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Carbon Catabolite Repression and Impranil Polyurethane Degradation in *Pseudomonas protegens* Strain Pf-5

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ABSTRACT

Polyester polyurethane (PU) coatings are widely used to help protect underlying structural surfaces but are susceptible to biological degradation. PUs are susceptible to degradation by *Pseudomonas* species, due in part to the degradative activity of secreted hydrolytic enzymes. Microorganisms often respond to environmental cues by secreting enzymes or secondary metabolites to benefit their survival. This study investigated the impact of exposing several *Pseudomonas* strains to select carbon sources on the degradation of the colloidal polyester polyurethane Impranil DLN (Impranil). The prototypic *Pseudomonas protegens* strain Pf-5 exhibited Impranil-degrading activities when grown in sodium citrate but not in glucose-containing medium. Glucose also inhibited the induction of Impranil-degrading activity by citrate-fed Pf-5 in a dose-dependent manner. Biochemical and mutational analyses identified two extracellular lipases present in the Pf-5 culture supernatant (PueA and PueB) that were involved in degradation of Impranil. Deletion of the *pueA* gene reduced Impranil-clearing activities, while *pueB* deletion exhibited little effect. Removal of both genes was necessary to stop degradation of the polyurethane. Bioinformatic analysis showed that putative Cbr/Hfq/Crc-mediated regulatory elements were present in the intergenic sequences upstream of both *pueA* and *pueB* genes. Our results confirmed that both PueA and PueB extracellular enzymes act in concert to degrade Impranil. Furthermore, our data showed that carbon sources in the growth medium directly affected the levels of Impranil-degrading activity but that carbon source effects varied among *Pseudomonas* strains. This study uncovered an intricate and complicated regulation of *P. protegens* PU degradation activity controlled by carbon catabolite repression.

IMPORTANCE

Polyurethane (PU) coatings are commonly used to protect metals from corrosion. Microbiologically induced PU degradation might pose a substantial problem for the integrity of these coatings. Microorganisms from diverse genera, including pseudomonads, possess the ability to degrade PUs via various means. This work identified two extracellular lipases, PueA and PueB, secreted by *P. protegens* strain Pf-5, to be responsible for the degradation of a colloidal polyester PU, Impranil. This study also revealed that the expression of the degradative activity by strain Pf-5 is controlled by glucose carbon catabolite repression. Furthermore, this study showed that the Impranil-degrading activity of many other *Pseudomonas* strains could be influenced by different carbon sources. This work shed light on the carbon source regulation of PU degradation activity among pseudomonads and identified the polyurethane lipases in *P. protegens*.

Microbiologically induced deterioration of polyurethane (PU) and other military-relevant coatings has been recognized as a critical sustainability and sustainment issue for several decades (reviewed in references 1 to 3). A wide variety of fungi and Gram-positive and Gram-negative bacteria have been shown to secrete enzymes capable of degrading PUs. Among bacteria, some of the most well-studied PU-degrading organisms are pseudomonads—Gram-negative, heterotrophic bacteria (2, 4–8). Several *Pseudomonas* species including *Pseudomonas chlororaphis*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* have been shown to degrade a model polyester PU, Impranil DLN (Impranil) (4–6). Three PU-degrading enzymes have been purified and characterized from some of these strains, including polyurethane esterases A (PueA) and B (PueB) from *Pseudomonas chlororaphis* (5, 9) and polyurethane lipase (PulA) from *P. fluorescens* (5, 9–11). Although they are often referred to as "polyurethanases," these enzymes are more accurately classified as extracellular lipases and esterases.

Enzyme secretion in bacteria is often dictated by environmental conditions. It has been observed that the expression of the extracellular proteome of bacteria can be affected by nutrient conditions as well as by growth phases (12–19). However, very little is known about how enzymes that effect polymer degradation are physiologically regulated in pseudomonads. Several studies have proposed that polyurethanase expression is constitutive (11),

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Bacterial strains		
P. aeruginosa PAO1	Wild type	53
P. fluorescens A506	Wild type	54
P. fluorescens SBW25	Wild type	55
P. fluorescens 204	Wild type (ATCC 17571)	56
P. fluorescens Pf0-1	Wild type	57
P. protegens pv. fluorescens Pf-5	Wild type	27
P. protegens CHA0	Wild type	58
P. protegens pv. chlororaphis BC2-12	Mutant of wild-type BC2; enhanced polyurethane degrading activity (ATCC 55729)	29
P. putida KT2440	Wild type	59
E. coli S17-1/λpir	K-12 strain; used for conjugative transfer of plasmids into Pf-5	60
Plasmid		
pMQ30	Allelic replacement vector	33

while others have reported that PU deterioration by both Gram-positive and Gram-negative bacteria is affected by the presence or absence of organic nutrients in the form of yeast extract (6, 20, 21). The biotechnology industry has investigated regulatory elements for PU-degrading enzymes in pseudomonads to optimize lipase production (reviewed in reference 22). For example, Makhzoum et al. published an extensive study on the factors affecting growth and extracellular lipase production by *P. fluorescens* 2D, a psychrotrophic bacterium that causes milk spoilage (18). Growth and lipase production in this strain were stimulated by organic acid carbon sources such as pyruvate. On the other hand, glucose (at 0.5%) completely inhibited lipase production, while supporting more growth than any other mono- or disaccharides. This was also true for *Pseudomonas fragi* cultures grown in ammonium sulfate-glucose medium formulations (23). In contrast, addition of glucose stimulated lipase activity in *P. aeruginosa* cultures (24).

In addition to saccharides and organic acids, iron(III) and other transition metals, triglycerides, fatty acids, growth phase, and the presence of the siderophore pyoverdin all have been reported to affect lipase production in *P. fluorescens* (18, 25, 26). Organic solvents and detergents, such as hexadecane and Tween 80, respectively, in low concentrations also have been shown to enhance lipase secretion in *Burkholderia glumae* (13). Collectively, these reports suggest that multiple factors and routes of extracellular lipase regulation, ranging from transcription to secretion, exist among different microorganisms.

The goal of our research was 2-fold: to understand how regulation of PU degradation occurs at the molecular level in *Pseudomonas protegens* Pf-5 and then to determine if a common regulatory mechanism of PU degradation exists among pseudomonads. We characterized the role of carbon sources and carbon catabolite repression (CCR) in the well-characterized *P. protegens* strain Pf-5 (27, 28), by identifying its two key PU-degrading enzymes, polyurethane esterases A (PueA) and B (PueB) and examining the phenotypic effects of mutations in these genes. We further determined how carbon sources influenced polyurethane degradation in *pueA* and *pueB* knockout strains. Lastly, we demonstrated the impact of medium conditions such as carbon sources and their concentrations on polyurethane degradation by various *Pseudomonas* strains.

