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Initial Characterization of Two Extracellular Autolysins from *Pseudomonas aeruginosa* PAO1

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Two extracellular autolysins have been detected in the spent culture supernatants of *Pseudomonas aeruginosa* PAO1 by using renaturing polyacrylamide gel electrophoresis. The two autolysins were isolated from the culture supernatant by trichloroacetic acid precipitation and were shown to have apparent molecular masses of 26 and 29 kDa. The 26-kDa autolysin first appears during the early exponential phase of growth and then declines sharply, while the 29-kDa autolysin first appears in the late exponential phase of growth and continues well into the stationary phase. Fractionation of whole cells indicated that the 26-kDa enzyme was also localized within the periplasm, with a lesser amount of activity associated with the cytoplasmic membrane. The 29-kDa autolytic activity was distributed within the cell equally between the periplasm and the cytoplasmic membrane. The pH optima of the isolated 26- and 29-kDa autolysins are 6.0 and 5.0, respectively. Further evidence from both protease susceptibility and inhibition studies confirms that these two extracellular autolysins isolated from *P. aeruginosa* PAO1 are separate and distinct.

Most eubacteria are mechanically stabilized by the shape-determining peptidoglycan, or murein, which consists of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid to which a short peptide is attached. Expansion of the bacterial cell wall during growth (42–44, 53) and splitting of the septum prior to cell separation (17, 24, 41, 42, 46) require enzymes that cleave some of the existing covalent bonds within the murein sacculus. These enzymes are collectively known as murein hydrolases (10, 17). Particular murein hydrolases, known as autolysins, can weaken the murein sacculus, leading to bacterial cell wall degradation and cell death. Four common types of autolysins have been recognized in bacteria, including the β-1,4-*N*-acetylmuramidases (i.e., lysozymes), the β-1,4-*N*-acetylglucosaminidases, the *N*-acetylmuramyl-L-alanine amidases, and several peptidases (41).

Murein hydrolases of few gram-negative bacteria have been studied in detail, and the murein hydrolases of *Escherichia coli* have been studied most extensively (18–20, 23, 29, 30, 32, 42). The autolysins of the opportunistic pathogen *Pseudomonas aeruginosa* have not been previously studied, although a DD-carboxypeptidase (38) and a DD-endopeptidase (3) have been described. *P. aeruginosa* has developed several mechanisms of resistance to oppose the action of most commonly used antibiotics; murein hydrolases are known to play a role in the antibiotic-induced lysis of bacteria (8, 15, 25, 40, 41, 46, 47). In the search for novel therapies for *P. aeruginosa* infections, we have initiated studies on the murein hydrolases of this important pathogen.

Renaturing polyacrylamide gel electrophoresis (PAGE) in substrate-containing gels has facilitated the study of autolysins in gram-positive bacteria (9, 21, 28, 37, 45). The method has recently been modified to examine the autolysins of a few select gram-negative pathogens, including those of *P. aeruginosa* (1). Eleven distinct lytic bands were detected in whole-cell sonicates of *P. aeruginosa* by this technique; the bands presumably correspond to the activities of individual autolysins. This paper describes the initial characterization of two extracellular and distinct autolysins isolated from *P. aeruginosa* PAO1.

Preparation of murein sacculi. An overnight starter culture of *P. aeruginosa* PAO1 (H103; serotype 05), kindly supplied by J. Lam, University of Guelph, was grown in 50 ml of tryptic soy broth (Difco Laboratories, Detroit, MI) and incubated at 37°C in a rotary shaker at 200 rpm. Tryptic soy broth (6 liters) was inoculated with 1.0 ml of the starter culture and incubated at 37°C in a rotary shaker at 150 rpm for 16 h before the cells were harvested by centrifugation (5,000 × *g*, 10 min, 4°C). Cells were washed twice with 25 mM sodium acetate (pH 6.2), subjected to centrifugation as before, and resuspended in 90 ml of 25 mM sodium acetate, pH 6.2. Murein sacculi were isolated and purified as previously described (5).

