

Polynucleotide Phosphorylase-Deficient Mutants of *Pseudomonas putida*

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In bacteria, polynucleotide phosphorylase (PNPase) is one of the main exonucleolytic activities involved in RNA turnover and is widely conserved. In spite of this, PNPase does not seem to be essential for growth if the organisms are not subjected to special conditions, such as low temperature. We identified the PNPase-encoding gene (*pnp*) of *Pseudomonas putida* and constructed deletion mutants that did not exhibit cold sensitivity. In addition, we found that the transcription pattern of *pnp* upon cold shock in *P. putida* was markedly different from that in *Escherichia coli*. It thus appears that *pnp* expression control and the physiological roles in the cold may be different in different bacterial species.

Polynucleotide phosphorylase (PNPase), which is encoded by *pnp*, is widely conserved among bacteria, with the notable exception of the *Mycoplasmataceae*, a family that includes the smallest free-living bacteria bearing a minimal set of genetic information. Homologous genes are found not only in plants, where the protein localizes in the chloroplasts (22), but also in *Caenorhabditis elegans*, *Drosophila melanogaster*, mice, and humans, as assessed by BLAST (1) analysis. In human cells PNPase has been localized in mitochondria and seems to be involved in cellular senescence and terminal differentiation (30, 35a).

PNPase degrades RNA phosphorolytically and processively in a 3'-to-5' direction and plays a central role in bacterial RNA degradation. In *Escherichia coli* PNPase may be found as part of a multiprotein machine, the degradosome, together with RNase E, the RNA helicase RhlB, and enolase (reference 11 and references therein). Despite its evolutionary conservation and the physiological relevance of RNA degradation, PNPase does not seem to be essential for survival of the bacterial species tested so far, possibly because of functional redundancy in RNA turnover and quality control pathways (8, 9, 14, 37, 38). Nevertheless, PNPase is essential for growth at low temperatures in the psychrotrophic mesophiles *E. coli*, *Bacillus subtilis*, and *Yersinia enterocolitica* (20, 31). A less severe growth defect at low temperatures has been observed in *pnp* mutants of *Salmonella enterica* (10). Additional phenotypes associated with *pnp* mutations include defective transformation competence, increased tetracycline sensitivity, and cell filamentation in *B. subtilis* (31, 42) and increased virulence in *S. enterica* (10). The pleiotropic effects of *pnp* mutations suggest that several functions are indirectly controlled by PNPase, possibly via regulation of mRNA processing and/or decay of a variety of genes. To detect common traits in the physiological roles of this

enzyme in bacteria, we identified, cloned, and mutagenized the *pnp* gene of the gram-negative soil bacterium *Pseudomonas putida* and analyzed its expression profile in different growth phases and after cold shock.

Identification and cloning of the *P. putida pnp* gene. The presence of a *pnp* homologue in *Pseudomonas* species was assessed by Southern blot hybridization of genomic DNA from *P. putida* TMB, *Pseudomonas fluorescens* N3, and *Pseudomonas aeruginosa* PAO11 (Table 1) digested with different restriction enzymes by using *E. coli pnp* DNA as a probe at a low stringency. In each species DNA fragments that hybridized with the heterologous probe were found (data not shown).

Attempts to clone the *pnp* gene by screening a *P. putida* TMB genomic bank in *E. coli* by using the specific *P. putida* probe cloned in pURF10 (Table 1 and Fig. 1) failed. We speculated that *P. putida pnp* or another nearby gene could be toxic to *E. coli*. We thus cloned and sequenced the locus by performing inverse PCR multiple times (23; data not shown). Within the 2.8-kb region sequenced (GenBank accession number Y18132), which is 98% identical to the equivalent region of the recently released *P. putida* KT2442 complete DNA sequence (accession number NC_002947), we found two open reading frames (ORFs) (Fig. 1A). The product of the upstream 89-codon ORF exhibited more than 80% similarity with the *E. coli* ribosomal protein S15. The product of the downstream 701-codon ORF exhibited 78.5% similarity with *E. coli* PNPase. Plasmid pRF17, which can express the putative *P. putida pnp* gene from the *plac* promoter, was able to complement the *E. coli pnp* mutant C-5641 (Table 1) for the growth defect at 15°C, although it was less efficient (i.e., smaller colonies were formed) than a plasmid carrying the *E. coli* gene (data not shown), thus indicating that the plasmid expressed a functional PNPase. We tentatively propose that GUG is the initiation codon, as suggested by the alignment with the *E. coli* protein.

