponential-phase cells produced less of the transcript than did that isolated from the stationary-phase cells, and yet the S1 band it produced was substantial (Fig. 6). Indeed, judging from the amount of the transcript product, the decrease in the message in exponential phase appeared to be less than the threefold decrease in β-galactosidase activity in this phase compared with the stationary phase. This raises the possibility of posttranscriptional regulation of this gene.

Deletion analysis was carried out to check the results of transcript mapping and to delineate the sequence within the 655-bp fragment required for the expression of this gene. As

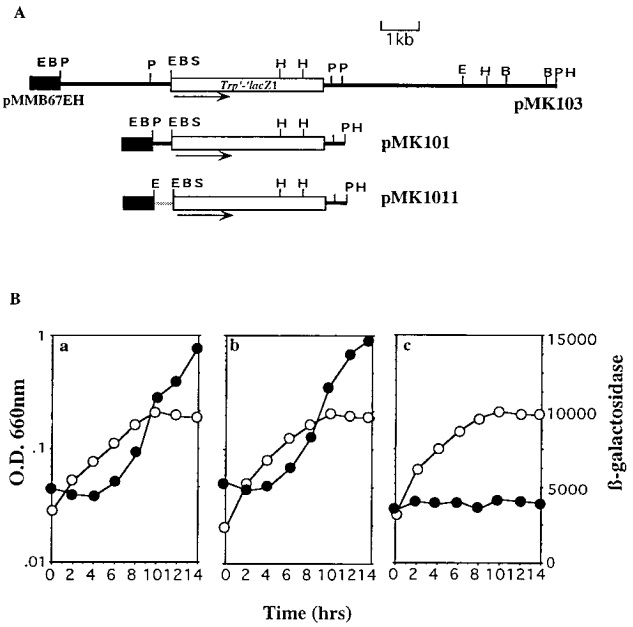

FIG. 4. (A) Restriction maps of mini-Tn*5*::*lacZ1*-containing insert DNA cloned from the mutant MK107. pMK1011 was constructed from pMK101 by removing 655-bp *Pst*I-*Eco*RI chromosomal DNA as described in Materials and Methods. Closed and open boxes represent the vector pMMB67EH and mini-Tn*5*::*lacZ1* fusion, respectively. Orientation of transcription is indicated by arrows. The stippled bar denotes the removed fragment. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sma*I. (B) β-Galactosidase synthesis during growth and stationary phase in *P. putida* MK1 containing pMK101 (a), pMK103 (b), or pMK1011 (c). Cells were grown on M9 medium plus 0.05% glucose. Open symbols denote growth, and closed symbols indicate β-galactosidase activity.

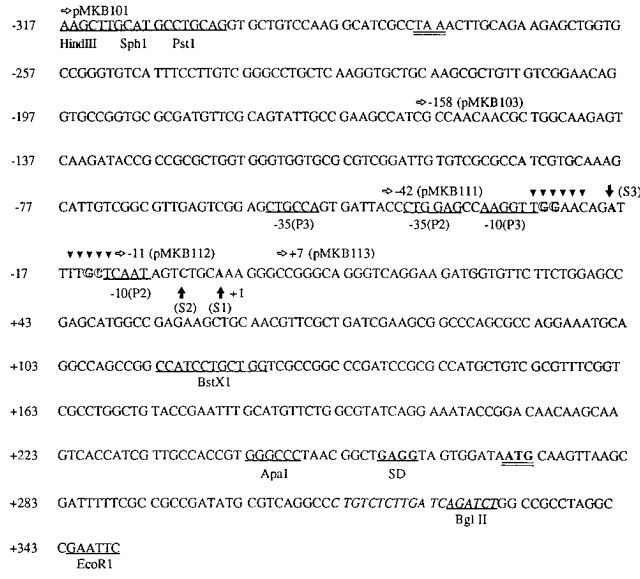

FIG. 5. Nucleotide sequence of the 655-bp *Pst*I-*Eco*RI fragment cloned from pMK101 into pUC19 (the *Hin*dIII site was derived from the vector). Numbering is with respect to S1, which is assigned +1. The σ^{54} consensus sequence, tGGaac-N5-ttGCt, is marked with triangles, and the GG and GC doublets are highlighted. The putative −35 and −10 regions of the Pribnow boxes (P2 and P3), the Shine-Dalgarno sequence, and the restriction sites are underlined. The putative translational stop and start sites are double underlined. The vertical arrows indicate transcription start sites. The horizontal arrows indicate the starting points of 5′ deletion clones. Italics indicate the site of the I end of mini-Tn*5*.

