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Lysophosphatidic acid inhibition of the accumulation of *Pseudomonas aeruginosa* PAO1 alginate, pyoverdin, elastase and LasA

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The pathogenesis of *Pseudomonas aeruginosa* is at least partially attributable to its ability to synthesize and secrete the siderophore pyoverdin and the two zinc metalloproteases elastase and LasA, and its ability to form biofilms in which bacterial cells are embedded in an alginate matrix. In the present study, a lysophospholipid, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate [also called monopalmitoylphosphatidic acid (MPPA)], which accumulates in inflammatory exudates, was shown to inhibit the extracellular accumulation of *P. aeruginosa* PAO1 alginate, elastase, LasA protease and the siderophore pyoverdin. MPPA also inhibited biofilm formation. The inhibitory effects of MPPA occur independently of *rpoS* expression and without affecting the accumulation of the autoinducers *N*-(3-oxododecanoyl) homoserine lactone and *N*-butyryl-L-homoserine lactone, and may be due, at least in part, to the ability of MPPA to bind divalent cations.

Keywords: monopalmitoylphosphatidic acid, cystic fibrosis

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that rarely causes pulmonary disease in healthy hosts, but which causes acute pneumonia in immunocompromised patients (Bodey *et al*., 1983). It is also associated with chronic pneumonia in cystic fibrosis patients (Bodey *et al*., 1983; Ojeniji, 1994). *P. aeruginosa* produces several virulence factors that contribute to its pathogenesis, including lipopolysaccharide (LPS) (Cryz *et al*., 1984; Tang *et al*., 1996), the two ADP-ribosylating enzymes exotoxin A (Vasil *et al*., 1993) and exoenzyme S (Nicas *et al*., 1985), the exoproteases elastase (Woods *et al*., 1982), LasA (Preston *et al*., 1997) and alkaline protease (Howe & Iglewski, 1984), a haemolytic and a non-haemolytic phospholipase C (Vasil *et al*., 1993), the siderophores pyochelin and pyoverdin (Cox, 1993), the surface-associated polysaccharide alginate (Baltimore, 1993), and pilus-associated adhesins responsible for binding to specific epithelial-cell receptors (Tang *et al*., 1995).

The expression of elastase, as well as the expression of a large number of other virulence factors, by *P. aeruginosa* is regulated by quorum sensing (Van Delden & Iglewski, 1998). In *P. aeruginosa* there are at least two different quorum-sensing systems, *las* (Gambello & Iglewski, 1991) and *rhl* (Ochsner & Reiser, 1995), which consist of two signal-generating synthetases (LasI/RhlI) and two cognate transcriptional regulators (LasR/RhlR). The major products of the peptides LasI and RhlI are the autoinducers *N*-(3-oxododecanoyl) homoserine lactone (OdDHL; also referred to as $3OC_{12}$-HSL or PAI-1) (Pearson *et al*., 1994) and *N*-butanoyl homoserine lactone (BHL; also referred to as C_4-HSL or PAI-2) (Pearson *et al*., 1995; Winson *et al*., 1995), respectively. These two quorum-sensing systems have been shown to be involved in biofilm differentiation (Davies *et al*., 1998). Biofilm differentiation plays an important role in

Abbreviations: BHL, *N*-butanoyl homoserine lactone; GFP, green fluorescent protein; LB, Luria broth; LPS, lipopolysaccharide; MPPA, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate (also known as monopalmitoylphosphatidic acid); OdDHL, *N*-(3-oxododecanoyl) homoserine lactone.

the protection of *P. aeruginosa* from the host-defence system and from the action of antibiotics (Costerton *et al.*, 1987). In addition, OdDHL interferes with the host immune system, where it specifically down-regulates the production of the cytokines IL-12 and TNF-α which support the bactericidal Th-1 milieu and protect the host, a finding which has to led the suggestion that the signal molecule is also an important virulence factor (Telford *et al.*, 1998).

In addition to being regulated by quorum-sensing systems, *Pseudomonas* spp. virulence factors are produced in response to a variety of environmental factors. For example, several of the extracellular virulence factors accumulate to maximal levels only under conditions of low iron in the growth medium, e.g. the siderophores pyochelin and pyoverdin, exotoxin A, and the exoproteases elastase and alkaline protease (Cox, 1993; Vasil & Ochsner, 1999). In addition, zinc has been shown to enhance pyoverdin production (Höfte *et al.*, 1993; Rossbach *et al.*, 2000), and zinc and calcium have been shown to be important for the efficient production and processing of elastase and the LasA protease (Brumlik & Storey, 1992; Olson & Ohman, 1992). Evidence also exists suggesting that limiting phosphate availability may also be an environmental signal in inducing phospholipase C synthesis (Shortridge *et al.*, 1992).