The *P. putida* PNPase gene exhibits a high degree of similarity with the *E. coli* gene and other homologous bacterial genes and has the characteristic four-domain organization (two RNase PH domains that form the duplicated core and the two C-terminal RNA binding domains, KH and S1) (41; Conserved

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TABLE 1. Bacterial strains and plasmids used

Bacterial strains or plasmids	Relevant characteristics ^a	Reference or source
Bacterial strains		
<i>E. coli</i> C-5641	Prototrophic, <i>pnp</i> ::Tn5-Km	35
<i>P. fluorescens</i> N3	Naf ⁺ Sal ⁺ /pN3	5
<i>P. aeruginosa</i> PAO11	<i>tfp-54 nal-19 fon-1</i>	B. W. Holloway Collection
<i>P. putida</i> TMB	Tmb ⁺ Tol ⁺ /pGB	3
<i>P. putida</i> KT2440	<i>hsdR hdsM</i> ⁺ Ben ⁺ Rif ^s	2
PPM101	<i>P. putida</i> TMB <i>pnp-1</i> (INS pKRF7A), Kan ^r Str ^r Suc ^s	This study
PPM103	<i>P. putida</i> KT2440 <i>pnp-1</i> (INS pKRF7A), Kan ^r Str ^r Suc ^s	This study
Plasmids		
pGEM-4Z	Cloning vector	Promega
pGEM-4Z-Km1	pHP45Ω-Km 2.2-kb <i>Bam</i> HI fragment containing the Ω-Km interposon cloned in <i>Bam</i> HI-digested pGEM-4Z	This study
pGeRF1	<i>P. putida pnp Eco</i> RI (698)/ <i>Xho</i> I (1268) fragment from pRF17 cloned in <i>Eco</i> RI/ <i>Sal</i> I-digested pGEM-4Z, 5' end of <i>pnp</i>	This study
pGZ119EH (HE)	Cloning vector, Cm ^r	29
pHP45Ω-Km	pBR322 derivative carrying the Ω-Km ^r interposon	15
pJB3Pnp2	2.5-kb <i>Sph</i> I/ <i>Sac</i> I fragment from pRF17, containing the <i>P. putida pnp</i> gene, cloned in <i>Sph</i> I/ <i>Sac</i> I-digested pJB3Tc19	This study
pJB3Tc19	Broad-host-range cloning vector, Ap ^r /Tc ^r , RK2 <i>oriT oriV</i>	6
pKNG101	Suicide cloning vector, R6K <i>ori</i> , RK2 (RP4) <i>mob</i> , <i>strA strB</i> (Sr ^r) <i>sacB</i> (sucrose sensitivity)	27
pKRF7A	pMA9 3.8-kb <i>Bam</i> HI/ <i>Hind</i> III fragment, carrying the central part of <i>P. putida pnp</i> gene interrupted by Ω-Km interposon, cloned in <i>Sma</i> I-digested pKNG101	This study
pMA5	<i>P. putida pnp</i> fragment 889-2452 obtained by PCR with <i>P. putida</i> genomic DNA by using the FG218 and FG219 primers, cloned in <i>Hinc</i> II-digested pGEM-4Z	This study
pMA9	Klenow-fragment-treated <i>Hind</i> III fragment of pGEM-4Z-Km1 containing the Ω-Km interposon cloned in <i>Hinc</i> II fragment of pMA5 interrupting the central fragment of <i>pnp</i>	This study
pRF14	<i>P. putida pnp</i> fragment 1089-2888 obtained by PCR with <i>P. putida</i> genomic DNA by using the FG231 and FG393 primers, cloned in <i>Sma</i> I-digested pGZ119-EH; contains the 3' end of <i>pnp</i>	This study
pRF16	<i>P. putida pnp</i> fragment 396-2243 obtained by PCR with <i>P. putida</i> genomic DNA by using the FG390 and FG172 primers, cloned in <i>Sma</i> I-digested pGZ119-EH; contains the 5' end of <i>pnp</i>	This study
pRF17	4.8-kb <i>Hind</i> III- <i>Eco</i> RI (partial digestion) fragment of pRF16 ligated to the 1.5-kb <i>Eco</i> RI- <i>Hind</i> III fragment of pRF14; contains the <i>P. putida pnp</i> gene (396-2857) reconstructed in pGZ119EH	This study
pURF10	215-bp <i>P. putida pnp</i> fragment amplified with degenerate primers FG143 and FG144, cloned in pUC18 <i>Sma</i> I	This study

^a Numbers are coordinates. See Table 2 for oligonucleotides.

Domain Database at <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). The linkage of *pnp* with the upstream gene *rpsO* is also a conserved feature (see the Clusters of Orthologous Groups database genomic context at <http://www.ncbi.nlm.nih.gov/COG/>). It is possible that expression of *P. putida* S15 ribosomal protein could be incompatible with the *E. coli* ribosome and could have been responsible for our failure to obtain clones carrying *pnp* in a genomic data bank. Comparison of the *E. coli* genomic map with the map deduced from the *P. aeruginosa* and *P. putida* KT2442 sequences also revealed that the homologous genomic organization extends to the upstream *nusA* operon, which in *E. coli* includes cold-induced genes (19, 26). This arrangement appears to be widely but not universally conserved among bacteria. In contrast, in *P. putida* *deaD* is not linked to *pnp*, whereas in *E. coli* this cold-induced gene (25) is located downstream of *pnp* and is cotranscribed with *pnp* during cold acclimation (43).

