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Three *Pseudomonas aeruginosa* strains with different protease profiles

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The proteolytic activity of three *Pseudomonas aeruginosa* strains, ATCC 27853 — a reference strain, and two clinical isolates was tested. The activity was examined after culturing the bacteria in two different growth media: the minimal M9 medium and rich Luria-Bertani broth (LB). Based on zymograms and protease activity specific assays, it was concluded that the reference strain produced three proteolytic enzymes in the LB medium: protease IV, elastase B and elastase A, while alkaline protease was only produced in the M9 medium. The clinical isolates of *P. aeruginosa* produced elastase B and alkaline protease when grown in the LB medium and the minimal M9 medium, respectively. PCR analysis confirmed the presence of both the *lasB* gene encoding elastase B and *aprA* coding for alkaline protease in the genomes of the three *P. aeruginosa* strains analyzed. The expression of these genes coding for two important *P. aeruginosa* virulence factors was dependent on the growth conditions in all the strains studied. The contribution of the extracellular proteinases to the virulence of *P. aeruginosa* strains used in this study was investigated using an insect model, the greater wax moth *Galleria mellonella*.

Key words: *Pseudomonas aeruginosa*, extracellular proteases, elastase B, alkaline protease, *lasB*, *aprA*, virulence

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INTRODUCTION

Pseudomonas aeruginosa is a common environmental Gram-negative opportunistic pathogen of diverse hosts including mammals, insects, nematodes and plants. The bacterium has been implicated in various human diseases, e.g. keratitis, pneumonias and burn wound infections (Dart & Seal, 1988; Jarvis & Martone, 1992; Pruitt *et al*., 1998). Different strains of *P. aeruginosa* secrete several extracellular proteolytic enzymes that have been implicated as virulence factors. They include protease IV, alkaline protease (aeruginolysin), and two elastases, LasA (staphylolysin) and LasB (pseudolysin) (Caballero *et al*., 2001). The proteases promote development of the bacteria within the infected host and interfere with the host immune system (Jarosz, 1995; Jin *et al*., 1996; Miyoshi *et al*., 2002; Hung *et al*., 2005; Hoge *et al*., 2010).

Elastase A (LasA) is a metalloproteinase with a molecular mass of approximately 22 kDa belonging to the β-lytic family of Zn-metalloendopeptidases. LasA possesses a high staphylolytic activity that lyses *Staphylococcus aureus* cells by cleaving the pentaglycine bridges within the peptidoglycan and may also enhance elastolysis by cleaving Gly-Gly peptide bonds abundant in elastin (Peters & Galloway, 1990; Toder *et al*., 1991; Kessler *et al*., 1993, 1997). This protease has been suggested as a secreted *P. aeruginosa* virulence factor in animal models of corneal and lung infections. LasA is responsible for shedding of the host cell surface proteoglycan syndecan-1 (Preston *et al*., 1997; Park *et al*., 2000).

Elastase B is a 33-kDa endopeptidase belonging to the thermolysin family (M4 peptidase); it hydrolyses the peptide bond at the amino side of the P1' amino acid residue, usually a hydrophobic one (Morihara *et al*., 1965; Matthews, 1988; Thayer *et al*., 1991; Miyoshi & Shinoda, 2000). Elastase B is involved in pathogenesis by degradation of human immunologically competent molecules. It is proven that LasB destroys complement components (Schultz & Miller, 1974), cytokines (Parmely *et al*., 1990), immunoglobulins IgA and IgG (Buret & Cripps, 1993; Maeda & Yamamoto, 1996), human airway lysozyme (Jacquot *et al*., 1985), proteinase-activated receptors (Dulon *et al*., 2005) and surfactant proteins A and D (Marienchecк *et al*., 2003).

Another protease involved in the virulence of *P. aeruginosa* is a metalloprotease, alkaline protease, which belongs to the serralysin family (aeruginolysin, APR) (Morihara *et al*., 1973; Okuda *et al*., 1990). Structural comparison has revealed that APR is homologous to the 50-kDa metalloproteinases secreted by *Serratia marcescens* and *Erwinia chrysanthemi* (Maeda & Morihara, 1995). Alkaline protease degrades casein and gelatin, and migrates in zymography gels as a protein of approximately 56 kDa (Caballero *et al*., 2001). APR is implicated in hydrolysis of many biologically important proteins including the α_1-proteinase inhibitor (Morihara *et al*., 1979), cytokines (Parmely *et al*., 1990), complement factors (Hong & Ghebrehiwet, 1992), laminin (Heck *et al*., 1986), matrix metalloproteinases (Twining *et al*., 1993), human γ-interferon and tumor necrosis factor-α (Horvat & Parmely, 1988; Parmely *et al*., 1990).

In contrast to the three metalloproteases mentioned above, protease IV is a serine endoprotease with a molecular mass of 26 kDa. The enzyme specifically cleaves substrates on the carboxyl side of lysine residues. Protease IV is capable of degrading a number of biologically important proteins of the mammalian immune system including immunoglobulins, elements of the complement system, fibrinogen and plasminogen (Engel *et al*., 1998).

