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Cloning and Mutational Analysis of the Gene for the Stationary-Phase Inducible Catalase (*catC*) from *Pseudomonas putida*†

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***Pseudomonas putida*, a bacterium that colonizes plant roots and enhances plant growth, produces three isozymes of catalase (A, B, and C) in stationary-phase cells. A catalase probe, generated by PCR analysis of *P. putida* genomic DNA with oligomers based on typical catalase sequences, hybridized to a genomic clone that expressed catalase C in *Escherichia coli*. The *catC* gene from this clone had a 2,133-bp open reading frame with a high level of identity to the stationary-phase-specific *E. coli katE*. Chromosomal mutants of *P. putida* deficient in catalase C, obtained by gene interruption with a *luxAB-npt* cassette, demonstrated enhanced *catC* transcription in stationary-phase cells and, upon exposure to phenol, in logarithmic-phase cells. The catalase C-deficient cells were not impaired in their ability to colonize roots of bean or wheat plants grown under sterile conditions.**

Catalases are involved as one of the mechanisms used to protect cells against the damage caused by reactive oxygen species to cellular components, including nucleic acids, lipids, and proteins (5). Challenge by reactive oxygen species occurs from external sources as well as from normal metabolism. Soil pseudomonads are exposed as they contact plant roots (9, 10), and certain soil fungi also produce hydrogen peroxide as a mechanism to compete with other organisms (12). Regulation of catalase activity, including alteration in expression of isozymes, may be important in surviving such challenge. The pseudomonad *Pseudomonas putida*, a root-colonizing isolate (3, 22), produced a single isozyme of catalase, catalase A, in logarithmic phase but three isozymes, catalases A, B, and C, in stationary phase (7–9). In order to understand the basis for the change in expression of the different isozymes, cloning of the individual genes for catalase was initiated.

Cloning of the gene for catalase C and expression in *Escherichia coli*. Two degenerate oligonucleotides [oligomer I, 5′-CG(G/T/C)GA(G/A)CG(G/T/C)AT(T/C)CC(C/G)GA(G/A)CG(G/T/C)GT(C/G)GT(C/G)CA(T/C)GCC-3′, and oligomer IV, 5′-(A/G)TA(G/C)(G/C/A)(A/T/C)(A/G)AACA(A/G/C)(A/C/T/G)CG(A/C/T/G)CC(T/C)TGCA(A/G)CA-3′] that were predicted to generate PCR products of about 900 bp from typical catalase genes were constructed (18). PCR analysis of genomic *P. putida* DNA with these primers gave a product of 890 bp that was purified by standard methods (1, 20), cloned into pBluescript SK$^+$ (Stratagene, San Diego, Calif.), and sequenced. The DNA sequence and the corresponding amino acid sequence of the product showed identity to other known catalase genes (Fig. 1).

The PCR product, when randomly labeled with digoxigenin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), hybridized to a single 6.5-kb *Pst*I fragment from the genomic DNA of *P. putida* (data not shown). Screening about 150 clones from a cosmid library of a partial *Bam*HI digest revealed 10 putative catalase-positive clones. One clone, pCatC-1, produced an 890-bp product upon PCR analysis with primers I and IV. The 6.5-kb *Pst*I fragment was subcloned into pBluescriptSK$^+$ (producing pCatCBS-1). Subsequent restriction analysis and cloning produced a 3.5-kb *Pst*I/*Hin*cII fragment in pCRII (Invitrogen, San Diego, Calif.) (pCatC-II) that contained the *P. putida catC* nucleic acid sequence (Fig. 1). The nucleotide and deduced amino acid sequences of *catC* of *P. putida* have the accession no. U82622 in the GenBank nucleotide sequence database. The open reading frame of 2,133 bp corresponds to a peptide of 711 amino acids (aa), giving a subunit molecular size of approximately 80 kDa. The deduced amino acid sequence for the *catC* product has the highest degree of identity (60%) with 732 aa of the stationary-phase-induced *Escherichia coli katE* gene product (16, 18). Alignments with other catalase sequences from pseudomonads and *E. coli* are shown in Fig. 1.

