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Requirement of Polyphosphate by *Pseudomonas fluorescens* Pf0-1 for Competitive Fitness and Heat Tolerance in Laboratory Media and Sterile Soil[▽]

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Knowledge of the genetic basis for bacterial survival and persistence in soil is a critical component in the development of successful biological control strategies and for understanding the ecological success of bacteria. We found a locus specifying polyphosphate kinase (*ppk*) and a nonpredicted antisense RNA (*iiv8*) in *Pseudomonas fluorescens* Pf0-1 to be necessary for optimal competitive fitness in LB broth culture and sterile loam soil. Pf0-1 lacking *ppk* and *iiv8* was more than 10-fold less competitive against wild-type Pf0-1 in sterile loam soil low in inorganic phosphate. Studies indicated that *ppk*, and not *iiv8*, was required for competitive fitness. No role for *iiv8* was identified. While a *ppk* and *iiv8* mutant of Pf0-1 did not have increased sensitivity to osmotic, oxidative, and acid stress, it was more sensitive to elevated temperatures in laboratory medium and during growth in sterile soil. *ppk* was shown to be part of the Pho regulon in *P. fluorescens*, being upregulated in response to a low external P_i concentration. Of importance, overproduction of polyphosphate in the soil environment appears to be more deleterious than production of none at all. Our findings reveal a new role for polyphosphate (and the need for proper regulation of its production) in competitive fitness of *P. fluorescens* in laboratory and soil environments.

Soils are complex environments, presenting microbial inhabitants with a range of challenges which must be met if they are to survive and persist. Nutrients, pH, water content, and temperature can all affect survival of bacteria in soil (44). An understanding of the ecological success of microbes in natural environments such as soil requires knowledge of the mechanisms underlying adaptation and persistence. This appreciation for adaptive mechanisms is also important for the successful use of microbes for environmental applications, such as biocontrol or bioremediation (29, 47). *Pseudomonas* species, which are frequently isolated from soil environments, have a large complement of regulatory genes which are thought to permit rapid responses to environmental changes (42). For example, the two-component pair *phoB-phoR* responds to change in the concentration of exogenous inorganic phosphate (P_i) (25, 46). The regulatory targets of such systems likely include genes that are critical for adaptation and thus are important for long-term success within a fluctuating environment.

Many *Pseudomonas* species are being investigated as potential biocontrol agents because of their ability to produce compounds that are inhibitory to plant-pathogenic fungi (10). In addition, some *Pseudomonas* species have plant growth-promoting activity independent of their antifungal activity, making them very attractive in biocontrol (45). Despite the frequency with which *Pseudomonas* species are isolated from soils and the interest in developing biocontrol applications, the determinants of their environmental success are not well characterized (8).

Genes that are upregulated in a particular environment are likely to be important for success in that environment (28). Indeed, a number of environmentally regulated genes, identified using in vivo expression technology (IVET), have been shown to be important for success in the environment from which they were isolated (for examples, see references 3, 8, 19, and 20). We have been investigating the genetic basis of persistence of the soil isolate *Pseudomonas fluorescens* Pf0-1. Using an IVET screen, we found 22 sequences upregulated during growth in sterile loam soil (40). Three of these IVET-identified genes were shown to be involved in colonization of sterile soil. Here we report the analysis of an additional IVET-identified locus, termed *iiv8* (40). The sequence upregulated in soil did not match any gene predicted in the Pf0-1 genome annotation (GenBank accession number CP000094) and was antisense to a predicted polyphosphate (poly P) kinase gene (Pfl01_5464). In a previous study, we demonstrated that a nonpredicted IVET-identified antisense gene specified a protein of importance in colonization of sterile soil (39). Kim and Levy (16) recently showed that at a sense/antisense locus, at which the antisense gene was identified by IVET, the sense gene had a role in soil fitness.

poly P is a polymer of inorganic phosphate found ubiquitously and is important for a number of processes in bacteria (reviewed in reference 36). For example, in *Escherichia coli*, loss of poly P production is associated with defects in stationary-phase survival and with tolerance of osmotic, oxidative, and heat stress (30). In *Vibrio cholerae* poly P is important for motility (33), surface attachment (27), and tolerance of low pH and oxidative and osmotic stress (12). Proposed functions of poly P include chelation of metals, phosphate storage, substitution for ATP, and maintenance of pH (reviewed in reference 18). In *Pseudomonas aeruginosa* PAO1, poly P plays a role in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
Bacterial strains		
E. coli		
DH5α λpir	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR* λ-*pir*	J. J. Mekalanos
S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* λ-*pir*; donor in conjugations	41
P. fluorescens		
Pf0-1	Wild type, Ap^r	5
Pf0-1Km^r	Pf0-1::mini-Tn7Km, Km^r	39
Pf0-1Sm^r	Pf0-1::mini-Tn7Sm, Sm^r	39
Pf0-1Δ8-*ppk*	Deletion of *iiv8* and the 1,655 bp of *ppk* which it overlaps (bases 6131357 to 6133054 of the Pf0-1 genome); Ap^r	This study
Pf0-1Δ*ppk*-full	Deletion of *ppk* (bases 6130799 to 6133021 of the Pf0-1 genome)	This study
Pf0-1Δ8-*ppk*-Km	Pf0-1Δ8-*ppk*::mini-Tn7km	This study
Pf0-1*ppk*Rev-1	Pf0-1Δ8-*ppk* reverted to wild type by allele exchange with pSR-8DFR	This study
Pf0-1*ppk*Rev-2	Pf0-1Δ8-*ppk* reverted to wild type by allele exchange with pSR-8DFR	This study
Pf0-1*ppk*Rev-Km	Pf0-1*ppk*Rev-1::mini-Tn7Km	This study
Pf0-1Δ*pst*	Pf0-1 with deletion of *pstSCAB-phoU*; Gm^r	25
Pf0-1Δ*phoB*	Pf0-1 with deletion of *phoB*; Gm^r	25
Pf0-1Δ*pst*Δ*phoB*	Pf0-1Δ*pst* with unmarked deletion of *phoB*; Gm^r	25
Pf0-1Δ*pst*Δ*ppk*	Pf0-1Δ*pst* with unmarked deletion of *iiv8* and *ppk*; Gm^r	This study
Plasmids		
pGEM-T Easy	Ap^r; cloning vector for PCR products	Promega
pHRB2	Plasmid derived from pUC18T-Tn*7*T; mini-Tn*7* carries Km^r	24
pME6000	Tc^r; pBBR1MCS derivative, mobilizable *Pseudomonas* vector	22
pME8comp	*iiv8* complementation construct in pME6000 (bases 6130970 to 6133214 of the Pf0-1 genome)	This study
pME-hemB	*hemB* complementation construct in pME6000 (bases 6132852 to 6134285 of the Pf0-1 genome)	This study
pMEppk	*ppk* complementation construct in pME6000 (bases 6130717 to 6134285 of the Pf0-1 genome)	This study
pSR47s	Km^r; *sacB*-containing suicide vector (Pi-requiring R6K replication origin)	
pSRΔiiv8	Clone of SOE-PCR product in pSR47s for allele exchange deletion of *iiv8-ppk* in Pf0-1	This study
pSR-8DFR	Clone of *hemB-ppk* in pSR47s for allele exchange replacement of Δ*iiv8-ppk* with wild-type sequence	This study
pUIC3	Universal IVET construct; *lacZY' bla* Tc^r; R6K origin	28
pUIC*ppk-lac*	pUIC3 carrying *ppk-lacZ* transcriptional fusion	This study

numerous traits including motility (32); biofilm formation, quorum sensing, and virulence (34); and carbenicillin tolerance and maintenance of normal cellular ultrastructure (7). In *E. coli*, poly P production is part of the Pho regulon (1), a suite of genes upregulated in response to low phosphate by the response regulator PhoB. When P_i is limiting, PhoB is phosphorylated by PhoR and can then activate the Pho regulon. When P_i is in excess, PhoR-mediated phosphorylation of PhoB is prevented by an interaction with the Pst (phosphate-specific transport) system. This regulatory system is widely conserved and has been demonstrated to be present in *P. fluorescens* Pf0-1. The Pho regulon is constitutively derepressed in *pst* mutants because of the inability to prevent phosphorylation of PhoB. *phoB* mutants cannot activate the Pho regulon, and hence, Pho regulon genes are repressed under all conditions (25).