Renaturing PAGE. The renaturing gel electrophoresis was similar to that described by Foster (9) with some modifications which will be described in detail elsewhere. Briefly, sodium dodecyl sulfate (SDS)-polyacrylamide separating gels (15% acrylamide, pH 8.8) containing 0.1% purified *P. aeruginosa* PAO1 murein sacculi were used to detect lytic activity. Following electrophoresis (20-mA constant current), gels were soaked for 30 min in deionized water at room temperature with gentle shaking. The gels were then transferred to 200 ml of 25 mM sodium phosphate (pH 7.0) with 0.1% Triton X-100 and 10 mM $MgCl_2$ (the renaturation solution, unless otherwise stated) and were gently shaken for 30 min at room temperature. The gels were then transferred to 250 ml of fresh renaturation solution and were incubated for 18 to 24 h at 37°C with gentle shaking. Following incubation, the gels were rinsed with deionized water, stained in 0.1% methylene blue in 0.01% KOH for at least 3 h, and destained in deionized water. Lytic activity was visualized as zones of clearing in the blue background. For presentation purposes, gels were photographed with Kodak 5360 B/W positive film and then printed onto Ilford RC paper. Thus, the clear bands in the blue background of the gels appear as dark bands in the white background of the photograph. The results shown in the photographs are representative, and all observations were confirmed in at least two separate experiments.

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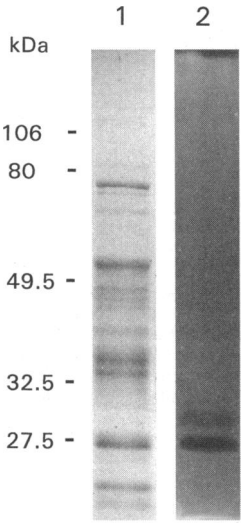

FIG. 1. SDS-PAGE of concentrated samples of *P. aeruginosa* PAO1 culture supernatant. Lane 1, gel slice of 15% acrylamide stained with Coomassie brilliant blue R-250; lane 2, gel slice of 15% acrylamide containing 0.1% *P. aeruginosa* murein sacculi stained with 0.1% methylene blue in 0.01% KOH following renaturation of separated proteins in 0.1% Triton X-100 and 10 mM $MgCl_2$. The mobilities of prestained low molecular weight markers are indicated on the left.

Profile of extracellular autolysins. Renaturing PAGE was used to examine the profile of autolysins liberated from *P. aeruginosa* PAO1 (Fig. 1). Tryptic soy broth (200 ml) was inoculated with 1.0 ml of the starter culture and grown as described above. Cells were collected by centrifugation (5,000 × *g*, 10 min, 4°C), and the supernatant was retained. The supernatant was precipitated with 10% trichloroacetic acid at 4°C for at least 1 h, the precipitate was recovered by centrifugation (15,000 × *g*, 10 min, 4°C), and the pellet was washed with acetone at −20°C, recovered as before, and allowed to air dry. The protein pellet was resuspended in 20 μl of nonreducing SDS-PAGE sample buffer (2) and boiled for 5 min prior to both SDS-PAGE and renaturing gel electrophoresis. Two autolysins were visualized in the supernatants of cultures harvested between the late exponential and the early stationary phases of growth. The two autolysins were estimated to have molecular masses of 26 and 29 kDa by comparison with prestained standards (Bio-Rad) of known sizes subjected to electrophoresis on the same gel. A discontinuous SDS-PAGE (26) profile of the same sample stained with Coomassie brilliant blue R-250 (Fig. 1) demonstrated the total protein in the culture supernatant.

The two extracellular autolysins were particularly resistant to permanent denaturation, allowing their visualization following electrophoresis. Both autolysins were precipitated from culture supernatant with 10% trichloroacetic acid and washed with acetone with no apparent loss in activity following renaturation. To ensure that the clearing zones in the gel were not an artifact of trichloroacetic acid precipitation, a solution of bovine serum albumin was precipitated with 10% trichloroacetic acid, washed with acetone, and subjected to electrophoresis on a sacculus-containing gel. No "lytic" activity was visualized on the gel following renaturation, although positive controls (26- and 29-kDa autolysins, hen egg white lysozyme) were renatured with full activity (data not shown). Boiling the two autolysins in the absence of SDS-PAGE sample buffer for extended periods of time (>1 h) prior to electrophoresis permanently denatured the two autolysins as indicated by the lack of recovered activity. Incubation of both autolysins with 10 μg of pronase per ml for 10, 30, and 60 min led to a progressive decrease in the activity of the 26-kDa autolysin but had a lesser effect on the activity of the 29-kDa autolysin. These results provide strong evidence that the clear zones in the stained gels corresponding to apparent molecular masses of 26 and 29 kDa are not artifacts of renaturing PAGE but do, in fact, reflect the activity of true enzymes.

