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ORIGINAL ARTICLE

Effect of insertional mutations in the *pueA* and *pueB* genes encoding two polyurethanases in *Pseudomonas chlororaphis* contained within a gene cluster

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Abstract

Aims: To better understand the role of PueA and PueB from *Pseudomonas chlororaphis* in polyurethane degradation, the present study was conducted to create insertional mutants in their respective genes.

Methods and Results: Growth kinetic studies showed that the *pue*A knockout mutant had a greater effect than the *pue*B knockout mutant. The *pue*A mutant had an 80% decrease in cell density from that of the wild type, while the *pue*B mutant had an 18% decrease in cell density. Polyurethane utilization followed Michaelis-Menten kinetics. The *pue*A and *pue*B mutants exhibited a 17% and 10% decrease respectively in growth rate using polyurethane when compared with the wild type.

Conclusions: In this present study, *pue*A and *pue*B, are shown to be part of an ABC transporter gene cluster that consists of seven open reading frames. Mutational analysis results suggest that PueA may play a more major role in polyurethane degradation than PueB based on cell density and growth rates.

Significance and Impact of the Study: The results from this study provide a starting point for the eventual enhancement and bioremediation of polyurethane waste. Understanding the role of polyurethane-degrading enzymes is useful for the creation of strains for this purpose.

Introduction

Polyurethanes (PU) are a large versatile class of synthetic polymers that have found widespread acceptance for use in a variety of applications that have impacted both military systems and the modern civilian economy (Dombrow 1957). These polymers were originally developed as an alternative to super-polyamides, are being used to replace the older polymers for several reasons. One reason is that the use of PUs can alleviate some of the environmental problems associated with the older polymers. For example, the United States Government has phased out the use of chlorinated rubber in marine and aircraft paints and coatings that previously contained environmentally hazardous volatile organic compounds (Hegedus *et al.* 1989; Reisch 1990).

As the initial production of PUs over 50 years ago, the stable nature of these polymers and other advantages they provide, have resulted in a dramatic increase in their use. One advantage with important environmental implications includes the discovery that PU is susceptible to various forms of degradation. Variation in degradation characteristics of various PU forms has been attributed to differences in physical properties, such as topology and chemical composition (Pathirana and Seal 1983).

In a previous report from this laboratory (Howard *et al.* 1999), growth of *Pseudomonas chlororaphis* was

observed within 9 h of batch culture in which PU was used as the sole carbon and energy source. When the bacterium was grown on PU agar plates, zones of clearing indicative of PU degradation were observed within 18 h. The enzymes from *P. chlororaphis* responsible for PU degradation were found to be secreted into the extracellular medium. Two of these enzymes, PueA (65 kDa) and PueB (60 kDa), have been cloned, sequenced and their encoded-proteins characterized (Stern and Howard 2000; Howard *et al.* 2001). Both genes were expressed in *E. coli*. The PueA enzyme was secreted and displayed a beta-zone of clearing on polyurethane agar plates while PueB was not secreted and displayed an alpha-zone of clearing of polyurethane agar plates. Both enzymes are temperature stable up to 100°C and exhibit esterase activity towards ρ-nitrophenyl substrates. This esterase activity is inhibited by the addition of 1 mmol l^{-1} phenylmethylsulfonylflouride (PMSF).

Herein, we report that the *pueA* and *pueB* genes are contained in a gene cluster similar to that of the group I lipase found in *Pseudomonas fluorescens*. Furthermore, we created kanamycin insertion mutations of *pueA* and *pue*B to study growth of the *P. chlororaphis* mutants on polyurethane.

Materials and methods

Cultures and plasmids

The bacteria and plasmids utilized in this research are listed in Table 1. *Pseudomonas chlororaphis* was obtained from a microbial consortium supplied by the Naval Research Laboratory, Washington, DC. *Escherichia coli* strains XL1-Blue and DH5α were obtained from Stratagene Cloning Systems (LaJolla, CA, USA). Plasmid pBBR1MCS-5 was obtained from Dr Kenneth Peterson at Louisiana State University Medical Centre, Shreveport, LA. *P. chlororaphis* was grown in either Luria-Bertani (LB) media at 30°C or Yeast extract salts (YES) medium, which was prepared as previously described (Crabbe *et al.* 1994). YES consists of the following: 7·5 mmol l^{-1} NH_4CL, 0·4% (wt vol^{-1}) gelatin, 0·002% yeast extract, 2% (vol/vol) Solution A (50 g KH_2PO_4 and 25 g K_2HPO_4 l^{-1}), 1% Solution B (50 g $MgSO_4{\cdot}7H_2O$ l^{-1}) and 0·2% Solution C (40 mg $CoCl_2{\cdot}6H_2O$, 28 mg $CuCl_2{\cdot}2H_2O$, 150 mg $FeCl_3{\cdot}6H_2O$, 2 g $MnCl_2{\cdot}4H_2O$, 26 mg $Na_2Mo_4{\cdot}2H_2O$ and 22 mg $ZnCl_2$ l^{-1}). *E. coli* XL1-Blue was grown in LB media at 37°C with constant shaking.