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Abbreviations: APR, alkaline protease; EDTA, ethylenediaminetetraacetic acid; LB, Luria-Bertani broth; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TLCK, tosyl-L-lysine chloromethylketone

The purpose of the present study was to characterize the proteolytic profile of two clinical isolates of *P. aeruginosa* (PA C124/9 and PA 02/18) and to determine the effects of culture conditions on the production of extracellular proteases by the strains. Our preliminary experiments indicated a relatively low level of total proteinase activity in culture supernatants of these strains. In parallel, as a reference strain, *P. aeruginosa* strain ATCC 27853 from culture collections was investigated. The level of total proteinases as well as specific activities of elastase A, elastase B, protease IV and alkaline protease produced by the tested strains under defined culture conditions were quantitatively examined. In addition, identification of *lasB* and *aprA* genes, encoding elastase B and alkaline protease, respectively, in the genomes of the strains was performed. To evaluate the contribution of the detected proteinases to the virulence of the clinical isolates, an insect model, the greater wax moth *Galleria mellonella,* was used. Larvae of this lepidopteran insect have been exploited as a model in numerous studies on virulence factors of different pathogens, including bacteria such as *P. aeruginosa* (Dunphy *et al*., 1986; Miyata *et al*., 2003), *Francisella tularensis* (Aperis *et al*., 2007), *Staphylococcus aureus* (Garcia-Lara *et al*., 2005), *Enterococcus fecalis* (Park *et al*., 2007), *Burkholderia cepacia* (Seed & Dennis, 2008), *Listeria monocytogenes* (Mukherjee *et al*., 2010) and *Legionella pneumophila* (Harding *et al*., 2012). Considerable analogies were reported between the virulence of *P. aeruginosa* in mice and *G. mellonella* larvae (Jander *et al*., 2000).

MATERIALS AND METHODS

Bacterial strains. A pyocyanin-producing *Pseudomonas aeruginosa* strain ATCC 27853, i.e. an isolate of moderate virulence to the 7th instar larvae of the greater wax moth *Galleria mellonella* (LD_{50} = 17 cells), and two clinical strains, PA C124/9 (PA9) and PA 02/18 (PA18) provided by Prof. E. A. Trafny (Department of Microbiology and Epidemiology, Military Institute of Hygiene and Epidemiology in Warsaw, Poland) were used in the study. The bacteria were grown overnight at 37°C in Luria-Bertani broth (LB broth, Sigma) or M9 minimal medium supplemented with monosodium glutamate (0.13 M), glycerol (0.1 M) and $CaCl_2$ (0.01 M).

Preparation of culture supernatants. In order to obtain culture supernatants, 5 ml of culture medium (LB or M9) in a tube was inoculated with a loop of bacterial cells and the bacteria were grown overnight at 37°C under constant rotation at 120 rpm. Then, supernatants were obtained by centrifugation at 8000 × *g* for 10 min at 4°C. The supernatant samples were stored at –20°C. The respective supernatants were determined: ATCC LB, PA9 LB and PA18 LB (strains ATCC, PA9 and PA18 grown in LB medium) and ATCC M9, PA9 M9 and PA18 M9 (strains ATCC, PA9 and PA18 grown in minimal medium M9).

Determination of total protease activity. Total protease activity of the culture supernatants was estimated using azocasein as a substrate with a modified method described by Kessler *et al.* (1982). The assay mixture contained 0.25 ml of azocasein (Fluka) (5 mg/ml) dissolved in water and 0.25 ml of the culture supernatant. After incubation at 37°C for 60 min, the reaction was stopped by addition of trichloroacetic acid (10%, 0.5 ml). Precipitated azocasein was then pelleted at 20000 × *g* for 10 min. The supernatant obtained (0.75 ml) was mixed with NaOH solution (0.5 M, 0.375 ml) and absorbance was determined at 450 nm using a Smart-Spec™3000 spectrophotometer (Bio-Rad). One unit (U) of proteolytic activity was defined as equivalent to an absorbance increase by 0.02 per h at 450 nm.

Elastin Congo red assay. The elastolytic activity of the culture supernatants was determined using elastin Congo red (ICN, Biomedicals Inc.) as a substrate with a modified method described by Caballero *et al.* (2001). Elastin Congo red (5 mg) in 0.49 ml of 10 mM Tris/HCl, pH 8.0 with or without EDTA (10 mM) was mixed with 0.01 ml of the culture supernatant. The reaction mixture (0.5 ml) was incubated at 37°C for 24 h, centrifuged at 10000 × *g* for 15 min and the absorbance was recorded at 490 nm using a Smart-Spec™ 3000 spectrophotometer.

Staphylolytic activity assay. Staphylolytic activity of the supernatants was tested with a procedure of Caballero *et al.* (2001). *Staphylococcus aureus* strain ATCC 25923 was cultured in LB overnight at 37°C and the bacteria were pelleted and resuspended in 0.25 ml of 25 mM of diethanolamine, pH 9.5. After heating at 100°C for 10 min, the heat-killed *S. aureus* cells were diluted to a final OD_{595} of 1.0. The assay was carried out at 37°C by adding 0.01 ml of the supernatant mixed with the diethanolamine buffer (0.09 ml) to 0.4 ml of the heat-killed cell suspension. Staphylolysis was determined by measuring the change in OD_{595} every 30 min for 90 min using a Smart-Spec™ 3000 spectrophotometer.