Renaturation optimization. The effect of different renaturation buffers on the activities of the 26- and 29-kDa autolysins following renaturing PAGE was examined. Late-exponential-phase culture supernatant was precipitated as described above, was loaded onto sacculus-containing gels, and following electrophoresis was separately incubated in the following buffers: 25 mM sodium phosphate (pH 7.0) in the absence and presence of either 0.1% Triton X-100, 10 mM $MgCl_2$, or both; 25 mM citrate-phosphate (pH 6.0) and 0.1% Triton X-100 with and without 10 mM $MgCl_2$; and 25 mM sodium acetate (pH 5.0) and 0.1% Triton X-100 with and without 10 mM $MgCl_2$. The following buffers containing both 0.1% Triton X-100 and 10 mM $MgCl_2$ were also used for renaturation: 25 mM sodium phosphate, pH 6.5 and 7.5; 25 mM citrate-phosphate, pH 5.0 and 7.0; 25 mM MES (morpholineethanesulfonic acid), pH 5.0 to 7.0; and 25 mM Tris-HCl, pH 7.0 to 9.0.

Little activity of either autolysin was visible when the renaturation buffer was significantly less than pH 7.0 (data not shown), even though the pH optima of the native autolysins were both acidic as determined by turbidometric assays (see below). Conversely, the two autolysins were active when the renaturation buffer was alkaline. $MgCl_2$ was required for the effective renaturation of only the 26-kDa autolysin; whereas $MgCl_2$ appears to inhibit the 29-kDa enzyme (see below), it did not negatively affect its renaturation. Triton X-100 was important for full activity of both autolysins. For most applications, the optimum conditions for restoring autolysin activity following renaturing PAGE were confirmed to be the same as those developed by Foster (9), i.e., 25 mM sodium phosphate (pH 7.0) with 0.1% Triton X-100 and 10 mM $MgCl_2$.

Temporal distribution of extracellular autolysins. A growth curve for *P. aeruginosa* PAO1 was established (Fig. 2B), clearly demonstrating the various phases of growth. Samples (1.0 ml) were removed from the growing culture at various times (4 to 25 h) after inoculation and subjected to centrifugation to remove cells (15,000 × *g*, 10 min, 4°C), and the supernatants were precipitated as described above. Renaturing PAGE revealed the temporal distribution of the autolysin activities (Fig. 2A). The activity of the 26-kDa autolysin increased and reached a maximum during the late exponential phase of growth and then declined sharply as the culture entered the stationary phase. This inactivation with time may reflect the susceptibility of the 26-kDa autolysin to proteases, as noted above. The 29-kDa autolysin first appeared during the mid-exponential phase of growth and continued to be active well into the stationary phase. Indeed, cultures have been left for up to 7 days with the active 29-kDa autolysin still present in the supernatant. Thus, the production and/or release of the 29-kDa autolysin temporally followed that of the 26-kDa autolysin, strongly indicating that the latter is not a degradation product of the larger enzyme. The temporal distribution of the two activities may suggest that the peptidoglycan of *P. aeruginosa* physically and/or chemically changes as the bacterium matures, similar to those changes which occur in *E. coli* (4, 6, 7, 11, 12, 34, 36, 48) and other organisms (13).