MATERIALS AND METHODS

Bacterial strains and materials. The strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were cultured in lysogeny broth-Miller (LB) or minimal medium M9 at 27°C with agitation at 200 rpm. *Escherichia coli* strain S17-1/λpir was cultured in LB at 37°C. Media were supplemented with carbon sources and the PU dispersion agent Impranil DLN (Impranil) (Bayer Materials Science, PA) as indicated. If not specifically indicated, M9 was supplemented with 20 mM sodium citrate (M9-citrate) or 20 mM glucose (M9-glucose). To prepare agar plates, M9 and LB media were solidified with 1.5% agar. *p*-Nitrophenyl palmitate (4-NPP) was purchased from Sigma-Aldrich, MO. Sequences of DNA primers used to create and verify Pf-5 deletion mutants (Integrated DNA Technologies, Inc., IA) are listed in Table 2.

Polyurethane degradation assays. To test the PU-degrading activity of cultures in agar plate assays, cells from 1.0-ml aliquots of overnight cultures grown in LB were pelleted by centrifugation, washed, and resuspended in 1.0 ml of 50 mM sodium potassium phosphate buffer (pH 7.4). Twenty microliters of the resultant cell suspension were placed onto M9 agar plates containing 3 g/liter Impranil and carbon sources as indicated. Plates were incubated for 48 h at 27°C. Zones of clearing around areas of bacterial growth indicated Impranil degradation.

A liquid-based assay using an Impranil dispersion was developed to quantitatively measure PU-degrading activity in cell-free supernatants. Culture samples were collected periodically during 14 h of growth. For each time point, cultures were centrifuged for 10 min at 12,800 × *g*, and supernatants were filter sterilized immediately using a 0.1-μm polyvinylidene difluoride (PVDF) filter (Millipore). Cell-free filtrates were stored at 4°C until further use. Similar to the assay described in reference 29, 20 μl of a 40 g/liter Impranil stock solution was added to 980 μl of the filtered supernatant samples. Clearing of the Impranil dispersion was monitored at 600 nm with a spectrophotometer (SpectraMax M3; Molecular Devices, CA). Impranil clearing rates, an indication of PU degradation, during exponential and early-stationary growth phases of the cultures were calculated over four time points within the first 45 min of the experiment.

Endpoint assays of Impranil clearing by late-stationary-phase cultures (22 to 24 h growth) were set up as described above without filtration. Clearing of the Impranil dispersion was measured as above periodically over a 24-hour period. Impranil clearing appeared to have approached maximum by 3 h postincubation (data not shown). The decrease in optical density at 600 nm (OD_{600}) after a 3-h incubation was used to calculate the levels of Impranil clearing as an indication of Impranil degradation. The OD of some samples, particularly those of supernatants of M9-glucose cultures, increased over time. These samples did not show any Impranil degradation based on ^{1}H nuclear magnetic

TABLE 2 Sequences of primers used to generate *pueA* and *pueB* deletion mutants

Primer name	Primer sequence (5′ to 3′)[a]
Allelic replacement primers	
Pf5pueALfor	tcgactgagcctttcgttttatttgatgcctggcagttccGAAGTCACTGAACGCAAGCG
Pf5pueALrev	ACAACAGAAGAGGCAATACCTGCCGGCGCGGCGCGAGGCC
Pf5pueARfor	GGCCTCGCGCCGCGCCGGCAGGTATTGCCTCTTCTGTTGT
Pf5pueARrev	ggaattgtgagcggataacaatttcacacaggaaacagctAAGGCGCCTGGTTCAAGGTC
Pf5pueBLfor	tcgactgagcctttcgttttatttgatgcctggcagttccGTTGTTCTTGTCCAGGCCCG
Pf5pueBLrev	AATAAAAAAGAGGAATGAGCCCCTCACCGATATCCAGCGC
Pf5pueBRfor	GCGCTGGATATCGGTGAGGGGCTCATTCCTCTTTTTTATT
Pf5pueBRrev	ggaattgtgagcggataacaatttcacacaggaaacagctGAGTTGCTGGTGCGGGTGCT
Knockout mutant verification primers	
Pf5pueATestF	AATTCGTTACGCACCTGCT
Pf5pueATestR	CTTCGCTAACCTGGCTTATGT
Pf5pueBTestF	CGTAGACCTGGGCATTGAAG
Pf5pueBTestR	CGGGAAACCCTGATTGAACT
pueAF	CGCTGGCGATCACCCTGTACTCC
pueAR	CTGGGAATTGCCGCTGCCGATCAG
pueBF	GCACCCACGACAAGCCCCAGG
puber	GTAGTCCACCGCACTATGGGCCG
rpoDF	GCCAACCTGCGTCTGGTGATCTCC
rpoDR	GCCGCGACGGTATTCGAACTTGTCC

[a] Uppercase letters correspond to sequences in the Pf-5 genome; lowercase letters correspond to sequences in the cloning vector pMQ30.

resonance (NMR) studies (as described in Results); therefore, the change in OD was set to 0.

Chemical characterization of Impranil degradation by NMR spectroscopy. Although Impranil clearing appeared to have approached a maximum by 3 h postincubation, the clearing reaction was carried out to 24 h before ^{1}H NMR analysis was performed (30). At the end of the incubation period, Impranil-containing samples and control samples that did not contain Impranil were centrifuged in microcentrifuge tubes at 14,000 rpm for 30 min. Clarified supernatants were transferred to new microcentrifuge tubes and stored at −80°C until analysis. Samples for NMR measurements of hydrolysis products were prepared using 490 μl of sample and 10 μl of a 0.28 M 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt aqueous solution as an internal standard (30). A sealed capillary filled with D_2O was added for NMR lock. The concentrations of the soluble hydrolysis products from Impranil were calculated using the average integrated area of two different ^{1}H NMR signals resulting from a single experiment. Each experiment was performed in biological triplicates, and the concentration was calculated using the average concentrations from all three replicates. The first NMR signal (if not obscured by signals from metabolic products) used to calculate the concentration was the background-subtracted triplet at δH 3.5 ppm, and the second signal was the collective area of the 3 singlets between δH 1.0 and 0.50 ppm. The average integrated area of the peak at δH 3.5 ppm (and supported by the area from the signals between δH 1.0 to 0.5 ppm) was standardized to the signal for the internal standard (δH of −0.25 ppm), which had a known concentration. The use of two different signals from the same NMR spectrum allowed redundancy in the calculation of the concentration of soluble hydrolysis products.