In the present study, evidence is presented which indicates that a specific lysophospholipid, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate [also called monopalmitoylphosphatidic acid (MPPA)], which is generated by the secretory phospholipase A2 and accumulates in inflammatory exudates (Fourcade *et al.*, 1998; Paya *et al.*, 1996), inhibits the extracellular accumulation of alginate, pyoverdin, elastase and LasA protease in cultures of *P. aeruginosa* PAO1 grown in Luria broth (LB).

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Preparation of LB containing MPPA. MPPA was purchased from Avanti Polar-Lipids. It was added to LB (EZ Mix Powder; Sigma) at a concentration of 500 μg ml^{-1}. The mixture was sonicated for 5 min in an ultrasonic water bath at room temperature, and then incubated overnight at 37 °C in a circulating water bath. After this incubation, undissolved MPPA was removed by centrifugation at 16000 ***g*** for 10 min at room temperature. The supernatant contains approximately 160 μM MPPA (Krogfelt *et al.*, 2000). LB containing 160 μM MPPA was diluted 1:1 with LB, to give LB containing 80 μM MPPA. The diluted LB was then diluted 1:1 with LB, to give LB containing 40 μM MPPA.

Determination of the zinc and iron content of the LB and the LB containing MPPA. The zinc and iron contents of the LB and the LB containing MPPA (80 μM) were determined by atomic absorption (CEIMIC). The zinc and iron concentrations in LB lot 98H8213 were 10·3 and 7·2 μM, respectively, and in lot 109H8206 they were 10·6 and 7·2 μM, respectively. In the LB (lot 98H8213) containing 80 μM MPPA, the zinc and iron concentrations were 5·7 and 3·6 μM, respectively.

Culture conditions and growth measurements. *P. aeruginosa* PAO1 was grown overnight in a rotary-shaking water bath at 37 °C to about 10^9 c.f.u. ml^{-1}. The next day, *P. aeruginosa* PAO1 was inoculated at 10^3 c.f.u. ml^{-1} into culture tubes (15 mm × 100 mm) containing 2 ml aliquots of LB or 2 ml aliquots of LB containing MPPA. Cultures were incubated by standing them in a water bath at 37 °C for periods of up to 48 h, as required. Growth was assayed by optical density measurements at 500 nm and, where indicated, by viable counts. In all experiments in which green fluorescent protein (GFP) synthesis was measured, *P. aeruginosa* PAO1 containing the plasmid pMRP17R$^-$, which expresses the GFP, was grown in the presence of carbenicillin (60 μg ml^{-1}), which prevents loss of the plasmid.

Quantification of GFP in liquid cultures. Expression of *lasB*::*gfp* was measured by detection of the green fluorescence with a Perkin Elmer LS-5B Luminescence Spectrometer (470 nm excitation wavelength; 515 nm emission wavelength). At the time of measurement, the background fluorescence emitted by un-inoculated LB was used as a control, and this value (routinely about 50) was subtracted from the subsequent sample values. Cultures were diluted fivefold in LB; the fluorescence emitted by these diluted cultures of *P. aeruginosa* PAO1(pMRP17R$^-$) was routinely about 250. The background fluorescence measured in fivefold-diluted LB-grown cultures of *P. aeruginosa* PAO1 without pMRP17R$^-$ was routinely about 50. In each experiment, fluorescence measurements of a fivefold-diluted 48 h LB-grown culture of *P. aeruginosa* PAO1(pMRP17R$^-$) diluted additionally over a 20-fold range generated a linear standard curve. This curve was used to calculate the relative amounts of fluorescence in each of the remaining cultures within that experiment.