Subcellular localization of autolysins. The peptidoglycan of gram-negative bacteria is somewhat protected from the envi-

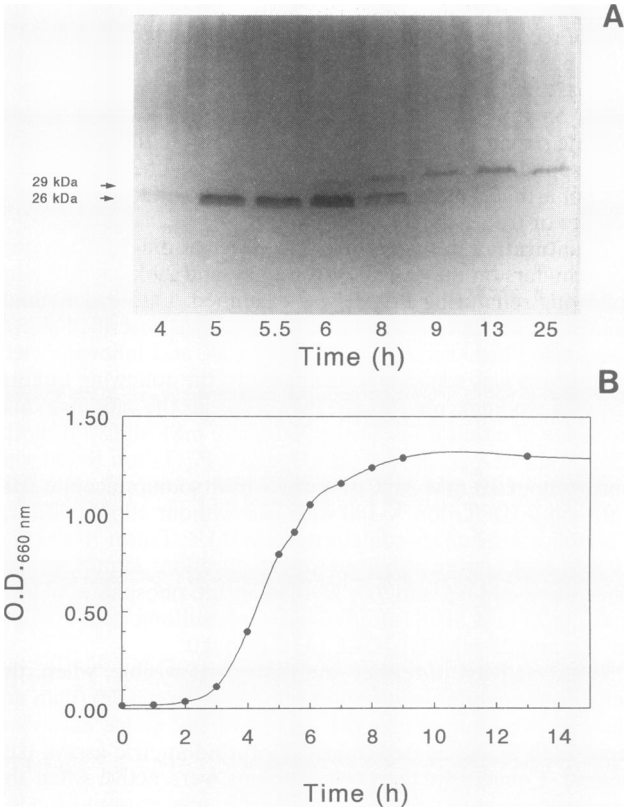

FIG. 2. Temporal distribution of the 26- and 29-kDa extracellular autolysins during batch culture of *P. aeruginosa* PAO1. (A) The spent medium of samples isolated as described for panel B, following removal of whole cells and debris by centrifugation, was treated with 10% trichloroacetic acid, and the precipitated proteins were washed with acetone at −20°C. The protein samples isolated at the indicated times were subjected to renaturing SDS-PAGE in a 15% acrylamide gel containing 0.1% murein sacculi as described in the legend to Fig. 1. (B) Tryptic soy broth (200 ml) was inoculated with *P. aeruginosa* PAO1 and incubated at 37°C. At 30-min intervals, the optical density at 660 nm ($O.D._{660nm}$) of 1.0-ml samples was determined and plotted against time.

ronment by the outer membrane, thereby limiting access to the peptidoglycan by truly extracellular autolysins. *P. aeruginosa* whole cells were fractionated to localize the 26- and 29-kDa autolysins within specific intracellular compartments. The fractionation of *P. aeruginosa* PAO1 was adapted from the procedure described by Lambert (27). Cells were grown to the late exponential phase, harvested by centrifugation (5,000 × *g*, 10 min, 4°C), washed twice with phosphate buffered saline (pH 7.4) (PBS), and recovered as before. The culture supernatant was retained and precipitated with 10% trichloroacetic acid (culture supernatant). The washed cells from 100 ml of culture were resuspended in 2 ml of 0.1-mg/ml polymyxin B, incubated for 30 min at 37°C, and subjected to centrifugation (10,000 × *g*, 10 min, room temperature). The supernatant was filtered through a 0.22-μm-pore-size membrane filter, and the filtrate was stored at −20°C (periplasmic fraction). The remaining cell pellet was resuspended in 22 ml of PBS containing 0.1 mM phenylmethylsulfonyl fluoride and subjected to sonication at 150 W for a total of 5 min. Following ultracentrifugation at 10,000 × *g* (30 min, 4°C), the supernatant was lyophilized and stored at −20°C (cytoplasmic fraction). For the isolation of

TABLE 1. Subcellular localization of autolysins[a]

Cellular fraction	Autolysin activity[b]	
	26 kDa	29 kDa
Culture supernatant	+++	+++
Cell homogenate	+	+
Cytoplasm	−	−
Cytoplasmic membrane	+	++
Periplasm	+++	++
Peptidoglycan-associated material	−	−
Outer membrane	−	−

[a] Autolytic activity was determined by renaturing SDS-PAGE. Samples of each cellular fraction were applied to the same SDS-PAGE gel containing *P. aeruginosa* murein sacculi, and electrophoresis was performed as described in the legend to Fig. 1. Relative measurements were based on the sizes of the clear zones in the methylene blue-stained gel created by the activities of the respective autolysins.