described in Materials and Methods, we constructed a promoter-probe vector (pMK301) for this purpose which facilitated cloning of small DNA fragments and minimized transcriptional read-through from the vector. Subfragments of the 0.66-kb chromosomal insert in pMK101 were prepared (Materials and Methods) and cloned into this vector to generate plasmids pMKB101 through -111 (Table 1 and Fig. 7). Starvation phenotype, i.e., β-galactosidase induction by starvation, of *P. putida* strains bearing these plasmids was determined in both glucose M9 and LB media.

Deletion of DNA upstream of the +7 (pMKB113) and −11 (pMKB112) nucleotides (Fig. 5 and 7) nearly abolished β-galactosidase activity in both the exponential and starvation phases, as predicted by transcript mapping. Deletion of DNA upstream of the −42 nucleotide (pMKB111) also nearly abolished β-galactosidase synthesis, but deletion above the −158 nucleotide (pMKB103) made no difference to promoter expression in growth or starvation, indicating that this upstream sequence had no role in the expression of the promoter.

The 42 nucleotides upstream of S1 retained in pMKB111 include σ^{54}-type P1, as well as σ^{70}-resembling P2; only P3 is missing (Fig. 5 and 7). Given the very low activity of P3 (Fig. 6), it is unlikely that the lack of activity of this fusion is due to its loss. Instead, a reasonable inference is that the sequence between the −158 and −42 nucleotides is required for the functioning of the σ^{54} promoter. This is consistent with the near-universal requirement of an upstream region for the activity of this type of promoter (26, 40a).

Other fusions provided information on the role of the sequence downstream of S1 in the regulation of this gene. Fusions pMKB105 through -108 lacking different regions between S1 and the translational start codon, including, in some cases, parts of the coding region of the gene (Fig. 5 and 7), retained starvation-mediated induction; however, there was an over twofold (in LB medium) or threefold (in M9-0.1% glucose medium) reduction in β-galactosidase synthesis compared with pMKB101 or -103. Thus, the region downstream of S1 also has a role in the regulation of this gene.

Transcript map and expression of the fusion in *rpoN* mutant background. The S1 start site is most likely due to the activity of the σ^{54} promoter (P1) since a canonical σ^{54}-recognized sequence is present at an optimal distance upstream of this site. However, a σ^{70}-type promoter is also present 8 nucleotides upstream of S1, and although its sequence is not particularly close to the consensus for such promoters, the possibility remains that S1 is controlled by it. To explore this possibility, we investigated the effect of an *rpoN* mutation.

The *rpoN* mutation is not available in strain ATCC 12633, and therefore, *rpoN*$^+$ and *rpoN* mutant KT2440 strains (23) were used. pMKB101 or pMKB103 (which both contain all the nucleotides implicated in regulation [Fig. 5 and 7]) was introduced into these strains, and transcript mapping was performed. This map in the KT2440 wild type was very similar to that shown in Fig. 6, but in the *rpoN* mutant background, the extension product corresponding to S1 was not detected (Fig. 8), confirming that S1, in fact, is σ^{54} dependent. Somewhat unexpectedly, we found that the S2 and S3 sites became stronger in the *rpoN* mutant background and that transcription from them increased upon starvation.

The *rpoN*$^+$ KT2440 strain carrying pMKB101 expressed nearly the same levels of β-galactosidase during growth and starvation as strain MK1 harboring this plasmid (Fig. 7 and 8), indicating that the change in the strain background did not affect expression of the fusion in this plasmid. In the *rpoN* mutant strain, both the basal and the starvation-induced levels