Transfer of plasmid pCatCBS-1, containing the 6.5-kb *Pst*I fragment in pBluescriptSK$^+$, to *E. coli katE katG* mutant UM255 caused expression of catalase activity, as detected by the spectrophotometric assay for degradation of hydrogen peroxide as described by Katsuwon and Anderson (7), but only in extracts from stationary-phase cells. Specific activity of UM255 containing pCatCBS-1 was 18.5 (±9.4) U/mg of protein, compared with 3.6 (±0.6) U/mg of protein for UM255. Protein concentrations of cellular extracts were determined by the bicinchoninic acid protein assay (Pierce Co., Rockford, Ill.). The catalase activity in complemented *E. coli* UM255 migrated to the same position on nondenaturing gel electrophoresis (7) as did catalase C from *P. putida.*

Creation of catalase C-deficient mutants in *P. putida*. In order to generate homologous exchange mutants and to monitor promoter activity of the gene, a novel *luxAB-npt* cassette was constructed in pCMYCK (Fig. 2). Sequences conferring kanamycin resistance (*npt*) from pRL648 (4) and promoterless *luxAB* from pRL1063a that retained the ribosomal binding site (23) were combined in pUC18, creating pUC18-*luxAB-npt*. The *luxAB-npt* cassette was removed from pUC18-*luxAB-npt* with *Eco*RI and *Sal*I and blunt-end ligated into pRL500 (4) at the *Bam*HI site to flank the promoterless *luxAB-npt* cassette by symmetrical multiple cloning sites, making pCMYCK a versatile construct for gene interruption and promoter analysis.

The construct pCatC-IIa, which contained a 2.4-kb *Hin*cII fragment, was interrupted by digestion with *Kpn*I and insertion of the *luxAB-npt* cassette (pCatC-*luxAB-npt*) (Fig. 2). The plasmid, pCatC-*luxAB-npt*, contained *catC* interrupted between aa 96 and 97 by the *luxAB-npt* marker, thus forcing luciferase activity to be regulated by the *catC* promoter (Fig. 2). Homol-

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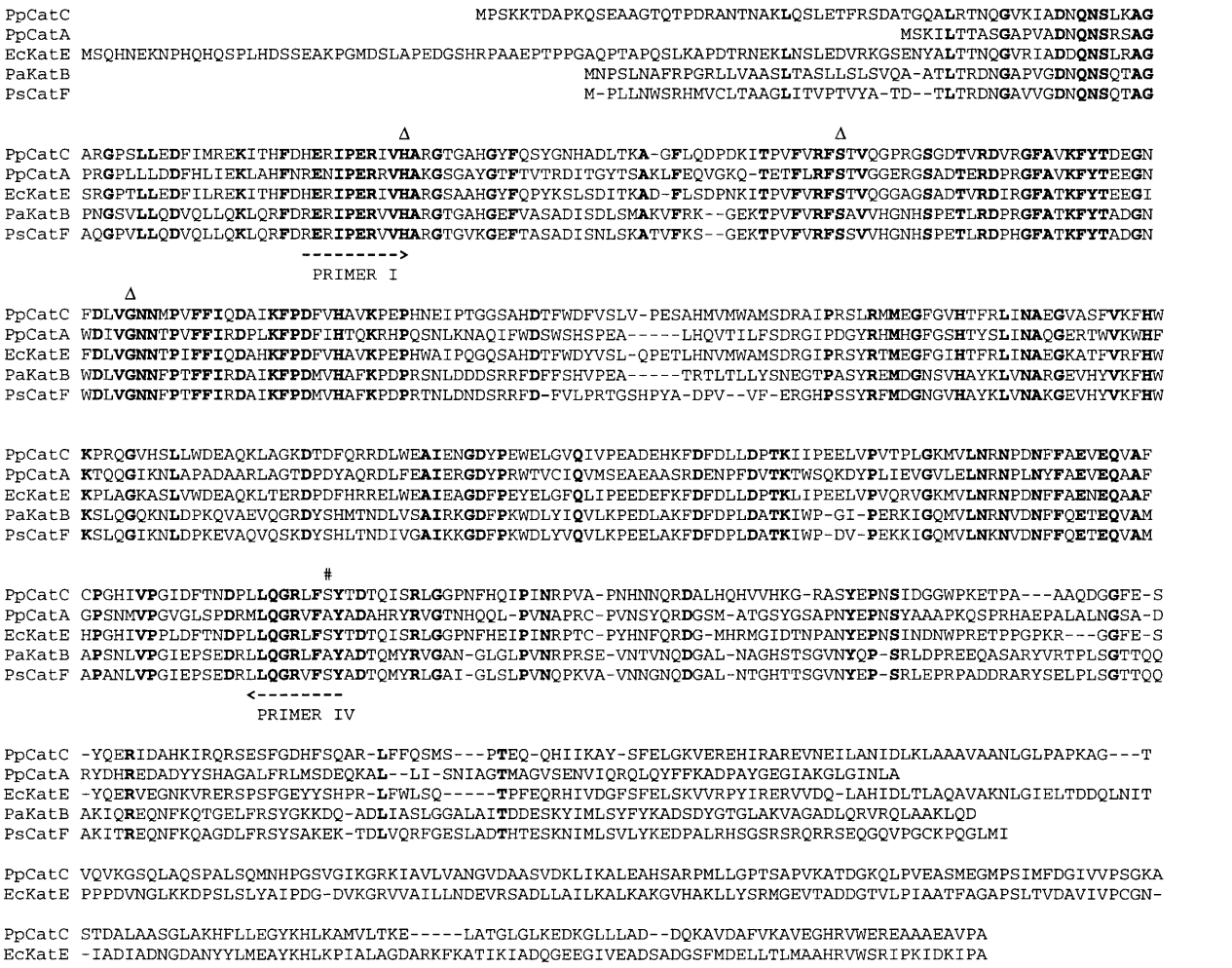