The objective of this study was to determine the importance of the overlapping gene pair *ppk* and *iiv8* in stress tolerance and fitness. Studies of bacteria in soil sought to extend our understanding of survival and fitness traits of soil bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas fluorescens* strains were grown at 30°C, and *Escherichia coli* strains were grown at 37°C. *E. coli* strains were routinely grown in LB medium (35), while *P. fluorescens* strains were cultured in LB or in *Pseudomonas* minimal medium (PMM) (17). For defined phosphate medium, MOPS (morpholinepropanesulfonic acid) minimal medium (26, 31) was used. MOPS medium was amended with either 2 mM (high-phosphate) or 0.14 mM (low-phosphate [25]) K_2HPO_4, creating media called MOPS-H and MOPS-L, respectively. For solid medium, agar was added to a final concentration of 1.5%. For motility assays, the agar concentration was 0.3%. The following antibiotics were used at the concentrations indicated: ampicillin, 50 μg/ml; streptomycin (Sm), 50 μg/ml; kanamycin (Km), 25 μg/ml; tetracycline, 15 μg/ml (for strains carrying replicating plasmids) or 7.5 μg/ml (for strains with plasmids integrated into the genome); gentamicin, 10 μg/ml. Plasmids were introduced into *P. fluorescens* strains by conjugation from *E. coli* S17-1. Briefly, donor and recipient strains were grown for 16 h. Cells were collected by centrifugation, washed twice in LB broth, and finally suspended in 200 μl of LB. Cells were mixed in a 1:4 donor-to-recipient ratio, and a 100-μl drop was placed onto the surface of an LB agar plate and incubated at 30°C for 8 h, after which the cells were scraped from the plate and plated onto appropriate medium to select the desired transconjugants.

DNA manipulations and sequencing. Recombinant DNA techniques were performed as described previously (35) or according to the supplier's instructions. Restriction enzymes and DNA-modifying enzymes were purchased from Invitrogen (Carlsbad, CA), New England Biolabs (Ipswich, MA), and Promega (Madison, WI). Plasmid DNA was extracted using a QIAprep Spin miniprep kit (Qiagen, Valencia, CA). DNA fragments were recovered from agarose gel slices using a QIAquick gel extraction kit (Qiagen). The PCR was carried out in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA), using *Taq* or Platinum *Taq* High Fidelity DNA polymerase (Invitrogen). PCRs to amplify DNA for cloning were all carried out using purified genomic DNA for the template (Wizard DNA isolation kit; Promega). Screening of mutants was carried out by colony PCR. When required, PCR products were cloned with pGEM-T Easy (Promega). Oligonucleotides were synthesized by IDT (Cor-

TABLE 2. Primers[a]

Primer	Sequence (5′–3′)	Purpose
8RT-R	CACTGGACGACCAAGATCAA	RT-PCR; 5′ RACE
8RT-F	AGCAGGTAGTCCGACAGGTG	RT-PCR; 3′ RACE
8GSP2	CGTTGCTGGTGAACAAGAGC	5′ RACE
8GSP3	GTCTGCTGCCGCGGATCAT	5′ RACE
8GSP5	GACGTCTTCGGAATCAAGC	3′ RACE
ppk-UIC-FWD	GGGagatctGCATAACTGCCGAAGAACGC	Construction of *ppk-lacZ*
ppk-UIC-REV	GGGactagtTACAGGCTGCTGTCATCCAG	Construction of *ppk-lacZ*
8D5F	CAATCGCTCCAGCAACGTCG	SOE-PCR deletion of *iiv8* and *ppk*; PCR to construct pSR-8DFR
8D5R	CCGTGCCGCTCAATTGTTAGCGCCGTTCACCCTGAAGAAG	SOE-PCR deletion of *iiv8* and *ppk*
8D3F	CTTCTTCAGGGTGAACGGCGCTAACAATTGAGCGGCACGG	SOE-PCR deletion of *iiv8* and *ppk*
8D3R	ACATCACCGTCGACGCACTG	SOE-PCR deletion of *iiv8* and *ppk*; PCR to construct pSR-8DFR
8D5F2	GACCGTGCATTGAGTAACG	Confirming deletion of *iiv8* and *ppk*
8D3R2	GCGTGAGAATGTGTTGACG	Confirming deletion of *iiv8* and *ppk*
8compF	GCGaagcttCAGCAGCTTCTTGCCTTC	Construction of pME8comp
8compR	GCGaagcttCTTCGTCTATCAGGTCAG	Construction of pME8comp
8D5F2H	GGGaagcttGACCGTGCATTGAGTAACG	Construction of pMEppk
hemF-H	GGGaagcttGTCATGCAGAAATTGTTGG	Construction of pMEppk

[a] Restriction sites engineered into the primers are shown in lowercase letters.

alville, IA). All custom primers are shown in Table 2. DNA sequences were determined by the Tufts University Core Facility (Boston, MA).

Construction of a *ppk iiv8* mutant. A *ppk-iiv8* deletion mutant was constructed by splicing-by-overlap extension–PCR (SOE-PCR) (11) and allele exchange between the suicide plasmid pSRΔiiv8 and the Pf0-1 genome to replace the wild-type sequence with a sequence in which nucleotides 6131357 to 6133054 were deleted. The suicide plasmid was introduced into Pf0-1 by conjugation, and single-crossover integration was selected by Km resistance. Sensitivity to sucrose, because of the plasmid-carried *sacB* gene, confirmed the presence of the plasmid. This strain was then grown for 24 h in LB broth in the absence of selection to allow a second recombination and loss of the suicide plasmid, and those strains were selected by plating dilutions from the second recombination culture on sucrose-containing medium. After plasmid loss was confirmed by sensitivity to Km, colonies possessing the desired deletion were identified by PCR. Primers for the SOE-PCR are listed in Table 2. This deletion spanned the transcribed region of *iiv8* identified by IVET (40), a possible open reading frame in which the transcribed sequence was found (bases 6131357 to 6133057 in the Pf0-1 genome), and the first 1,665 bases of *ppk* (the full length is 2,226 bases). The resulting strain was called Pf0-1Δ8-*ppk*. To provide a selectable marker to allow Pf0-1Δ8-*ppk* to be distinguished in competition experiments, mini-Tn*7*Km (24) was allowed to insert into the unique Tn*7* integration site downstream of *glmS* in Pf0-1Δ8-*ppk*, producing strain Pf0-1Δ8-*ppk*-Km.

