Biodegradation (2012) 23:561–573
DOI 10.1007/s10532-011-9533-6

ORIGINAL PAPER

Growth of *Acinetobacter gerneri* P7 on polyurethane and the purification and characterization of a polyurethanase enzyme

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Received: 10 August 2011 / Accepted: 26 December 2011 / Published online: 7 January 2012

Abstract A soil microorganism, designated as P7, was characterized and investigated for its ability to degrade polyurethane (PU). This bacterial isolate was identified as *Acinetobacter gerneri* on the basis of 16 s rRNA sequencing and biochemical phenotype analysis. The ability of this organism to degrade polyurethane was characterized by the measurement of growth, SEM observation, measurement of electrophoretic mobility and the purification and characterization of a polyurethane degrading enzyme. The purified protein has a molecular weight of approximately 66 kDa as determined by SDS-PAGE. Substrate specificity was examined using p-nitrophenyl substrates with varying carbon lengths. The highest substrate specificity was observed using p-nitrophenylpropanate with an activity of 37.58 ± 0.21 U mg^{-1}. Additionally, the enzyme is inhibited by phenylmethylsulfonylfluoride and by ethylenediamine-tetra acetic acid. When grown on Impranil DLN™ YES medium, a lag phase was noted for the first 3 h which was followed by logarithmic growth for 5 h. For the linear portion of growth between 5 and 9 h, a μ value of 0.413 doublings h^{-1} was calculated. After 9 h of incubation the cell number dramatically decreased resulting in a chalky precipitate. Measurements of electrophoretic mobility indicated the formation of a complex between the PU and *A. gerneri* P7 cells. A hybrid zeta potential had been generated between the cells and polyurethane. Further evidence for a complex was provided by SEM observation where cells appeared to cluster along the surface of polyurethane particles and along edges of polyurethane films. Occasionally, the cells established an anchor-like structure that connected the cells to polyurethane particles.

Keywords Polyurethanase · *Acinetobacter* · Biodegradation

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Introduction

Polyurethanes (PU) are ubiquitous in many aspects of modern life. They represent a class of polymers that are widely used in the medical, automotive and industrial fields. Polyurethanes are favorably characterized by their substantial tensile strength and high melting points which enables them to be extremely durable (Bayer 1947). They have proven to be excellent replacements of plastics because of their resistance to degradation by water, oils, and solvents (Saunders and Frisch 1964). Polyurethanes possess a number of chemical and physical properties, including an excellent ability to adhere to a variety of substances, a natural resistance to abrasion and weathering, and possession of electrical properties which, collectively,

enables them to be very favorable for industrial purposes (Saunders and Frisch 1964; Urbanski et al. 1977; Fried 1995). Depending on the specific chemical structures of the polyisocyanates and polyols, PU can be synthesized in a variety of forms that range from flexible to rigid and from low density to solid elastomer. The chemical composition of PU necessitates them to be classified as a mixed polymer rather than a pure plastic. Polyisocyanate polyaddition, which is responsible for the versatility of PU, is distinct from polymerization and polycondensation that is associated with the production of synthetic polymers. By varying the isocyanate and polyol composition, chemists can synthesize an enormous diversity of PU materials, including flexible polyester PU. When the polyol is a polyester resin, the product is polyester PU.

The global consumption of plastic in 1997 totaled approximately 145 million tons with polyurethanes comprising a 5% share which resulted in PU ranking fifth in global consumption of plastic (Uhlig 1999). Over three-fourths of the global consumption of PU is in the form of foams. In the United States alone, the production of PU increased from 45,000 tons in 1960 to 2,722,000 tons in 2004. Plastics have reached a level of over 10% by weight and 30% by volume of solid waste in landfills of the US and Japan. The increasing presence of polyurethanes is one of the primary reasons for the rapid depletion of landfills. Their persistence in landfills is also adding to the growing problems of water and surface waste litter, which has raised concerns about non-degradable products and, consequently, has promoted an increased interest in the development of products that are degradable. There is also a current emphasis on the development of new alternatives for the reduction of waste (Kawai 1995).

One of the processes that are being developed for the degradation of plastics focuses on bioremediation, which consists of biological remedies for the reduction of pollution. Biological means have been employed since the early 1900s to treat non-refractory wastes such as sewage. The treatment of hazardous and refractory waste (associated with chemical/industrial activities) through bioremediation is relatively recent and is still in the developmental stages. There is a definitive need to develop effective and meaningful bioremediation techniques for hazardous and refractory waste, such as: determining how these compounds are metabolized by existing organisms, investigating new microorganisms, and developing novel metabolic capabilities through the use of genetic engineering. A basic understanding of the biological processes associated with chemical degradation will enhance the development of new bioremediation techniques (Shannon and Unterman 1993).

A fundamental comprehension of the mechanisms of polyurethane degradation should enable investigators to develop a more efficient technique for the biodegradation of polyurethane. As the production and use of polyurethane increases annually, there is a need for efficient and environmentally friendly techniques designed for its disposal. Lipolytic bacteria associated with a previous study (Howard et al. 2010), and obtained from the decomposition of swine carrion were isolated and characterized. One of the isolates investigated during that study, P7, is capable of degrading polyurethane. The objectives of the current research are to characterize P7 and to elucidate its polyurethane-degrading system.

Materials and methods

Media and culture conditions

From a previous study (Howard et al. 2010), enumeration of lipolytic *Acinetobacter* isolates were screened for their ability to degrade polyurethane. Only one isolate, P7, had the capability of degrading polyurethane and was further studied.