FIG. 1. Similarity of deduced amino acid sequences of the *catC* and *catA* products from *P. putida* (PpCatC and PpCatA, respectively) with the products of *katE* from *E. coli* (EcKatE), *katB* from *Pseudomonas aeruginosa* (PaKatB) (2), and *catF* from *Pseudomonas syringae* (PsCatF) (14). Gaps introduced to obtain maximal sequence alignment are indicated by dashes, and identical amino acids are in boldface. Amino acids postulated to be involved in the active sites are indicated (△), as are amino acids postulated as critical in forming the ligand for heme (#). Amino acid sequences for the degenerate primers used in DNA amplification for pseudomonads are underlined and designated primer I and primer IV. Sequence comparisons were obtained by using the Wisconsin Sequence Analysis programs BESTFIT and PILEUP of the Genetics Computer Group (Madison, Wis.).

ogous exchange of the interrupted *catC* gene into the genome of the wild-type *P. putida* was achieved by using the *sacB-sacR* system (19), which produces sensitivity to 5% sucrose, to expel the tetracycline resistance plasmid pCPP54 used as a shuttle vector (Fig. 2). Briefly, cells of DH5α, containing the tetracycline resistance *sacR-sacB* plasmid pCPP54 (D. Bauer, Cornell University) harboring the interrupted *catC* gene, were mixed with recipient wild-type, nalidixic acid- and rifampin-resistant *P. putida*, by using helper plasmid pRK2073. Transconjugants were established on Luria broth (LB) agar amended with nalidixic acid (50 μg/ml), rifampin (20 μg/ml), tetracycline (20 μg/ml), and kanamycin (25 μg/ml). After transfer to LB agar containing nalidixic acid, rifampin, and kanamycin and incubation for 48 h, colonies were serially diluted and plated onto LB agar containing nalidixic acid, kanamycin, and 5% sucrose to screen for putative homologous recombinants that were sensitive to tetracycline because of the loss of the pCPP54 plasmid. Southern analysis of the putative *P. putida* CatC mutant, CatC-461, revealed the predicted hybridization bands with the 890-bp *catC* PCR probe and with a 2.4-kb *luxAB* probe (data not shown).

TABLE 1. Specific activities of catalase in extracts from wild-type cells and the catalase C-deficient mutant, CatC-461, at various stages of growth[a]

Cell type	OD_{600}	Specific catalase activity (SE) (U/mg)	Catalase isozyme(s)
Wild type	0.55	13.3 (3.0)	A
	0.78	60.8 (2.8)	A
	1.02	64.4 (4.2)	A
	1.69	50.0 (2.6)	A
	>2.50	94.2 (17)	A, B, and C
CatC-461	0.57	14.5 (2.9)	A
	0.78	59.0 (1.0)	A
	1.17	64.4 (3.4)	A
	1.72	53.2 (2.2)	A
	>2.50	50.4 (7.9)	A and B

[a] Cells were grown in LB medium at 25°C. At various times up to 48 h, cells were pelleted and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.8). Cells were lysed by using a French press and subsequently centrifuged at 12,000 × *g* for 30 min to pellet cellular debris. Protein, catalase activities, and isozymes were then determined according to the method of Katsuwon and Anderson (7). Data are means and standard errors for two to four independent experiments.