Soil growth and competition assays. The soil used in these experiments was a gamma-irradiated fine loam from Sherborn, MA, as described previously (6). Bacterial strains were grown for 16 h in LB with appropriate antibiotics. Cells were diluted to approximately 1×10^5 CFU/ml in sterile distilled H_2O. For survival experiments, 1 ml of diluted cell suspension was mixed with 5 g of soil, achieving a water-holding capacity of approximately 50%. For competition experiments, cultures were adjusted to equal values of optical density at 600 nm prior to dilution, and then 500 μl of each diluted competing strain was combined and mixed with soil as described for the survival experiments. Inoculated soil samples were transferred to a 15-ml conical polypropylene Falcon tube. The initial recoverable population was established by removal of 0.5 g of soil after 30 min and recovery and enumeration of bacteria from that sample. Briefly, 1 ml of sterile water was added to the 0.5-g soil sample, followed by vigorous vortexing for 30 s. After the soil particulates had settled, bacteria in suspension were recovered and enumerated by CFU determination. This method has been described elsewhere (39, 40). The initial populations of wild-type and mutant strains were approximately equal. Populations were monitored over time by extraction of bacteria from the soil and determination of numbers by counting colonies on the appropriate selective medium (39, 40). The wild-type strain in competition experiments was Pf0-1Sm^r. In wild-type versus wild-type control assays, Pf0-1Sm^r was competed with Pf0-1Km^r. The competitive index (CI) is the ratio of mutant to wild type at a given time point divided by the initial mutant/wild-type ratio.

Competition experiments in LB medium. Competition experiments were carried out in 12.5 ml of LB in 125-ml flasks, in a shaking water bath at 150 rpm. Strains for competition experiments were grown for 20 h in LB with appropriate selection and then washed in fresh LB. Competition cultures were established as 1:1,000 dilutions of the washed cells. A 100-μl sample was immediately removed, diluted, and plated on selective medium to allow enumeration of each competitor in the culture. Samples (100 μl) were then removed, diluted, and plated on selective medium each for 24 h for 5 days. The CI was calculated as described for soil competition experiments.

Construction of complementation plasmids. Plasmids bearing the genes for complementation were constructed in the vector pME6000. PCR was used to amplify bases 6130970 to 6133214 for *iiv8* (primers 8compF and 8compR) and bases 6130717 to 6134285 for *ppk* (primers 8D5F2H and hemF-H). The genomic regions amplified were chosen so that the native promoters would be included. The PCR products were cloned into the HindIII site of pME6000 by using restriction enzyme sites incorporated into the primer sequences.

Restoration of *ppk* by allele exchange. Pf0-1Δ8-*ppk* was restored to the *ppk*$^+$ genotype for soil experiments. DNA spanning the deletion in Pf0-1Δ8-*ppk* was amplified by PCR using primers 8D5F and 8D3R, cloned in pGEM-T Easy, and then cloned in the NotI restriction site of pSR47s. The resulting clone (pSR-8DFR) was transferred to Pf0-1Δ8-*ppk* by conjugation. Allele exchange was used to replace the Δ*iiv8-ppk* region with the wild-type sequence, generating strains Pf0-1*ppk*Rev-1 and Pf0-1*ppk*Rev-2 (independently selected). The allele exchange was confirmed by PCR with primers 8D5F2 and 8D3R2 and by confirming restored poly P production.

Stress tolerance assays. (i) Heat tolerance. Heat tolerance during growth in culture medium was tested by starting 15-ml cultures in the appropriate medium with a 1:50 dilution of a 16-h culture grown in the same medium. The 15-ml cultures were grown for 24 h at 30°C and then transferred to 45°C. Samples (100 μl) were periodically taken, serially diluted in 10-fold increments, and plated to determine CFU/ml. To examine heat tolerance during growth in soil, the soil was inoculated with single strains as described above for soil growth assays, and the initial recoverable population was determined. After 2 days, 0.5 g of soil was removed for CFU determination and the remainder of the soil was subjected to the various temperatures used. The population was ascertained for each of the next 2 days.

(ii) Osmotic stress. Cultures (15 ml) were started in MOPS-H and MOPS-L by 1:50 dilution of cells (washed in MOPS-L) that had grown for 16 h in MOPS-H. After 20 h of growth, the bacteria from 15-ml cultures were recovered by centrifugation and then suspended in the same MOPS medium amended with NaCl at concentrations ranging up to 2.5 M. Controls were suspended in medium lacking the NaCl supplement. Bacterial populations were monitored over 3 h by plating to determine the CFU/ml.

(iii) Oxidative stress. Cultures (15 ml) in MOPS-H and MOPS-L were started as described above for osmotic stress. After 20 h of growth, H_2O_2 was added to

final concentrations ranging up to 150 mM, and populations were monitored over 3 h by plating to determine the CFU/ml.

(iv) Acid stress. Acid stress experiments were carried out following the same method as that for the osmotic stress experiments, except that medium was adjusted to pH 4.5 instead of being amended with NaCl. Populations were monitored over 3 h by plating to determine the CFU/ml.

Extraction and quantification of poly P. poly P was extracted and measured essentially as described previously (4). A standard poly P curve was made by serially diluting (10-fold increments) a solution of a known quantity of sodium poly P (Sigma-Aldrich, catalog no. 305553) and adding 100-μl aliquots to 900 μl of 6-mg/liter toluidine blue O dye (Sigma-Aldrich) in 40 mM acetic acid. After incubation at room temperature for 15 min, A_{530} and A_{630} levels were measured, and the amount of poly P was expressed as the ratio of A_{530} to A_{630}.

For poly P quantification in cells, bacteria were grown for 16 h in LB, 1 ml of culture was washed with MOPS-L, and the appropriate media were inoculated to achieve a 1:50 dilution of bacteria. At required time points, 1 ml of culture was removed for poly P extraction, and a 100-μl sample was taken for CFU determination. Cells from the 1-ml aliquot were collected by centrifugation, suspended by being vortexed in prewarmed (95°C) guanidine thiocyanate lysis buffer (4 M guanidine thiocyanate in 500 mM Tris-HCl [pH 7]), and incubated at 95°C for 5 min, after which 30 μl of 10% sodium dodecyl sulfate, 500 μl of 95% ethanol, and 10 μl of Glassmilk (Qbiogene catalog no. 1001-404) were added. Samples were mixed by vortexing and then briefly centrifuged in a bench top centrifuge (15 s, maximum speed) to pellet the Glassmilk. Pellets were suspended in New Wash buffer (5 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM EDTA, 50% ethanol) and pelleted again. The New Wash buffer treatment was repeated twice more. The pellet was then suspended in 100 μl of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 1 mg/ml of DNase I and RNase A. Samples were incubated at 37°C for 30 min, and then the Glassmilk was pelleted and suspended in 300 μl of a 1:1 mix of guanidine thiocyanate buffer and 95% ethanol. The Glassmilk was again pelleted and then suspended in New Wash buffer. This wash was repeated once. poly P was eluted from the Glassmilk by suspending the pellet in 50 μl of 50 mM Tris-HCl (pH 8) and incubating it at 95°C for 2 min. After the Glassmilk was pelleted, the supernatant was collected. The elution was repeated twice more, and the three 50-μl eluates were pooled. poly P was quantified by adding 10-, 25-, or 50-μl samples to 900 μl of toluidine blue O dye and bringing the final volume to 1 ml. After 15 min at room temperature, the A_{530}/A_{630} was determined and compared to the standard curve.