Table 1 Bacterial strains and plasmids used

Bacterial strains/plasmids	Relevant characteristics	Source or reference
Pseudomonas chlororaphis	wild type polyurethanase	Howard *et al.* (1999)
Escherichia strain XL1-Blue	recA1 end A1 gyr A96thi-1 hsdR17 (rk–, mk+) sup E44 relA1 lac-F′ [pro AB+ lac I– laczΔm15 Tn10 (tet R)] F-end A1 hsd R17 (rK–, mK+) Sup E44 thi-1 rec A1 Gyr A96 relA1λ-	Stratagene
Plasmids pUC4 K	colE1 amp^r Kan^r	Jordan and Ogren (1981)
pPU2	pBluescript SK- (puc19 ampR orif1 colE1 lacz) 5·7 kb EcoRI pueA gene	Stern and Howard (2000)
pPU13	pBluescript SK (puc19 amp^r orif1 colE1 lacz) 5·7 kb EcoRI pueB gene	Howard *et al.* (2001)
pT7-6 ES3·2	colE1 amp^r PT7 3·2 kb SalI-EcoRI pueA gene	Stern and Howard (2000)
pBBR1MCS-5	broad-host-range cloning vector gentamicin resistant	Kovach *et al.* (1995)
pPueA-1	pBBR1MCS-5 + 3·2 kb SalI-EcoRI pueA gene	This study
pBSESs2·8	pBluescript SK +2·8 kb *Sst*I-*Eco*RI pueA gene	This study
pBSESs2·8:: Kan^r	pBSESs2·8 + 1·5 kb *Pst*I Kan^r gene from pUC4K	This study
pPueB-1	pBBR1MCS-5 + 1·7 kb *Bam* HI–*Xho* I pueB gene	This study
pBSPueB-1	pBluescriptSK$^-$ +1·7 kb *Bam* HI–*Xho* I *pue* B gene	This study
PBSPueB-2	pBSPueB-1 + 1·5 kb *Pst*I Kan^r gene	This study

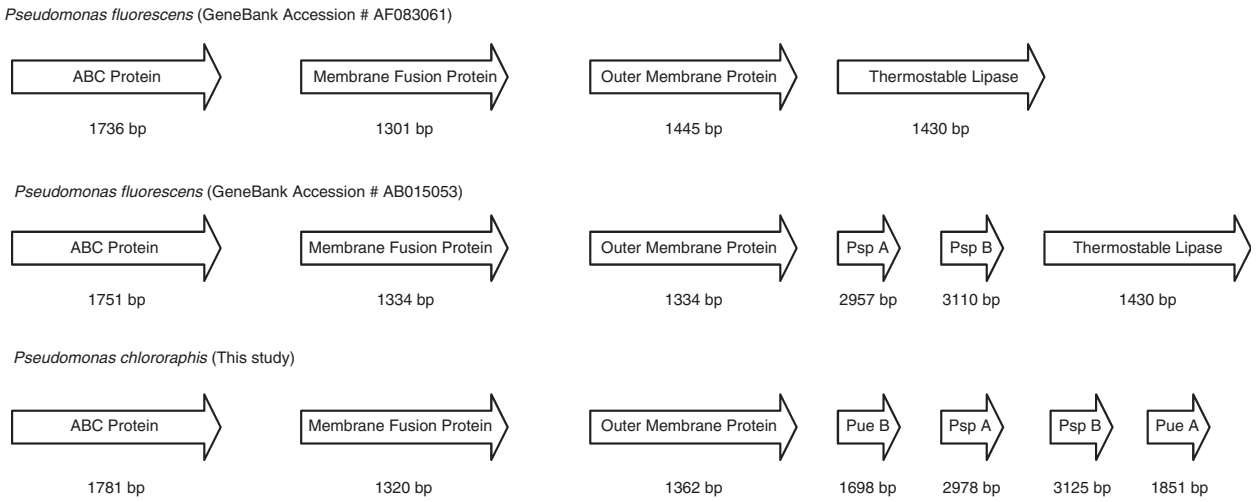

Figure 1 Comparison of the gene clusters from two strains of *Pseudomonas fluorescens* and the PUase gene cluster from *Pseudomonas chlororaphis*. The ABC Reporter Protein, Membrane Fusion Protein and Outer Membrane Protein are involved in Type I translocation of the extracellular protein. The PspA and PspB proteins are serine protease homologues.

DNA isolation

Chromosomal DNA was extracted using an Ultra Clean soil DNA isolation kit purchased from MoBio Laboratories, Inc. (Solano Beach, CA, USA). Plasmid DNA was extracted according to the protocol for Fast Plasmid Mini Prep (MoBio Laboratories, Solano Beach, CA, USA) as described by the manufacturer.