HPLC analysis of glucose and citrate consumption by liquid-phase cultures. Aliquots of cell-free culture supernatants were analyzed using high-performance liquid chromatography (HPLC) for the amount of carbon source consumed during the growth of *P. protegens* Pf-5 in M9 medium. A Varian Prostar HPLC system equipped with a refractive index (RI) detector and diode array detector (DaD) was used for each sample. The flow rate was 0.2 ml/min at 40°C using water as the mobile phase. The chromatography column was a conditioned 8 μm (300 × 7.7 mm) Agilent PL Hi-Plex column (H+). The concentrations of citrate and glucose were determined using external standards monitored at 213 nm with the DaD detector and with the RI detector, respectively.

General lipase activity assay. Extracellular lipase activity was assessed using 4-NPP as the substrate. Bacteria were grown in minimal medium M9 supplemented with 20 mM succinate at 27°C with agitation for 22 h. Cells were pelleted at 12,000 × *g* for 30 min at 4°C to obtain clarified supernatants. For the assay, 495 μl of clarified supernatant was mixed with 5 μl of 25 mM 4-NPP (dissolved in dimethyl formamide [DMF]). The reactions were carried out at room temperature and monitored at 405 nm over 30 min. The rate of 4-NPP hydrolysis was calculated based on the extinction coefficient of 1.78×10^4 M^{-1} cm^{-1} (31).

Protein assays. Protein concentrations were determined with a Bradford-based assay (Bio-Rad, CA) using the microassay procedure for microtiter plates. A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA) was employed to measure protein concentrations in concentrated culture supernatants. Pierce bovine serum albumin standards (Thermo Fisher Scientific) were used to generate standard curves for determination of protein concentrations in samples.

PAGE and zymogram analysis. To identify the proteins responsible for PU biodeterioration in Pf-5, a combination of nondenaturing (i.e., native) PAGE, zymogram analysis, and standard SDS-PAGE was conducted. Cell-free supernatants from overnight cultures grown in M9 medium containing 20 mM citrate were found to contain PU-degrading activity. An overnight culture (16 ml) was centrifuged (10 min at 12,800 × *g*), and the resultant supernatant was filtered through a 0.1-μm PVDF filter. The filtrate was supplemented with 2 mM dithiothreitol (DTT) and concentrated approximately 130-fold in an Amicon Centricon concentrator with a molecular mass cutoff of 50 kDa that had been passivated with 5% Triton X-100. Concentrates were washed with 8 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM DTT and 0.1% Triton X-100. Samples were subjected to native PAGE using a 3 to 12% NativePAGE Novex bis-Tris gel according to the manufacturer's instructions (Invitrogen). The resulting protein gels were stained with colloidal blue or used for zymograms; proteins from the NativePAGE gel slices were also extracted for further analysis by standard denaturing SDS-PAGE.

For zymograms, a native protein gel containing samples of interest was washed with 50 mM Tris-HCl buffer (pH 8.0) for 15 min and subsequently placed on top of the zymogram gel (50 mM Tris-HCl [pH 8.0], 0.7% agarose, 1.5 g/liter Impranil, 0.1% Triton X-100, and 0.1 mM

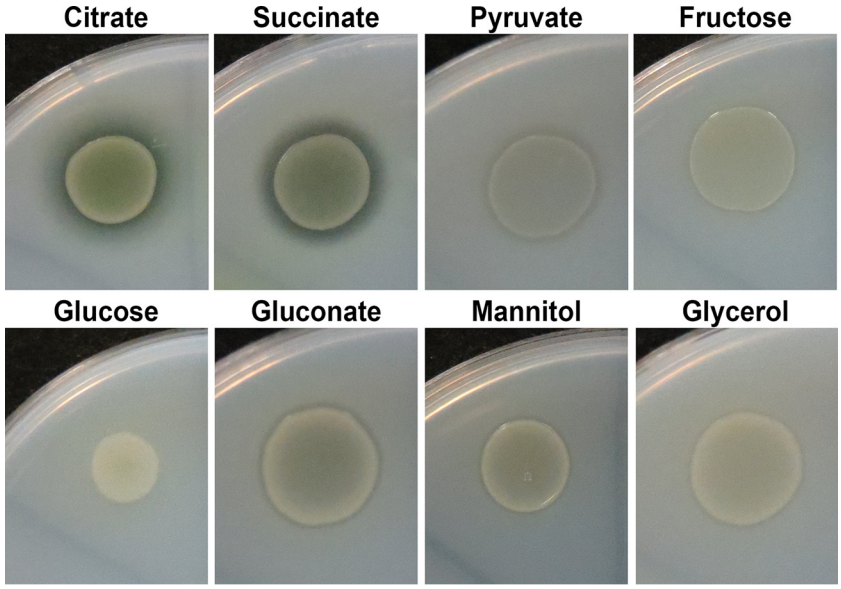

FIG 1 Effects of carbon sources on polyurethane degradation by *P. protegens* strain Pf-5. *P. protegens* strain Pf-5 was grown on M9 agar plates supplemented with Impranil and different carbon sources for 48 h. Degradation of Impranil resulted in visible zones of clearing.

$CaCl_2$). The zymogram was incubated overnight at room temperature and analyzed for clearing the following day.

To resolve proteins within the native protein gel that corresponded to zones of clearing in zymogram analysis, gel slices were excised and incubated in 100 μl of 50 mM Tris-HCl (pH 8.0) containing 2 mM DTT and 0.1% Triton X-100 overnight to elute proteins in these regions. The resulting eluates were concentrated by overnight precipitation with ice-cold acetone (80% final concentration) at −20°C. Precipitated proteins were resuspended in 25 μl of LDS sample buffer containing reducing agent (Invitrogen). SDS-PAGE was performed using a 4 to 12% bis-Tris gel and morpholineethanesulfonic acid (MES) buffer according to the manufacturer's instructions (Invitrogen). After electrophoresis, the gel was stained using colloidal blue (Invitrogen).

Protein identification. Identification of proteins in the SDS-PAGE gel bands was performed by the University of Cincinnati Proteomics Laboratory according to the protocol described elsewhere (32). In brief, selected protein bands were excised from an SDS-PAGE gel and subjected to tryptic digests. The resulting peptides were analyzed using matrix-assisted laser desorption ionization–time of flight mass spectrometry and tandem mass spectrometry (MALDI-TOF-TOF MS/MS) for sequence determination. Protein identification was performed using the MASCOT search algorithm (Matrix Science, Boston, MA).