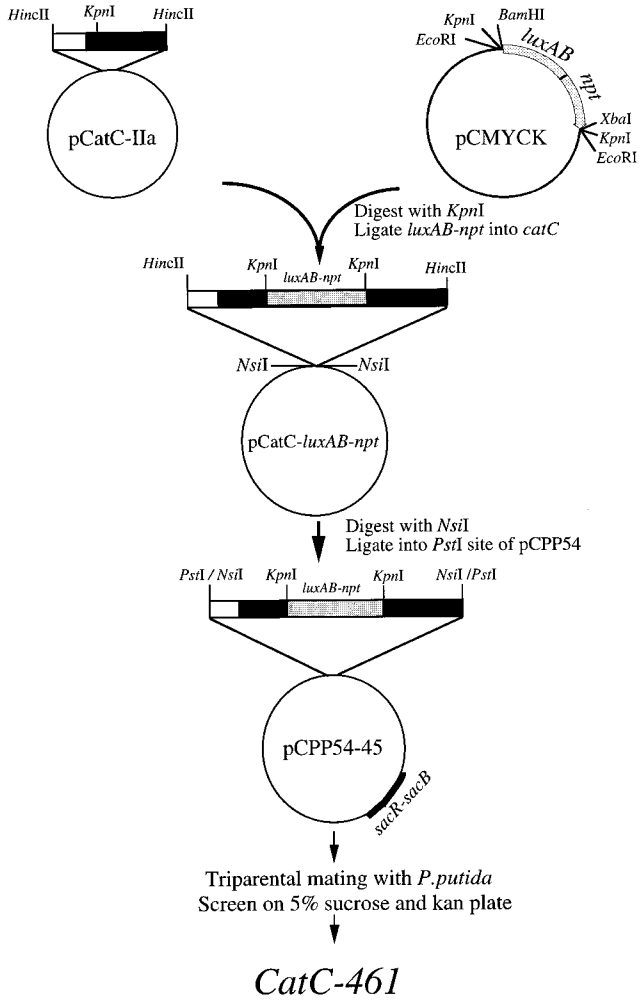

FIG. 2. The *luxAB-npt* cassette vector, pCMYCK, and the strategy for homologous marker exchange mutagenesis to obtain a *catC*-deficient mutant of *P. putida* (CatC-461). kan, kanamycin.

Properties of the catalase C mutant. Catalase C activity was not detected, even in stationary-phase cells of the mutant CatC-461 (Table 1; Fig. 3). Northern analysis of stationary-phase cells showed a band of hybridization of the expected size with the *catC* probe in wild-type but not in mutant cells. Catalase C activity was restored by complementation of *P. putida* CatC-461 with plasmid pCatC-1 through triparental mating with pRK2073 as a helper and selection for nalidixic acid-, rifampin-, tetracycline-, and kanamycin-resistant cells (Fig. 3). Catalase C activity was expressed only in stationary phase, for the wild type and complemented mutant (Fig. 3). Wild-type cells, mutant cells, and complemented mutants each produced catalase A throughout growth phase and produced catalase B in stationary phase (Fig. 3). Total catalase activities in CatC-461 were similar to wild-type levels, except in late-stationary-phase cells, where they were lower (Table 1). Both the CatC-deficient *P. putida* mutant, CatC-461, and the complemented *P. putida* mutant, Comp461, grew at wild-type rates in KB or LB broth at 25°C with shaking at 220 rpm for up to 48 h.

Mutant CatC-461 expressed luciferase activity, measured by adding *n*-decanol (Sigma Chemical Company, St. Louis, Mo.) (10 μl of 1.0% [vol/vol] *n*-decanol in 95% ethanol) to 0.5-ml samples prior to measurement of luminescence for 10 s in a Berkhold Luminometer (Berthold Analytical Inc., Nashua, N.H.), at a level that increased gradually throughout logarithmic phase (Fig. 4A) but increased markedly at the onset of stationary phase (optical density at 600 nm [OD_{600}] = 2.0). However, increased luciferase activity from the *catC* mutant was observed in logarithmic-phase cells grown to an OD_{600} of 0.4 in LB within 30 min after addition of phenol at 200 ppm (Fig. 4B). The *catC* promoter activity in the phenol-treated cells was about five- to sixfold greater than in nontreated control cells. The addition of phenol did not affect growth rates of the cells as determined by OD_{600} values.