The P7 isolate was grown at 30°C in either Luria–Bertani (LB) medium which contained 5 g yeast extract, 10 g NaCl, and 10 g tryptone per 1 L dH_2O or in Yeast extract salts (YES) medium that was prepared as previously described (Crabbe et al. 1994). When using defined YES medium, the medium excluded the addition of both gelatin and yeast extract. Yes medium consisted of three stock solutions: Solution A (50×) 50 g K_2HPO_4 and 25 g KH_2PO_4 per liter; Solution B (100×) $MgSO_4{\cdot}7\ H_2O$ per liter; and Solution C (1,000×) 1.0 g $MnCl_2{\cdot}4\ H_2O$, 14 mg $CuCl_2{\cdot}2\ H_2O$, 11 mg $ZnCl_2$, 20 mg $CoCl_2{\cdot}6\ H_2O$, 13 mg $Na_2Mo_4{\cdot}2H_2O$, and 75 mg $FeCl_3{\cdot}6\ H_2O$ per 500 ml. Solutions B and C were filtered sterilized and stored at room temperature. Solution A was diluted 1:50 with distilled water and amended with 0.4 g NH_4Cl, 4.0 g gelatin, and 20 mg yeast extract per liter. YES medium was supplemented with Impranil DLN™ (Bayer Co., Pittsburg, PA) which provided a final concentration of

3.0 g l^{-1}. Impranil DLN™ is polyurethane that has been made from a poly hexane/neopentyl adipate polyester and hexamethylene diisocyanate).

Phenotypic characterization

Phenotypic identification initially was based upon Gram staining, a catalase test, and an oxidase test, that was followed by a biochemical characterization of the microbes by means of the Biolog system (Hayward, CA) and 16S rDNA sequencing.

DNA sequencing

PCR amplifications of the 16S rDNA were carried out using the universal primers: 27F (5′-AGAGTTTGA TYMTGGCTCAG-3′) and 1525R (5′-AGAAAGGA GGTGATCCAGCC-3′). The nucleotide sequences were determined by the Pennington Biomedical Research Center (Baton Rouge, LA) and analyzed by the BLAST program in GenBank (Altschul et al. 1997). The DNA sequence of 16S rDNA was deposited in GenBank under the accession number GU082482.

Phylogenetic analysis

The DNA sequences were aligned by means of the Greengenes program (DeSantis et al. 2006), while the phylogenetic and molecular evolutionary analyses were conducted by MEGA version 4 (Tamura et al. 2007). *Acinetobacter* sequences that were available from the GenBank database (Altschul et al. 1997) were used. *Pseudomonas immobilis* functioned as an out-group for the rooted tree. Phylogenetically distinct clusters were assigned on the basis of a bootstrap value >90%.

Determination of bacterial growth

A 5 ml LB-broth culture was inoculated with the microbes and incubated at 30°C with constant shaking for 12 h. YES media was prepared with an addition of Impranil DLN™(3 g l^{-1}). The media (100 ml) was inoculated with 100 µl from the LB broth culture. Cell number was determined by the use of a Coulter Multisizer IIe instrument (Coulter Scientific Instruments, Inc., Hialeah, FL) that was fitted with a 30 µm aperture. Growth studies were run in triplicate.

Scanning electron microscopy

One drop of a bacterial suspension was placed on a glass cover slip that had been coated with a 0.1% (v v^{-1}) solution of poly-L-lysine. After a 10 min incubation period, the cover slip was rinsed 3 times in 0.1 M sodium cacodylate buffer (pH 7.4) and then immersed in a similarly buffered solution of 4% (v v^{-1}) glutaraldehyde. Subsequent to their fixation at 4°C for 1 h, the bacteria were rinsed 3 times with fresh buffer for 10 min each. The bacteria were post-fixed for 1 h at room temperature by immersion of the cover slip in a 0.1 M sodium cacodylate buffered solution (pH 7.4) of 1% (w v^{-1}) osmium tetroxide. The cells were rinsed 3 times with distilled water, dehydrated in a graded series of ethanol, critical-point-dried with liquid carbon dioxide, and sputtered coated with gold. The cells were examined with an FEI 20XL scanning electron microscope at 20 kV.

Electrokinetic measurements

Electrophoretic mobility was determined with a Beckman-Coulter Delsa Nano C Particle Analyzer (Fullerton, CA). Readings were taken in triplicate with an accumulation time of 10 s each and the instrument was set for a water based solution. Zeta potentials were calculated with a viscosity of 0.8878 cP, a refractive index of 1.3328, and a dielectric constant of 78.3. Samples that were analyzed included the isolate P7 which was grown for either 1, 2, or 3 days in YES medium that had been supplemented with 1% (wt v^{-1}) polyurethane. In addition, measurements were obtained for the isolate P7 that had been grown in LB broth overnight and for the YES medium that had been supplemented with 1% (wt v^{-1}) polyurethane without the addition of bacterial cells.

Enzyme purification

A 500 ml culture of the P7 isolate was grown in YES medium that had been supplemented with 1% (wt vol^{-1}) polyurethane. The culture was centrifuged at 5,000×*g* for 15 min at 4°C. All remaining manipulations were also conducted at 4°C. $(NH_4)_2SO_4$ was added to the supernatant to provide 50% saturation. The precipitated proteins were collected by centrifugation (10,000×*g*, 15 min) and suspended in a 50 mM phosphate buffer at pH 7.0. Dialysis was performed in

a 50 mM phosphate buffer at pH 7.0 to remove the ammonium sulfate from the protein extract. To isolate the protein(s) of interest anion-exchange chromatography was employed. A solution of the concentrated protein was passed over a DEAE-Sepharose CL-6B column (3 × 14 cm) with a bed volume of 50 ml, which had been previously equilibrated with a 50 mM phosphate buffer at pH 7.0. A linear step-wise gradient of 0.1–1.0 M NaCl, was applied and the fractions were collected for each concentration. All of the fractions were tested for polyurethanase activity by a radial diffusion assay. When assaying for enzyme activity, wells of 1.0 cm diameter were cut and charged with 100 ml of enzyme. The plates were incubated at 30°C for 18–20 h. Agar plates contained 50 mM phosphate buffer (pH 7.0) and 1% Impranil. Polyurethanase activity was monitored for zones of clearing. The polyurethane-degrading enzyme was determined to be 95% homogenetic as indicated by SDS-PAGE.