Protease IV assay. Protease IV activity of the culture supernatants was specifically measured using the chromogenic peptide Chromozym PL (tosyl-Gly-Pro-Lys-*p*-nitroanilide, Sigma) and a modified method described by O'Callaghan *et al.* (1996). The culture supernatants (0.01 ml) were mixed with 40 μg of Chromozym PL in a buffer (0.49 ml) consisting of 50 mM Tris/HCl, pH 8.0, and 150 mM NaCl, with or without 2 mM TLCK (tosyl-L-lysine chloromethylketone, Sigma). The reaction mixture (0.5 ml) was incubated at 37°C for 30 min. Release of *p*-nitroanilide due to cleavage at lysine was measured at 410 nm using a Smart-Spec™3000 spectrophotometer.

Alkaline protease activity assay. The alkaline protease activity was tested using a modified method described by Howe & Iglewski (1984). Samples containing 10 mg of Hide powder azure (Sigma) dissolved in a buffer (0.75 ml) consisting of 20 mM Tris/HCl, pH 8.0 and 1 mM $CaCl_2$ were mixed with 0.25 ml of the culture supernatants. The reaction mixtures (1 ml) were incubated at 37°C for 1 h with constant rotation. The undissolved substrate was removed by centrifugation at 4000 × *g* for 5 min. The absorbance of the reaction mixtures was then determined at 595 nm. Protease activity was expressed in terms of protease units per milliliter (U/ml), where one unit is equivalent to an increase in OD_{595} of 1.0 per h at 37°C.

Zymography analysis of protease profiles. Casein or gelatin zymography was conducted following the procedures described by Caballero *et al.* (2001). Samples of the culture supernatants (30 μl) were electrophoresed under non-reducing conditions using a 10% SDS–PAGE with 0.1% casein or gelatin, at 4°C. The gels were then soaked twice in 2.5% Triton X-100 for 15 min and incubated at 37°C for 24 h in either casein gel substrate buffer (50 mM Tris/HCl, pH 7.6, 100 mM NaCl) or gelatin gel substrate buffer (50 mM Tris/HCl, pH 8.0, 10 mM $CaCl_2$ 1 μM $ZnCl_2$, 150 mM NaCl). The gels were stained for 60 min in 0.2% amido black and then destained in 10% acetic acid.

DNA isolation and PCR amplification. Total genomic DNA was obtained from the three *P. aeruginosa* strains according to the method described by Pitcher *et*

al. (1989) and its concentration was estimated by electrophoresis with a molecular mass DNA marker. PCR amplifications for both elastase B and alkaline protease genes were performed using 100 ng of DNA and RED-Taq Ready Mix PCR Reaction Mix (Sigma) in a 50 μl final volume. Each PCR mixture contained 1.5 U of *Taq*I polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, and 0.4 μM of each primer. Primers lasBF2 (forward, 5'-CCAGCCCGCTGACCCACAAGCTGTA-3') and lasBR1 (reverse, 5'-CATTCCTTCCTGGAGTGCYRGCCG-3') were used to amplify a 650-bp fragment of the *lasB* gene using the following temperature profile: initial denaturation at 94°C for 4 min, 25 cycles of denaturation (1 min at 94°C), annealing (40 s at 54°C) and extension (72°C for 60 s), and a final extension at 72°C for 4 min. The amplification of a 1580-bp-long fragment for the alkaline protease gene *aprA* was carried out using the primer pair aprA-F (forward, 5'-CCTGATCKGGCCGATAACTGCAAT-3') and aprA-R (reverse, 5'-GGAAGACASCTATCAATTCGAACAG-3') and PCR conditions as above, with the exception of the annealing temperature (46°C) and extension time (2 min). PCR products for both genes were separated by 1.5% agarose gel electrophoresis using Tris-borate buffer containing 90 mM Tris base, 90 mM boric acid 90 mM and 2.5 mM EDTA, pH 8.

Determination of lethal activity of *P. aeruginosa* culture supernatants. For *in vivo* experiments, the 7th instar *G. mellonella* larvae ($n = 10$) were injected with different doses of the culture supernatants. LB broth or M9 medium were used for control injections. After the treatment, the larvae were kept at 28°C in the dark on sterile Petri dishes and were observed for the occurrence of death over a period of 48 h. The number of insects dead and alive for each dose was used to calculate the LD_{50} of the tested supernatants according to the arithmetic method of Reed & Muench (1938). The median lethal doses, LD_{50}, were expressed for each bacterial strain as the ratio of total proteolytic activity units/larva (U/larva) producing death of 50% of larvae. The experiment was repeated five times.

Statistical analysis. The data are presented as means ± standard deviation (S.D.) for at least three experiments. In order to compare two means, statistical analysis was performed by Student's *t*-test.