Bioinformatic analysis. Protein sequences were obtained from the protein database at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Homologous proteins were identified using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (PSI-BLAST) (blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequence alignments and percent identity determinations were performed using EMBOSS Stretcher software (EMBL-EBI) (www.ebi.ac.uk).

Creation of genetic knockout mutants. In-frame deletions of *pueA* and *pueB* genes were created according to published protocols with minor modification (33). Briefly, 1,000 bp of both up- and downstream regions of either *pueA* or *pueB* were PCR amplified. The 5′ region of the upstream PCR product and the 3′ region of the downstream PCR product contained sequences overlapping portions of the cloning vector pMQ30 into which they would be inserted. In addition, the 3′ region of the upstream PCR product and the 5′ region of the downstream PCR product contained sequences overlapping each other. The PCR products were cloned into linearized pMQ30 via homologous recombination with the aid of a Gibson assembly kit (New England BioLabs, MA). The resulting allelic replacement constructs (ARCs) were introduced into Pf-5 by conjugation through *E. coli* strain S17-1/λpir. The exoconjugants were selected in the presence of 30 μg/ml gentamicin (to select for Pf-5 merodiploids) and 20 μg/ml nalidixic acid (to eliminate S17-1/λpir). Subsequently, the Pf-5 merodiploids were grown in the presence of 50 mg/ml sucrose to force the removal of integrated ARC, which in turn resulted in deletion of the *pueA* or *pueB* gene and the gentamicin-resistant cassette. To create the *pueAB* double-knockout mutant, the *pueB* single-knockout mutant was subjected to another round of deletion protocol to remove the *pueA* gene from the chromosome.

RESULTS

Induction and repression of polyurethane-degrading activity of *P. protegens* strain Pf-5. We previously reported that a zone of clearing around the *P. protegens* strain Pf-5 colony was visible when this strain was grown on minimal medium M9 agar supplemented with 10 mM citrate and Impranil, an indication of polyurethane hydrolysis (34). We further investigated the impact of carbon sources on polymer degradation by Pf-5 using the Impranil agar plate-clearing assays (Fig. 1). Impranil clearing was mostly promoted when Pf-5 was grown on a minimal medium M9 agar plate in the presence of 20 mM citrate or succinate, while little to no clearing was observed when 20 mM glucose was supplied. Supplementation with other carbon sources resulted in an intermediate phenotype. To further define the effects of carbon sources that might be involved in pathways for the degradation of polyester polyurethanes, we focused on citrate and glucose due to their clear relationship with degradation. We first examined the effects of carbon source concentrations on Impranil clearing. In M9-Impranil agar plate assays with various concentrations of citrate, Impranil clearing was observed at all citrate concentrations with the greatest degree of clearing observed on plates containing 20 mM or 40 mM citrate (see Fig. S1A in the supplemental material). In contrast, no zones of clearing were observed when Pf-5 was grown on plates containing 20 mM or

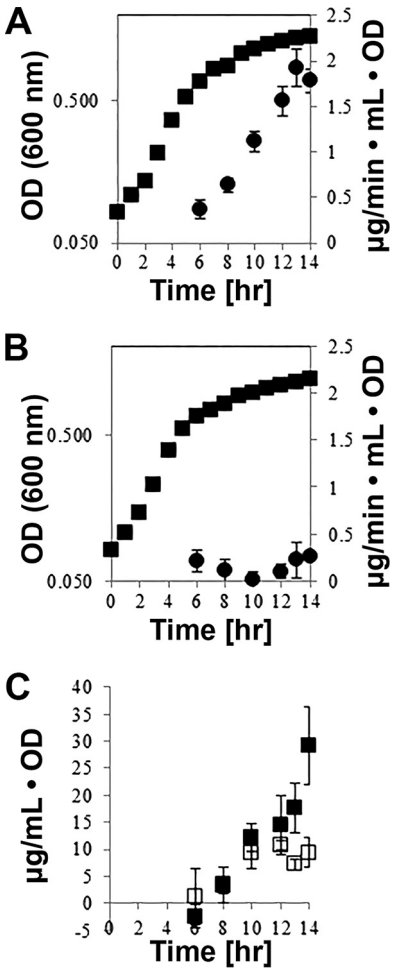

FIG 2 Relationship between growth state, protein secretion, and Impranil-clearing activities. (A and B) Growth (■) and Impranil-clearing activity (●) in cultures of *P. protegens* strain Pf-5 grown in M9 medium containing 20 mM citrate (A) or 20 mM glucose (B) over 14 h. Cell-free supernatants from culture samples were assayed for Impranil clearing as a measure of PU degradation. PU degradation rates were calculated over four time points within the first 45 min of the Impranil-clearing assay. Cultures were grown in triplicate, and individual supernatant samples were assayed twice. (C) Protein concentrations in supernatant samples from cultures of strain Pf-5 grown in M9 medium with 20 mM citrate (■) or 20 mM glucose (□) normalized to the OD of the cultures.

higher glucose levels (see Fig. S1B in the supplemental material). Insignificant levels of Impranil clearing were visible from Pf-5 cultured on M9-Impranil plates containing 5 mM or 10 mM glucose after 48 h of incubation.

It is possible that the effects of carbon sources on Pf-5 Impranil-clearing activity might be due to differences in the level of growth when Pf-5 is cultured on different carbon sources on agar plates. To address this possibility, we monitored the growth and polyurethane-degrading activity of strain Pf-5 in planktonic cultures containing either 20 mM citrate (M9-citrate) or 20 mM glucose (M9-glucose) as a carbon source (Fig. 2). Strain Pf-5 grew at similar rates in citrate ($0.532 \pm 0.056\ h^{-1}$) and glucose ($0.521 \pm 0.006\ h^{-1}$) and reached comparable optical densities (Fig. 2A and B). A liquid-based assay was used to determine the Impranil-clearing activity of cell-free culture supernatants using Impranil colloid suspensions (Fig. 2A and B). The Impranil-clearing activity started to increase at the end of the exponential growth and reached higher levels in supernatants from citrate-grown cultures than in those from glucose-grown cultures. Supernatants of citrate-grown cultures also contained higher protein concentrations than glucose cultures. Elevated extracellular protein concentrations correlated with the increase of Impranil-clearing activity as the culture transitioned into stationary phase (Fig. 2C).