[b] +++, maximal autolytic activity; ++, less than maximal activity; +, minimal activity; −, no activity.

cytoplasmic and outer membrane preparations, cells from 500 ml of culture were resuspended in 22 ml of PBS containing 0.1 mM phenylmethylsulfonyl fluoride and were subjected to sonication as described above. A 1.0-ml sample of this sonicated material was stored at −20°C (cell homogenate). Whole cells were removed from the sonicate by centrifugation (5,000 × *g*, 5 min, 4°C), and the membrane fractions suspended in the supernatant were recovered by ultracentrifugation (100,000 × *g*, 30 min, 4°C). The membrane pellet was washed twice with PBS and recovered each time by ultracentrifugation as before, and the cytoplasmic membrane fraction was solubilized by the addition of 22 ml of 2% Sarkosyl. Following incubation at room temperature for 30 min, the solubilized cytoplasmic membrane fraction was separated from the insoluble outer membrane fraction by ultracentrifugation (100,000 × *g*, 30 min, room temperature). The supernatant was lyophilized and stored at −20°C (cytoplasmic membrane fraction). The outer membrane pellet was resuspended in 22 ml of 2% Sarkosyl, recovered by ultracentrifugation as before, and resuspended in 22 ml of 10 mM Tris-HCl (pH 8.0) containing 2% SDS. Residual insoluble components (peptidoglycan) were removed by ultracentrifugation as before, and the supernatant was lyophilized and stored at −20°C (outer membrane fraction). The peptidoglycan pellet was washed twice with 10 mM Tris-HCl (pH 8.0) containing 2% SDS, subjected to ultracentrifugation as before, resuspended in 10 mM Tris-HCl (pH 8.0), lyophilized, and stored at −20°C (peptidoglycan-associated fraction). Renaturing PAGE was used to detect the 26- and 29-kDa autolysins in the various fractions (Table 1). The 26-kDa autolytic activity was greatest in the periplasm, with a lesser amount of activity associated with the cytoplasmic membrane. The 29-kDa autolytic activity was distributed more or less equally between the periplasm and the cytoplasmic membrane. Thus, both the 26- and 29-kDa autolysins were active within the periplasm, where growth of the sacculus and splitting of the septum by murein hydrolases must, in fact, occur. No autolytic activity was seen in either the peptidoglycan-associated or the outer-membrane associated material, further supporting a primary location of the two autolysins either with the cytoplasmic membrane or free within the periplasm. Normal SDS-PAGE with Coomassie brilliant blue R-250 staining of the subcellular fractions demonstrated both that the fractionation successfully isolated the different cellular compartments and that the total protein concentrations examined were consistent between fractions (results not shown).

P. aeruginosa secretes numerous proteins into the culture medium; it is not yet clear whether the autolysins are truly secreted or whether their presence extracellularly may have some other significance. To our knowledge, the 26- and 29-kDa autolysins from *P. aeruginosa* PAO1 are the first reported examples of extracellular autolysins from a gram-negative bacterium.

Determination of pH optima. Early-exponential-phase and late-stationary-phase culture supernatants were precipitated with 30% ethanol and 25% methanol, respectively, to isolate the 26- and 29-kDa autolysins. Precipitates were removed by centrifugation (20,000 × *g*, 30 min, 4°C) and were stored at −20°C. Autolysin activity was measured by the turbidometric assay of Hash (16) and was defined as the change in optical density at 660 nm per hour of incubation. The isolated 26- and 29-kDa autolysins were incubated with murein sacculi (0.3 mg/ml) suspended in buffers of various pH to determine their pH optima. Buffers included 50 mM sodium citrate, pH 2.5 to 4.0; 50 mM sodium acetate, pH 3.5 to 5.5; 50 mM MES, pH 5.0 to 7.0; 50 mM citrate-phosphate, pH 5.5 to 7.0; 50 mM sodium phosphate, pH 6.5 to 7.5; 50 mM Tris-HCl, pH 7.5 to 9.0; and 50 mM glycine, pH 9.0 to 11.0. Sodium azide at 0.1% was added to the suspensions to prevent any contaminating microbial growth. The pH optimum of the 26-kDa autolysin was 6.0 in 50 mM citrate-phosphate buffer, while that of the 29-kDa enzyme was 5.0 in 50 mM acetate buffer (Fig. 3). A decrease in activity of the 26-kDa autolysin appeared to be associated with 50 mM MES buffer, pH 5.0 to 7.0, suggesting that this buffer may be an inhibitor of this autolysin. Because of this anomaly, MES buffers were not used for the determination of pH optima. The difference in the pH optima provides further evidence that the 26- and 29-kDa autolysins are distinct enzymes.