The *catC*-deficient mutant colonized the roots of dark red kidney bean and wheat plants to the same extent as did the wild-type strain. The procedures for determining colonization

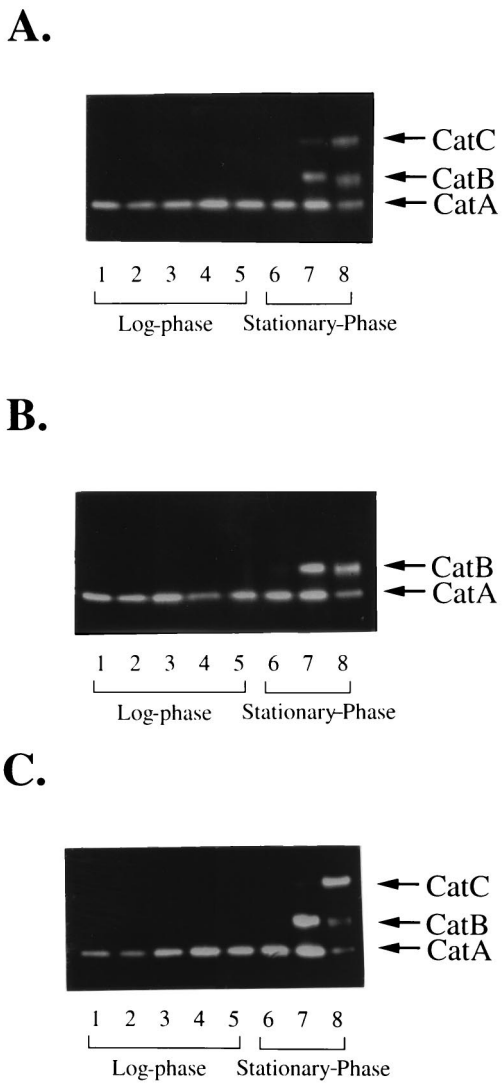

FIG. 3. Nondenaturing polyacrylamide gel electrophoresis of cell extracts from wild-type *P. putida* Corvallis (A), the CatC mutant (CatC-461) (B), and the CatC-complemented mutant (CatC-Comp) (C). Bacteria were grown aerobically in LB at 27°C with shaking at 250 rpm. Cultures (50 ml) were inoculated with 100 μl of cultures previously grown to an OD_{600} of 2.0. At various stages of growth, cells were harvested and extracts were prepared as described in the text. One unit of catalase activity (determined spectrophotometrically) was applied to 7.5% polyacrylamide nondenaturing gels, and staining for catalase activity was done. Lanes: 1, OD_{600} = 0.10; 2, OD_{600} = 0.26; 3, OD_{600} = 0.60; 4, OD_{600} = 1.3; 5, OD_{600} = 1.75; 6, OD_{600} = 2.0; 7, OD_{600} = 2.5; 8, OD_{600} = >2.5.