Electrophoresis

Sodium dodecyl sulfate (SDS)-PAGE was performed as described by Laemmli (1970) with a 15% (wt v^{-1}) polyacrylamide resolving gel. Proteins were denatured by the addition of 2-mercaptoethanol and heating to 100°C for 5 min. Proteins were visualized by silver staining.

Protein concentration determination

Protein concentrations were measured according to the method of Bradford (1976), with bovine serum albumin as a standard using BioRad low range standards (Bio-Rad Laboratories, Hercules, CA).

Enzyme assays

Once the protein concentration was determined, substrate specificities could be ascertained. Esterase activity was calculated with the use of ρ-nitrophenyl-acetate, ρ-nitrophenyl-butyrate, ρ-nitrophenyl-propionate, ρ-nitrophenyl-caprolate, and ρ-nitrophenyl- caprylate solutions at 8 mM concentrations of the substrate in a 50 mM phosphate buffer at pH 7.0. One unit of esterase activity was defined as the amount of enzyme that produced 1 μmole of ρ-nitrophenol per minute. All of the enzyme assays were determined to be linear with respect to time and protein concentration. The enzyme activity using 2 μg of protein was calculated for all the substrate specificities through the use of the following equation (Deshpande et al. 1984):

$$\text{U mg}^{-1} = (\Delta A_{420\text{nm}})(\text{vol})/(\text{Ec})(\Delta T)(\text{mg protein})$$

where ΔAbs_{420nm} is the change in absorbance at 420 nm, vol is the volume of the assay, Ec is the molar extinction coefficient of 18.5 ml μmol^{-1} cm^{-1} for ρ-nitrophenol produced, ΔT is the change in time that is expressed in minutes, and mg protein is the amount of enzyme in the milligrams assayed. All assays were performed in triplicate.

Enzymatic activity was assayed at room temperature at various pH values (4.0–11.0) to determine the optimal pH. The buffers used for the pH ranges 4.0–6.0, 6.0–7.0, and 7.0–11.0 were 50 mM sodium acetate, 50 mM phosphate, and 50 mM Tris–HCl.

Results

Characterization of bacterial isolate

Lipolytic bacteria associated with the decomposition of swine carrion were investigated previously (Howard et al. 2010). These isolates were screened for their ability to degrade polyurethane. One of the isolates, P7, was observed to degrade polyurethane and was further investigated in this study. The ability of this microbe to degrade Impranil DLN™ was tested by growth on a LB agar plate that was supplemented with 1% Impranil DLN™. After incubation at 30°C for 48 h, the plate was observed for zones of clearing (Fig. 1).

Data obtained from a BLAST analysis that employed a 16S rDNA sequence (1,404 nt) closely matched the P7 isolate to the type strain *Acinetobacter gerneri* 9A01 (AF509829) (98%) and DSM 14967 (EF611410) (99% identity). Phylogenetic and molecular evolutionary analyses implied that isolate P7 is more closely related to *A. gerneri* (accession # AF509829) than to *A. grimontii* (accession # AM410706). In addition, the results indicate P7 is clustered with the environmental *Acinetobacter* spp. rather than those that are implicated with clinical infections, the so-called *A. calcoaceticus-A. baumannii* complex (Fig. 2).

Isolate P7 was determined to be a gram-negative, oxidase negative, catalase positive, coccobacillus that

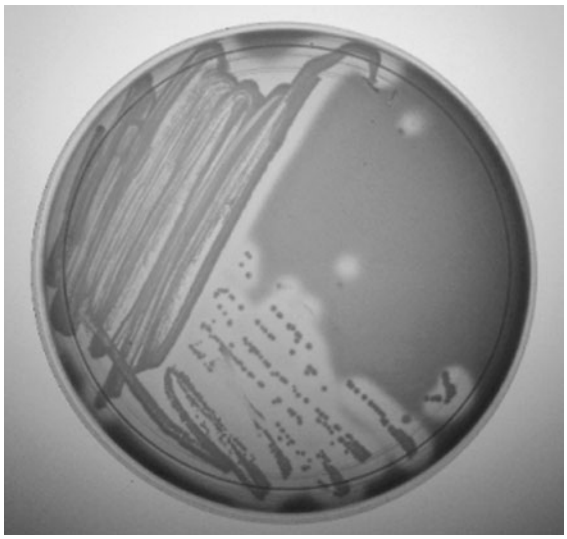

Fig. 1 Growth of *Acinetobacter gerneri* P7 on a LB plate supplemented with 1% Impranil DLN™ with zones of clearing around the edges of the colony showing polyurethane degradation

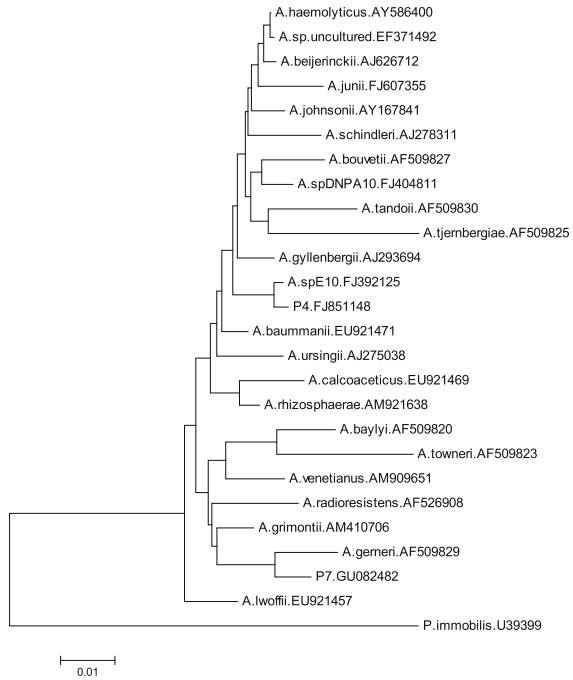

Fig. 2 Phylogenetic and molecular evolutionary analyses of 16S rDNA sequences of *Acinetobacter* species. Analysis was conducted using MEGA version 4 (Tamura et al. 2007). DNA sequences were aligned using the Greengenes program (DeSantis et al. 2006). *Acinetobacter* sequences available from the GenBank database (Altschul et al. 1997) were used. *Pseudomonas immobilis* was used as out-group for the rooted tree. Phylogenetically distinct clusters were assigned based on a bootstrap value >50%