Inhibition of autolysins. The inhibition of the 26- and 29-kDa autolysins was determined as described for the pH optimization with murein sacculi suspended in 50 mM citrate-phosphate buffer (pH 6.0) and 50 mM acetate buffer (pH 5.0), respectively; however, the residual activity of each autolysin was expressed as a percentage of the activity of each autolysin incubated with murein sacculi suspended in buffer alone. The potential inhibitors and their tested concentrations are listed in Table 2. Of the components for renaturation of gels, Triton X-100 greatly increased the activities of both autolysins, whereas $MgCl_2$ inhibited the 29-kDa autolysin. MES buffer (50 mM) at pH 6.0 inhibited the 26-kDa autolysin, confirming the results obtained with pH optimization. Both Ca^{2+} and Hg^{2+} had an effect similar to that of Mg^{+} in that they also inhibited the 29-kDa autolysin. Li^{+} and Na^{+} were equally inhibitory to both autolysins. EDTA completely inhibited both autolysins. Heavy metal ions have previously been shown to inhibit the autolysins of *Bacillus subtilis* 168 (9) and *Staphylococcus aureus* (45), while the soluble lytic transglyosylase of *E. coli* is stimulated by Mg^{2+} (15, 18). Different peptidoglycan components and some peptidoglycan analogs were examined as potential inhibitors of activity. *N*-Acetylglucosamine was inhibitory to the 26-kDa autolysin and less so to the 29-kDa autolysin. Gluconolactone was equally inhibitory to both autolysins. Neither autolysin was inhibited by penicillin G.

Discussion and conclusions. *P. aeruginosa* was an uncommon cause of infection until the widespread use of antibiotics; in the past thirty years, however, *P. aeruginosa* has become an etiologic agent in an increasing proportion of nosocomial infections (33). Today, *P. aeruginosa* is the third most common cause of nosocomial infections after *E. coli* and *S. aureus*, accounting for approximately 10% of all hospital-acquired infections (39). Particularly susceptible are those patients who