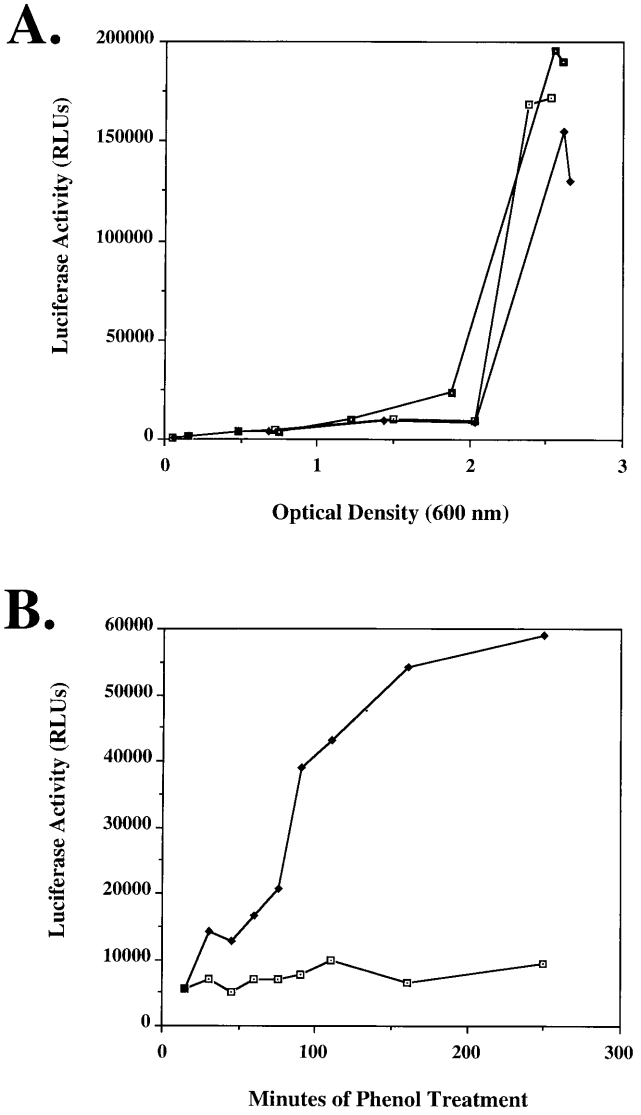

FIG. 4. Analysis of luciferase expression during the growth of catalase C-deficient mutant CatC-461 (A) and during treatment with phenol (B). (A) CatC-461 cultures were grown in LB broth at 25°C with shaking at 200 rpm. Aliquots (0.5 ml) were analyzed for luciferase activity at various times. Light emission was measured by using a Berthold Luminometer, and light emission is represented as relative light units (RLUs). The data are from three independent experiments. (B) CatC-461 cultures were grown as described for panel A and divided into two when they reached an OD_{600} of 0.4; luciferase activity was measured at various time points after the addition of 200 ppm of phenol to one of the cultures. ◆, phenol added; ⊡, no addition.

involved inoculating suspensions of 10^9 CFU of bacteria/ml onto surface-disinfested seed, prepared and planted in sterile vermiculite in enclosed boxes as described by Buell and Anderson (3). Cells were recovered from the roots at 1 and 2 weeks after growth at 25°C and were enumerated on LB agar. On bean roots, CFU were $(113 \pm 80) \times 10^6$/g for the wild type and $(50 \pm 25) \times 10^6$/g for the mutant at 1 week. At 2 weeks, CFU for the wild type were $(31 \pm 10) \times 10^6$ and CFU for the mutant were $(43 \pm 15) \times 10^6$. On wheat roots, the wild type colonized at $(28 \pm 15) \times 10^6$ CFU/g and the CatC-461 mutant colonized at $(35 \pm 15) \times 10^6$ CFU/g after 2 weeks.

Conclusion. Our data indicate that catalase C, one of three isozymes expressed by *P. putida*, is produced by a distinct genetic locus that is expressed in stationary phase. The gene has a relatively large open reading frame, like those of the stationary-phase catalase from *E. coli* encoded by *katE* and genes from fungi (11). The promoter from this pseudomonad gene is functional in *E. coli*, with expression also in stationary phase, indicating that the regulatory sequences function in both organisms.

A novel system based upon plasmid pCMYCK as a vector with the *sacB-sacR* eviction system was successful in generating homologous exchange mutants with *catC*. We have used the system successfully for other genes in our laboratory (13). Loss of catalase C activity did not impair growth in LB or KB media into stationary phase or alter the ability of the strain to colonize roots under otherwise sterile growth conditions. Thus, during growth under these conditions, the activities provided by the other catalases (CatA and CatB) expressed by *P. putida* apparently were adequate.

Transcriptional analysis of the *catC* gene by measurement of luciferase activity shows a correlation between onset of detectable catalase C activity and onset of stationary phase. Luciferase activity from the *catC* promoter also increased when phenol was administered to cells in the logarithmic growth phase. Because *P. putida* degrades phenol by oxidative mechanisms that could generate oxidative stress (6, 17), the sixfold induction of the *P. putida catC* promoter activity in the presence of phenol suggests that catalase C plays a protective role. A similar role in stationary-phase cells may be associated with expression of catalase C. Studies with *E. coli* indicate that the regulation of expression of catalase genes is one of the strategies that protect stationary-phase cells from mutations induced by oxidative stress (15, 22). Our studies with the *catC* gene confirm the usefulness of the promoter fusion technique we have developed using *luxAB* as a marker and *npt* for selection.

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