RNA isolation, RT-PCR, and rapid amplification of cDNA ends (RACE). RNA was extracted from *P. fluorescens* Pf0-1 using an RNeasy minikit and on-column DNase I digestion (Qiagen). RNA so recovered was treated with RQ1 DNase (37°C, 1 h) (Promega) and subsequently purified using an RNeasy minikit column. First-strand synthesis for reverse transcription-PCR (RT-PCR) was carried out using Superscript III (Invitrogen) and gene-specific primers (8RT-F for *ppk* and 8RT-R for *iiv8*) (Table 2), at 52°C for 1 h. The cDNAs for both *iiv8* and *ppk* were amplified by PCR using primers 8RT-F and 8RT-R. A negative control consisting of a reverse transcriptase-free reaction mixture was used in each experiment.

5′- and 3′-RACE experiments were carried out using Invitrogen 5′- and 3′-RACE system protocols, except that prior to 3′ RACE, total RNA was treated with *E. coli* poly(A) polymerase (Ambion). The gene-specific primers used were 8RT-R, 8GSP2, and 8GSP3 (for 5′ RACE) and 8RT-F and 8GSP5 (for 3′ RACE) (Table 2).

Measuring *ppk* transcription by β-galactosidase assay. A *ppk-lacZ* transcriptional fusion was constructed to measure expression of *ppk*. Bases 6132852 to 6134419 of the Pf0-1 genome, which include the *hemB* gene and the first 170 bases of *ppk*, were amplified by PCR (primers ppk-UIC-FWD and ppk-UIC-REV; Table 2). The product was cloned into the BglII and SpeI sites of plasmid pUIC3, which carries a promoterless *lacZ* gene. The resulting plasmid, pUIC*ppk-lac*, cloned in *E. coli* S17-1, was transferred to *Pseudomonas* strains by conjugation. Transconjugants were selected on tetracycline-containing PMM agar. Since pUIC3 and its derivatives do not replicate in *Pseudomonas* (28), transconjugants have a single copy of the plasmid integrated in the genome of the recipient by a single homologous recombination event, resulting in strains carrying *ppk-lacZ* in a near-native genetic context, with *ppk* transcription from its native promoter. Strains for the assay were grown for 16 h in MOPS-H medium, before 1 ml of culture was removed and washed twice in MOPS-L medium. The appropriate fresh medium was then inoculated with washed cells using a 1:50 dilution. β-Galactosidase assays were carried out essentially as described by Miller (23), using 20 μl of chloroform and 10 μl of 0.1% sodium dodecyl sulfate to permeabilize the cells. Data were expressed as pmol of orthonitrophenol produced per 10^3 CFU per min of reaction.

Statistical analysis. Data were analyzed with a *t* test using Graph Pad Prism version 4.

RESULTS

The *iiv8-ppk* locus is important for competitive fitness in soil and laboratory culture. DNA sequence analysis revealed that *iiv8* is located opposite the *ppk* gene in *P. fluorescens* Pf0-1. Since *iiv8* was upregulated in a soil environment (40), we reasoned that the *iiv8-ppk* locus may be important for fitness in soil. Moreover, *ppk* has been linked to a number of stress response phenotypes including survival in the stationary phase during laboratory culture (15, 30) and survival of *Mycobacterium tuberculosis* in macrophages (43). We therefore created a Pf0-1 derivative (Pf0-1Δ8-*ppk*) with this locus deleted (see Materials and Methods) and added a Km^r marker to facilitate selection after competition experiments with the Pf0-1 wild type bearing Sm^r. As shown in Fig. 1A, loss of the *iiv8-ppk* locus resulted in reduced competitive fitness in soil. After 9 days of competition, the CI for the Km^r *iiv8-ppk* mutant dropped to less than 0.05 compared to a value of approximately 1.0 for a Km^r wild-type strain in competition with the Sm^r Pf0-1 (Fig. 1A).

A "mock" CI for monocultures was calculated by combining data from Pf0-1Δ8-*ppk*-Km and Pf0-1Sm^r monocultures. This mock CI demonstrated that when Pf0-1Δ8-*ppk*-Km was grown alone in sterile soil, no growth defect was detected, compared to Km^r Pf0-1 grown under the same conditions (Fig. 1A). Since stationary-phase defects have been seen in *ppk* mutants of other species (e.g., reference 15), we asked whether the competitive defect was confined to soil or was a more general phenotype associated with Pf0-1Δ8-*ppk*-Km. Pf0-1Δ8-*ppk*-Km was cocultured with Sm^r Pf0-1 in LB broth for 5 days. As seen in the soil experiments, Pf0-1Δ8-*ppk*-Km was unable to compete with the wild type (Fig. 1B). Notably, the mutant population was comparable to that of the wild type for 2 days, after which the *iiv8 ppk* mutant population declined dramatically, resulting in very low CI values of about 0.0005 (Fig. 1B). These data show that the competitive defect is not limited to soil and not associated with a failure to reach a high population at the start of the experiment or the result of a lower initial growth rate than that of Pf0-1. The competitive defect of Pf0-1Δ8-*ppk*-Km in culture and in sterile soil was reversed when the *ppk-iiv8* deletion was reverted to wild type by allele replacement (strain Pf0-1ppkRev; see Materials and Methods), thus confirming the importance of the *ppk-iiv8* locus in competitive fitness (Fig. 1). As shown in Fig. 2, the *ppk* deletion also results in removal of the last 15 bases of the upstream gene, *hemB*. Experiments with a different *ppk*-null mutant (Pf0-1Δ*ppk*-full) confirmed that the *hemB* 3′ deletion was not responsible for the fitness defects (data not shown).

The *iiv8-ppk* locus produces overlapping transcripts. We tested whether production of both *iiv8* and *ppk* transcripts could be detected after growth in PMM, using strand-specific RT-PCR (data not shown; Table 2 shows primer sequences). Products were readily detected, indicating that genes are located on both strands of this single stretch of DNA. While *ppk* is well characterized in other organisms and generally well conserved, the *iiv8* gene is novel. Using 5′ and 3′ RACE, we found that the transcribed *iiv8* gene spans bases 6131885 to