Assays for pyoverdin and proteolytic activity in culture supernatants. Culture supernatants for assays of proteolytic activity and the presence of pyoverdin were prepared by centrifugation (16000 ***g*** for 10 min at room temperature). The relative amounts of pyoverdin present in culture supernatants were measured at A_{380} (Haas *et al.*, 1991). The proteolytic activity of the culture supernatants was measured on nutrient broth casein agar plates [Nutrient agar (Difco) plus 1% skim milk powder] containing 100 μg streptomycin sulfate ml^{-1} (to prevent growth of residual bacteria). Supernatant samples (50 μl) were added to wells within the plates, which had been made by removing agar plugs (6·5 mm diameter). The plates were incubated for 24 h at 37 °C, after which time the diameter of the zone of casein hydrolysis was measured. To compare the relative amounts of proteolytic activity within the culture supernatants (on the basis of 50 μl aliquots) from each experiment, a standard curve of proteolytic activity was generated. This was done by diluting a fresh LB-grown culture supernatant with LB such that the diameters (mm) of the zones of casein hydrolysis obtained from 50 μl aliquots of the diluted LB-grown culture related to proteolytic activities of 100, 80, 60, 40, 20, 10, 5 and 2·5%. Standard curves for the actual proteolytic activities (mm) versus the $\log_{10}$ of the theoretical activities (%) were linear.

Immunoblot analysis of LasB and LasA. Washed pellets from 2 ml cultures of *P. aeruginosa* PAO1 were resuspended in 2 ml of fresh LB and sonicated as described previously (Zhou *et al.*, 1997). Sonicates (500 μl) and cell-free filtered (0·22 μm pore size) supernatants (500 μl) were mixed with 100 μl of 6× concentrated sample buffer (0·35 M Tris/HCl, pH 6·8; 10% SDS; 6% 2-mercaptoethanol; 30% (v/v) glycerol; 0·012% bromophenol blue). The mixtures were boiled for 5 min and

Table 1. Bacterial strains and plasmids used in this study

Bacteria/plasmid	Relevant genotype/phenotype	Source/reference
P. aeruginosa		
PAO1	Wild-type	Toder *et al.* (1994)
PAO-B1	*lasB*::Ω of PAO1	Toder *et al.* (1994)
PAO-MW20	*rpoS* mutant of PAO1; Gm^R	Whitely *et al.* (2000)
Plasmids		
pMH297	OdDHL monitor, *lasB*::*luxCDABE* P_{lac}::*lasR*; Ap^R	Charlton *et al.* (2000)
pSB536	BHL monitor, *ahyR ahyI*::*luxCDABE*; Ap^R	Swift *et al.* (1997)
$pMRP17R^-$	*lasB*::*gfp* transcriptional fusion, contains the *lasB* promoter, its upstream transcriptional regulatory region, and *gfp* in pEX1.8 (Pearson *et al.*, 1997); Ap^R	M. R. Parsek

samples (40 µl) were loaded alongside pre-stained protein standards (BioRad). Samples were separated on 10% acrylamide gels and were transferred to nitrocellulose membranes by standard methods (Gallagher, 1999; Gallagher *et al.*, 1997). Blots were treated with 1:8000 dilutions of either rabbit anti-elastase serum (Olson & Ohman, 1992) or rat anti-LasA serum (Olson & Ohman, 1992). The chemiluminescent detection of LasB or LasA was performed using the appropriate secondary antibody and the protocol supplied with the ECL chemiluminescence Western-blotting kit (Amersham Pharmacia Biotech). Relative amounts of the 33 kDa elastase protein made in the presence and absence of MPPA were determined by densitometry (Molecular Dynamics Personal Densitometer SI).

Extraction of autoinducers. Autoinducers were isolated from 2 ml static cultures of *P. aeruginosa* PAO1 grown at 37 °C. The cultures were homogenized by vortexing, and the cells were pelleted by centrifugation. Cell-free supernatants were extracted twice in glass test tubes with 3 ml acidified ethyl acetate (100 µl glacial acid per 100 ml). The solvent was removed by evaporation under a gentle stream of nitrogen gas. The dried residues were re-dissolved in 100 µl of ethanol and stored at −20 °C.

Assay of autoinducer concentration. The concentrations of the autoinducers in the extracted cultures were determined by a bioluminescent assay. OdDHL and BHL activities were assayed by use of *E. coli* JM109-based monitor strains harbouring pMH297 and pSB536, respectively (Table 1). Overnight cultures of the autoinducer monitor strains were subcultured at an OD_{450} of 0·05 in AB minimal medium (Clark & Maaløe, 1967) supplemented with 0·1% glucose and Casamino acids. The cultures were grown with shaking (200 r.p.m.) at 37 °C. At OD_{450} 0·3, the cultures were split into aliquots and each aliquot was placed in a 5 ml glass test tube. Autoinducer extracts were added (2 µl per 1·0 ml culture), and the cultures were further incubated for 2 h at 37 °C with shaking (200 r.p.m.). A 100 µl sample of each culture was retrieved for measurement of its bioluminescence (1253 Luminometer, Bio-Orbit Oy, Turku, Finland) and its OD_{450} value. The autoinducer activity was calculated as the specific bioluminescence. The autoinducer concentration in the extracted cultures was determined from a standard curve generated by use of pure autoinducer standards.