Cloning and sequencing

DNA sequencing was carried out by automated microsequencing performed in the Sequencing Laboratory of the Biotechnology Centre at the University of Illinois at Urbana-Champaign. Both strands were sequenced and the correct DNA alignments were deduced using Sequencher 3·0. Homology searches were conducted against the GenBank and EMBL databases by using the BLAST program. Two primers Pchlo-F (5′-ACAAGAACAACCAGCAGAAATACAACC-3′) and Pchlo-R (5′-CTCCAGCCTTTACCGGAATGTAGG-3′) targeted at positions 7244 to 7270 and 9900 to 9880 within the PU gene cluster, respectively, were designed to amplify the gap between partially sequenced PspA and PspB (Fig. 1a). PCR reaction mixture (50 μl) contained 50 ng of genomic DNA 100 ng of each primer, 200 μmol l^{-1} of each dNTP, 2·5 U of Herculase Hot Start DNA polymerase (Strategene, LaJolla, CA, USA) and 5% DMSO. The PCR thermal profiles consisted of denaturation at 98°C 1 min^{-1}, annealing 56°C 1 min^{-1} and extension 72°C 3 min^{-1}. The approximately 1700 bp PCR product was cloned into the TA cloning vector pGEMT–Easy (Promega, Madison, WI, USA). Five clones checked for the right sized inserts and subsequently sequenced as described above. Genome-walking PCR was used to obtain the sequence for the putative ABC transporter upstream of the Outer membrane protein (Fig. 1). The procedure for genome-walking PCR was as described by the manufacturer using the Universal Genome Walker kit; (Clontech Laboratories, Mountain View, CA, USA). Three genome-walking experiments were conducted to fully decipher the sequence. A total of six reverse primers (Table 2) were designed and in conjunction with

Table 2 List of designed primers used for genome walking putative ABC transporter gene

Experiment	Reaction	Primer	Target position in PU operon
Walk 1	Primary PCR	5′-CAGGATCCAGCCCATGCGGGCGAAAAAG-3′	1901-1875R
	Nested PCR	5′-CTCTTCGATCATCATGTTGCTGTCCTT-3′	1838-1812R
Walk 2	Primary PCR	5′-CAGCTTGGCCGAACTCCATTGCTT-3′	933-910 R
	Nested PCR	5′-CATATCGCCCTTGATCACCAGCAA-3′	831-808 R
Walk 3	Primary PCR	5′-GAACTGGCGAATATGGGTCAAAT-3′	408-385 R
	Nested PCR	5′-TTGCGCTCGAACGCGGCCTGGTA-3′	344-322 R

manufacturer's adapter specific primers AP1 (5′-GTAATACGACTCACTATAGGGC-3′) and AP2 (5′-ACTATAGGGCACG CGTGGTC-3′) to generate the entire ABC transporter sequence. All designed oligonucleotides were synthesized using standard methods with an automated DNA/RNA synthesizer (Sigma Genosys, St Louis, MO, USA). An aliquot (500 ng) of *P. chlororaphis* genomic DNA was completely digested separately in three reactions with *Pvu*I, *Eco*RV and *Stu*I. The digested DNA was ethanol precipitated and re-dissolved in 20 μl of TE buffer. The purified DNA from each library was ligated into genome walker adapters. The reaction mixtures (25 μmol l^{-1} genome walker adaptor, 1·6 μl 10X ligation buffer, 3 U of T4 DNA ligase and 4 μl of the purified digested DNA from each library) were incubated at 16°C overnight. The ligation reaction was stopped by heating reaction mix at 70°C for 5 min and subsequently 72 μl of TE buffer was added to each reaction mixture. Each library was used as a template for PCR amplification using known primer sequences based on segments of the *P. chlororaphis* ABC transporter on combination with manufacturer supplied adaptor primers. Each 50 μl PCR reaction contained 1 μl of ligated template, 100 ng of each primer, 200 μmol l^{-1} of each dNTP, 2·5 U of Herculase Hot Start DNA polymerase (Strategene, LaJolla, CA, USA) and 5% DMSO. The PCR thermal profiles consisted of denaturation at 98°C 1 min^{-1}, annealing 56°C 1 min^{-1} and extension 72°C 3 min^{-1}. PCR amplification was performed in a programmable thermal cycler (Applied Biosystems 3700; Foster City, CA, USA). The largest PCR fragment obtained from any of the three libraries was ligated into a pGEM-T Easy vector DNA (Promega), and the product was used in transforming *E. coli* JM109 cells using the heat shock method (42°C for 45 s). The insert was subsequently sequenced as described above.

Nucleotide sequence accession number

The complete nucleotide sequence of the gene cluster has been deposited at GenBank/EMBL/DDBJ database under the following accession number: EF175556.

Mutant construction

The2·8 kb *Eco*RI–*Sst*I DNA fragment containing the *pueA* gene within the plasmid pT7-6 ES3·2 (Stern and Howard 2000) was subcloned into the cloning vector pBluescript SK using the restriction endonucleases *Eco*RI-*Sst*I yielding pBSESs2·8. The construct containing the *pue*A gene was interrupted by inserting the 1·5 kb kanamycin resistance gene (Kan^r) cut from pUC4 K with *Pst*I into the *Pst*I site of *pueA* yielding pBSESs2·8::Kan^r. The resulting suicide vector containing the *pueA* gene interruption was subsequently transformed using a GenePulser II from BioRad (Herculers, CA, USA) into *P. chlororaphis*. The electroporation conditions consisted of an overnight culture of *P. chlororaphis* grown in LB broth. Cells were washed in double distilled water with 10% (vol/vol) glycerol. Cells were suspended in 10% (vol/vol) glycerol with a cell density of 10^{10} cells ml^{-1} and 40 μl was used along with 100 ng DNA for electroporation. A 0·2 cm cuvette gap was used with 2·5 kV voltage, a field strength of 12·5 kV cm^{-1}, 25 μF capacitor, 200 Ω resistor and a time constant of 4·5 ms. After electroporation, the cells were incubated for 6 h in LB medium, Kanamycin was added to a final concentration of 50 μg ml^{-1}, and incubation continued for 18 h. Cells (50 μl) were plated on selective medium. After 2 days, colonies were transferred to fresh medium. Correct replacement of the wild-type gene with the mutated gene was confirmed by the Southern blot analysis. In addition, the 3·2 kb *Eco*RI–*Sal*I DNA fragment containing the *pueA* gene within the plasmid pT7-6 ES3·2 was cloned into pBBR1MCS-5 using the restriction endonucleases *Eco*RI–*Sal*I creating the shuttle vector pPueA-1.