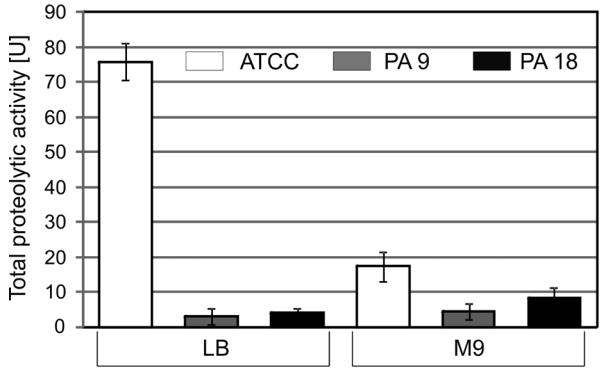

Figure 1. Comparison of total protease activity of culture supernatants of three *P. aeruginosa* strains.
Bacteria were cultured in LB or M9 medium for 24 h at 37°C. Protease activity was measured with azocasein as a substrate (Materials and Methods). All values represent means ± S.D. of at least ten independent experiments. ATCC, reference strain; PA9 and PA18, clinical isolates.

RESULTS

Analysis of the total extracellular protease activity in *P. aeruginosa* culture supernatants

Total proteinase activity in culture supernatants of three *P. aeruginosa* strains, ATCC 27853 — a reference strain, and two clinical isolates was determined. The activity was examined after culturing the bacteria in two different media: the minimal medium M9 and the rich LB broth after 24-h incubation at 37°C. The total proteolytic activity was evaluated with the azocasein assay.

Figure 1 presents the total protease activity produced by *P. aeruginosa* strains grown in the above-mentioned media. The highest proteolytic activity was observed for the ATCC strain growing in both LB and the minimal medium. However, the bacteria growing in LB exhibited a 5-fold higher proteolytic activity than those growing in the M9 medium. Among the clinical strains, the highest activity was observed for the PA18 strain growing in the M9 medium, but it was lower by about 60% than the activity displayed by the ATCC strain in the same medium. In the other samples (PA9 LB, PA18 LB and PA9 M9) only a very low level of activity was observed (approximately 5% of the activity of ATCC LB). These results indicated that the production of extracellular proteases by *P. aeruginosa* was medium- and strain-dependent.

Zymographic analysis of *P. aeruginosa* extracellular proteases

The *P. aeruginosa* culture supernatants were examined by gelatin or casein zymography to differentiate the activities of protease IV, elastase B (LasB), elastase A (LasA) and alkaline protease. As can be seen in Fig. 2, the gelatin zymograms revealed different protease profiles depending on the strain and medium (LB or M9).

Gel electrophoresis (SDS/PAGE) under non-reducing conditions demonstrated three patterns of gelatinase activity in LB culture supernatants based on differences in electrophoretic mobility. One type, represented by the ATCC strain, was characterized by a combination of activities corresponding to molecular masses of > 200 kDa and 160 kDa. Another type, represented by the PA9 strain was characterized by activities at 120 kDa, and the PA18 strain exhibited activity corresponding to an apparent molecular mass of 160 kDa (Fig. 2). It has been reported that protease IV produces a proteolytic band of approximately 200 kDa in gelatin and casein gels, where-

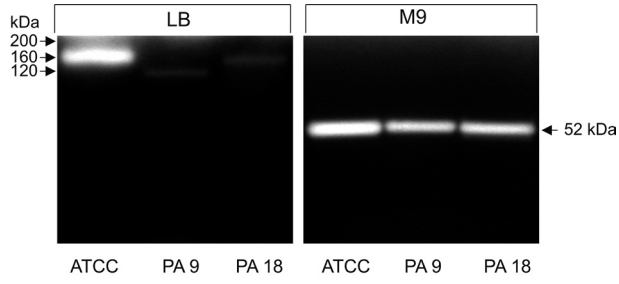

Figure 2. Gelatin zymogram analysis of culture supernatants of *P. aeruginosa* strains grown in LB and M9 medium.
Culture supernatants were electrophoresed under non-reducing conditions in a 10% polyacrylamide gel containing 0.1% gelatin. The gels were washed in Triton X-100, incubated at 37°C for 24 h in specific buffer and stained in amido black as described in Materials and methods. The zymograms shown are typical representatives of at least five independent experiments. ATCC, reference strain; PA9 and PA18, clinical isolates.

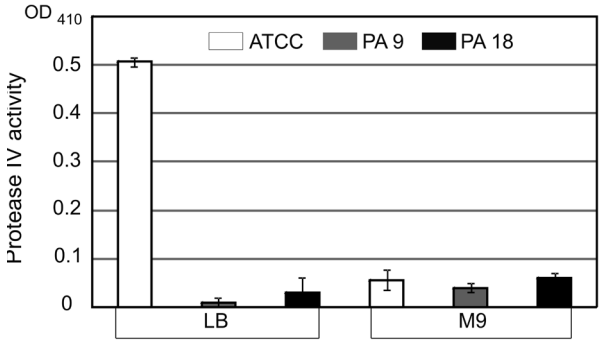

Figure 3. Protease IV activity of culture supernatants of *P. aeruginosa* strains grown in LB and M9 medium.
Culture supernatants were incubated with Chromozym PL as a substrate (see Materials and Methods). Cleavage of the chromogenic peptide was measured by an increase in OD_{410}. All values represent the mean ± S.D. of three independent experiments. ATCC, reference strain; PA9 and PA18, clinical isolates.