Biofilm formation. This was assessed using the procedure of O'Toole *et al.* (1999). Twenty-four-well Nunclon polystyrene culture plates (Nunc, Roskilde, Denmark) containing 2 ml LB or 2 ml LB containing 80 µM MPPA were inoculated, as described above. Following 28 or 48 h of incubation at 37 °C, the wells were washed four times with LB and stained with crystal violet. After washing to remove excess stain, the remaining crystal violet dye was solubilized with 2·5 ml of 95% ethyl alcohol. The solubilized dye was then diluted twofold and the level of crystal violet recovered was assessed in a spectrophotometer at 580 nm.

Alginate determination. Ten static 2 ml LB-grown cultures of *P. aeruginosa* PAO1 (incubated for 48 h at 37 °C) were pooled and added to 60 ml of 0·9% sterile saline. The mixture was vortexed extensively and centrifuged at 10000 ***g*** for 12 min at room temperature. The supernatant was saved and the pellet was resuspended in 5 ml of 0·9% sterile saline, vortexed extensively and then centrifuged as above. The first and second supernatants were combined, ethanol precipitated and then assayed for alginate, as described by May & Chakrabarty (1994). The pellet was assayed for protein (see below) using the Lowry method. Ten static 2 ml *P. aeruginosa* PAO1 cultures grown in LB containing MPPA (80 µM) were assayed for the presence of alginate and protein in the same manner as the LB-grown cultures.

Protein determinations. Statically grown 2 ml cultures of *P. aeruginosa* PAO1 were centrifuged at room temperature for 10 min at 10000 ***g***. The cell pellets were resuspended in 1 ml of saline and were precipitated with 1 ml of 10% (w/v) trichloroacetic acid (TCA). The precipitates were centrifuged at room temperature for 10 min at 10000 ***g***, and were resuspended in 100 µl of 1 M NaOH. Supernatants from the static cultures were filtered free of *P. aeruginosa* PAO1 (0·22 µm pore-size filters), TCA-precipitated and then resuspended in 1 M NaOH, as described above. Protein determinations of the cell and supernatant fractions were performed using the Lowry method.

Isolation of LPS and immunological detection. O-serotyping was performed at the Statens Seruminstitut, as described by Liu *et al.* (1983). LPS was isolated from 48 h static cultures of *P. aeruginosa* PAO1, as described previously (Coyne *et al.*, 1994). LPS samples were analysed for O5 antigen by immunoblotting SDS polyacrylamide gels, as described previously (Coyne *et al.*, 1994).

Statistics. Student's *t*-test was used to evaluate the effect of MPPA on *P. aeruginosa* PAO1 growth, pyoverdin accumulation and exoprotease synthesis.

RESULTS

Growth, presence of pyoverdin, and exoprotease production in shake and static cultures of *P. aeruginosa* PAO1

In a preliminary set of experiments, bacterial growth, the presence of pyoverdin and exoprotease production were compared after 24 and 48 h incubation at 37 °C in shake (20 ml) and static (2 ml) LB-grown cultures inoculated with *P. aeruginosa* PAO1 at 10^3 c.f.u. ml^{-1}. Whereas growth in the shake cultures (OD_{500}) was about twice that of the static cultures, pyoverdin production in the shake cultures was only half that observed in the static cultures. Cell-free supernatants of the shake cultures exhibited no exoprotease activity on casein agar plates, whereas cell-free supernatants of the static cultures could be diluted 20- to 30-fold and still exhibited exoprotease activity (data not shown). In addition, LB-grown static cultures of *P. aeruginosa* PAO1 allowed the formation of a distinct biofilm. For these reasons, we used static LB-grown cultures for the studies detailed below.