A single promoter upstream of *rpsO* (at positions 185 to 230, with a putative transcription start point at position 220) was predicted by the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) with a score of 0.95. The 250-nucleotide (nt) *rpsO-pnp* intergenic region of *P. putida* TMB did not exhibit any significant similarity with the equivalent *E. coli* region that was the same length, nor were similar sequences found by BLAST analysis of 228 bacterial genome sequences available at the ENTREZ search and retrieval system (<http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez>

/genom_table.cgi); exceptions were the homologous region in *P. putida* KT2440 (100% identity) and a ca. 100-nt sequence upstream of the *Pseudomonas syringae* and *P. fluorescens pnp* putative genes. Two large hairpin structures within the first two-thirds of the intergenic region, which are potential RNase III cut

TABLE 2. Oligonucleotides used

Oligonucleotide	Sequence ^a	Coordinates ^b
FG143	CCXRRXMGXMGXGARATHGGXCA YGG	395-404 ^c
FG144	CCCATXGCDATXCCXGXCXGSGXG CYTT	458-467 ^c
FG172	ACCGGCTACCTGAAGTCCA	2243-2224
FG231	GTCTGCACCGTGGTTTCC	1089-1106
FG390	TGATCCGTATGGTCAACCACCGTCGT AAGCTGC	396-430
FG393	TTTGAAGGGTTTTCTCTATTGCGTTAC GGTTCG	2888-2857
FG432	GAAAGCTTATTAGCGACGCAGGCCCA	501-483 (<i>Hind</i> III 5' tail)
FG243	AGAAACCCTTGCTGGATCAGCCTG	918-894

^a Oligonucleotide sequences are 5' to 3', and the IUB/GCG code is used. Bases missing in the *P. putida* sequence are underlined.

^b Coordinates in GenBank accession number Y18132.

^c Degenerate primer FG143 and FG144 correspond to amino acids 395 to 404 and 458 to 467, respectively, of *E. coli* PNPase (SwissProt accession no. P05055) and bracket the region from position 1978 to position 2123 of *P. putida*, as assessed a posteriori.