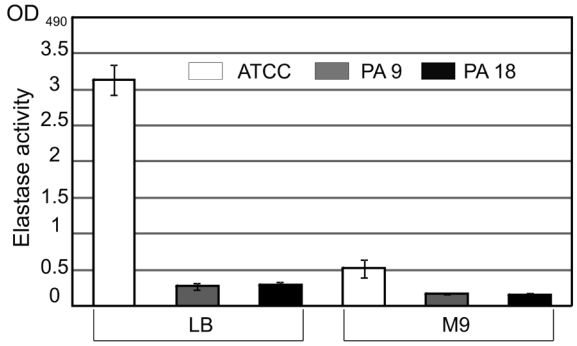

Figure 5. Elastase activity of culture supernatants of *P. aeruginosa* strains.
Culture supernatants were incubated with elastin Congo red as described in Materials and Methods. After pelleting insoluble materials optical density of the solutions was determined at 490 nm. The data presented are derived from five determinations and all values represent the mean ± S.D.

as the band of 160 kDa corresponds to elastase B and/or elastase A activity (Caballero *et al.*, 2001).

In contrast, for the three strains growing in the M9 medium, the zymogram revealed the presence of only one band with an apparent molecular mass of 52 kDa (Fig. 2). It corresponded to the molecular mass of *P. aeruginosa* alkaline protease (Caballero *et al.* 2001). It was concluded that the strains grown in the minimal synthetic medium produced mainly alkaline protease (Fig. 2). Similar results were obtained when the supernatants were examined using casein zymography (data not shown).

Specific enzyme assays

We used specific activity assays to confirm the presence of particular proteases detected in the zymography gels.

The Chromozym PL degradation assay was performed specifically for protease IV. The results obtained clearly indicated that only the LB culture supernatant of strain ATCC exhibited a strong affinity to the chromogenic peptide Chromozym PL, a specific substrate for protease IV (Fig. 3). In the presence of 2 mM TLCK, a selective inhibitor of serine proteases, its activity was completely inhibited (data not shown).

To verify whether the *P. aeruginosa* strains studied secreted LasA, the staphylolytic activity of the supernatants was assayed using heat-killed *S. aureus*. The activity of elastase A was detected only in the LB supernatant of strain ATCC. It was evidenced by a rapid decrease of the optical density of the *S. aureus* suspension during the first 30 min of incubation in the presence of this culture supernatant. The optical density of the other samples remained indistinguishable from that of the control sample (Fig. 4).

On the other hand, the LasB activity identified using elastin Congo red was observed in the culture supernatants of all the *P. aeruginosa* strains studied (Fig. 5). Definitely, the highest activity was detected for the ATCC strain grown in the LB medium. The activity was nearly six-fold higher than that for this strain grown in the M9 medium. Significantly lower elastase B production was observed for the clinical strains in the LB medium, and only minimal cleavage of elastin Congo red was detected in the M9 supernatants of both clinical strains. In the presence of 10 mM EDTA, an inhibitor of metalloproteinases, the activity of the enzyme was completely inhibited (data not shown).

The three *P. aeruginosa* culture supernatants obtained from the minimal medium were analysed by the hide powder azure degradation assay (Materials and Methods). The results indicated that all samples were able to hydrolyze hide powder azure, a substrate for *P. aeruginosa* alkaline protease (Fig. 6). The highest activity was determined in the supernatant of strain ATCC, while in the

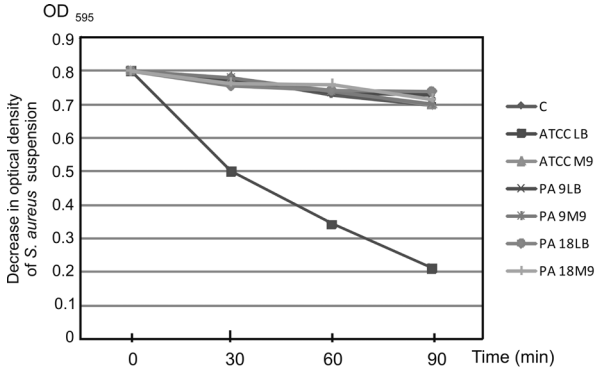

Figure 4. Staphylolytic (LasA) activity of culture supernatants of *P. aeruginosa* strains.
Culture supernatants in diethanolamine buffer were incubated with heat-killed *S. aureus* cell suspension (see Materials and Methods). Lysis of bacterial cells was determined by measuring the change in OD_{595}. The data presented are typical representatives of five determinations. C, control, bacterial cells incubated alone.

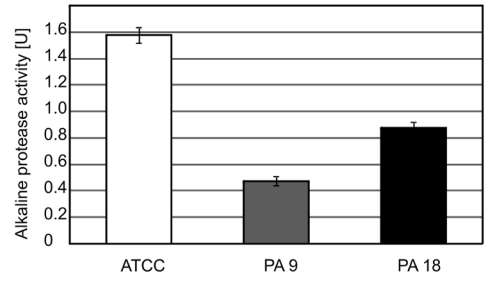

Figure 6. Alkaline protease activity of culture supernatants of P. aeruginosa strains grown in M9 medium.
Samples containing hide powder azure and culture supernatants were incubated for 1 h (Materials and Methods). Proteolytic activity was expressed in terms of protease units per milliliter (U/ml). The data presented are derived from three determinations and all values represent the mean ± S.D.

Table 1. Summary of proteolytic profiles of *P. aeruginosa* strains analyzed in this study.