Effect of MPPA on the growth of *P. aeruginosa* PAO1 and on the extracellular accumulation of pyoverdin and exoprotease

Specific phospholipids enhance the activity of β-lactam antibiotics against LB-grown *P. aeruginosa* PAO1 and against LB-grown *P. aeruginosa* strains isolated from the sputum of cystic fibrosis patients (Krogfelt *et al.*, 2000). The most active phospholipid is MPPA (Krogfelt *et al.*, 2000). Experiments were performed to determine whether MPPA has any effect on *P. aeruginosa* PAO1 growth and on pyoverdin and exoprotease production. LB-grown cultures of *P. aeruginosa* PAO1 (2 ml), inoculated at about 10^3 c.f.u. ml^{-1}, were grown statically at 37 °C for 48 h in the presence of 40 and 80 μM MPPA. The presence of 40 μM MPPA in the growth medium had no effect on *P. aeruginosa* PAO1 growth nor on the accumulation of extracellular pyoverdin; however, extracellular exoprotease activity was reduced by about 10-fold ($P < 0{\cdot}05$) (Table 2). In the presence of 80 μM MPPA, the *P. aeruginosa* PAO1 doubling time was increased by about 1·5-fold (3·62 h when grown in LB alone compared to 5·33 h when grown in LB + MPPA) (Fig. 1), the final growth yield (measured at OD_{500}) was reduced by 40 % ($P < 0{\cdot}001$), the extracellular accumulation of pyoverdin was reduced by 67 % ($P < 0{\cdot}001$), and extracellular protease activity was undetectable (Table 2). In control experiments, MPPA (80 μM) was added to the cell-free supernatants containing exoprotease activity and the mixture was incubated at 37 °C for 24 h. Under these conditions, MPPA had no effect on exoprotease activity, ruling out the possibility that the exoproteases were inactivated by MPPA.

P. aeruginosa PAO1 is known to secrete at least two exoproteases, elastase (encoded by *lasB*) and LasA (encoded by *lasA*). To determine which exoprotease was detected by the casein agar plate assay, the following experiment was performed. *P. aeruginosa* PAO-B1, a *lasB* mutant of our PAO1 strain (Toder *et al.*, 1994), was grown statically in 2 ml LB cultures for 48 h at 37 °C. The culture supernatant was then assayed for proteolytic activity in the wells of casein agar plates, as described in Methods. *P. aeruginosa* PAO-B1 cell-free supernatants did not shown any protease activity when placed in wells in casein agar plates, although this strain is known to synthesize LasA (Toder *et al.*, 1994). In contrast, *P. aeruginosa* PAO1 cell-free supernatants could be diluted greater than 20-fold and still showed proteolytic activity. These data suggest that the only exoprotease being detected on casein plates with wells containing the *P. aeruginosa* PAO1 cell-free culture supernatants, derived from 2 ml statically grown cultures, was elastase. Hence, the exoprotease activity on casein agar plates will be referred to as elastase activity from this point on. However, it should be noted that although it appears elastase is the only protease detected on casein agar plates, we do not mean to imply that elastase is the only protease synthesized in static LB-grown cultures of *P. aeruginosa* PAO1. In fact, as described below, although not detected on casein agar plates, *P. aeruginosa* PAO1 produces LasA protease in static LB cultures.