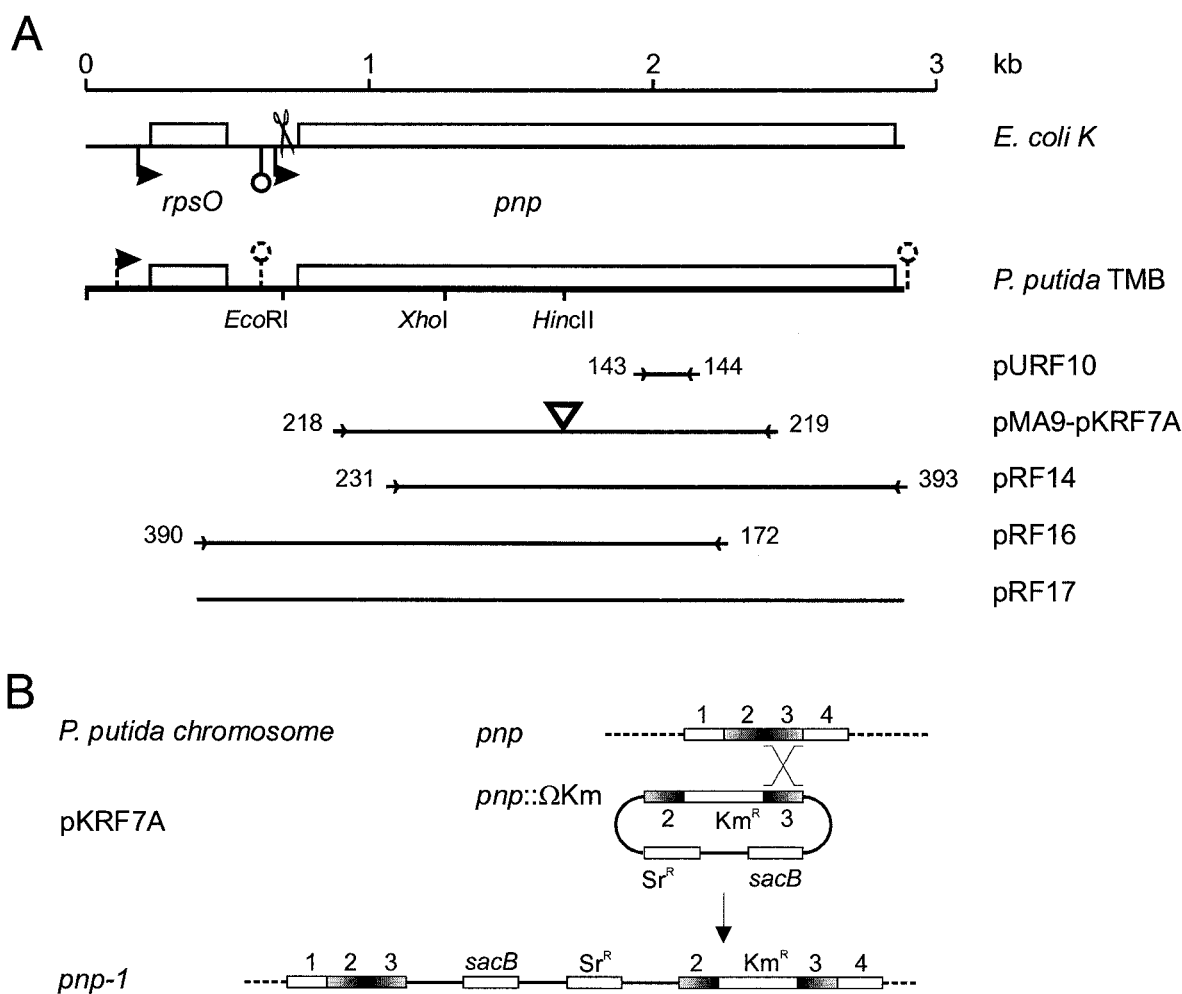


FIG. 1. (A) Map of *P. putida* TMB *rpsO-pnp* region. The *E. coli* K-12 region is included at the top for comparison. Relevant restriction sites and cloned regions are shown; the corresponding plasmids (designations on the right) are described in Table 1. Promoters and intrinsic terminators are indicated by bent arrows and lollipops, respectively (dashed lines indicate that such features are putative). The terminator downstream of *pnp* is outside the sequence established in this work and was inferred from the complete genomic sequence of *P. putida* KT2442 (accession number NC_002947). Scissors indicate the RNase III cut site in the *E. coli* mRNA. Primers (Table 2) are indicated by arrowheads flanked by the oligonucleotide numbers and delimit the regions amplified by PCR and cloned. The triangle in pMA9 and pKRF7A indicates the Ω -Km cassette cloned into the *HincII* site. The fragment cloned in pURF10 was obtained by PCR of *P. putida* TMB genomic DNA with degenerate primers FG143 and FG144 (Table 2) designed with two amino acid motifs (PK/GRREIGHG and KAA/PVAGIAMG) conserved in the PNPases of *E. coli* (SwissProt accession number P05055), *Photobacterium luminescens* (P41121), *Haemophilus influenzae* (P44584), *B. subtilis* (P50849), and *Streptomyces antibioticus* (Q53597). (B) Mutagenesis of *P. putida pnp* gene. An internal fragment of *pnp* (regions 2 and 3) interrupted by a kanamycin resistance cassette and cloned in the suicide plasmid pKNG101 (giving pKRF7A) recombined with homologous region 3 of the chromosomal gene, producing a merodiploid with two mutant *pnp* alleles (*pnp-1* mutation in PPM101 and PPM103).

sites, were predicted by MFOLD analysis performed at <http://bioinfo.math.rpi.edu/~zukerm> (32, 44). Computer analysis (TERMINATOR, Wisconsin GCG package) revealed a putative intrinsic transcription terminator in the middle of the *rpsO-pnp* intergenic region, ending 125 nt downstream of *rpsO*. Another terminator ended 47 nt downstream of the *pnp* coding region in *P. putida* KT2442 (Fig. 1), immediately outside the strain TMB region sequenced.

Isolation and characterization of *P. putida pnp* mutants. The procedure used to create *pnp* mutants by allelic substitution is shown in Fig. 1B. The internal fragment of the *pnp* gene from nt 889 to 2,473 interrupted by the Ω -Km interposon (15) in the *HincII* site (nt 1682) was cloned in the suicide plasmid vector pKNG101, which can replicate in *E. coli* but not in *Pseudomo-*

nas (27) (Table 1). The resulting plasmid, pKRF7A, was transferred by conjugation, essentially as described previously (28), into both *P. putida* KT2440 and TMB. *P. putida* exconjugants, selected for the ability to use benzoate as a sole carbon and energy source and for kanamycin resistance, were also streptomycin resistant and sucrose sensitive, as expected after a single crossover in one homology region on either side of the Km^R cassette. In all 15 clones analyzed recombination occurred within the 3' region of *pnp* (region 3 in Fig. 1B), as assessed both by Southern blotting and by PCR analysis (data not shown). We attempted to obtain second-site recombinants to get rid of the integrated plasmid vector by selecting for sucrose resistance on LD agar plates (17) containing 4% NaCl, 6% sucrose, and 50 μ g of kanamycin per ml. However, all clones obtained