Medium	Strain	Las B activity*	Las A activity**	protease IV activity**	alkaline protease activity***
LB	ATCC	100	+	+	–
	PA9	8.7	–	–	–
	PA18	9.3	–	–	–
M9	ATCC	16.8	–	–	100
	PA9	5.5	–	–	29.7
	PA18	4.8	–	–	55.7

*Results are given as percentages of the reference strain ATCC LB activity (100%) calculated on the basis of specific activity assay; **Activity of LasA and protease IV as detected in zymography gels as well as on the basis of specific activity assays; ***Results are given as percentages of the reference strain ATCC M9 activity (100%) calculated on the basis of specific activity assay; (+) positive reaction (gelatinase activity); (-) negative reaction (no gelatinase activity).

case of clinical strains PA9 and PA18, the activity was lower by 70% and 45%, respectively.

In general, there was a reasonably good correspondence between the results presented in the zymograms and the protease activity specific assays. Based on the above results we concluded that the ATCC strain produced three proteolytic enzymes in the LB medium: protease IV, elastase B and elastase A, but alkaline protease only in the M9 medium. The zymogram of the ATCC M9 culture supernatant did not show elastase B activity, although minimal cleavage of elastin Congo red was detected using the specific assay. In contrast, the clinical strains of *P. aeruginosa* produced elastase B and alkaline protease when grown in the LB and M9 medium, respectively (Table 1).

Identification of *lasB* and *aprA* genes in *P. aeruginosa* strains

To confirm the production of LasB and alkaline protease, whose activity was detected by zymography and specific assays, by the clinical isolates, attempts were made to identify the corresponding genes in their genomes. In order to identify the *lasB* and *aprA* genes in the genomes of the two *P. aeruginosa* clinical isolates (PA9 and PA18) and the reference strain ATCC 27853, *in silico* sequence analysis of these genes from several *P. aeruginosa* strains deposited at the GenBank database were performed. Then, sequence alignment for both *lasB* and *aprA* genes was performed, which facilitated choosing the most conserved sequence regions for primer design. The primer set used for *lasB* identification allowed amplifying a 0.65-kb DNA fragment containing the 3'-end of the gene. We obtained PCR products of the same length for the *P. aeruginosa* isolates PA9 and PA18, and the strain ATCC 27853 (Fig. 7). These results confirmed the presence of the *lasB* gene encoding elastase B in the genomes of all the three *P. aeruginosa* strains analyzed. Additionally, the presence of the second gene, *aprA* coding for the alkaline protease, was confirmed in the genomes of these strains. Using primers specific for *aprA*, we obtained 1.58-kb long amplicons encompassing the whole gene for alkaline protease for both the PA9 and PA18 isolates and the reference strain (Fig. 7).

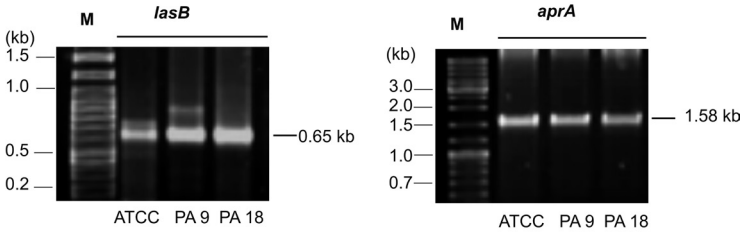

Figure 7. PCR identification of lasB and aprA genes in genomes of *P. aeruginosa* strains.
Total genomic DNA of the strains studied was prepared and PCR amplifications were performed as described in Materials and Methods. PCR products for *lasB* and *aprA* genes were then separated by 1.5% agarose gel electrophoresis. M, DNA size marker.

Toxicity of *P. aeruginosa* culture supernatants toward *G. mellonella* larvae

To evaluate the contribution of elastase B and alkaline protease to the *P. aeruginosa* virulence, the toxicity of the culture supernatants toward 7th instar *G. mellonella* larvae was determined and LD_{50} values of the studied supernatants were calculated. The observed differences in the toxic effect of the culture supernatants were expressed for each bacterial strain as the ratio of the total proteolytic activity units/larva (U/larva).

The *P. aeruginosa* culture supernatants were toxic for *G. mellonella* larvae and an evident relationship was observed between the proteolytic activity of the supernatants and their LD_{50} values. There was no mortality in the control group. The culture supernatants of the ATCC strain exhibited a stronger toxicity to *G. mellonella* larvae than the corresponding culture supernatants of either clinical strain. It is noteworthy that the proteins secreted by *P. aeruginosa* strains into the minimal medium were much more toxic toward the caterpillars than those released into the LB broth. Median lethal doses, LD_{50}, of the M9 culture supernatants were about 50-fold and 22–32-fold lower in comparison to the LD_{50} values calculated for the corresponding LB culture supernatants of the ATCC strain and the both clinical isolates, respectively. The LD_{50} values of the LB culture supernatants, containing mainly elastase B activity, were determined as 0.1, 0.32 and 0.45 U/larva, respectively, for the ATCC strain, and the clinical strains 9 and 18. For the M9 culture supernatants exhibiting alkaline protease activity, the LD_{50} values were calculated as 0.002, 0.01 and 0.02 U/larva for the ATCC strain, and the clinical isolates 9 and 18, respectively. The affected insects exhibited typical symptoms of *P. aeruginosa* infection. All larvae killed by the supernatant injections became black indicating strong activation of the prophenoloxidase system, which mediated melanization. Those results suggested that alkaline protease, besides elastase B, con-

tributed considerably to the virulence of the *P. aeruginosa* strains studied.