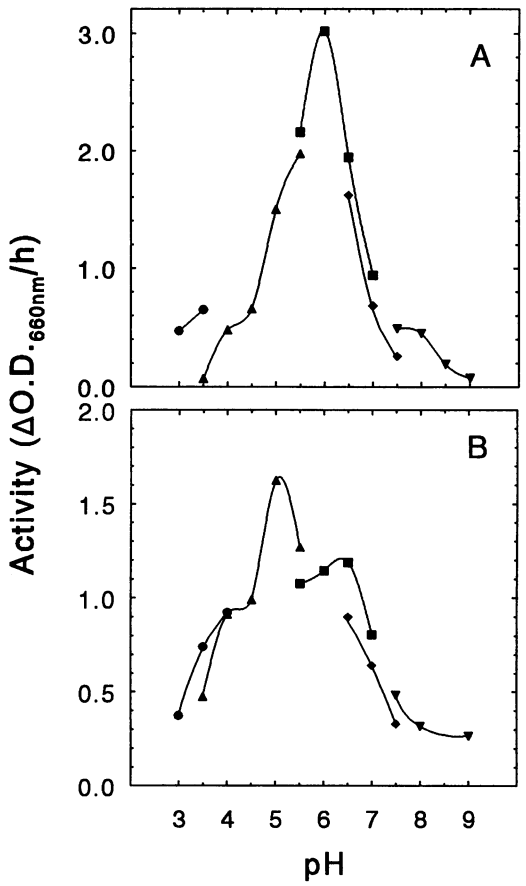

FIG. 3. Effect of pH on the initial activities of the isolated 26- and 29-kDa extracellular *P. aeruginosa* PAO1 autolysins. The 26-kDa (A) and 29-kDa (B) autolysins (125 μg/ml; precipitated from separate samples of culture supernatant by 30% ethanol and 25% methanol, respectively) were assayed for activity by the turbidometric assay of Hash (16) with 0.3 mg of *P. aeruginosa* murein sacculi per ml as the substrate at 37°C in 50 mM sodium citrate, pH 3.0 to 4.0 (●); 50 mM sodium acetate, pH 3.5 to 5.5 (▲); 50 mM citrate-phosphate, pH 5.5 to 7.0 (■); 50 mM sodium phosphate, pH 6.5 to 7.5 (◆); and 50 mM Tris-HCl, pH 7.5 to 9.0 (▼).

are immunosuppressed or individuals with cystic fibrosis or chronic lung diseases, with thermal injuries, or with severe trauma (54).

Gentamicin is an aminoglycoside antibiotic effective against *P. aeruginosa*; its mode of action may be to inhibit protein synthesis via binding to ribosomes, although recent data suggest that it may also induce bacteriolysis by perturbation of the outer membrane (22). A variety of permeabilizers of the *P. aeruginosa* outer membrane have been identified and classified into three groups: (i) divalent cation chelators, such as EDTA; (ii) polycations, such as the aminoglycosides; and (iii) large monovalent cations, such as Tris (14). Beveridge and coworkers have shown by thin-section electron microscopy the disruption of the *P. aeruginosa* peptidoglycan directly beneath outer membrane blebs caused by the aminoglycosides gentamicin and amikacin (31, 52). Such limited hydrolytic activity would require the specific activation or deregulation of a specific and/or localized autolysin(s). Metals have been shown to affect the activity of several autolysins (9, 35, 45), and they may be responsible for their regulation (29, 30). Hence, their removal

TABLE 2. Effects of various potential modulators of autolytic activity[a]

Potential modulator (final concn)	Autolysin activity (%)	
	26 kDa	29k Da
None	100	100
$MgCl_2$ (10 mM)	90	50
Triton X-100 (0.1%)	190	245
$MgCl_2$ (10 mM) and Triton X-100 (0.1%)	175	160
MES (50 mM)[b]	55	90
LiCl (200 mM)	30	0
NaCl (200 mM)	20	30
$MgCl_2$ (10 mM)	90	50
$CaCl_2$ (10 mM)	75	40
$ZnCl_2$ (10 mM)	40	30
$HgCl_2$ (1 mM)	65	0
EDTA (10 mM)	0	0
N-Acetylglucosamine	25	75
Glucosamine (10 mM)	60	60
Gluconolactone (10 mM)	35	35
Galactosamine (10 mM)	65	105
Maltose (10 mM)	55	40
Cellobiose (10 mM)	65	80
D-Alanine (10 mM)	65	40
Diaminopimelic acid (10 mM)	45	70
Gentamicin sulfate (15 μg/ml)	75	60
Penicillin G (1.0 mg/ml)	100	105

[a] Modulators/inhibitors were added to reaction mixtures to the final concentrations indicated. Reaction mixtures contained either 50 mM citrate-phosphate (pH 6.0) for the 26-kDa autolysin assays or 50 mM sodium acetate (pH 5.0) for the 29-kDa autolysin assays, unless stated otherwise. Initial rates of ΔA_{660} were determined with *P. aeruginosa* peptidoglycan as the substrate and are expressed as a percentage of the reaction without modulator.

[b] The activities of the 26-kDa autolysin and the 29-kDa autolysin were compared by using 50 mM MES, pH 6.0 or pH 5.0, respectively.

by chelators such as EDTA or displacement by aminoglycosides may account for the localized hydrolysis of peptidoglycan directly below an outer membrane bleb. Alternatively, perturbation of the outer membrane may activate these enzymes, since the activity of an *E. coli* *N*-acetylmuramyl-L-alanine amidase has been shown to be modulated by lipid-protein interactions (49–51). It seems obvious that further detailed examination of the autolysins present in *P. aeruginosa* will lead to a better understanding of cell wall physiology and may eventually bring about novel therapies for *P. aeruginosa* infections.

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