ranged from 1.0 to 1.5 μm by 1.5 to 2.5 μm in its dimensions. The microbes were typically found in pairs or in clusters and did not display the presence of flagella. A phenotypic description of P7 matched that of the known type strain *A. gerneri* 9A01 and was distinguished from that of type strain *A. grimontii* 17A04 as noted in Table 1. Growth for isolate P7 occurred at 30, 37 and 41°C. Isolate P7 hydrolyzed the following: Tween 40, Tween 80, Wesson oil, pyruvate, acetate, α-ketobutyric acid, α-ketoglutaric acid, D-L-lactate, propionate, succinate, proline, L-phenylalanine, phenylacetate, and citrate. In addition, isolate P7 was unable to metabolize gelatin, sheep's blood, L-histidine, L- leucine, and L-aspartate.

Growth of bacterial isolate on polyurethane

The complexity of the bacteria-polyurethane interaction was more apparent when the *Acinetobacter* was grown on polyurethane in a liquid media. Growth of the bacterium on polyurethane as the sole carbon source in defined YES medium was evident but slow, on the order of days. Growth of the bacterium on polyurethane in the presence of peptone and/or yeast extract in normal YES medium was much faster, on the order of hours. In either instance, prolonged incubation resulted in the appearance of a chalky precipitate of residual mass that appeared resistant to further degradation. A Coulter Multisizer IIe was used to measure the change in bacterial cell number as the substrate was being utilized (Fig. 3). When grown on a 1% Impranil DLN™ YES medium, a lag phase growth was noted for the first 3 h, which was followed by logarithmic growth for 5 h that resulted in an ultimate cell density of $66.682 \times 10^6 \pm 2.237 \times 10^5$. After 9 h of incubation, the cell number dramatically decreased to $15.005 \times 10^6 \pm 2.485 \times 10^5$. For the linear portion of growth between 5 and 9 h, a μ value of 0.413 doublings h^{-1} was calculated.

Bacterial cell: polyurethane complex formation

Acinetobacer gerneri P7 formed a chalky precipitate when grown on polyurethane liquid medium, indicating cell binding to the PU colloidal particles.

Table 1 Phenotypic characteristics of isolate P7 and other *Acinetobacter* type species

Characteristic	P7	*gerneri*	*grimontii*	*baylyi*	*towneri*	*bouvetii*	*tandonii*	*tjernbergiae*
41°C	+	+	+	+	+	+	−	−
37°C	+	+	+	+	+	+	+	−
Gelatin hydrolysis	−	−	−	−	−	−	−	−
Hemolysis: sheep blood	−	−	−	−	−	−	−	−
Acid from glucose	+	+	+	+	−	−	−	−
Utilization of:								
L-Phenylalanine	+	+	−	−	−	−	+	−
L-Histidine	−	−	+	+	−	+	+	+
L-Aspartate	−	−	−	+	−	+	+	+
L-Leucine	−	−	−	−	−	−	−	−
b-Alanine	+	+	−	−	−	−	−	−
Phenylacetate	+	+	−	+	−	−	−	−
Citrate	+	+	+	+	−	−	−	−
Tween 40	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+
Succinate	+	+	+	+	+	+	+	+
Proline	+	+	+	+	+	+	+	+

Phenotypic description of type species from Carr et al. (2003)

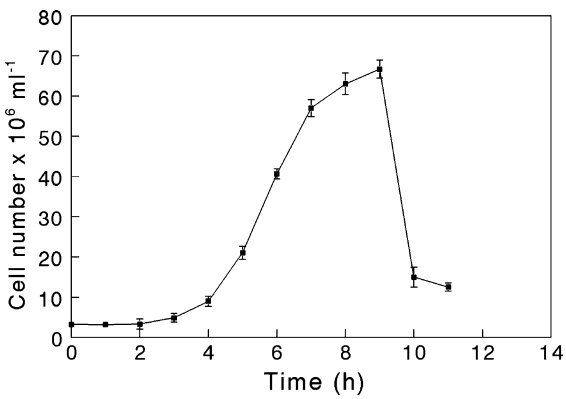

Fig. 3 Bacterial growth of *Acinetobacter gerneri* P7 in YES media supplemented with 1% (wt v^{-1}) Impranil DLN™ monitored with a Coulter Multisizer IIe instrument fitted with a 30 μm aperture. Growth studies were run in triplicate