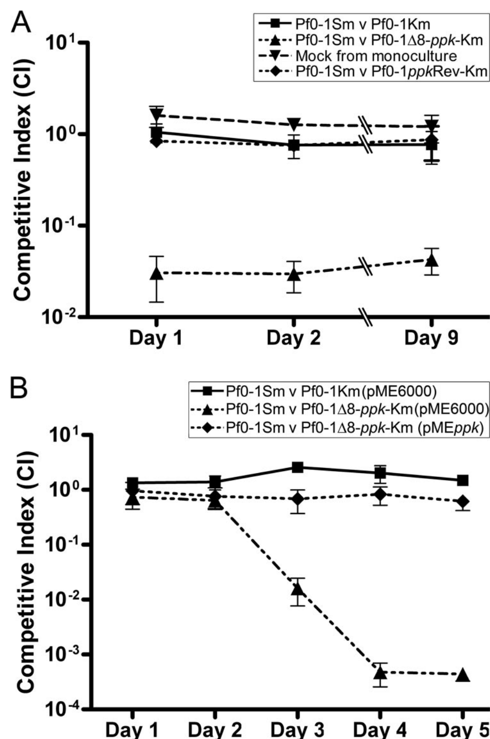

FIG. 1. Competition between Pf0-1 wild-type and *ppk* mutant strains. (A) Competition in sterile loam soil. Soil was inoculated with equal numbers of each competing bacterium. Bacteria were recovered from 0.5 g of soil 1, 2, and 9 days after inoculation and enumerated on media containing Sm or Km. A mock CI was derived from soil experiments in which Pf0-1 and the *ppk* mutant were grown separately, to show the absence of growth defects in monoculture (inverted triangles). The CI of Pf0-1Δ8-*ppk* was significantly different from those of Pf0-1, Pf0-1*ppk*Rev-Km, and the mock experiment ($P < 0.05$). Note that the *x* axis is not continuous. (B) Competition in LB culture. LB (15 ml) was inoculated with equal numbers of each competing strain. Bacteria were enumerated by diluting a 100-μl sample and plating it on medium containing Sm or Km. All strains carried the plasmid vector pME6000, except where the complementation plasmid is indicated. The CI of Pf0-1Δ8-*ppk* was significantly different from that of Pf0-1 and the complemented strain. For both panels, the CI was calculated by dividing the ratio of mutant to wild type on a particular day by the initial ratio. The *y* axis on both panels is log plotted. Means and standard errors of the means of at least three replicate experiments are shown. Note the different scales of the two panels.

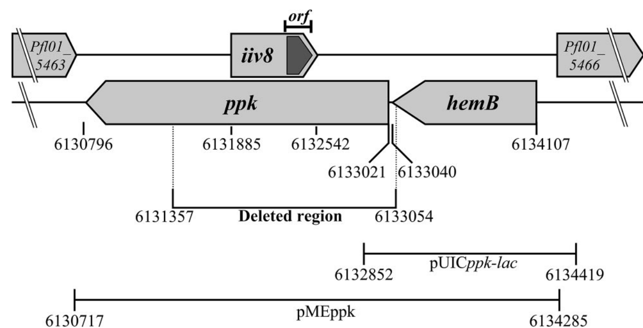

FIG. 2. Organization of the *ppk* and *iiv8* locus in Pf0-1. The *ppk* (Pfl01_5464), *hemB* (Pfl01_5465), and *iiv8* genes as well as the positions of flanking genes (Pfl01_5463 and Pfl01_5466) are shown. In the arrow representing *iiv8*, the embedded small dark arrow shows the longest open reading frame (*orf*) in the transcribed region. The region deleted in Pf0-1Δ8-*ppk* (bases 6131357 to 6133054) is indicated, as are the regions cloned in plasmids pUIC*ppk-lac* and pMEppk. Parallel diagonal lines through the Pfl01_5463 and Pfl01_5466 arrows indicate that the full length is not shown. Nucleotide positions in the Pf0-1 genome are shown for the genes, deleted region, and cloned regions.

6132542, producing a transcript of 658 bases (Fig. 2). It is unlikely that the transcript is translated into a protein since the longest open reading frame that starts and finishes within this region is only 180 bases long (bases 6132309 to 6132488 of the Pf0-1 genome). If translated, it would produce a small 59-amino-acid protein, and the mRNA would also have a 424-base 5′ untranslated sequence (Fig. 2). These data suggest that *iiv8*, the gene opposite *ppk*, probably specifies a noncoding RNA molecule, although the possibility that a small protein is also produced has not been excluded.

Loss of *ppk* is responsible for the competitive defect in laboratory culture. To distinguish between loss of *ppk* and loss of *iiv8* as the cause of the competitive defect shown by Pf0-1Δ8-*ppk*-Km, we carried out complementation experiments in LB culture, where the maintenance of complementing plasmids could be easily controlled and verified. Two different constructs were tested, one encoding both *iiv8* and *ppk* (pMEppk) and the other encoding just *iiv8* (pME8comp). Pf0-1Δ8-*ppk*-Km carrying each plasmid was competed against the Sm^r Pf0-1 carrying the parent plasmid pME6000. While carriage of pME8comp did not improve the fate of Pf0-1Δ8-*ppk*-Km (data not shown), possession of pMEppk resulted in a more-than-1,000-fold improvement relative to Pf0-1Δ8-*ppk*-Km (Fig. 1B). The plasmid pMEppk did not completely restore the competitive fitness of the mutant because of a growth defect associated with cells carrying pMEppk but did confer a significant improvement in the CI of Pf0-1Δ8-*ppk*-Km, relative to Pf0-1Δ8-*ppk*-Km carrying the plasmid vector (Fig. 1B). A truncated version of pMEppk which included the complete *hemB* sequence (pME-hemB) failed to complement the competitive fitness defect (data not shown). We conclude from these data that loss of *ppk*, not *iiv8*, in Pf0-1 results in reduced competitive fitness.

***ppk* is required for poly P accumulation.** To verify that poly P is produced by Ppk in Pf0-1 and is thus important for fitness, we measured accumulation of poly P by the wild type and Pf0-1Δ8-*ppk*. MOPS-L and MOPS-H minimal media were used for the initial poly P accumulation assays, since those media have limited (MOPS-L) and sufficient (MOPS-H) concentrations of P_i for derepression and repression of the Pho regulon, respectively. These media have previously been used for poly P studies (26, 31). When the strain was grown in MOPS-H, accumulation of poly P by Pf0-1 was easily measured, whereas poly P accumulation during growth in MOPS-L medium was close to the lower limit of detection (data not shown). In contrast, Pf0-1Δ8-*ppk* failed to accumulate detectable poly P in either medium. The poly P defect was reversed by replacing the region carrying the *ppk* deletion with a wild-type sequence, by allele exchange (Fig. 3A). The presence of plasmid pMEppk impaired growth of Pf0-1Δ8-*ppk* relative to strains carrying the plasmid vector alone (data not shown) but led to greater poly P accumulation than that observed in Pf0-1, probably because of expression of *ppk* from a multicopy plasmid (Fig. 3B). The parent plasmid pME6000 did not comple-

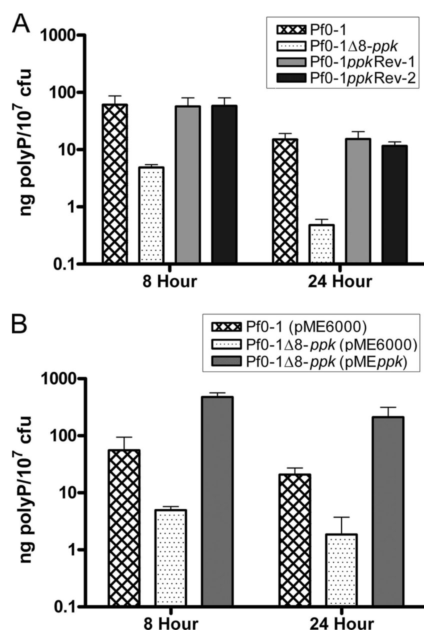

FIG. 3. Accumulation of poly P under high-P_i conditions. (A) poly P accumulation by Pf0-1, Pf0-1Δ8-*ppk*, and two independent isolates of Pf0-1Δ8-*ppk* reverted to wild type by allele replacement (strains Pf0-1*ppk*Rev-1 and Pf0-1*ppk*Rev-2) after 8 and 24 h of growth. Loss of *ppk* results in a greater-than-10-fold reduction in measurable poly P. Replacing the *ppk* deletion with wild-type sequence (Pf0-1*ppk*Rev strains) completely restores poly P accumulation. (B) Complementation of *ppk* by the plasmid pMEppk. Pf0-1 and Pf0-1Δ8-*ppk* carrying the plasmid vector pME6000 accumulate poly P to approximately the same level as do plasmid-free strains (A). Pf0-1Δ8-*ppk* carrying pMEppk accumulates almost 10 times more poly P than does Pf0-1. Plasmids used in the complementation experiments were maintained by including tetracycline in the medium. Means and standard errors of the means of at least two replicates are shown. Data are expressed as the quantity of poly P (in ng) per 10^7 CFU.