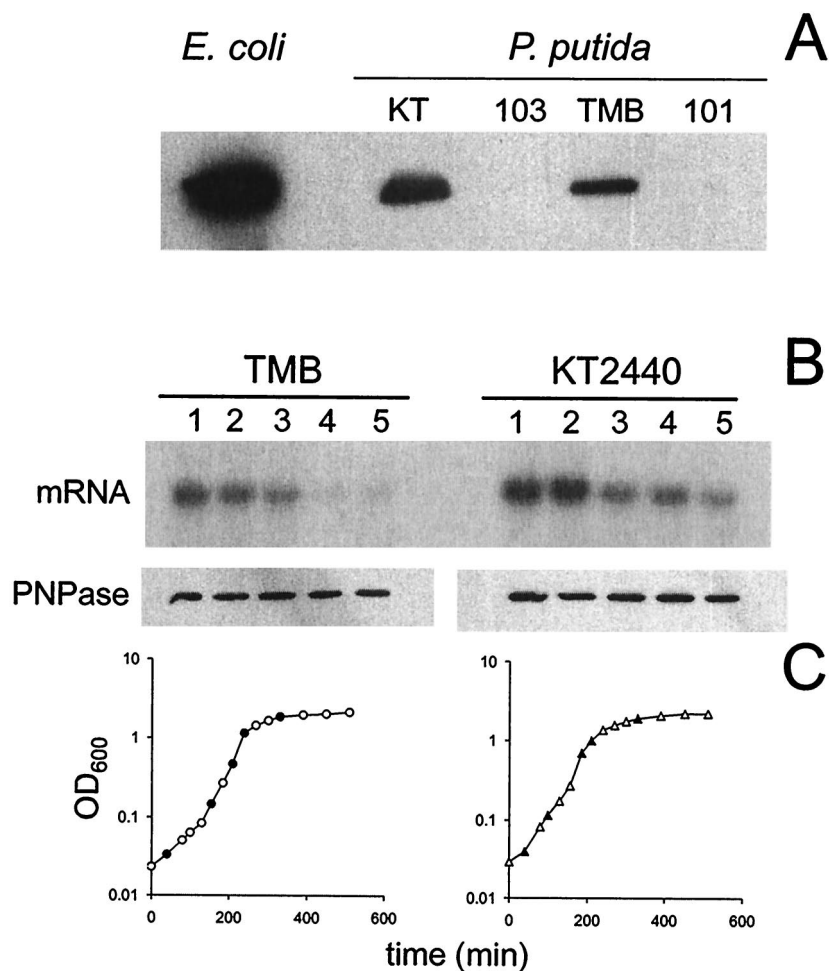


FIG. 2. (A) Western blot analysis of PNPase in wild-type and mutant *P. putida* strains. Cultures of *E. coli* and *P. putida* strains KT2440 (KT), PPM103 (103), TMB, and PPM101 (101) were grown in LD broth at 30°C (*P. putida* strains) or 37°C (*E. coli*) to an optical density at 600 nm of 0.4 and analyzed by Western blotting by using polyclonal antibodies raised against *E. coli* PNPase (a generous gift from A. J. Carpousis). Equal portions (1.5 μ g) of proteins were loaded in all lanes. Immunoreactive bands were revealed by using the Amersham ECL Western blotting reagent. (B) Expression of *pnp* in different culture growth phases at 30°C. *pnp* mRNA and PNPase from samples of *P. putida* TMB and KT2440 cultures in different growth phases were analyzed by Northern and Western blotting, respectively, as described previously (43). The size of the mRNA is about 2.2 kb. (C) Growth curves. Solid symbols indicate the times when the samples analyzed in panel B were taken. OD₆₀₀, optical density at 600 nm.

in several independent experiments retained the vector Sr^r marker, indicating that the loss of sucrose sensitivity was not due to excision of the inserted plasmid via homologous recombination. It should be noted that in the single-crossover clones the efficiency of plating on sucrose was 10⁻² to 10⁻³, and thus sucrose tolerance selection was not very effective in this system.

Insertion of plasmid pKRF7A by recombination in region 3 generated two mutant *pnp* alleles, one truncated at the C terminus and the other missing the N terminal region; therefore, the recombinants were *pnp* mutants. However, the allele truncated at the C terminus was predicted to encode a 578-amino-acid peptide missing the KH and S1 RNA binding domains but retaining the putative catalytic core. Our failure to obtain simple allelic substitutions suggests that *pnp* is an essential gene in *Pseudomonas* and that the 3'-truncated allele expresses the essential function(s). One thing that supports this hypothesis is that in *E. coli* PNPase-RNase II double mutants are not viable (14), and in *P. putida* RNase II seems to be

missing. (However, the same situation occurs in *B. subtilis* [13], in which *pnp* is not essential [31].) We therefore tested two single-crossover strains, PPM101 and PPM103 (TMB and KT2440 derivatives, respectively) for PNPase activity. The phosphorylolytic activity in crude extracts was below the level of detection for both mutants, as measured by the photometric assay described by Fontanella et al. (16) (in comparison, the activity for both wild-type *P. putida* strains was 0.05 μ M min⁻² and in *E. coli* it was 0.2 μ M min⁻²). *P. putida* PNPase readily reacted with anti-*E. coli* PNPase polyclonal antibodies in Western blotting assays; however, no truncated polypeptide could be detected in the *pnp* mutants (Fig. 2A). These results confirm that PPM101 and PPM103 are PNPase-deficient mutants and support the idea that *pnp* is not an essential gene in *P. putida*. The lack of a truncated peptide in the mutants was somewhat surprising since the 3' interrupted gene could be transcribed from its natural promoter (see below). It is possible that the transcript of the truncated gene is functionally inactivated very