Measurements of electrophoretic mobility (Table 2) revealed *A. gerneri* P7 had a net negative charge that corresponds to a zeta potential of −13.32 ± 1.06 mV and a particle size of 1,211.83 ± 76.86 nm. Colloidal polyurethane had a strongly negative charge with a zeta potential of −45.60 ± 1.51 mV and a particle size of 114.07 ± 1.22 nm. Complex formation between the PU and cells after 1 day resulted in a zeta potential of −17.60 ± 1.06 mV with an overall particle size of 1,455.7 ± 92.48 nm. After 3 days of exposure, an increase in the zeta potential of −19.32 ± 1.22 mV was observed with an overall particle size of 1,758.67 ± 72.61 nm. Further evidence for a complex formation between the PU and P7 cells was obtained by scanning electron microscopy (Fig. 4). The solution of polyurethane consisted of amorphous particles of various dimensions (Fig. 4a). Cells of P7 were typically found in pairs or in clusters that ranged from 1.0 to 1.5 μm by 1.5 to 2.5 μm (Fig. 4b). After 24 h of exposure with PU, clusters of P7 cells were detected on individual polyurethane particles (Fig. 4c) and on the surface of large aggregates of the particles. Occasionally, anchor-like appendages linked microbes to polyurethane particles. After 2 days of exposure, the polyurethane appeared to be in a more homogeneous state; however, individual particles were still detected. In a manner similar to day 1, the microbes were clustered along the surface of

Table 2 Electrokinetic measurements of bacterial complex formation between P7 cells and Impranil

Media sampled	Zetapotential (mV)	Electophorectic mobility (cm^3 v s^{-1})	Particle size (nm)
Impranil	−45.60 ± 1.51	−3.557 ± 0.117	114.07 ± 1.22
Acinetobacter P7 cells[a]	−13.32 ± 1.06	−1.082 ± 0.083	1,211.83 ± 76.86
Day 0[b]	−20.79 ± 1.31	−1.621 ± 0.102	113.2 ± 3.0
Day 1[b]	−17.60 ± 1.06	−1.373 ± 0.057	1,455.7 ± 92.48
Day 2[b]	−18.72 ± 0.29	−1.453 ± 0.034	1,791.03 ± 65.66
Day 3[b]	−19.32 ± 1.22	−1.506 ± 0.096	1,758.67 ± 72.61

Electrophoretic mobility was determined with a Beckman-Coulter Delsa Nano C Particle Analyzer (Fullerton, CA). Readings were taken in triplicate with an accumulation time of 10 s each, and the instrument was set for a water based solution

[a] *Acinetobacter* P7 was grown in LB broth overnight

[b] Samples analyzed were *Acinetobacter* P7 grown either for 1, 2, or 3 days in YES medium supplemented with 1% (wt v^{-1}) polyurethane

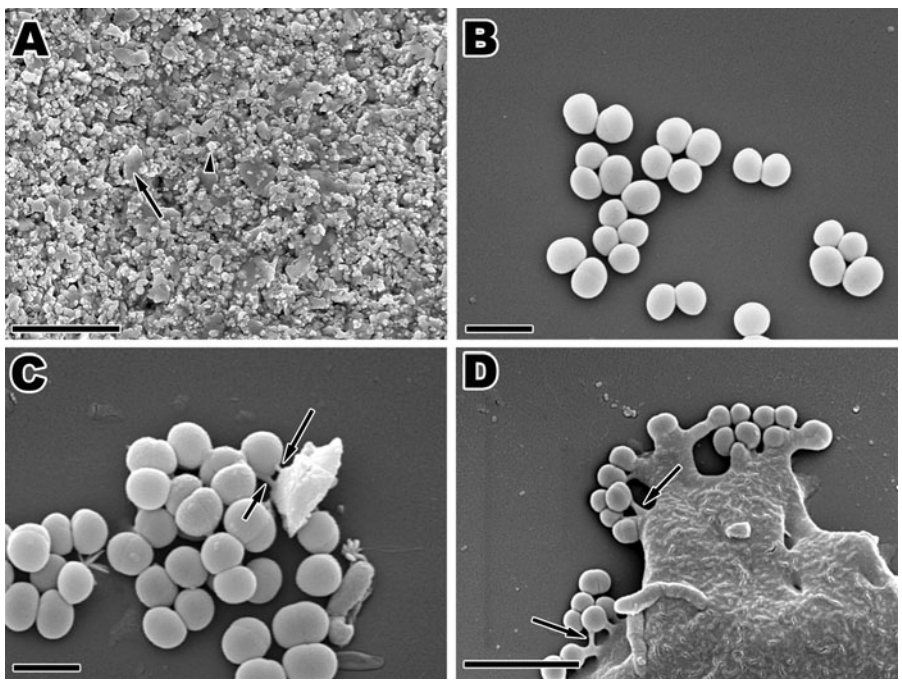

Fig. 4 Microscopic evidence revealing *Acinetobacter gerneri* P7 and polyurethane form a complex. **a** Scanning electron micrograph of Impranil (polyurethane) before exposure to bacterial cells. *Line scale* represents a length of 3 μm. **b** Scanning electron micrograph of bacterial cells before exposure to polyurethane illustrating rod shape of 1.3 microns in length and which usually cluster in pairs. *Line scale* represents a length of 1 μm. **c** Scanning electron micrograph of bacterial culture following a 1 day exposure to polyurethane. *Line scale* represents a length of 1 μm. **d** Scanning electron micrograph of bacterial culture following a 2 day exposure to polyurethane. *Line scale* represents a length of 2 μm

the particles and especially along the edges of polyurethane films. Anchor-like appendages extended from some of the microbes to the polyurethane (Fig. 4d).

Protein purification and characterization

The PUase enzyme from *A. gerneri* P7 was purified to apparent electrophoretic homogeneity as indicated by

the appearance of a single band on a SDS-PAGE gel (Fig. 5). A plot of log MW versus R_f for the SDS-PAGE gel indicated the presence of a 66 kDa protein. The purified enzyme displayed PU degradation as revealed by a radial diffusion assay where Impranil was used as the substrate (Fig. 6). In addition to the PUase activity, catalytic properties of the purified protein revealed esterase activity. Through the use of ρ-nitrophenylacetate as an esterase substrate, the purified protein displayed a specific activity of 16.31 ± 1.77 U mg^{-1}. The enzyme also exhibited activity against ρ-nitrophenylpropionate (37.58 ± 0.21 U mg^{-1}), ρ-nitrophenylbutyrate (14.3 ± 0.54 U mg^{-1}), while no activity was detected for either ρ-nitrophenylcaproate or ρ-nitrophenylcaprylate (Fig. 7). The enzyme activity was heat labile after 10 min at 100°C and the esterase activity was inhibited 50% by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). In addition, the enzyme was inhibited by the addition of 3 mM EDTA (0.35 ± 0.1 U mg^{-1}), while the addition of 5 mM $CaCl_2$ increased enzyme activity (43.61 ± 3.51 U mg^{-1}).