A DNA fragment encoding the *pueB* gene of *P. chlororaphis* was obtained by PCR amplification of this fragment using pPU13 plasmid DNA as template (Howard *et al.* 2001). The forward primer (with a *Bam* HI site shown in bold), was 5′-CG **GGATCC** GGAGGCCTTGCCGACCGCAGG-3′ and the reverse primer (with a *Xho* I site shown in bold,), was 5′-GC**CTCGAGG**GGCTGCGGGGCCTCTAGCGC-3′. PCR was carried out with the ExTaq kit (Takara, Madison, WI, USA) as recommended by the manufacturer. The reaction mixture contained 100 ng of template DNA and 60 ρmol of each oligonucleotide primer. PCR was carried out for 30 cycles of 95°C denaturing for 1 min, 68°C annealing for 5 min. The resulting product, 1·7 kb DNA fragment, was digested with *Bam*HI and *Xho*I and ligated into *Bam*HI–*Xho*I sites of pBluescript SK creating the suicide vector, pBSPueB-1, and into pBBR1MCS-5 creating the shuttle vector pPueB-1. The 1·7 kb PCR DNA fragment containing the *pueB* gene in pBSPueB-1 was interrupted by inserting the kanamycin resistance gene (Kan^R) cut from pUC4 K with *Pst*I into the *Pst*I site of *pueB* yielding pBSPueB-2. The suicide vector containing the *pueB* gene interruption was transformed into *P. chlororaphis*. After electroporation, the cells were incubated for 6 h in LB medium, Kanamycin was added to a final concentration of 50 μg ml^{-1}, and incubation continued for 6 h. Cells (50 μl) were plated on selective medium. After 1 day, colonies were transferred to fresh medium. Correct replacement of the wild-type gene with the mutated gene was confirmed by the Southern blot analysis.

Southern blot analysis

Chromosomal DNA from *P. chlororaphis* wild type and each mutant was digested to completion with *Eco*RI. The digested DNA fragments were fractionated by agarose gel electrophoresis (0·7%, wt/vol) using TAE buffer. The DNA fragments were transferred to a Nytran membrane by capillary action. The 5·7 kb *Eco*RI fragment from pPU2 (Stern and Howard 2000) containing the *pueA* gene, the 1·7 kb *Xho*I-*Bam*HI fragment from PCR containing the *pueB* gene, or the 1·5 kb *Pst*I fragment from pUC4 K containing the Kan^R gene served as probes. The DNA fragments were labelled and detection carried out using Amersham Life Sciences AlkPhos labelling and detection kit following manufacturer's instructions. Hybridizations were carried out overnight at 55°C. Membranes were washed for 30 min with 0·5X SSC/0·1% SDS at 55°C prior to autoradiography.

Western blot analysis

Western blot analysis was performed using an anti-PueA antibody and an anti-PueB raised in rabbit and a goat anti-rabbit IgG (vector). VECTASTAIN® ABC-Amp™, a colorimetric immunodetection kit, was used in the analysis (Vector Labs, Burlingame, CA, USA). Extracellular proteins from culture supernatant (8 μg) from the wild type and mutant *P. chlororaphis* were fractionated on a 12% (wt/vol) SDS-PAGE then transferred to a PVDF membrane by electrophoresis at 25 V for 12 h. The membrane was incubated and signals detected according to kit specifications.

Growth kinetics

Growth kinetic studies were performed on the wild type *P. chlororaphis*, the *P. chlororaphis* mutant (*pueA*::Kan^R), the *P. chlororaphis* complement (*pueA*::Kan^R pPueA-1) and the *P. chlororaphis* multicopy *pueA* (pPueA-1) at 30°C with constant shaking. YES media was used with the addition of varying concentrations of Impranil DLN™ (Bayer, Pittsburgh, PA, USA). Impranil DLN is water dispersible polyurethane made from a poly hexane/neopentyl adipate polyester and hexamethylene diisocyanate. The concentrations of Impranil DLN™ used were: 9·0, 6·0, 3·0, 1·5, 0·75, 0·54, 0·375 and 0·18 mg ml^{-1}. Each concentration was prepared in triplicate. The media were then inoculated with 100 μl from the LB broth culture. Cell number was determined using a Coulter Multisizer IIe instrument (Coulter Scientific Instruments, Inc., Hialeah, FL, USA) fitted with a 30 μm aperture. Values for K_s and μ_{max} for polyurethane utilization were elucidated. The above growth kinetic studies were repeated on the *P. chlororaphis* mutant (*pueB*::Kan^R), the *P. chlororaphis* complement (*pueB*::Kan^R pPueB-1) and the *P. chlororaphis* multicopy *pueB* (pPueB-1).