DISCUSSION

It has been reported that the *P. aeruginosa* entomopathogenic strain used in this study as a reference strain can produce four extracellular proteases, namely protease IV, elastase A, elastase B and alkaline protease (Morihara *et al.*, 1965; Peters & Galloway, 1990; Baumann *et al.*, 1993; Engel *et al.*, 1998), and these findings have been confirmed in our investigations. However, the gelatin zymography analysis revealed the presence of different protease profiles depending on the *P. aeruginosa* strain as well as on the medium used for bacterial culture.

The proteolytic band with a molecular mass above 200 kDa, detected by us in zymography, corresponded to protease IV activity. This is consistent with the results presented by Caballero *et al.* (2001), who reported that the enzyme aggregated under non-reducing conditions into a high-molecular-mass-complex (> 200 kDa). This serine proteinase was produced by the entomopathogenic strain ATCC in the LB medium only. Likewise protease IV, the activity of elastase A was detected in our studies only in the LB culture supernatant of the ATCC strain. In contrast, all three *P. aeruginosa* strains exhibited LasB activity. Elastase B produced by the ATCC strain in the LB medium migrated as a protein band of approximately 160 kDa, while the LB supernatants of the clinical isolates generated zymography bands of different molecular masses, i.e. 160 kDa (PA18) or 120 kDa (PA9). Other authors reported that elastase B migrated in gelatin zymography under non-reducing conditions as bands corresponding to apparent molecular masses of 116 or 163 kDa (Engel *et al.*, 1997; Stepińska *et al.*, 2010). Furthermore, the apparent sizes of elastase B in polyacrylamide gels were dependent on the concentration of gelatin and polyacrylamide in the gels (Hummel *et al.*, 1995). As demonstrated by Caballero *et al.* (2001), the 160 kDa bands in gels with casein represented the proteolytic activity of LasB, whereas in the gelatin gels that band represented the combined activity of LasA and LasB. It was also reported that elastase B aggregated into a complex of high molecular mass, approximately 160 kDa. The two elastase B bands of molecular masses 160 kDa and 120 kDa detected by us could reflect aggregation of different monomer numbers or aggregate formation with other proteins. Lomholt *et al.* (2001) postulated that the two mobility variants of elastase B in gelatin gels could reflect different conformations of the mature proteins resulting from differences in the *lasB* gene sequence or the formation of sequence-dependent non-covalently linked oligomers.

The gelatinase activity of the *P. aeruginosa* M9 culture supernatants showed that all the strains studied produced alkaline protease selectively only under those conditions. That protease was not produced in the LB medium. This finding is in agreement with data of Cryz & Iglewski (1980), who found that production of alkaline protease was strain-variable and medium-dependent. The molecular mass of alkaline protease was calculated as 48.4 kDa on the basis of purified enzyme migration in SDS/PAGE however, the enzyme was reported to migrate under non-reducing conditions as a band corresponding to an apparent molecular mass of 53 kDa in 7.5–10 % gelatin gels, which is consistent with our results (Twining *et al.*, 1993; Engel *et al.*, 1997).

Although all three *P. aeruginosa* strains tested in this study produced elastase B and alkaline protease, the activity level of these extracellular proteinases differed significantly between the strains. The proteinases of the clinical strains exhibited considerably lower activity in comparison to the ATCC strain enzymes. The detected differences in the activity level could be due to point mutations in the relevant genes affecting the enzyme activity. A detailed analysis of complete *lasB* and *aprA* gene sequences in the *P. aeruginosa* strains studied, currently under way in our laboratory, will provide data for amino acid sequence analysis of the proteinases which will allow solving this question.

In this study, we showed that the *P. aeruginosa* clinical strains produced only two proteinases, elastase B and alkaline protease, and their expression was dependent on the composition of the media. In addition, the proteinase profiles of both clinical strains differed from the proteinase pattern of the *P. aeruginosa* ATCC strain. The observed differences could result from diverse requirements for expression of proteolytic activities by the strains studied.

Numerous studies have shown that *P. aeruginosa* isolates differ in expression of two virulence factors, elastase B and alkaline proteinase (Jagger *et al.*, 1983; Woods *et al.*, 1986; Schmidtchen *et al.*, 2001). An analysis of different *P. aeruginosa* clinical isolates performed by Rumbaugh *et al.* (1999) suggested that both the infection site as well as the duration of infection influenced the virulence of the bacteria by altering production of extracellular virulence factors. It was reported that, despite the fact that the *lasA* and *lasB* genes were present in all 145 *P. aeruginosa* isolates examined, 17 isolates did not exhibit elastase A activity, whereas 4 isolates failed to express elastase B (Lomholt *et al.*, 2001).

The possible mechanism responsible for altering the production of these *P. aeruginosa* virulence factors is different regulation of *lasB* and *aprA* expression. *P. aeruginosa* is known to contain a complex network of regulatory genes that influence the production of different virulence factors in response to changing environmental stimuli (Rumbaugh *et al.*, 1999). A number of environmental factors have been shown to influence the production of *P. aeruginosa* exoproducts, e.g., the production of exotoxin A, elastase B and alkaline protease is independently regulated by iron (Bjorn *et al.*, 1979; Shigematsu *et al.*, 2001).