Both agar plate- and liquid Impranil-clearing assays are rapid and straightforward methods to determine if an organism can affect the light-diffractive properties of the Impranil colloids. To confirm that clearing of Impranil suspensions equates to hydrolysis of polyurethane polymers, we used ^{1}H NMR to detect the presence of Impranil hydrolysis products in Impranil-clearing assay samples (30). The Impranil-degrading activities of late-stationary-phase (22 h) cell-free culture supernatants were examined. Supernatants of Pf-5 grown in M9-citrate contained substantial Impranil-clearing activity ($0.131 \pm 0.005\ OD_{600}$ change/OD culture), while those from M9-glucose medium did not exhibit any activity. Similar to clearing results, Pf-5 M9-citrate but not M9-glucose culture supernatants produced detectable hydrolysis products (0.52 ± 0.04 mM) at the end of the Impranil-clearing assays (see Fig. S2 in the supplemental material). These results confirm that the disappearance of Impranil colloids was the consequence of PU polymer hydrolysis.

We suspected that the preferential metabolism of one carbon source over another might affect the amount of Impranil degradation by Pf-5. Therefore, the concentrations of glucose and citrate remaining in the culture supernatants containing 20 mM citrate with increasing levels of glucose were assessed by HPLC to determine how much of each carbon source was metabolized. As more glucose was supplemented into M9-citrate cultures, the amount of glucose utilization increased (see Fig. S3A in the supplemental material). Conversely, an inverse relationship was observed between citrate consumption and glucose addition (see Fig. S3B in the supplemental material). Most of the citrate was depleted in cultures containing 0 or 1.25 mM glucose. Although citrate utilization was reduced in cultures containing ≥2.5 mM glucose, very little citrate was consumed in cultures containing 10 and 20 mM glucose.

These results strongly indicate that glucose is the preferential carbon source in planktonic cultures. We hypothesized that the presence of glucose would repress the Impranil-degrading activity by Pf-5 induced by citrate. To test this hypothesis, we cultured strain Pf-5 in M9 medium containing 20 mM citrate supplemented with various amounts of glucose. Addition of increasing concentrations of glucose to M9-citrate resulted in a dose-dependent decrease of Impranil-degrading activity in the culture supernatants (Fig. 3A). We also observed a positive correlation between citrate consumption and the levels of Impranil clearing (Fig. 3B). These results indicate that glucose actively suppresses the induction of Impranil-degrading activity in *P. protegens* strain Pf-5.

Polyurethane-degrading enzymes in *P. protegens* strain Pf-5. To identify PU-degrading extracellular enzymes from strain Pf-5, sterile-filtered supernatant from an overnight culture of strain Pf-5 grown in M9-citrate medium was concentrated and subjected to nondenaturing (i.e., native) PAGE (Fig. 4A). The resulting gel was used to obtain a zymogram that revealed two zones of polyurethane clearing (Fig. 4B). Proteins in the clearing zones and regions in between were eluted from a native gel and analyzed

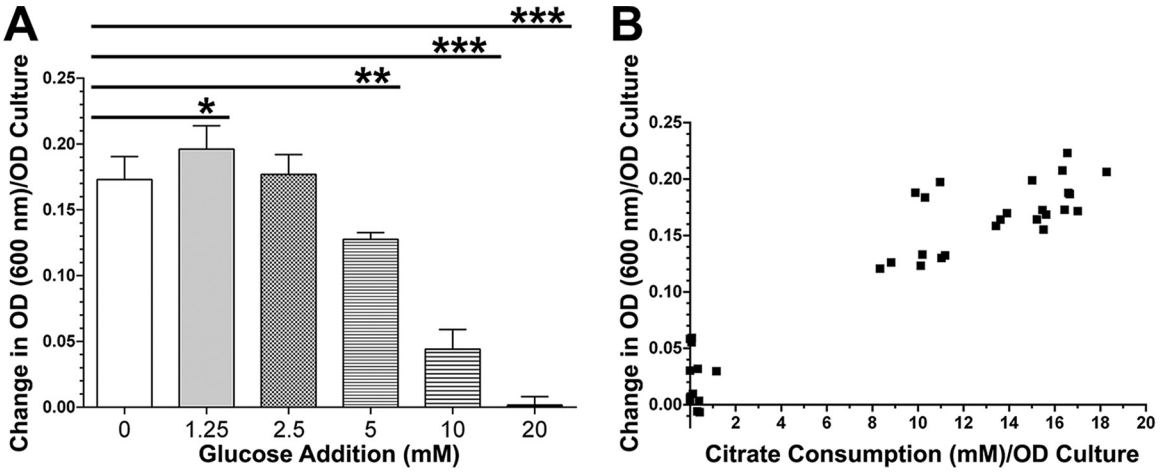

FIG 3 Glucose-inhibited induction of PU-degrading activity in strain Pf-5 late-stationary-phase culture supernatants. (A) Cultures of strain Pf-5 were grown for 22 h in M9-citrate medium supplemented with various concentrations of glucose. Cell-free supernatants of these cultures were subsequently incubated with Impranil to determine the levels of PU degradation. (B) The levels of citrate consumption were correlated with the degrees of Impranil clearing in Pf-5 cultures from panel A to assess correlations between citrate utilization and Impranil clearing. *, $P < 0.05$; **, $P = 0.0001$; ***, $P < 0.0001$.

using SDS-PAGE (Fig. 4C). Each region from the native PAGE gel contained distinct protein banding patterns. The major bands (bands 1A, 1B, 6A, and 6B in Fig. 4C) from regions corresponding to the clearing zones identified by the zymogram were subjected to mass spectrometry analyses. Four proteins were identified in these regions (Table 3). Six tryptic peptides from protein band 6A were identical to sequences of the annotated polyurethane esterase A (*pueA*) gene products from *P. chlororaphis* strain O6 and *P. protegens* strain Pf-5 (see Table S1 in the supplemental material). Similarly, 6 tryptic peptides from protein band 1B were found to be identical to sequences of the annotated *pueB* gene product from *P. protegens* strain Pf-5 (see Table S2 in the supplemental material).