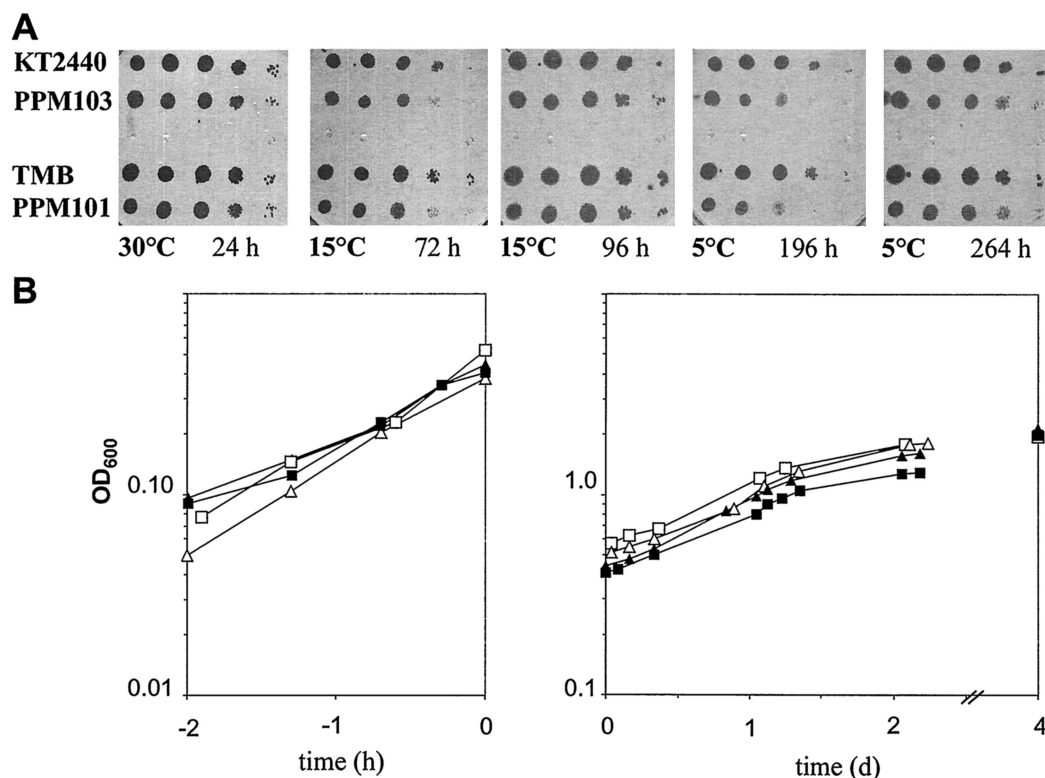


FIG. 3. Growth of *P. putida pnp* mutants at low temperatures. (A) Overnight cultures (grown in LD broth at 30°C with aeration) of the wild-type and *pnp* mutant strains indicated on the left were serially diluted (10^0 to 10^{-4}) in Bertani tray wells, replica plated onto five LD agar plates, and incubated at different temperatures and for different times, as indicated below the images, until visible colonies were observed. (B) The same strains were grown in LD broth at 30°C to an optical density at 600 nm (OD_{600}) of about 0.5 and then shifted to 9°C (time zero). The optical density at 600 nm is plotted versus time in hours before the temperature downshift (left graph) or in days after the temperature downshift (right graph). Symbols: □, TMB; ■, PPM101; △, KT2440; ▲, PPM103.

rapidly and/or that the truncated peptide is particularly labile and is promptly degraded (for example, following tmRNA-mediated tagging) (18).

In contrast to what was observed in *E. coli* (4, 31, 43), *P. putida pnp* mutants could grow at low temperatures. As shown in Fig. 3A, the efficiency of plating for both mutants was comparable to that for the wild-type control strains at temperatures as low as 5°C, although the colonies appeared later and were smaller than the wild-type colonies. This was confirmed in liquid cultures; at 30°C, the generation times of the mutants were about 60% longer than those of the wild-type strains (about 1 h). After a temperature downshift to 9°C, both the wild-type and *pnp* mutant *P. putida* strains continued to grow with comparable generation times (about 22 h) and, unlike *E. coli* (25, 34), did not exhibit acclimation growth arrest (Fig. 3B).

pnp expression during growth at 30°C and upon cold shock.

In *E. coli*, regulation of PNPase appears to be quite complex at both optimum and low growth temperatures. At 37°C PNPase controls its own expression by promoting degradation of its RNase III-processed mRNA (24, 39, 40), whereas upon cold shock this autogenous regulation seems to be alleviated and leads to a substantial transient increase in *pnp* mRNA. During the first 1 h of cold adaptation the *pnp* transcript level increases more than 10-fold, while transcription termination is suppressed and polycistronic transcript species appear. Later, the

transcript profile returns to the preshift condition. In contrast, the PNPase level slowly increases up to two- or threefold in cold-adapted cells, when transcript abundance returns to an almost preshock level (4, 33, 43). Such an expression pattern implies that *pnp* mRNA, unlike the mRNA encoding true cold-induced proteins (19, 21), is not efficiently translated during cold acclimation. Control of the *pnp* mRNA level is also exerted in *E. coli* at 37°C in a growth phase-dependent manner, and this mRNA is more abundant in early-exponential-phase cultures than in late-exponential-phase cultures. The PNPase level, however, remains constant throughout the culture growth phases, suggesting that there is translational control (43).