lasB::*gfp* expression in the presence of MPPA

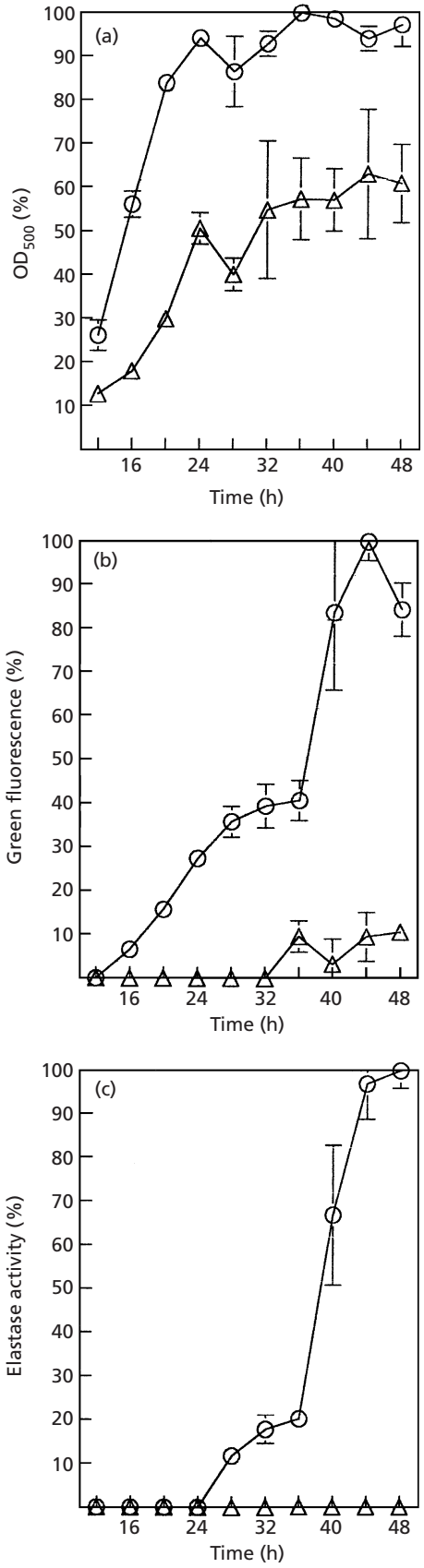
To investigate the effect of MPPA on the transcription of *lasB*, *a lasB*::*gfp* transcriptional fusion, containing the regulatory region upstream of *lasB* (Rust *et al.*, 1996; Dr

Table 2. Effect of MPPA on *P. aeruginosa* PAO1 growth, pyoverdin production and exoprotease accumulation

Cultures were grown statically for 48 h at 37 °C.

Growth medium	Growth (OD_{500})	Pyoverdin (A_{380})	Exoprotease (%)
LB ($n = 15$)	1·53 ± 0·09	1·69 ± 0·18	100 ± 2·60*
LB + MPPA (40 μM; $n = 6$)	1·69 ± 0·40	1·41 ± 0·42	9·10 ± 6·00
LB + MPPA (80 μM; $n = 15$)	0·92 ± 0·12	0·56 ± 0·20	0

* One hundred percent exoprotease activity corresponds to a 20·0 mm in diameter zone of casein hydrolysis (including the 6·5 mm diameter of the well). The exoprotease activity in cultures grown in the presence of 40 μM MPPA was determined using a standard curve, as described in Methods.



Matthew R. Parsek, personal communication) and supplied to *P. aeruginosa* PAO1 *in trans* on pMRP17R$^-$ (Table 1), was used to evaluate the activity of the *lasB* promoter. The data from one of two experiments performed with essentially identical results are illustrated in Fig. 1. The LB-grown *P. aeruginosa* PAO1 (pMRP17R$^-$) cultures entered stationary phase about 24 h post-inoculation (Fig. 1a). The *lasB*::*gfp* fusion was expressed in two cycles. The first cycle of *lasB*::*gfp*-directed GFP synthesis began between 12 and 16 h post-inoculation, and continued for about 12 h (Fig. 1b). The second cycle of GFP synthesis began between 36 and 40 h post-inoculation, and stopped between 44 and 48 h post-inoculation (Fig. 1b). The accumulation of active extracellular elastase followed the pattern of *lasB*::*gfp* expression, except that extracellular elastase accumulation lagged several hours behind the start of the first cycle of transcription (compare Fig. 1b, c), suggesting either that the *lasB*::*gfp* transcriptional fusion does not accurately reflect first cycle *lasB* transcription or that first cycle *lasB* transcripts are post-transcriptionally regulated. Post-transcriptional iron regulation of *lasB* expression has, in fact, been demonstrated (Brumlik & Story, 1998). In any case, the first cycle of *lasB*::*gfp* expression accounted for 40 % of the total GFP synthesis and 20 % of the total elastase activity (compare Fig. 1b, c). The second cycle of *lasB*::*gfp* expression and the extracellular accumulation of elastase were essentially simultaneous (compare Fig. 1b, c).