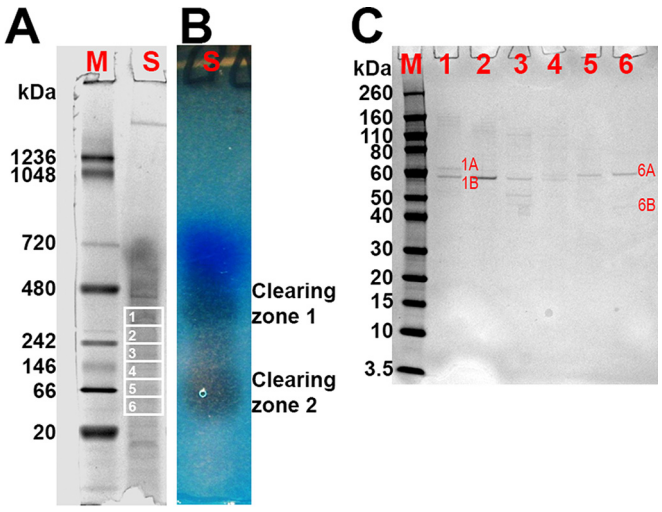

FIG 4 Zymogram analysis and identification of proteins with PU degradation activity. (A and B) NativePAGE with concentrated supernatant from a Pf-5 culture containing 103 μg of protein (A) and corresponding zymogram (B). M, NativeMARK protein standard; S, supernatant sample. (C) SDS-PAGE analysis of eluates 1 to 6 obtained from NativePAGE gel slices 1 to 6. M, Novex Sharp protein standard. Proteins in bands 1A, 1B, 6A, and 6B were identified using mass spectrometry analysis.

To better understand the prevalence of these proteins among *Pseudomonas* species, we searched the NCBI nonredundant protein database for proteins homologous to both Pf-5 PueA and PueB in pseudomonads (Table 4). We also compared the amino acid sequences of PueA and PueB from strain Pf-5 with these homologs, including known polyurethanases PueA and PueB from *P. chlororaphis* (Table 4). Pf-5 PueA showed the highest amino acid sequence identity with *P. protegens* strain CHA0 lipase, class 3 (99.5%), *P. fluorescens* strain Pf0-1 lipase, class 3 (88.5%), and *P. chlororaphis* O6 polyurethanase A (82.0%). Pf-5 PueB showed the highest amino acid sequence identity with CHA0 lipase (100%), Pf0-1 triacylglycerol lipase (77.1%), and O6 triacylglycerol lipase (67.4%). The PueA homologs in *P. fluorescens* strains A506 and SBW25 and the PueB homolog in strain SBW25 were shorter than their counterparts in strain Pf-5 based on primary sequences. No homologs were identified in *P. aeruginosa* strain PAO1 or *Pseudomonas putida* strain KT2440.

Genome analysis in *P. protegens* strain Pf-5. The impact of carbon sources on the Impranil-degrading activity by Pf-5 might be due to the levels of *pueA* and *pueB* gene expression. Reverse transcription-quantitative PCR (qRT-PCR) analyses of RNA from Pf-5 grown in either M9-citrate or M9-glucose were performed. While the expression of both *pueA* and *pueB* transcripts was detected, the levels of *pueA* and *pueB* expression were similar under both growth conditions (data not shown).

We subsequently analyzed the genome sequence of strain Pf-5 in an attempt to uncover the putative regulatory mechanisms for these genes. Adenine-rich sequence motifs located upstream of

TABLE 3 Identification of proteins in SDS-PAGE by mass spectrometry

Band	Protein	Accession no.	Molecular mass (kDa)
1A	Flagellar hook-associated protein (FlgK)	YP_258744	70.6
1B	Polyurethanase B (PueB)	YP_260310	59.1
	Flagellar hook-associated protein (FlgK)	YP_258744	70.6
6A	Polyurethanase A (PueA)	YP_260307	64.9
6B	Isocitrate dehydrogenase	YP_260988	45.3

TABLE 4 Amino acid sequence comparison of PueA (YP_260307, 617 aa) and PueB (YP_260310, 561 aa) of *P. protegens* strain Pf-5 with derived protein sequences from known genomes of strains investigated in our studies[a]

Pseudomonas strain	Identity with PueA[b]	%[b]	Identity with PueB[b]	%[b]
P. protegens CHA0	Lipase, class 3 (617 aa, YP_008000506)	99.5	Lipase (561 aa, YP_008000509)	100
P. fluorescens Pf0-1	Lipase, class 3 (617 aa, YP_348417)	88.5	Triacylglycerol lipase (562 aa, YP_348416)	77.1
P. chlororaphis O6	Polyurethanase A (617 aa, WP_009049212)	82.0	Triacylglycerol lipase (566 aa, EIM14910)	67.4
P. fluorescens A506	Lipase (474 aa, YP_006324104)	66.1	Lipase (569 aa, YP_006323649)	69.1
P. fluorescens SBW25	Lipase (469 aa, YP_002872716)	65.9	Lipase (469 aa, YP_002872716)	48.3
P. aeruginosa PAO1	Alkaline metalloprotease (479 aa, NP_249940)	31.1	Alkaline metalloprotease (479 aa, NP_249940)	30.3
P. putida KT2440	No significant identity (<10 %)	NA[c]	Hemolysin-type calcium binding bacteriocin (3,619 aa, NP_744706)	10

[a] PueA: YP_260307, 617 amino acids (aa); PueB YP_260310, 561 aa.
[b] Proteins with the highest homology were identified with NCBI blastp. Percent identities shown here were obtained using pairwise alignment (Stretcher algorithm).
[c] NA, not applicable.

both *pueA* (5′ AACAACAG 3′) and *pueB* (5′ AATAAAAA 3′) genes in the Pf-5 genome matched the consensus binding site (5′ AANAANAA 3′) of Hfq/Crc, key regulatory proteins of the catabolite control system found in pseudomonads. The presence of *cbrA* and *cbrB* genes in the Pf-5 genome supports the existence of the CbrB/Hfq/Crc catabolite control system in this strain. Furthermore, we were able to identify a potential Crc homologue, exodeoxyribonuclease III (YP_263115.1), among the Pf-5 coding sequences. In addition, two regions of noncoding sequences in the Pf-5 genome that exhibited high sequence identities (69% and 86%) to the crcZ gene from *P. aeruginosa* PAO1 were identified, suggesting the presence of regulatory RNA encoding genes (see the "Identification of Putative Cbr/crc Catabolite Repression System in *P. protegens* Pf-5" in the supplemental material).