As shown in Fig. 2B (upper gel), both *P. putida* KT2440 and TMB at 30°C expressed a 2.2-kb transcript that hybridized to the *pnp*-specific probe; the length of this transcript is consistent with the distance from the major 5' end detected by primer extension (see below) and the predicted transcription terminator downstream of *pnp*.

The signal was strong in the early exponential phase, and the intensity decreased with increasing cell density. Western blot analysis with anti-PNPase antibodies, however, revealed that the total amount of PNPase protein was constant at all optical densities (Fig. 2B, lower gel). Therefore, the expression pattern of the *P. putida pnp* gene throughout exponential growth at the optimum growth temperature paralleled the profile ob-

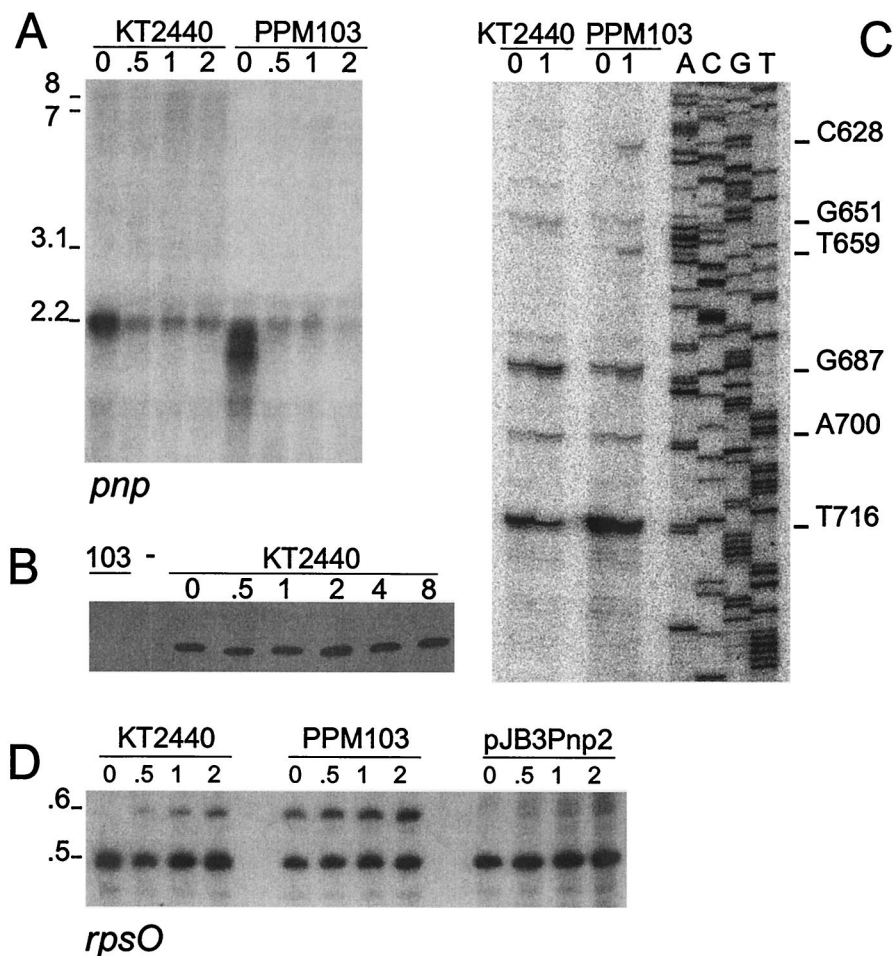


FIG. 4. Expression profile of *pnp* and *rpsO* upon cold shock. Cultures of KT2440 (*pnp*⁺), PPM103 (*pnp*), and PPM103 containing pJB3Pnp2 (a plasmid expressing *P. putida pnp* from the *plac* promoter) were grown in LD broth at 30°C with aeration to an optical density at 600 nm of 0.4, quickly cooled to 9°C in an ice-water bath, and transferred to 9°C in a refrigerated water bath with aeration. Aliquots were obtained immediately before the temperature downshift (zero time) and at different times after the temperature downshift (indicated in hours above the lanes); RNA was extracted as described previously (12) and processed as follows. (A) Northern blot analysis of *pnp* transcripts. Fifteen micrograms of RNA was resolved by 1.5% agarose gel electrophoresis and analyzed by Northern blot hybridization with the radiolabeled riboprobe *pnp* transcribed with T7 polymerase from pGERF1, as previously described (7, 12). The approximate sizes of the RNAs (in kilobases) are indicated on the left. (B) Expression of PNPase upon cold shock. Western blotting with PNPase polyclonal antibodies was performed by using 1- μ g portions of total *P. putida* protein extracts. Lane 103 contained a sample of PPM103 (*pnp*) extract. (C) Primer extension of *pnp* transcripts. Oligonucleotide FG243 labeled at the 5' end with ³²P was hybridized to 10 μ g of total RNA, extended with reverse transcriptase, and electrophoresed in a 6% polyacrylamide sequencing gel. The sequencing reactions (lanes A, C, G, and T) were performed by using the FG243 primer and plasmid pRF17 as a template. The coordinates of the 5' end signals are indicated on the right. (D) Northern blot analysis of *rpsO* transcripts. RNA was resolved by 5% acrylamide denaturing gel electrophoresis and analyzed by Northern blot hybridization with radiolabeled oligonucleotide FG432.

served at 37°C in *E. coli* (43) at both the RNA and protein levels.