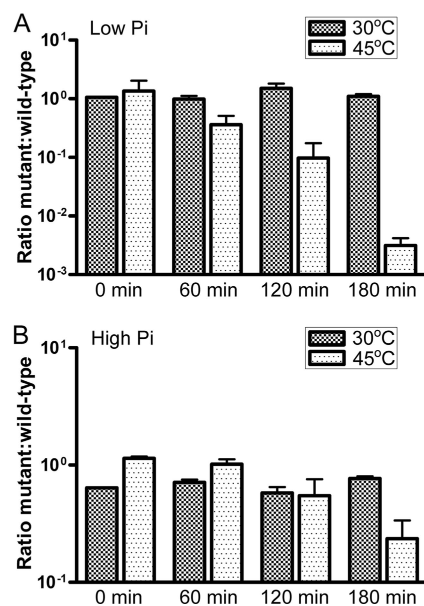

FIG. 4. Effect of temperature stress on survival of Pf0-1Δ8-*ppk* in high- and low-P_i laboratory media. (A) Pf0-1 and Pf0-1Δ8-*ppk* were grown as monocultures in MOPS-L at 30°C prior to cultures being exposed to 45°C for 180 min. Pairs of bars at each time point show the ratios of surviving Pf0-1Δ8-*ppk* to Pf0-1 for control cultures left at 30°C compared to those shifted to 45°C. The ratio of surviving Pf0-1Δ8-*ppk* to Pf0-1 at 45°C decreases over time, showing the effect of the *ppk* deletion on temperature sensitivity. In contrast, in control experiments that remained at 30°C, no such decline was seen. The differences between the heat exposure and control data are significant ($P < 0.05$) at 120 and 180 min after heat exposure began. (B) As described for panel A except that cultures were grown in the high-P_i MOPS-H medium. After 180 min, there was a significant difference between the control and heat exposure experiments ($P < 0.05$), although the magnitude of the difference is far smaller in the high-P_i medium than in the low-P_i medium (A). In both panels, data shown are the means and standard errors from at least three independent experiments. Note that the *y* axis scales are not the same in panels A and B.

ment the *ppk* mutation (Fig. 3B), and plasmid pME8comp bearing *iiv8* also failed to restore poly P accumulation to Pf0-1Δ8-*ppk* (data not shown), demonstrating that *ppk*, not *iiv8*, was required for poly P accumulation.

A role for poly P in stress tolerance of Pf0-1. The *ppk* mutant of Pf0-1 did not show reduced tolerance to osmotic, oxidative, or acid stress conditions in either MOPS-L or MOPS-H medium (data not shown). In MOPS-L medium, loss of *ppk* led to increased sensitivity to elevated temperature (Fig. 4A), an effect that was apparent but much less profound in the P_i-sufficient MOPS-H medium (Fig. 4B). Since temperature fluctuations are likely to be experienced in natural soil environments, we examined the effect of increased temperature on wild-type and *ppk* mutant Pf0-1 during monoculture growth in sterile soil (Fig. 5). As was seen in the culture-based experiments, the absence of *ppk* was associated with decreased tolerance to elevated temperature. After 1 day of exposure to the increased temperature, the population of Pf0-1Δ8-*ppk* was only ~10% of that of a Pf0-1 monoculture in soil. The Pf0-1 population had declined by 1 order of magnitude, compared with 2 orders of magnitude for Pf0-1Δ8-*ppk*. Although sustained exposure to 42°C caused a decline in both wild-type and mutant populations, this decline was more rapid in Pf0-1Δ8-*ppk*, implicating production of poly P in tolerance to heat stress.

Motility of *P. fluorescens* is not impaired in the *ppk* mutant. Motility and chemotaxis were found to be important for survival and spread of Pf0-1 in live soil (21). In *P. aeruginosa*, loss of poly P is associated with motility defects. We examined motility in Pf0-1Δ8-*ppk* as a potential explanation for the soil fitness defect. In contrast to *P. aeruginosa*, no motility difference was seen between Pf0-1 and Pf0-1Δ8-*ppk* on swimming agar containing 1% tryptone and 0.5% NaCl. These experiments, and those of Rashid and Kornberg (32), were carried out on the surface of rich medium in which the level of P_i was not determined but was likely to be high. Given the influence of P_i concentration on both accumulation of poly P and expression of the *ppk* gene (see below), the effect of P_i concentration on swimming motility of Pf0-1 was examined. On MOPS-H and MOPS-L swimming agar plates (0.3% agar), no substantial difference in motility was observed in comparison of wild-type and *ppk* mutant strains (data not shown).

***ppk* transcription is elevated under P_i-limiting conditions.** poly P production in *P. aeruginosa* is induced in P_i-limiting conditions (7). To examine the impact of external P_i on *ppk* expression in Pf0-1, we constructed a *ppk-lacZ* fusion in plasmid pUIC3 and integrated the resulting construct (pUIC*ppk*-

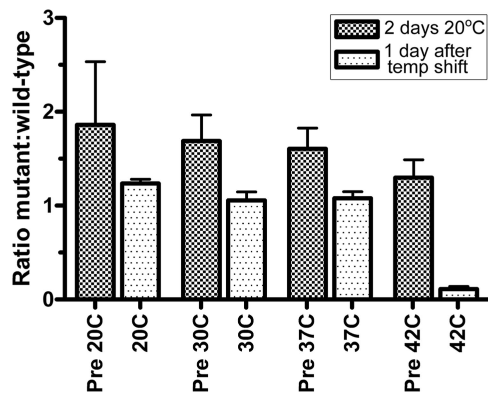

FIG. 5. Effect of temperature stress on survival of Pf0-1Δ8-*ppk* in sterile soil. Soil samples were inoculated with either Pf0-1 or Pf0-1Δ8-*ppk*. Bacteria were allowed to grow for 2 days at 20°C prior to a temperature shift. Data are shown as the ratio of surviving (CFU/0.5 g of soil) Pf0-1Δ8-*ppk* to Pf0-1. Bars indicate the ratios for experiments prior to temperature change (bars labeled "Pre") and after 1 day at the new temperature (20 [control], 30, 37, or 42°C). When the ratios before and after temperature shifts are compared, the effect of 42°C on Pf0-1Δ8-*ppk* is significant ($P < 0.05$), whereas no other temperature caused a significant decline relative to Pf0-1. Data shown are the means and standard errors from at least three independent experiments.