Results

Sequence analysis

We previously cloned and sequenced two polyurethanase genes, *pueA* (Stern and Howard 2000) and *pueB* (Howard *et al.* 2001) from *P. chlororaphis* in *Escherichia coli*. In the present study, we identified a gene cluster resembling a binding-protein-dependent ABC transport system in *Pseudomonas chlororaphis* in connection with *pueA* and *pueB*. Seven open reading frames, spanning 14 686 bp were delineated (Fig. 1). Each gene was compared with entries in the GenBank protein sequence database to predict its function (Table 3). Sequence alignment of the ORF 1 showed that the *abc1* had a high identity to ATP binding cassette (ABC) transporters found in *Pseudomonas fluorescens* Pf-5 (YP260313) (99%), *P. fluorescens* Pf0-1 (YP260311) (85%), *P. fluorescens* 33 (BAA36463) (82%), *P. tolaasii* (CAA07699) (84%) and *Serratia marcescens* (BAA08631) (63%). The hydropathy profile of the amino acid sequence indicated that ABC lacked a signal sequence and had five highly hydrophobic domains corresponding to putative transmembrane segments in the N-terminal

Table 3 Gene cluster from *Pseudomonas chlororaphis* containing the polyurethanases PueA and PueB*

ORF	Gene	rbs (position)	Start (position)	Stop (position)	aa length	Mol. wt. (kDa)
ORF 1	*abc1*	AGGAA (12–17)	ATG (28)	TGA (1809)	594	63·7
ORF 2	*mfp1*	AAGGA (1812–1816)	ATG (1824)	TAA (3143)	440	48·6
ORF 3	*omp1*	AAGAA (3136–3140)	ATG (3146)	TAG (4507)	454	50·9
ORF 4	*pueB*	AAGAAGGA (4544-4550)	ATG (4551)	TGA (6254)	568	60
ORF 5	*pspA*	AAGAGA (6432–6437)	ATG (6448)	TGA (9426)	993	103·5
ORF 6	*pspB*	AAGG (9488–9491)	ATG (9492)	TAG (12617)	1042	107·7
ORF 7	*pueA*	AGAAGA (12705–12710)	ATG (12720)	TGA (14570)	617	64·7

*The complete nucleotide sequence of the gene cluster has been deposited at GenBank/EMBL/DDBJ database under the following accession number: EF175556.

half. There was an ATP-binding consensus sequence (GXXGXGKS) located in the central portion, amino acids 367–374. Sequence alignment of ORF 2 showed that the *mfp1* had a high identity to membrane fusion proteins found in *P. fluorescens* Pf-5 (YP260312) (98%), *P. fluorescens* Pf0-1 (YP348412) (89%), *P. fluorescens* 33 (BAA36464) (83%), the *eprE* of *P. tolaasii* (CAA07700) (85%) and the *lipC* of *Serratia marcescens* (BAA08632) (44%). Sequence alignment of ORF 3 showed that the *omp1* had a high identity to outer membrane proteins found in *P. fluorescens* Pf-5 (YP260311) (99%), *P. fluorescens* Pf0-1 (YP348413) (86%), *P. fluorescens* 33 (BAA36465) (81%), the *eprF* of *P. tolaasii* (CAA07701) (78%) and the *lipD* of *Serratia marcescens* (BAA25796) (46%). Sequence alignment of ORF 4 showed that the *pueB* had a high identity with group I lipases (Howard *et al.* 2001). Sequence alignment of ORF 5 showed that the *pspA* had a high identity to serine proteases found in *P. fluorescens* Pf-5 (YP260309) (98%), *P. fluorescens* Pf0-1 (YP348414) (66%),the *pspA* of *P. fluorescens* 33 (BAA36466) (66%), the *eprS* of *P. tolaasii* (CAA07702) (65%) and the *ssp-h1* of *Serratia marcescens* (BAA33454) (38%). Sequence alignment of ORF 6 showed that the *pspB* had a high identity to serine proteases found in *P. fluorescens* Pf-5 (YP260308) (93%), *P. fluorescens* Pf0-1 (YP348415) (75%), the *pspB* of *P. fluorescens* 33 (BAA36467) (74%), the *eprS* of *P. tolaasii* (CAA07702) (37%) and the *ssp-h1* of *Serratia marcescens* (BAA33455) (71%). Sequence alignment of ORF 7 showed that the *pueA* had a high identity with group I lipases (Stern and Howard 2000).

Southern blot analysis

The *pueA* gene was insertionally inactivated, resulting in the mutant *P. chlororaphis pueA*::Kan^r. Correct replacement of the wild-type *pue*A gene with the mutated *pue*A gene was confirmed by Southern blot analysis (data not shown). The *pue*A gene resides on a 5·7 kb *Eco*RI restriction endonuclease DNA fragment (Stern and Howard 2000). Upon insertion of the 1·5 kb Kan^r cartridge into the *pue*A gene, the new DNA fragment has a molecular size of 7·2 kb. When the genomic DNA of the wild type and mutant strains of *P. chlororaphis* was probed with the *pue*A gene the corresponding 5·7 kb and 7·2 kb fragments were observed. In addition, when the Kan^r gene was used as the probe, this DNA fragment did not hybridize with the wild type genomic DNA but did hybridize with a 7·2 kb DNA fragment of the mutant.