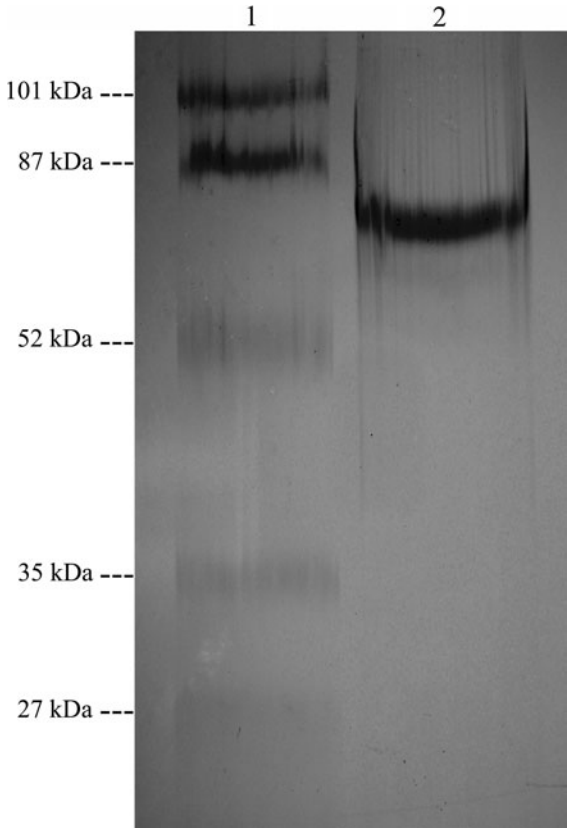

Fig. 5 Silver stain of a SDS-PAGE gel of the purified protein. Lane 1 is the molecular weight marker BioRad low range standards and lane 2 is 5 μg of the purified protein

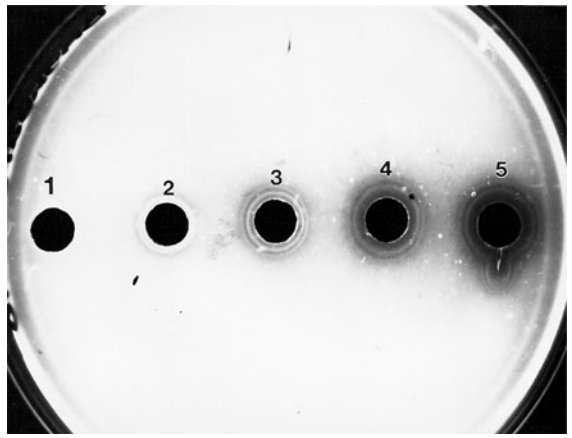

Fig. 6 Raidal diffusion assay preformed on different protein fractions Zones of clearing depict various degrees of polyurethanase activity. Protein fractions 4 and 5 were pooled and used for further purification and analysis

Using 8 mM ρ-nitrophenylpropionate as a substrate, the purified enzyme was assayed at various temperatures and pH. When the purified enzyme was assayed in 50 mM phosphate buffer and at a pH of 7.0, the optimum temperature was determined to be 37°C (90.77 ± 0.14 U mg^{-1}) (Fig. 8). Enzyme activity was detected between 25°C (37.58 ± 0.21 U mg^{-1}) and 55°C (17.02 ± 0.13 U mg^{-1}). In addition, the optimum pH was determined to be 8.0 at 25°C

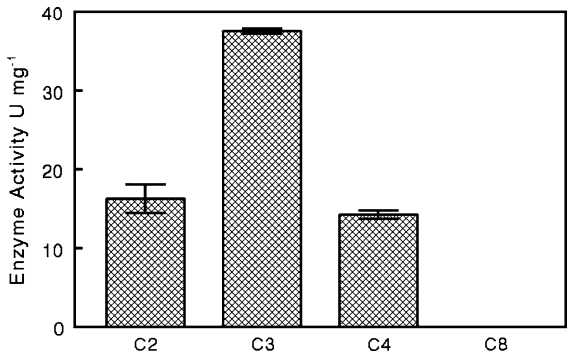

Fig. 7 Chain length selectivity of the PUase from *A. gerneri* P7. Activity measurements were performed using *p*-nitrophenyl esters with different chain lengths (8 mM *p*-nitrophenyl ester, 50 mM phosphate buffer, pH 7.0 at 25°C). Substrates used were: ρ-nitrophenyl-acetate (C2), ρ-nitrophenyl-propionate (C3), ρ-nitrophenyl-butyrate (C4), and ρ-nitrophenyl- caprylate (C8). Activities are the average performed in triplicate

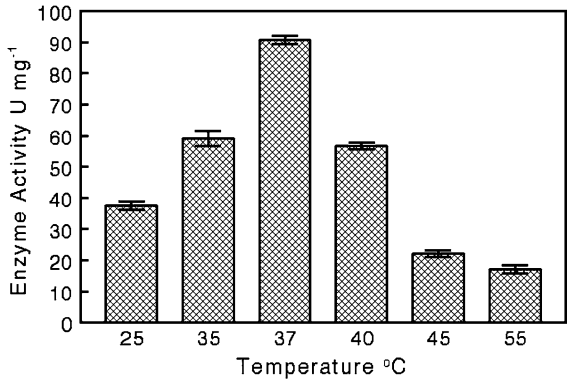

Fig. 8 Effect of temperature on the PUase from *A. gerneri* P7. The effect of temperature on enzyme activity was determined spectrophotometrically at various temperatures (8 mM *p*-NP propionate as the substrate in 50 mM phosphate buffer, pH 7.0). Activities are the average performed in triplicate

(75.17 ± 0.4.01 U mg^{-1}). Enzyme activity was not detected at pH 10.0 (Fig. 9).