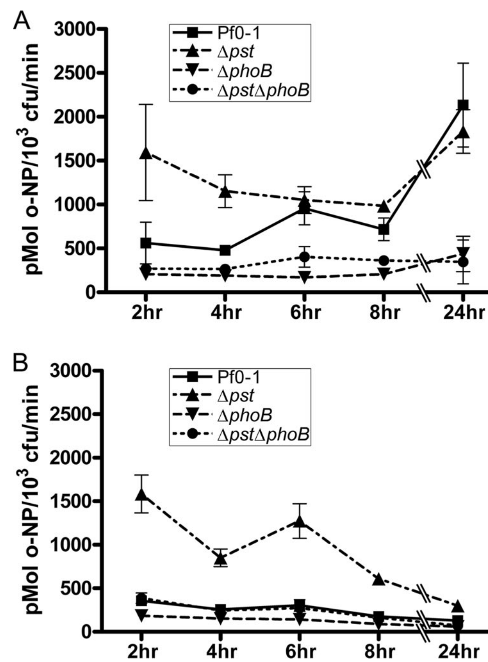

FIG. 6. Pho regulon control of transcription of a *ppk-lacZ* fusion in low- and high-P_i media. Expression of a *ppk-lacZ* transcriptional fusion was monitored over time in wild-type and Δ*pst*, Δ*phoB*, and Δ*pst* Δ*phoB* mutants of Pf0-1. (A) Under low-P_i conditions (0.14 mM), *ppk* is induced in the wild type and the Δ*pst* mutant but cannot be induced in strains lacking the Pho regulon regulator PhoB. (B) High-P_i conditions (2 mM) result in repression of *ppk* in Pf0-1, indicating Pho regulon control. Expression of *ppk* in the Δ*pst* mutant is higher than that in the wild type, indicating induction of Pho regulon genes by PhoB. Over time, *ppk* expression is reduced in the *pst* mutant, indicating additional regulation of *ppk*. In both panels, means and standard errors of the means of at least three replicates are shown. The *x* axes are not continuous. Data are presented as the pmol of *o*-nitrophenol released from *o*-nitrophenyl-β-D-galactopyranoside per 10^3 CFU, per min.

lac) into the native *ppk* locus in *P. fluorescens* Pf0-1. Transcription of *ppk* during growth in low- and high-P_i media was measured. During growth in high-P_i media, containing either high or limiting amino acid supplements, *ppk-lacZ* transcription remained reasonably constant at between 100 and 300 pmol *o*-nitrophenol/10^3 CFU/min over a 24-h period (Fig. 6B). In contrast, in low-P_i media, *ppk-lacZ* transcription began to increase by 4 h of growth, reaching around 2,000 pmol *o*-nitrophenol/10^3 CFU/min by 24 h (Fig. 6A). Thus, *ppk* is expressed at a relatively low level in high-P_i media, but this level is sufficient to allow accumulation of poly P in the cells (Fig. 3). In P_i-limiting media, *ppk* expression is elevated ~10-fold, suggesting positive regulation in response to low phosphate (Fig. 6).

***ppk* is part of the Pho regulon in *P. fluorescens* Pf0-1.** PhoB, the positive regulator of Pho regulon genes in P_i-limiting environments, is important for accumulation of poly P under certain stress conditions in *E. coli* (1) and for heterologous expression of *Klebsiella aerogenes ppk* in *E. coli* (13). The observations above show that *ppk* in Pf0-1 is regulated in response to P_i concentration, but definitive studies of regulation of *ppk* as part of the Pho regulon in *Pseudomonas* species are lacking. We sought to determine whether *ppk* was part of the Pho regulon in *P. fluorescens* as an initial step toward linking environmental sensing with fitness in soil, given the low concentration of phosphorus (10 ppm, wt/wt) in the soil used in this study (6).

We measured expression of the *ppk-lacZ* transcriptional fusion in *pst* and *phoB* deletion mutants of Pf0-1. Consistent with predictions, *ppk* transcription in Pf0-1Δ*pst* was generally equivalent to that seen in the wild type under phosphate-limiting conditions (Fig. 6A), and *ppk* transcription was elevated in the Δ*pst* strain during growth in high-P_i media (Fig. 6B). Transcription of *ppk* in the Δ*phoB* mutant remained at or below the level of that observed in the wild type under high-P_i medium conditions and was significantly lower in low-P_i conditions because of an inability to activate the Pho regulon. These data indicate that *ppk* is regulated by the Pho regulon system in Pf0-1. However, there are indications that *ppk* regulation is influenced by additional mechanisms. In high-P_i medium, *ppk* transcription in Pf0-1Δ*pst* is elevated during exponential growth but declines over time. By the 8-h time point, *ppk* transcription in Pf0-1Δ*pst* is less than threefold higher than in Pf0-1, and by 24 h, expression in Pf0-1Δ*pst* is only a little more than double that in Pf0-1. These data reveal an additional mechanism that suppresses *ppk* transcription in stationary-phase cells growing in high-P_i medium, which is not dependent on Pst.

Overproduction of poly P reduces competitive fitness in soil. Since the *ppk* deletion mutant showed reduced competitive fitness in soil, we hypothesized that strains capable of overproducing poly P might have a competitive advantage. Pf0-1Δ*pst*, which accumulates approximately sixfold more poly P than does Pf0-1 because of derepression of the Pho regulon, was competed against Pf0-1. Contrary to predictions, Pf0-1Δ*pst* had a severe competitive defect relative to Pf0-1. One possible explanation for this finding is that derepression of the Pho regulon results in other, unknown changes that impair fitness.

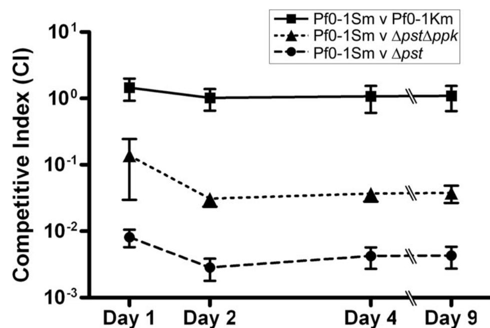

FIG. 7. Effect of deletion of *ppk* on Pf0-1Δ*pst* competition with Pf0-1 in sterile loam soil. The average CI of Pf0-1Δ*pst* was approximately 0.008, 0.003, 0.004, and 0.004 on days 1, 2, 4, and 9, respectively. When Δ*ppk* was introduced into Pf0-1Δ*pst*, the CI of the resulting double mutant improved significantly, by approximately 10-fold at each time point ($P < 0.05$). Means and standard errors of the means of at least six replicates are shown. Note that the *x* axis is not continuous.

Alternatively, producing too much poly P could be deleterious. The CI of Pf0-1Δ*pst* against Pf0-1 in soil was 10-fold lower than that of Pf0-1Δ8-*ppk*-Km (Fig. 7 and 1A). We predicted that if the latter possibility were true, deletion of *ppk* in Pf0-1Δ*pst* would improve the competitive fitness to approximately the same level as that of Pf0-1Δ8-*ppk*-Km, whereas if the former were true, loss of *ppk* would have minimal impact. The prediction was tested by introducing the *ppk* mutation used to create Pf0-1Δ8-*ppk*-Km into Pf0-1Δ*pst* (resulting in Pf0-1Δ*pst*Δ*ppk*) and carrying out soil competition experiments against Pf0-1. We observed a 10-fold improvement in the CI of Pf0-1Δ*pst*Δ*ppk* compared to that of Pf0-1Δ*pst* (Fig. 7), indicating that an environmentally responsive control of poly P synthesis is important for competitive fitness in soil.