In contrast, upon a temperature downshift to 9°C, the level of the 2.2-kb transcript expressed by the wild-type strains substantially decreased (Fig. 4A). Nevertheless, the level of PNPase remained constant, as determined by Western blotting (Fig. 4B). In the mutant, the truncated *pnp* gene produced a smeared signal whose intensity at 30°C was greater than the intensity of the wild-type signal, whereas upon cold shock the intensity decreased, as observed in the wild type. Therefore, expression of *P. putida pnp* in the cold seemed peculiar in that the level of the *pnp* transcript decreased significantly immediately after the temperature shift, whereas the PNPase level remained constant for several hours, suggesting that *pnp*

mRNA is translatable during cold acclimation. In addition, in contrast to *E. coli* (43), transcription antitermination did not seem to occur (only very faint 7- to 8-kb smeared signals, which were detected better with longer autoradiographic exposures, appeared 1 h after a temperature downshift). This finding nicely correlates with the lack in *P. putida* of the cold-inducible *dead* gene downstream of *pnp*.

We also determined the 5' end of the *pnp* transcripts by primer extension analysis (Fig. 4C). With RNA from cells grown at 30°C up to the mid-exponential phase, several signals were observed, and the major signals mapped 35 and 63 nt upstream of the putative start codon. One hour after a temperature downshift to 9°C the intensity of the *pnp*-proximal signal decreased, while the distal signal became stronger. In

the *pnp* mutant the *pnp*-proximal signal was more intense than that in the wild type, whereas the distal signal was fainter; as in the wild type, 1 h after a temperature downshift to 9°C the intensity of the *pnp*-proximal signal decreased, while the distal signal became stronger and additional signals appeared. Although the primer extension analysis was not quantitative, the data are in agreement with Northern blotting data.

In *E. coli pnp* may be transcribed from the following two promoters: P1, located upstream of *rpsO*, by reading through a Rho-independent terminator; and P2, located in the *rpsO-pnp* intergenic region. Both transcripts are efficiently processed by RNase III at a site downstream of P2 (36). The upstream *rpsO* and downstream *pnp* monocistronic transcripts are then processed further. In the equivalent *P. putida* region a single promoter upstream of *rpsO* was predicted. Whether a second promoter is present in the intergenic region remains to be ascertained; however, the multiple 5' ends found by primer extension analysis suggest that there is extensive endonucleolytic mRNA processing in the *rpsO-pnp* intergenic region, which appears to be modulated both by PNPase and by cold shock. As in *E. coli*, such endonucleolytic processing appears to be very efficient, since we could not detect dicistronic transcripts (expected to be 2.7 kb long) by Northern blotting.

Overall, our data suggest that PNPase may negatively control the abundance of *pnp* mRNA at the optimum temperature but does not seem to be responsible for its downregulation in the cold. It may be concluded that the *pnp* expression pattern in *P. putida* during cold acclimation differs from that observed in *E. coli* at the level of mRNA and at the level of protein abundance.

***rpsO* transcription analysis.** To test whether the transcript profile observed in response to cold shock was specific for *pnp*, we analyzed the transcription pattern of the adjacent upstream gene, *rpsO*. At 30°C a single signal at ca. 0.5 kb was detected for the wild-type strains, and this signal remained constant in the cold acclimation phase. However, a ca. 0.6-kb RNA, possibly a precursor of the 0.5-kb transcript, appeared, and its intensity progressively increased during cold shock. In contrast, in the mutant strains the 0.6-kb signal was present also at 30°C, suggesting that PNPase is involved in maturation of the *rpsO* transcript. Accordingly, the longer signal disappeared when the mutant was complemented by the *P. putida pnp* gene expressed from the pJB3Pnp2 plasmid (Fig. 4D). This preliminary transcription analysis of the *rpsO* transcripts indicated that PNPase plays a significant, albeit not indispensable, role in the processing of the main *rpsO* mRNA from a longer precursor.

In conclusion, the patterns of *pnp* regulation upon cold shock in *E. coli* and *P. putida* are markedly different, and *P. putida pnp* mutants did not show cold sensitivity, unlike other previously studied gram-positive and gram-negative organisms (19). It thus appears that involvement of PNPase in cold adaptation is not a universal feature of (mesophilic) bacteria and that control of PNPase expression in the cold evolved independently in different lines. Whether this reflects more general differences in cold adaptation mechanisms in *Pseudomonas* remains open to further investigation.

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