Discussion

Acinetobacter spp. are gram-negative oxidase negative, catalase positive, non-motile, non-fermentative coccobacilli easily isolated from soil, water, sewage, human skin and many foodstuffs (Towner 1997; Knight et al. 1995). It has been estimated that at least 0.001% of the total cultivable heterotrophic, aerobic population in soil and water are acinetobacters (Baumann 1968). Acinetobacters resemble saprophytic pseudomonades in their ability to use a wide variety of organic compounds as carbon and energy sources, including many aromatic compounds. Members of the genus *Acinetobacter* are ubiquitously distributed in nature. While the majority of strains of the described species have been isolated from clinical sources, many also include environmental strains. Overall, the ecology of species belonging to the genus *Acinetobacter* is not well elucidated. Taxonomically, the genus *Acinetobacter* has a rather long and complicated history. According to the description assigned to each genomic species (Bouvet and Grimont 1986), Genospecies 2 was named *A. baumannii*, Genospecies 4 was named *A. haemolyticus*, Genospecies 5 was named *A. junii*, and Genospecies 7 was named *A. johnsonii*. The relationship among these groups based on 16 s rRNA-gene sequence analysis revealed that genomic species 4 and 7 clustered together and genomic species 2 and 5 clustered together (Ibrahim et al. 1997).

In a previous study (Howard et al. 2010), the microbial diversity, succession, and lipolytic activity of the soil bacterial community associated with a swine carcass throughout its decomposition was investigated. *Acinetobacter* was found to be the predominate microbe isolated in that study, with 55% of the isolates identified as *Acinetobacter*. One of the isolates from that study, P7, was observed to have the capacity to degrade polyurethane. To the best of our knowledge, the only other report concerning PU degradation by an *Acinetobacter* sp. is by El-Sayed et al. (1996), who isolated several species of bacteria capable of degrading PU military aircraft paint, one of which was identified as *Acinetobacter calcoaceticus.* The present study is designed to characterize and definitively identify isolate P7 and to elucidate the polyurethane-degrading system of this microbe.

Phenotypic description of P7 matches that of the known type strain *Acinetobacter gerneri* 9A01 (Carr et al. 2003), and BLAST analysis using the 16S rDNA sequence closely matched the P7 isolate with *A. gerneri* (99% identity). Phylogenetic and molecular evolutionary analyses indicates that isolate P7 is closely related to *A. gerneri* (accession # AF509829) and clusters with the environmental *Acinetobacter* spp. rather than the more clinically relative so-called *A. calcoaceticus-A. baumannii* complex. Based upon phenotypic and phylogenetic characterization, the polyurethane degrading isolate is designated as *Acinetobacter gerneri* Strain P7.

Several studies have reported on the ability of three pseudomonades to utilize polyester PU as the sole source of carbon and energy. *Pseudomonas chlororaphis* exhibited a lag phase growth for the first 3 h then was followed by logarithmic growth for 6 h with a μ_{max} of 1.32 doublings h^{-1} and a cell density of $2.31 \times 10^9 \pm 0.87$ cells ml^{-1} (Howard et al. 1998). Two genes encoding polyurethanase activity from *P. chlororaphis* have been cloned in *Escherichia coli* (Stern and Howard 2000; Howard et al. 2001). Upon cloning and expressing PueA and PueB from *P. chlororaphis* in *E. 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 participate (Jaeger et al. 1994; Persson et al.

1989; Winkler et al. 1990). *Pseudomonas fluorescens* exhibited similar results, with cellular growth observed for the first 9 h with a μ_{max} of 1.61 doublings h^{-1}, reaching a cell density of $3.05 \times 10^9 \pm 0.14$ cells ml^{-1} (Howard and Blake 1999). One gene encoding a 48 kDa polyurethanase has been cloned and expressed in *E. coli* (Vega et al. 1999). The PulA amino acid sequence also showed a high identity with Group I lipases. Nakajima-Kambe et al. (1995, 1997) reported a strain of *C. acidovorans* that could utilize solid polyester PU as the sole source of carbon and nitrogen. These authors describe the role of a membrane-bound esterase (62 kDa) in PU degradation (Akutsu et al. 1998). The structural gene, *pudA*, for the PU esterase was cloned in *E. coli* and the predicted amino acid sequenced displayed characteristics of serine hydrolases.