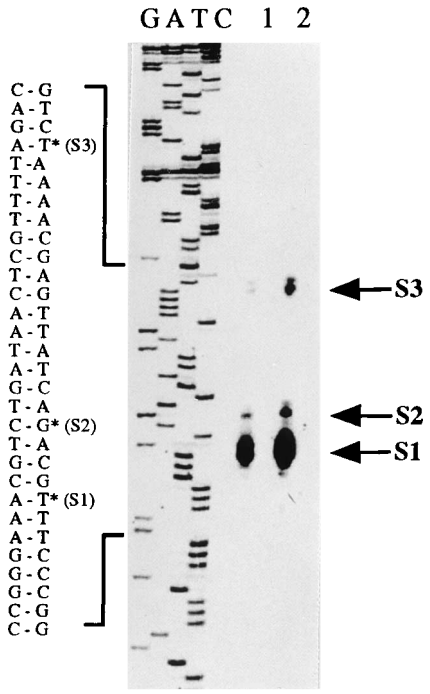

FIG. 6. Primer extension mapping of the starvation gene of the mutant MK107. RNA was isolated from the mutant (Materials and Methods), and 20 μg was hybridized with primer 103, which is complementary to 63 to 86 bp downstream of S1. Lanes 1 and 2 represent extension products from exponential- and stationary-phase cells, respectively. Lanes G, A, T, and C provide the sequence ladder.

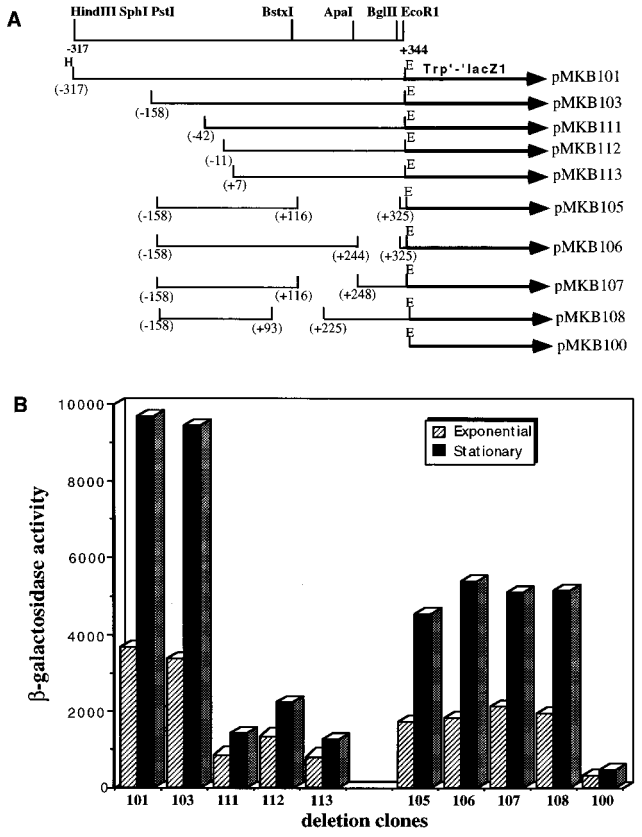

FIG. 7. (A) Restriction map of the 667-bp *Hin*dIII-*Eco*RI fragment of pMKU101 and subclones derived therefrom. Numbering is as in Fig. 5. Numbers in parentheses indicate the sites of deletion. All constructions are described in Materials and Methods. pMKB100, which has no chromosomal insert DNA, was used as a negative control. (B) β-Galactosidase activity of various deletion clones. Cells were cultured in LB medium. Exponential-phase (▨) and stationary-phase (■) samples were analyzed.

of the fusion were decreased; however, the starvation-dependent induction remained (Fig. 8).

DISCUSSION

To our knowledge, this is the first report of isolation of mutants which are impaired in starvation survival in any species of *Pseudomonas*. Molin and coworkers have shown that *P. putida* synthesizes some 72 new carbon starvation proteins over the first 24 h of starvation, which results in the development of a general cellular resistant state (12, 13). Some of these proteins are of the Pex (12) type and are essential for starvation survival. We assume that the defect in mutants MK107 and MK114 affects this class of proteins. The wild type remained fully viable for the first 19 days of starvation, as opposed to the mutants, which showed viability loss almost from the outset (Fig. 3). Thus, the affected genes appear to have a role in the remarkable capacity of *P. putida* to completely resist starvation for prolonged periods. The biochemical role of these proteins is not known. The promoter that we have characterized in MK107 controls an operon of at least two open reading frames, the first of which bears a high degree of identity in the N terminus region to the FLhF protein of *Bacillus subtilis*, which is involved in flagellar biosynthesis (6a, 22a).