DISCUSSION

Our data show the importance of poly P for competitive fitness of *P. fluorescens* in a soil environment, extending findings from others that poly P is important for survival of *Salmonella* bacteria, *Shigella* species, and *E. coli* in laboratory media (15, 30). The partial relief of the soil fitness defect of Pf0-1Δ*pst* by deletion of *ppk* strongly suggests that, even in the low-P_i soil environment used here, proper Pst-dependent regulation of *ppk* is necessary for fitness. The requirement for proper regulation of *ppk* in soil may reflect a nonuniform chemical composition of a particular soil type. The phosphate in our soil may be unequally distributed, resulting in a variety of niches with different amounts of phosphate available. The competition in soil requires dynamic adaptation to these different niches, and loss of the ability to regulate *ppk* diminishes the adaptive ability, leading to the observed competitive defect.

These experiments, putting mutants in competition with otherwise isogenic wild-type bacteria, were performed in sterile soil. This simple system is useful for revealing competitive defects, but its lack of complexity presumably leads to an underestimation of the severity of defects in soil survival. Live soil is likely to be a more difficult competitive environment because of the large number of different organisms present and their metabolic products and ecologic niches. In such a situation, we predict that the requirement for fitness genes would be even more pronounced than that seen in sterile soils. Furthermore, in natural environments there are fluctuations in physical parameters such as temperature. Besides a competitive defect associated with loss of *ppk*, there is the loss of tolerance to elevated temperature. Thus, in a natural setting, *ppk* is likely to play a dual role in survival: enabling adaptation to transient temperature increases and promoting competitive fitness.

The experiments on transcription of *ppk* in low- and high-P_i environments demonstrate that *ppk* is regulated in response to exogenous P_i, through PhoB, consistent with its being a member of the Pho regulon (25). Loss of *pst* leads to elevated expression of *ppk* in both high- and low-P_i environments, while loss of *phoB* results in an inability to induce *ppk* transcription. These assays point to additional mechanisms of regulation, since after 24 h in a high-P_i environment, *ppk* transcription in the Δ*pst* mutant decreases, inconsistent with the presumed constitutive derepression expected in Δ*pst* strains. Ghorbel et al. (9) suggested that in *Streptomyces lividans* there might be an additional level of regulation of *ppk*, since they could detect a small amount of Ppk in old cultures of a PhoP mutant, supporting our suggestion that phosphate regulation is only part of the regulatory picture. This additional complexity may be part of the explanation for the requirement for proper regulation of *ppk* discussed above.

One potential mechanism for regulation of *ppk* in Pf0-1 is via an antisense RNA. The *ppk* locus was isolated by virtue of the increased expression of the noncoding antisense gene *iiv8* during growth in soil. While no role for *iiv8* has yet been elucidated, we suggest that, by hybridizing with the *ppk* transcript, *iiv8* might affect the stability of the message, thus exerting a regulatory influence. If *ppk* is downregulated by *iiv8*, one might expect reduced competitive fitness, but such a situation would not be beneficial in natural environments. We propose that any antisense regulation of *ppk* would act as a secondary mechanism, in addition to that being controlled as part of the Pho regulon, to reduce poly P production in regions of soil where overproduction would be too costly. This possibility is being studied.

The role played by poly P in cell physiology is not clear and has raised much speculation. It has been suggested that the high-energy phosphoanhydride bonds linking the phosphates provide an energy store and that poly P is a reserve phosphate supply. The importance of poly P in nutrient-limiting conditions such as soil and long-term broth culture may reflect a need to utilize a phosphate store for normal cellular processes and as an alternative to ATP for energy. Recently, poly P was shown to be the source of phosphate for phosphorylation of the stress response regulator MprA in *Mycobacterium smegmatis* (43). It may be that the analogous stress response pathways in Pf0-1 depend on poly P in a similar manner, adding an additional possible explanation for the effect of *ppk* deletion on competitive fitness. poly P has been linked to induction of *rpoS* expression in *E. coli* (38) and accumulation of *rpoS* transcripts in *Pseudomonas chlororaphis* (14). It has been suggested that the absence of poly P-mediated control of *rpoS* in *ppk* mutants contributes to elevated sensitivities to stress conditions (15, 38). However, in *V. cholerae*, loss of *ppk* did not lead to reduced expression of RpoS, but the *ppk* mutant exhibited defects in tolerance to acid, osmotic, and oxidative stress when grown in low-P_i medium (12). Thus, reduced *rpoS* expression

does not fully explain the defects associated with loss of poly P. As in *V. cholerae*, it has been shown that *rpoS* expression is not dependent on *ppk* in *P. aeruginosa* (2), and some preliminary evidence shows that *rpoS* transcription in Pf0-1 is not dependent on poly P, arguing against the suggestion that reduced *rpoS* expression explains poly P-associated phenotypes.

A large body of research points to the importance of poly P, and the range of phenotypes associated with *ppk* mutations is broad. However, poly P-deficient mutants of different species do not consistently present the same phenotypes and typically have defects in one or more of those outlined above. In the related bacterium *Pseudomonas aeruginosa*, poly P is required for swimming and swarming motility (32). In at least six other bacterial species, including bacteria as dissimilar as *Vibrio cholerae*, *Bacillus cereus*, and *Myxococcus xanthus*, motility is defective in poly P mutants (27, 33, 37, 48). It should be noted that a *ppk* mutant of a *V. cholerae* strain different than that used by Ogawa et al. (27) showed no motility defect (12).

There is no obvious way to predict which phenotypes will be influenced by poly P. Indeed, in *P. aeruginosa*, besides defects in motility, loss of poly P has been associated with reduced biofilm formation (32, 34) and susceptibility to carbenicillin (7). These phenotypes are not associated with the Pf0-1 *ppk* mutant (unpublished data), and sensitivity to heat stress and reduced competitive fitness phenotypes have not been reported for *ppk* mutants of *P. aeruginosa*. Thus, even between related bacteria the importance of poly P varies.

Our study of *ppk* in Pf0-1 has added new insight into the spectrum of phenotypes associated with poly P. In Pf0-1, poly P is important for competitive fitness in a nonlaboratory environment (sterile loam soil). The loss of poly P production by Pf0-1 results in a narrow spectrum of phenotypic changes (Fig. 1, 4, and 5), which is in contrast to the impact of *ppk* mutations in several other bacteria including *P. aeruginosa* (7, 32, 34), *V. cholerae* (12, 27), and *E. coli* (30). While expression of *ppk* is phosphate regulated and *ppk* is a member of the Pho regulon, it appears that there are additional factors involved in regulation of *ppk*. Finally, we present evidence that a mutant that overproduces poly P (Pf0-1Δ*pst*) was more compromised in terms of competitive fitness in soil than was an isogenic *ppk* deletion mutant (Pf0-1Δ*pst*Δ*ppk*) which fails to produce poly P. The ability to respond appropriately to environmental signals which regulate poly P production and/or expression of poly P-regulated genes is critical for soil survival of *P. fluorescens* Pf0-1.

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