Growth of *Acinetobacter gerneri* P7 is unlike those reported for the pseudomonades. The results observed in the present study are more similar to those reported by Blake and Howard (1998) for a species of *Bacillus*. The pattern of degradation involved the binding of cells to the polymer with a subsequent flocculant formation. Growth of *A. gerneri* P7 and of *Bacillus* sp. on a solid medium resulted in the visual disappearance of the polyurethane. The complexity of the bacteria-polyurethane interaction was more apparent when the microbes were grown on a polyurethane liquid medium. Incubation of the *Bacillus* sp. in media supplemented with polyurethane resulted in the appearance of a chalky precipitate that appeared to be resistant to further degradation. A follow up study (Rowe and Howard 2002) revealed that when grown on 1% Impranil DLN™, a lag phase growth was noted for the first 5 h which was followed by logarithmic growth for 8 h, reaching a cell density of $2.60 \times 10^8 \pm 1.17$ cells ml^{-1}. *A. gerneri* P7 also forms a chalky precipitate when grown on polyurethane liquid medium. When grown in liquid medium containing Impranil, *A. gerneri* P7 displayed a lag phase growth for the first 3 h, which was followed by logarithmic growth for 5 h and resulted in an ultimate cell density of $66.682 \times 10^6 \pm 2.237 \times 10^5$. An analysis of the electrophoretic mobility of *A. gerneri* P7 indicates that the *A. gerneri* P7 has a net negative charge that corresponds to a zeta potential of −13 mV and a particle size of 1.2 μm; whereas, colloidal polyurethane has a strongly negative charge with a zeta potential of −45 mV and a particle size of 114 nm. Complex formation, after 3 days exposure between PU and cells, results in a zeta potential of −19 mV and an overall particle size of 1.76 μm. Blake and Howard (1998) reported that *Bacillus* cells have a relatively weak net negative charge corresponding to a zeta potential of −6 mV. Colloidal polyurethane has a strongly negative charge with a zeta potential of −42 mV. Complex formation between the PU and cells results in a zeta potential of −20 mV. These electrophoretic mobility data indicate that the peaks that are associated with the free polyurethane and the free bacterial cells are replaced by a single peak that possesses the size and charge properties anticipated for a complex of bacterial cells with the strongly negatively charge polyurethane. This should result in polyurethane-coated cells, which are not free in solution and therefore not detectable.

Ishii et al. (2004) noted the ability of *Acinetobacter* sp. Strain Tol 5 to adhere to the substratum of polyurethane in the form of multilayer cell clusters. The authors suggest that the strong adhesive property of Tol 5 is caused by "co-adhesion", the subsequent adhesion of cells to cells that has adhered to the polyurethane substratum. Both anchor-like structures and peritrichate fibrils extended from the Tol 5 cells. Images obtained by scanning electron microscopy indicate that *A. gerneri* P7 cells adhere to polyurethane that is present in both a particulate form and as a film. In a manner that is similar to that described for *Acinetobacter* sp. Strain Tol 5 microbes that adhere to polyurethane through anchor-like structures, the *A. gerneri* P7 microbes are also capable of attaching to the polyurethane substrate through anchor-like extensions, although the vast majority of cells adhere directly to the polyurethane without the assistance of an anchor.

A 66 kDa protein purified from *A. gerneri* P7 during this investigation displays PUase activity as determined from a radial diffusion assay which reveals a zone of clearing with Impranil serving as the substrate. In addition, the enzyme exhibits esterase activity with the highest activity towards *ρ*-nitrophenylpropionate (37.58 ± 0.21 U mg^{-1}). The enzyme is inhibited by PMSF, thus indicating that this protein is similar to Group I lipase that contains a serine hydrolase motif as observed in the pseudomonad PUases. Also, the enzyme activity is inhibited by EDTA and Ca^{+2} ions do increase activity. In addition, the enzyme activity is heat labile unlike that observed

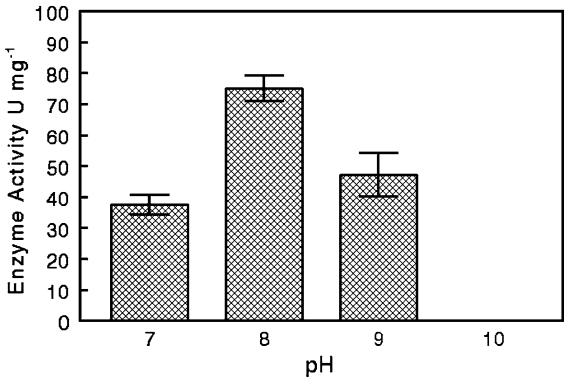

Fig. 9 pH effect of substrate hydrolysis. Activity measurement was performed spectrophotometrically in various buffer containing 3 mM *p*-NP propionate as a substrate at different pH values and at 25°C. Activities are the average performed in triplicate

for the pseudomonads (Stern and Howard 2000; Howard et al. 2001). The characteristics of this protein are unlike previous lipase enzymes studied from other *Acinetobacter* sp. whose molecular mass is smaller, has optimum temperatures of 55°C and at a pH of 10 (Kok et al. 1993, 1995a, b; Sullivan et al. 1999; Han et al. 2003). *Acinetobacter* spp. has gained increased recognition in recent years as a pathogenic microbe which has the potential to cause severe nosocomial infections in critically ill patients (Bergogne-Bérézin and Towner 1997). Strains from genomic species 2 (*Acinetobacter baumannii*), 3, and 13 sensu Tjernberg and Ursing (13TU) (Berlau et al. 1999) are frequently isolated from clinical specimens and are often associated with nosocomial outbreaks (Bergogne-Bérézin and Towner 1997). They are grouped together with genomic species 1 (*Acinetobacter calcoaceticus*), in the so-called *A. calcoaceticus-A. baumannii* complex (Gerner-Smidt 1992; Gerner-Smidt et al. 1991). Other *Acinetobacter* strains are also infrequently isolated from patients, although both *Acinetobacter junii* and *Acinetobacter johnsonii* have been reported to be involved in cases of septicemia (Bernards et al. 1997; Seifert et al. 1993). *Acinetobacter baumannii* has emerged as one of the more troublesome pathogens for health care institutions globally. Its clinical significance, especially over the last 15 years, has been propelled by its remarkable ability to up-regulate or acquire resistance determinants, making it one of the microbes threatening the current antibiotic era. *Acinetobacter baumannii* strains that are resistant to all known antibiotics have now been reported, signifying a sentinel event that should be acted upon promptly by the international health care community (Peleg et al. 2008). The results of the present study indicate the ability of an *Acinetobacter* spp. to bind and degrade polyurethane, which signifies a potential form of infection caused by these opportunistic pathogens in conjunction with medical devices constructed from polyurethane.

Acknowledgments This research was funded by the National Science Foundation (EPSCoR #53279) and by the Southeastern Louisiana University Research Initiative Program.

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