The *pueB* gene was also insertionally inactivated, resulting in the mutant *P. chlororaphis pueB*::Kan^r. Correct replacement of the wild-type *pue*B gene with the interrupted *pue*B gene was confirmed by the Southern blot analysis (data not shown). The *pue*B gene resides on a 5·7 kb *Eco*RI restriction endonuclease DNA fragment (Howard *et al.* 2001). Upon insertion of the 1·5 kb Kan^r cartridge into the *pue*B gene, the new DNA fragment has a molecular size of 7·2 kb. When the genomic DNA of the wild type and mutant strains of *P. chlororaphis* was probed with the *pue*B gene the corresponding 5·7 and 7·2 kb fragments were observed. In addition, when the Kan^r gene was used as the probe, this DNA fragment did not hybridize with the wild type genomic DNA but did hybridize with a 7·2 kb DNA fragment of the mutant.

Immunoblot analysis

The mutant and wild type strains of *P. chlororaphis* were analysed for the presence of PueA and PueB. Proteins were fractionated by 12% SDS-PAGE followed by electrotransfer to PVDF membranes. Western blot analysis was performed using either a rabbit anti-PueA primary antibody or a rabbit anti-PueB primary antibody and a goat antirabbit secondary antibody and colorimetric, immunodetection kit. Immuno-detection revealed that the PueA protein (65 kDa) and the PueB protein (60 kDa) are present in the wild type and not in the mutants of *P. chlororaphis* (Fig. 2).

Growth of *pueA* and *pueB* mutant

P. chlororaphis wild type and derivatives when grown on 1% Impranil DLN^{TM} YES medium exhibited a lag phase growth for the first 2 h that was followed by logarithmic growth for 6 h (Table 4). The wild type and complement reached approximately the same maximum density; whereas, the mutant had an 80% decrease from that of the wild type and the multicopy *pueA* had a 65% increase from that of the wild type. Values for K_s and μ_{max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0·18 to 9·0 mg ml^{-1} (Table 4). Polyurethane utilization followed Michaelis–Menten kinetics. The mutant exhibited a decrease in growth rate using polyurethane when compared with the wild type, whereas the complement restored the growth rate back to wild type levels and the multicopy *pueA* cell line had an increase in growth rate when compared with the wild type.

The PueB mutant had an 18% decrease in cell density whereas, the complement and multicopy PueB derivatives had an increase in cell densities, 26% and 40% respectively (Table 4). Values for K_s and μ_{max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0·18 to 9·0 mg ml^{-1} (Table 4). Polyurethane utilization followed Michaelis–Menten kinetics. The mutant exhibited a 10% decrease in growth rate using

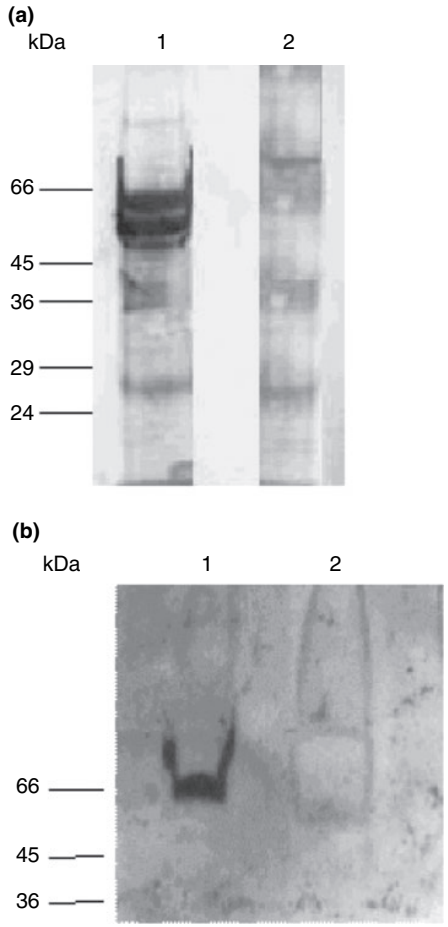

Figure 2 (a) Western blot analysis of PueA protein (65 kDa). Lane1, colorimetric immunodetection of the PueA protein from *Pseudomonas chlororaphis*. Lane 2, colorimetric immunodetection revealed no presence of PueA from *Pseudomonas chlororaphis* mutant. (b) Western blot analysis of PueB protein (60 kDa). Lane 1, colorimetric immunodetection of the PueB protein from *Pseudomonas chlororaphis* wild type. Lane 2, colorimetric immunodetection revealed no presence PueB from *Pseudomonas chlororaphis* mutant.

polyurethane when compared with the wild type, whereas the complement restored the growth rate back to wild type levels and the multicopy *pueB* cell line had an 8% increase in growth rate when compared with the wild type.

Discussion

Pseudomonas chlororaphis has been shown to utilize polyurethane as a sole carbon and energy source (Howard *et al.* 1999). Two genes encoding polyurethanase activity from *P. chlororaphis* have been cloned in *E. coli* (Stern and Howard 2000; Howard *et al.* 2001). Both genes can be expressed in *E. coli*. However, the PueA enzyme is secreted in the recombinant *E. coli* and displays a beta-zone of clearing on polyurethane agar plates while PueB is not secreted in the recombinant *E. coli* and displays an alpha-zone of clearing on polyurethane agar plates. In addition, PueB has been noted to display esterase activity towards ρ-nitrophenylacetate, ρ-nitrophenylpropionate, ρ-nitrophenylbutyrate, ρ-nitrophenylcaproate and ρ-nitrophenylcaprylate while PueA has been reported to display esterase activity only towards ρ-nitrophenylacetate and ρ-nitrophenylpropionate. In a related study (Wales and Sagar 1988), two types of polyurethanase activities were observed from *Asperigillus terreus*, which were attributed to endopolyurethane and exopolyurethane activity. The endopolyurethane activity resulted in a decrease in the degree of polymerization through the cleaving of bonds along the polymer chain. The authors hypothesized that the endo-enzyme activity accounted for the alpha-zone of clearing where a weakly translucent zone was observed. Exopolyurethane activity, which is involved in the removal of successive monomer units from the ends of the polymer chains, could account for the visible clearing seen in the beta-zone clearing. Both PueA and PueB confer esterase activity towards ρ-nitrophenyl substrates,