The mutated gene in strain MK107 is transcribed primarily by σ^{54} in the wild-type strain. The major reverse transcript product corresponds to a start site which is preceded by a perfect σ^{54}-recognized sequence, and the 13-nucleotide distance between the promoter and the start site is also optimal for these types of promoters. Further, this transcript product is not detectable in a σ^{54}-deficient background. This is the first instance in which a gene concerned with carbon starvation survival is shown to be regulated by a σ^{54} promoter. In *E. coli*, none of the promoters regulating the *pex* type of genes involved in increased cellular resistance to starvation is known to be σ^{54} regulated; they are regulated rather by σ^{38} or σ^{32} (20, 25, 27, 40). σ^{54} does regulate the nitrogen starvation gene of *E. coli*, glutamine synthetase (*glnA* [9, 26]), but this gene is not involved in conferring increased cellular resistance to starvation in this bacterium and has a role only in escape from nitrogen starvation (34, 38).

The activation of *glnA* in *E. coli* depends on an upstream sequence, which senses nitrogen starvation through a two-component system involving the proteins NtrB and NtrC (also referred to as NRII and NRI [26]). The latter, with the participation of the former, becomes phosphorylated when the cell experiences nitrogen deprivation and in this state can bind to the DNA sequence upstream of the σ^{54} promoter of *glnA*. This binding is required for the formation of an open complex around the transcription start site of *glnA*, which Eσ^{54} alone cannot bring about (26, 40a).

Several genes in *Pseudomonas* species are σ^{54} regulated (24, 30, 51, 52), and there is evidence that modification of NtrC-like proteins is necessary for the transcription also of these genes. As in *E. coli*, this modification usually involves phosphorylation. Thus, AlgR1 phosphorylation is required for the σ^{54}-regulated *algC* gene transcription (54), and PilR phosphorylation is required for σ^{54}-dependent pilin synthesis (19) in

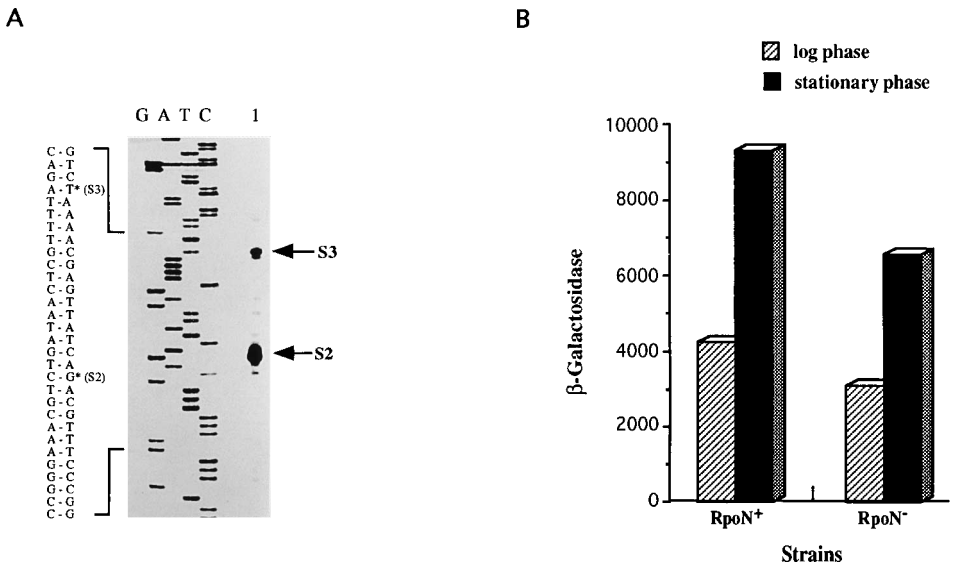

FIG. 8. (A) Transcript map of *rpoN* mutant of strain KT2440 containing pMKB101. Five micrograms of RNA from stationary-phase cells was hybridized with primer 103, and the extension products were determined. (B) β-Galactosidase activity in the exponential and stationary phases of *rpoN*$^+$ and *rpoN* mutant strains of KT2440 containing pMKB101.

Pseudomonas aeruginosa. However, the XylR protein of *P. putida*, which functions like NtrC in toluene metabolism, is apparently activated directly by binding to low-molecular-weight substrates of xylene catabolism (30).