The synthesis and subsequent secretion of elastase B is regulated by quorum sensing as it is part of the *las* and *rhl* regulons in *P. aeruginosa*. The two AHL-dependent QS systems (*las* and *rhl* systems) regulate overlapping sets of genes and play a central role in the pathogenicity of *P. aeruginosa*. The expression of numerous cellular and secreted virulence factors, including alkaline protease, LasA and LasB proteases, exotoxin A, pyocyanin and lectins is regulated by the *las* and *rhl* systems. In addition to the AHL-dependent quorum sensing system, expression of the *lasB* gene is also under regulation by the AQ-dependent QS system (Williams & Camara, 2009).

It was shown by Duan & Surette (2007) that expression of both QS systems in terms of magnitude as well as timing was dependent on the growth conditions, influencing expression of the QS-regulated virulence factors. For example, expression of the *aprA* gene encoding alkaline protease in the M9 minimal medium and diluted LB medium started already at the exponential phase, whereas in the rich LB medium the expression started at the early stationary phase. It was also reported that the expression levels of both QS systems were addi-

tionally influenced by iron deprivation and oxygen limitation (Duan & Surette, 2007). Exotoxin A production is regulated by the level of iron in the growth medium, the incubation temperature, and the presence of certain nutrients within the growth medium (Frank & Iglewski, 1988).

In the genomes of the two clinical isolates as well as the reference strain of *P. aeruginosa* used in our study, genes *lasB* and *aprA* encoding elastase B and alkaline protease, respectively, were identified. The expression of those genes, coding for two important *P. aeruginosa* virulence factors, was dependent on the growth conditions in all the strains studied. Elastase B activity and alkaline protease activity were only detected in the rich LB medium and the minimal M9 medium, respectively. This essential difference could result from induction of the stringent response in the bacteria grown in the minimal medium. The stringent response occurs in reaction to starvation for a variety of nutrients, e.g. amino acids and fatty acids, and enables a broad-scale adaptation of the bacteria to stressful conditions (Jain *et al*., 2006). The role of the stringent response in the regulation of expression of virulence factors in *P. aeruginosa* has been demonstrated using a *relA* mutant unable to produce RelA, one of the enzymes responsible for the synthesis of the alarmone (p)ppGpp In the *relA* mutant a decrease in elastase and an increase in pyocyanin production were reported (Erickson *et al*., 2004). Moreover, a *relA spoT P. aeruginosa* double mutant, unable to produce both enzymes involved in (p)ppGpp synthesis, RelA and SpoT, was avirulent. The synthesis of virulence factors, such as elastase, pyocyanin, and siderophores was impaired in this double mutant (Vogt *et al*., 2011). In addition, *relA* mutants exhibited reduced virulence, while *relA spoT* double mutants were avirulent in *Drosophila melanogaster* feeding assay (Erickson *et al*., 2004; Vogt *et al*., 2011).

The fact that all the strains examined in this study contain the genes for elastase B and alkaline protease suggests that these enzymes are conserved and could confirm their role as important *P. aeruginosa* virulence factors. A great contribution of these extracellular proteinases to the virulence of the entomopathogenic as well as both clinical *P. aeruginsa* strains tested in this study was confirmed using a well established insect model organism, *G. mellonella*. Interestingly, the culture supernatants containing alkaline protease were considerably more toxic towards insect larvae in comparison to those containing mainly elastase B, pointing to the importance of alkaline protease in overcoming mechanisms of immune response. However, in a discussion on the toxicity of the supernatants toward caterpillars, the role of other *P. aeruginosa* virulence factors, not tested in this study but most probably released into the culture medium along with the proteinases, should also be taken into consideration. As revealed in our previous paper, the LB and M9 culture supernatants of both clinical strains, despite containing elastase B and alkaline protease activity, respectively, hardly degraded *G. mellonella* hemolymph proteins and peptides *in vitro*. In contrast, after incubation in the presence of the ATCC strain culture supernatants, a strong proteolytic degradation of the larvae hemolymph polypeptides was detected (Andrejko *et al*., in press). Those results together with the toxicity study presented in this paper indicate that mainly extracellular proteinases could be involved in the toxicity of the ATCC strain supernatants toward *G. mellonella*, while for the toxicity of the clinical strains'supernatants most probably additional secreted virulence factors are responsible. For example, Miyata *et al*. (2003) have demonstrated a significant role of *P. aeruginosa* ADP-ribosyltransferase (ExoT) and phospholipase (ExoU) in killing of *G. mellonella* larvae.

Expression of virulence factors in *P. aeruginosa* is under comprehensive regulation and expression *in vitro* does not necessarily reflect the expression during infection. Certain strains may require additional signals or interactions that may not be present *in vitro* for expression of these genes. Since laboratory analyses of bacterial isolates need not fully reflect the true *in vivo* conditions, future studies should also include determinations of virulence factors *in situ*. The extracellular proteinases of *P. aeruginosa* strains used in this study are under further investigation in our laboratory using an insect model organism, *G. mellonella*.

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