Table 4 Growth kinetic analysis of *P. chlororaphis* and its derivatives using polyurethane as the sole carbon source*

Strain	μ_{max}	Doubling time (min)	K_s (mg ml^{-1})	Cell density (cells ml^{-1})
P. chlororaphis (wild type)	1·32	31·5	0·800	$2{\cdot}31 \times 10^8 \pm 0{\cdot}87$
P. chlororaphis pueA::Kan^r	1·09	38·2	0·917	$4{\cdot}66 \times 10^7 \pm 0{\cdot}13$
P. chlororaphis pueA::Kan^r (pPueA-1)	1·41	29·5	0·710	$2{\cdot}86 \times 10^8 \pm 0{\cdot}09$
P. chlororaphis (pPueA-1)	1·54	27·0	0·649	$3{\cdot}85 \times 10^8 \pm 0{\cdot}98$
P. chlororaphis pueB::Kan^r	1·19	34·9	0·893	$2{\cdot}35 \times 10^8 \pm 0{\cdot}148$
P. chlororaphis pueB::Kan^r (pPueB-1)	1·37	30·4	0·735	$3{\cdot}59 \times 10^8 \pm 0{\cdot}187$
P. chlororaphis (pPueB-1)	1·41	29·5	0·781	$3{\cdot}99 \times 10^8 \pm 0{\cdot}813$

*The concentrations of Impranil DLN^{TM} used were: 9·0, 6·0, 3·0, 1·5, 0·75, 0·54, 0·375 and 0·18 mg ml^{-1}. Each concentration was prepared in triplicate. No growth was observed in YES medium without addition of Impranil DLN.

are temperature stable up to 100°C and the esterase activity is inhibited by addition of 1 mmol l^{-1} PMSF.

Upon cloning PueA (Stern and Howard 2000) and PueB (Howard *et al.* 2001) from *P. chlororaphis* in *Escherichia coli*, the recombinant proteins were noted to have a high homology to group I lipases. This family of lipases and other serine hydrolases, are characterized by an active serine residue that forms a catalytic triad in which an aspartate or glutamate and a histidine also participate (Persson *et al.* 1989; Winkler *et al.* 1990; Jaeger *et al.* 1994). Sequence analysis of the two-polyurethanase genes revealed that both encoded proteins contain serine hydrolase-like active site residues (G-H-S-L-G) and a C-terminal nonapeptide tandem called repeat in toxin (RTX), (G-G-X-G-X-D-X-X-X) repeated three times. group I lipases lack an N-terminal signal peptide but instead contain a C-terminal secretion signal. The secretion of these enzymes occurs in one step through a three-component, ATP-binding cassette (ABC) transporter, Type I secretion system (Arpigny and Jaeger 1999). Proteins secreted by Type I systems typically exhibit two features: (i) an extreme C-terminal hydrophobic secretion signal located within the last 60 amino acids that is not cleaved as part of the secretion process and (ii) glycine-rich RTX motifs. The RTX repeats form a β-roll structure stabilized by Ca^{2+} ions coordinated between adjacent coils of the β-roll. These motifs are thought to be important for proper presentation of the secretion signal to the secretion machinery, but their exact role is controversial.

In the present study, we identified a gene cluster resembling a binding-protein-dependent ABC transport system in *Pseudomonas chlororaphis* in connection with PueA and PueB, which are involved in polyurethane degradation. The identified ABC transport system consists of three components: an ATPase- binding protein (ABC), an integral membrane protein (MFP), and an outer membrane protein (OMP). The ABC pathway has been shown to mediate translocation of an alkaline protease in *Pseudomonas aeruginosa* (Doung *et al.* 1994). Also, the ABC pathway has been shown to be involved in secretion of a lipase from *Serratia marcescens* (Akatsuka *et al.* 1995), which is located separately from the lipase gene on the chromosome and secretes protease, lipase and S-layer proteins (Kawai *et al.* 1998). A gene cluster (accession number AF083061) was identified for an ABC transporter specific for a lipase in *Pseudomonas fluorescens* SIK W1 (Ahn *et al.* 1999) and a similar gene cluster (accession number AB015053) was identified in *Pseudomonas fluorescens* 33 for a lipase gene and two serine proteases (Kawai *et al.* 1999). Interestingly, when the two ABC exporter gene clusters of *Pseudomonas fluorescens* are compared with the ABC exporter gene cluster of the one found in *Pseudomonas chlororaphis*, a unique gene arrangement is observed (Fig. 1). It appears that the novel gene arrangement observed is a combination of the two *P. fluorescens* gene clusters, and may have resulted through either a rearrangement or an insertional event between the two ABC gene clusters observed in *P. fluorescens*.