In the presence of 80 μM MPPA, *P. aeruginosa* PAO1 also entered stationary phase at about 24 h post-inoculation, and reached a level of growth of about 60 % of the stationary-phase level obtained when grown in LB only, as measured at OD_{500} (Fig. 1a) and by viable counts [48 h incubation in LB alone, c.f.u. ml^{-1} = $5{\cdot}23 \times 10^8$ ($\pm 1{\cdot}70 \times 10^8$, $n=4$); 48 h incubation in LB + MPPA, c.f.u. ml^{-1} = $3{\cdot}35 \times 10^8$ ($\pm 0{\cdot}64 \times 10^8$, $n=4$)]. The expression of *lasB*::*gfp*, as measured by GFP synthesis, began between 32 and 36 h post-inoculation, and stopped within 4 h at a level 10-fold lower than that observed in cultures grown in LB only (Fig. 1b), despite a less than twofold decrease in the growth yield (Fig. 1a). As in previous experiments, in the presence of 80 μM MPPA, active extracellular elastase did not accumulate (Fig. 1c). Therefore, these data suggest that MPPA in the culture medium resulted in the inhibition of *lasB* promoter activity, when compared to cultures grown in LB only.

Fig. 1. (a) Effect of MPPA on *P. aeruginosa* PAO1 growth. An OD_{500} value of 1·55 was equivalent to 100 %. LB alone, ○; LB + MPPA (80 μM), △. (b) Effect of MPPA on *lasB*::*gfp* expression. A green fluorescence reading (at 515 nm) of 164 was equivalent to 100 %. *P. aeruginosa* PAO1(pMRP17R$^-$) cultures were grown in LB (○) and in LB containing 80 μM MPPA (△). (c) Effect of MPPA on exoprotease accumulation. A zone of elastase activity of 19·5 mm (including the 6·5 mm diameter of the well) in diameter on casein agar plates was equivalent to 100 %. LB alone, ○; LB + MPPA (80 μM), △. In each graph the data points represent the mean ± SD for four separate 2 ml static cultures.

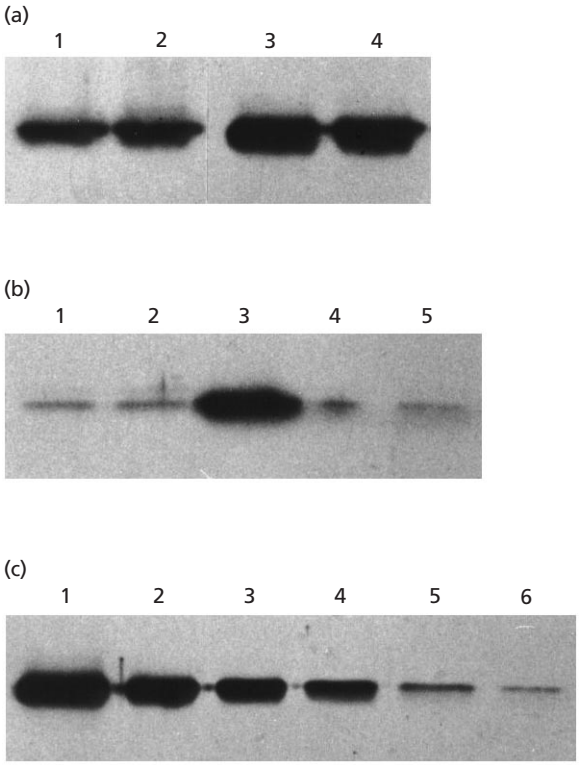


Fig. 2. Immunoblot analysis of *P. aeruginosa* PAO1 elastase production in the presence and absence of MPPA. (a) *P. aeruginosa* PAO1 grown in LB. Lanes 1 and 2, cell extracts from two independent cultures; lanes 3 and 4, cell-free supernatants from the same two independent cultures. (b) *P. aeruginosa* PAO1 grown in LB containing 80 μM MPPA. Lanes 1 and 2, cell extracts from two independent MPPA cultures; lane 3, LB cell-free supernatant [same as (a, lane 4)]; lanes 4 and 5, cell-free supernatants from the same two independent MPPA cultures. (c) Immunoblot analysis of serial twofold dilutions of the LB cell-free supernatant of (a, lane 4). Lanes: 1, undiluted; 2, diluted twofold; 3, diluted fourfold; 4, diluted eightfold; 5, diluted 16-fold; 6, diluted 32-fold.

P. aeruginosa PAO1 grown in the presence of 80 μM MPPA synthesizes a low level of inactive elastase

Elastase is synthesized as a 53 kDa pre-proenzyme which is processed to a 51 kDa inactive proenzyme as it translocates across the inner membrane (Iglewski *et al*., 1990; Kessler & Safrin, 1988). Proelastase is then further processed to its mature 33 kDa form in the periplasm, but remains non-covalently bound to its 18 kDa propeptide and remains inactive (Kessler & Safrin, 1988; Kessler *et al*., 1998). The propeptide–elastase complex is then secreted from the periplasm across the outer membrane by the *P. aeruginosa* type II general Xcp secretion pathway (Martinez *et al*., 1998; Tommassen *et al*., 1992). Elastase is activated extracellularly by degradation of the propeptide (Kessler *et al*., 1998).