Our deletion analysis data strongly suggest the need for an upstream sequence for the functioning of the σ^{54} promoter of the starvation gene affected in strain MK107. It is thus reasonable to speculate that an auxiliary, NtrC-type protein may be involved in sensing the carbon status of the environment. Upon carbon starvation, either through increase in the levels of this protein, and/or its modification by phosphorylation or other means, its binding to the upstream region may be facilitated, thereby increasing this σ^{54}-dependent *pex* gene promoter transcription. This possibility is now under investigation.

The two Pribnow boxes found in the starvation gene regulatory region (Fig. 5) had only a minimal role in its transcription in the wild-type background, as indicated by the transcript maps. These promoters are overlapped by a particularly strong σ^{54} consensus site, indicating that Eσ^{54} would bind to this site with high affinity. Such binding would inhibit transcription initiation from the Pribnow boxes by steric hindrance, preventing their expression. Our results support this possibility in that transcription from these promoters increased markedly in an *rpoN* mutant background. The situation is reminiscent of the *pilE* gene of *Neisseria gonorrhoeae*. In the promoter region of this gene also, a σ^{54} consensus sequence overlaps a Pribnow box, and in the absence of the activator protein, σ^{54} decreases the basal levels of *pilE* transcription, presumably by steric hindrance of the Pribnow box (5). This is consistent with the finding of Reitzer and Magasanik that the binding of Eσ^{54} to GlnAp2 could repress an adjacent weak σ^{70} promoter (44a).

The Pribnow boxes of our starvation gene exhibited induction upon carbon starvation in a σ^{54}-deficient background, although it was less pronounced than the σ^{54}-mediated induction of this gene in the wild type. Two aspects of this phenomenon are worth considering. The first is the possible mechanism of the Pribnow box induction in the *rpoN* mutant strain upon starvation. Many Pribnow boxes in *E. coli* can be induced by carbon starvation through an increase in the cellular concentration of the cyclic AMP (cAMP)-cAMP receptor protein complex (46, 47), and it is possible that, in the absence of the steric hindrance of σ^{54}, a similar mechanism operates in *P. putida*. Such a putative mechanism may involve cAMP or some other signal compound.

The other question with respect to the Pribnow box promoters is their possible physiological role. Our data indicate that they have little role unless σ^{54} is absent. σ^{54} levels have not been reported to fluctuate in *P. putida*, but they do so in *Caulobacter crescentus* (6), and may, under certain conditions, do so also in *P. putida*. If so, the results suggest that the Pribnow boxes are present to ensure that this gene is expressed also when σ^{54} is scarce or absent. The expression of the mutated gene in MK107 may be important enough for the overall survival of *P. putida* so as to necessitate use of multiple mechanisms for its expression under different conditions.

Deletion of sequences downstream of the S1 start site (Fig. 5 to 7) decreased expression of our starvation gene promoter, indicating that the intervening sequence between S1 and the coding region plays a role in its expression. It is possible that this sequence interacts with the upstream region of S1, facilitating interaction between the activator protein-bound enhancer region and the Eσ^{54}-bound start site by bringing about DNA bending. This mechanism evidently operates in the regulation of *algD*, which contains an integration host factor-binding site between the transcriptional start site and the ATG start codon (53). However, the starvation gene of MK107 did not contain such a site. Nevertheless, it is possible that another sequence could perform a similar role. Alternatively, the S1 downstream region may have a role in posttranscriptional regulation.

A final point of interest for the cloning of a starvation promoter from *P. putida* may be noted. We have constructed *E. coli* strains in which, because the *tmo* genes are controlled by starvation promoters, growth is dissociated from the expression of toluene monooxygenase (28, 37). This enzyme complex (encoded by the *tmo* gene cluster) can degrade trichloroethylene (TCE), which is a common environmental pollutant. We

have shown that our recombinant *E. coli* strains can degrade TCE during no growth or very slow growth. Such strains have a considerable potential advantage in in situ bioremediation. Their use may significantly reduce the amount of nutrients that need to be added to natural environments, where currently TCE bioremediation relies on the activity of resident wild-type bacteria. These resident bacteria can express their TCE-degrading potential only during rapid growth (48). Current methods of in situ bioremediation thus typically involve introduction of large amounts of nutrients into the environment. *E. coli*, of course, is not indigenous to environments requiring bioremediation. However, the cloning of a starvation promoter from *P. putida* has now opened the way to construct the desired recombinant strains from indigenous bacteria. We are initiating studies to evaluate the efficacy and ecological effects of recombinant strains which contain the starvation promoter sequence spliced to TCE-degrading genes.

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