In the present study, correct replacement of the wild-type *pue*A gene with the mutated *pue*A gene was confirmed by the Southern blot analysis. In addition, the Western blot analysis was performed using cell extracts from mutant and wild type strains of *P. chlororaphis* for determining the presence of PueA and PueB. Immunodetection revealed that the PueA and PueB proteins are present in the wild-type *P. chlororaphis* and that the PueA protein was inactivated in the PueA mutant *P. chlororaphis* and the PueB protein was inactivated in the PueB mutant *P. chlororaphis*. Thus, growth studies were performed to compare the effects of the PueA-deficient strain and the PueB deficient strain with the wild type strain in polyurethane utilization.

Pseudomonas chlororaphis wild type and its PueA derivatives when grown on 1% Impranil DLN™ YES medium exhibited a lag phase growth for the first 3 h then was followed by logarithmic growth for 6 h. The wild type reached a cell density of $2{\cdot}31 \times 10^8 \pm 0{\cdot}87$. The PueA mutant, *P. chlororaphis pue*A::Kan^r, had an 80% decrease in cell number ($4{\cdot}66 \times 10^7 \pm 0{\cdot}13$) whereas, both the complement, *P. chlororaphis pue*A::Kan^r pPueA-1 and *P. chlororaphis* pPueA-1 had an increase in cell densities, $2{\cdot}86 \times 10^8 \pm 0{\cdot}09$ (25% increase) and $3{\cdot}85 \times 10^8 \pm 0{\cdot}98$ (65% increase) respectively. The results obtained from the cell densities of each strain were reflected in the growth kinetic studies. Values for K_s and μ_{max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0·18 to 9·0 mg ml^{-1}. *P. chlororaphis* wild type exhibited a μ_{max} of 1·32 whereas, the PueA insertional mutant, *P. chlororaphis pue*A::Kan^r, exhibited a μ_{max} of 1·09. It would be hypothesized that a deletion of the *pue*A gene would result in a decrease in growth rate. However, the large decrease in growth obtained from the insertional mutant may indicate that PueA plays a more major role when compared with PueB in polyurethane degradation by *P. chlororaphis*. When multiple copies of the *pue*A gene were introduced into either the wild type, *P. chlororaphis* pPueA-1, a μ_{max} value of 1·54, or the mutant, *P. chlororaphis pue*B::Kan^r, pPueA-1, a μ_{max} value of 1·41, was obtained. An increase in growth rate seems plausible since more PueA produced from the added plasmid would reflect more polyurethane degraded, resulting in an increase in the amount of nutrients available to the cells.

The PueB mutant, *P. chlororaphis pue*B::Kan^r, had an 18% decrease in cell number ($2{\cdot}35 \times 10^8 \pm 0{\cdot}148$) whereas, both the complement, *P. chlororaphis pue*B::Kan^r pPueB-1 and *P. chlororaphis* pPueB-1 had an increase in

cell densities, $3{\cdot}59 \times 10^8 \pm 0{\cdot}187$ and $3{\cdot}99 \times 10^8 \pm 0{\cdot}813$ respectively. The results obtained from the cell densities of each strain were reflected in the growth kinetic studies. Values for K_s and μ_{max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0·18 to 9·0 mg ml^{-1}. *P. chlororaphis* wild type exhibited a μ_{max} of 1·31. When multiple copies of the *pue*B gene were introduced into the wild type, *P. chlororaphis* pPueB-1, a μ_{max} value of 1·41 was obtained which was similar to the complement, *P. chlororaphis pue*B::Kan^r pPueB-1, μ_{max} value of 1·37. An increase in growth rate seems plausible since more PueB produced would reflect more polyurethane degraded resulting in an increase in the amount of nutrients available to the cells. However, these values are small and may indicate that PueB plays a minor role when compared with PueA in polyurethane degradation by *P. chlororaphis*. The insertional mutant, *P. chlororaphis pue*B::Kan^r, displayed a μ_{max} value of 1·19. Again, it would be hypothesized that the deletion of the *pue*B gene would result in a decrease in growth rate however, this small variation compared with the wild type suggests that degradation of polyurethane by *P. chlororaphis* may be more dependent on PueA.

In this study, we identified a gene cluster containing seven open reading frames resembling a binding-protein-dependent ABC transport system in *Pseudomonas chlororaphis* in connection with PueA and PueB. The two-polyurethanase genes have previously been shown to have a high similarity with group I lipases (Stern and Howard 2000; Howard *et al.* 2001). Group I lipases utilize the type I secretion pathway (Doung *et al.* 1994). This pathway is a system consisting of three different proteins, which form a pore-like structure extending from the cytoplasm through the inner and outer cell membranes and into the extracellular space (Pugsley 1993). Comparison of the gene clusters involving a group I lipase in connection with a binding-protein-dependent ABC transport system from two strains of *Pseudomonas fluorescens* and the PUase gene cluster from *Pseudomonas chlororaphis* indicate that the gene cluster in *P. chlororaphis* may have arisen from an insertion and/or rearrangement of the two gene clusters found in *P. fluorescens*. Analysis from the creation of knock-out mutants in *pueA* and *pue*B suggests that degradation of polyurethane by *P. chlororaphis* may be more dependent on PueA than on PueB. The results from this study provide a starting point for the eventual enhancement and bioremediation of polyurethane waste.

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