Determination of the roles of Pf-5 PueA and PueB in polyurethane biodeterioration in strain Pf-5. To determine if *pueA* and *pueB* are solely responsible for Impranil-degrading activity in Pf-5, we created in-frame deletion mutants of *pueA* (Pf5Δ*pueA*), *pueB* (Pf5Δ*pueB*), and both genes (Pf5Δ*pueAB*). Deletion of the respective genes in each knockout mutant was verified by PCR (see Fig. S4 in the supplemental material). None of the deletion mutants exhibited any defects in their viability or growth compared to those for the wild-type strain (data not shown). Mutants of strain Pf-5 were grown in M9-citrate medium, and the cell-free culture supernatants were tested for Impranil-degrading activity (Fig. 5A). Deletion of the *pueA* gene (Pf5Δ*pueA*) resulted in a greater than 50% reduction in Impranil-degrading activity in culture supernatants based on clearing. Deletion of the *pueB* gene (Pf5Δ*pueB*) did not affect the ability of strain Pf-5 to degrade Impranil. Removal of both genes from the chromosome (Pf5Δ*pueAB*) almost completely abolished the Impranil-degrading activity of Pf-5 culture supernatants. We further examined the mutants in M9-glucose medium to assess regulation of PueA or PueB activities by glucose (Fig. 5A). Similar to the wild-type Pf-5, none of the mutants expressed Impranil-degrading activities when cultured in M9 minimal medium with glucose as the sole carbon source.

The Impranil-clearing reactions of Pf-5 mutants grown in M9-citrate culture supernatants were also analyzed by ^{1}H NMR to assess for the levels of detectable Impranil hydrolysis products (Fig. 5B). Comparable to the Impranil-clearing results, the culture supernatant of the *pueB* deletion mutant (Pf5Δ*pueB*) hydrolyzed Impranil to a level similar to that of wild-type Pf-5. Abolishment of PueA expression in Pf5Δ*pueA* severely affected Impranil hydrolysis. Deletion of both *pueA* and *pueB* genes completely abrogated the ability of this strain to hydrolyze Impranil.

In addition to the PU activity assays from above, we also determined general extracellular lipase activity of strains of Pf-5 and its mutants using the 4-NPP hydrolysis assays (Fig. 5C). Cell-free culture supernatants of the wild-type strain of Pf-5 and the Pf5Δ*pueB* mutant showed approximately 28 and 20 nmol/min/OD culture lipase activity, respectively. However, deletion of *pueA* (Pf5Δ*pueA*) or both genes (Pf5Δ*pueAB*) resulted in an almost complete loss of extracellular lipase activity in the culture supernatants (0.67 and 0 nmol/min/OD culture, respectively).

Screening of carbon sources for impact on polyurethane-degrading ability of *Pseudomonas* strains. After revealing how different carbon sources affected the PU-degrading ability of *P. protegens* strain Pf-5, Impranil agar plate-clearing assays were subsequently used to investigate the impact of carbon sources on various *Pseudomonas* species (Fig. 6). While *P. putida* KT2440 and *P. fluorescens* SBW25 showed no Impranil clearing during the time frame of the investigation, all other strains exhibited Impranil-clearing activity to various degrees. *P. fluorescens* strains A506 and Pf0-1 and *P. aeruginosa* strain PAO1 showed minimal Impranil clearing. Extensive Impranil clearing was observed with *P. protegens* strains Pf-5, CHA0, and BC2-12. The type of carbon source used in the growth medium had a strong impact on the ability of the strains to degrade polyurethane. In *P. protegens* strains, Impranil clearing was mostly promoted when they were grown in the presence of citrate and succinate, while little to no clearing was observed when glucose was supplied. The lack of Impranil clearing in the presence of glucose was also observed with *P. fluorescens* strains A506 and *P. aeruginosa* PAO1. With the exception of *P. fluorescens* SBW25 and *P. putida* KT2440, growth in pyruvate-containing medium resulted in Impranil clearing in all strains tested.

DISCUSSION

In order to thrive and survive, microorganisms often secrete enzymes or secondary metabolites in response to environmental conditions. Many secreted enzymes from fungal and bacterial organisms also possess the ability to degrade PUs of various formulations (4, 6, 7, 35–38). Genes that encode enzymes capable of degrading PU *ex vivo* from some of these organisms have also been identified and cloned (5, 9, 11). Comparison of these gene sequences revealed that the gene products from these diverse organisms all contain a lipase domain and are predicted to be secreted. We report here that strain Pf-5 also secretes lipases capable of degrading Impranil.

Although several PU-degrading lipases have been character-

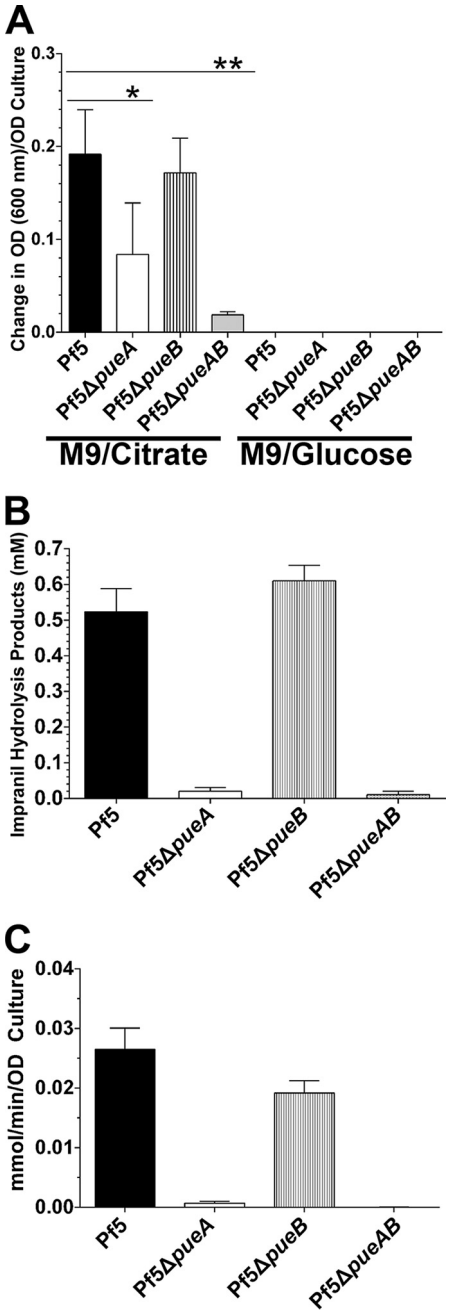

FIG 5 Involvement of polyurethane lipase PueA and PueB in PU-degrading activity and their regulation. (A) Cell-free supernatants of Pf-5 and *pueA* or *pueB* mutants (Pf5Δ*pueA*, Pf5Δ*pueB*, and Pf5Δ*pueAB*) grown in M9-citrate or M9-glucose medium were assayed for their abilities to clear Impranil. Results shown are average of 3 experiments with triplicate samples per experiment. (B) Impranil colloids were added to cell-free supernatants of Pf-5 and *pueA* or *pueB* mutants (Pf5Δ*pueA*, Pf5Δ*pueB*, and Pf5Δ*pueAB*) grown in M9-citrate medium. After 24 h of incubation, clarified samples were analyzed by ^{1}H NMR for the presence of hydrolysis products. (C) Cell-free supernatants of M9-citrate cultures were also incubated with 4-NPP to assess general lipase activities of the strains. *, $P < 0.001$; **, $P < 0.0001$.

ized on a biochemical level, to date, only the *pueA* and *pueB* genes in *P. chlororaphis* have been formally demonstrated through insertional mutation analysis to be responsible for Impranil degradation in the context of an organism (39). Using zymogram techniques and gene deletion mutants, we identified the products of *pueA* and *pueB* genes, annotated as polyurethanase A and polyurethanase B (28), respectively, in *P. protegens* strain Pf-5 to be responsible for the degradation of the polyester PU Impranil.

Using 3 different assays, we showed that PueA protein plays a major role in Impranil clearing, while PueB, although also able to degrade Impranil, contributes less to these activities. However, deletion of both genes was required to abolish the Impranil-degrading activity of strain Pf-5. We previously reported that Pf-5 preferentially hydrolyzes the ester components of Impranil in a solidified film in a manner similar to 4-NPP hydrolysis (34). Of note is that Pf5Δ*pueA*, which presumably still secretes PueB, did not hydrolyze 4-NPP, a prototypic substrate for lipases. It is conceivable that PueA has higher intrinsic activity for hydrolyzing Impranil and 4-NPP than does PueB. Alternatively, PueA might be synthesized and secreted at higher levels than PueB in wild-type Pf-5. Our results demonstrated a critical role for PueA and PueB in Impranil degradation, but it must be noted that we still observed a low level of Impranil-clearing activity by the culture supernatant of the *pueAB* double-knockout mutant (Pf5Δ*pueAB*) grown in citrate-containing medium. This might be due to a small degree of cell lysis in the culture, releasing other intracellular enzymes that attack PU. We have observed that metabolism of citrate by both wild-type Pf-5 and the mutants leads to an alkaline pH shift in the growth medium (ranges from 7.7 to 8.3), and thus it was possible that the residual Impranil clearing observed in Pf5Δ*pueAB* culture supernatants might be due, in part, to base hydrolysis (34). Alternatively, this activity might be due to other unidentified extracellular enzymes that were not identified through zymogram analysis. The Pf-5 genome also contains a *P. aeruginosa* PAO1 *lipA* homolog (PFL0617) which encodes a putative secreted triacylglycerol lipase unrelated to PueA and PueB. It is possible that Pf-5 LipA contributed to the residual activity in Impranil clearing.

Pseudomonas spp. are heterotrophic bacteria that are well adapted to living in diverse environments. They possess versatile cellular physiologies that permit metabolic adjustments, allowing them to utilize a large variety of carbon and nitrogen sources and to degrade many types of organic compounds. Carbon catabolic repression allows pseudomonads to adapt their metabolism to a specific carbon source in order to maximize assimilation of that specific carbon source (40). It is also known that carbon catabolite repression controls enzyme secretion (41, 42) and various metabolic pathways in *Pseudomonas*. It has been found that the Cbr/Hfq/Crc system, Cyo terminal oxidase, and the PTSNtr system are involved in such regulation (40, 43, 44). The Cbr/Hfq/Crc regulatory system involves small RNAs that scavenge the mRNA binding protein complex, Hfq/Crc, resulting in a block of translation of their targeted mRNA (45–52). The identification of putative Hfq/Crc binding sites upstream of *pueA* and *pueB* genes, the lack of differences in *pueA* and *pueB* transcript levels (data not shown), and the effects of carbon source types on Pf-5 Impranil degradation suggest that the expression of PueA and PueB might be regulated at the posttranscriptional level by carbon catabolite repression. This mode of regulation may be common to many pseudomonads, as indicated by this study as well as those of other authors (6, 20, 21). Further study on the regulation and biochemical modes of action for these proteins might greatly benefit our understanding of microbiologically induced polymer coatings biodeterioration.

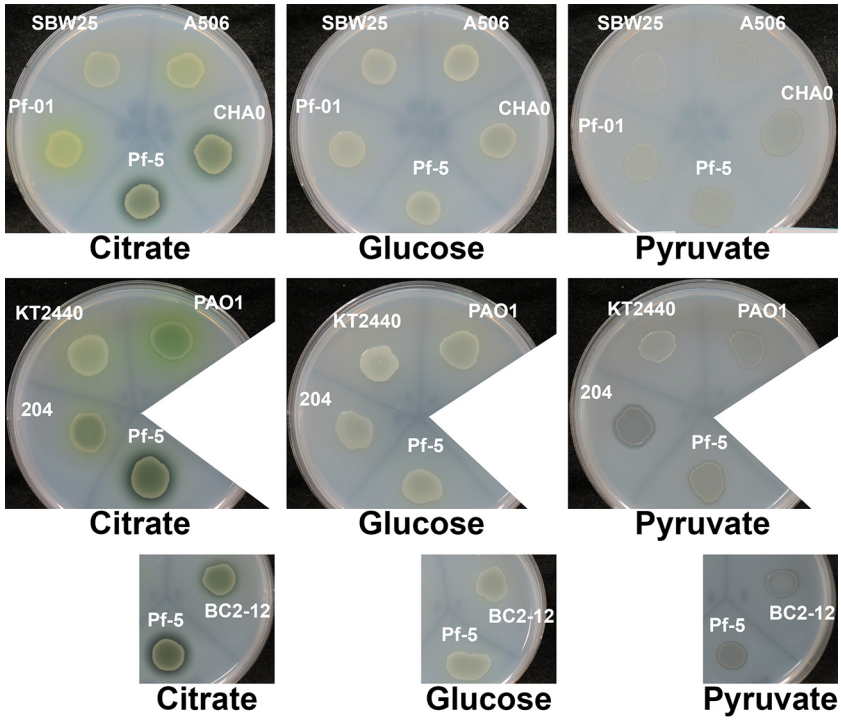

FIG 6 Effects of carbon sources on polyurethane degradation. Various *Pseudomonas* strains were grown on M9 agar plates supplemented with Impranil and different carbon sources for 48 h. Degradation of Impranil resulted in visible zones of clearing. Strain Pf-5 was used as an internal reference on all plates.

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