To examine the effect of MPPA on translational and processing events, the relative amounts of intracellular and extracellular elastase in 48 h static *P. aeruginosa* PAO1 cultures incubated in the presence and absence of MPPA (80 μM) were determined by immunoblotting. Neither intracellular nor extracellular unprocessed pre-proelastase (53 kDa) and proelastase (51 kDa) were detected in control *P. aeruginosa* PAO1 LB-grown cultures or in cultures grown in the presence of 80 μM MPPA (data not shown). In 48 h LB-grown cultures, the intracellular level of the 33 kDa elastase protein was about 40 % that of the extracellular level (determined by densitometry, also compare Fig. 2a, c). For cultures grown in the presence of MPPA (80 μM), intracellular levels of the 33 kDa elastase protein were found to be about 7 % those of the intracellular levels detected in cultures grown in LB (determined by densitometry, also compare Fig. 2a–c). When corrected for differences in growth, the intracellular levels of the 33 kDa elastase protein in the presence of MPPA were 11·7 % of those detected in the LB-grown cultures. Extracellular 33 kDa elastase protein was also detected in cultures grown in the presence of 80 μM MPPA, but at levels of about 6 % those detected in supernatants of cultures grown in LB (determined by densitometry, also compare Fig. 2b, c), 10 % when corrected for differences in growth. Thus, it appears that 80 μM MPPA inhibited both the intracellular and extracellular accumulation of the 33 kDa elastase protein by about 10-fold. In addition, it appears that the 33 kDa elastase present in MPPA supernatants was present in an inactive form.

Activation of exoprotease in MPPA-supplemented culture supernatants

Since LB culture supernatants could be diluted 20- to 30-fold and still demonstrated elastase activity on casein agar plates, and since proelastase is thought to be activated by elastase (Kessler *et al*., 1998), an attempt was made to activate the small amount of inactive elastase in the MPPA culture supernatants by adding low levels of active elastase to supernatants from cells grown in the presence of MPPA. More specifically, cell-free supernatants derived from *P. aeruginosa* PAO1 cultures grown in the presence of 80 μM MPPA were treated with a 20-fold-diluted cell-free supernatant derived from a *P. aeruginosa* PAO1 culture grown in LB, incubated for 1 h at 37 °C and assayed for proteolytic activity. The 20-fold-diluted cell-free LB supernatant alone showed little protease activity on casein agar plates exclusive of the 6·5 mm well diameter (2·70 ± 0·88 mm, $n = 8$). As expected, no elastase activity was detected in MPPA supernatants. An increase in elastase activity was, however, observed ($P < 0{\cdot}001$) in the MPPA supernatants that had been treated with the 20-fold-diluted LB cell-free supernatant (5·53 ± 1·51 mm, $n = 8$). The additional elastase activity observed was of the order of 5 % of that normally obtained in LB cultures. No increase in activity was detectable if the MPPA supernatant was heated (100 °C) for 30 min before addition of the diluted LB supernatant. Similarly, no activation occurred if the LB supernatant was heated before being added to the MPPA supernatant (data not shown). In addition, even when diluted only fivefold, *P. aeruginosa* PAO-B1 cell-free supernatants (see above)

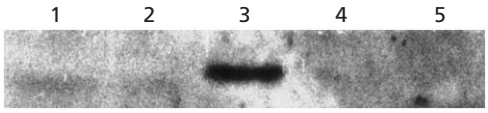


Fig. 3. Immunoblot analysis of *P. aeruginosa* PAO1 LasA production in LB and in LB containing 80 μM MPPA. Lanes 1 and 2, cell extracts from two independent MPPA cultures; lane 3, LB cell-free supernatant; lanes 4 and 5, cell-free supernatants from the same two independent MPPA cultures.

failed to activate inactive elastase in cell-free *P. aeruginosa* PAO1 MPPA supernatants; however, 20-fold-diluted LB supernatants of *P. aeruginosa* PAO1 did activate the inactive elastase. Furthermore, cell-free supernatants derived from *P. aeruginosa* PAO1 LB-grown cultures failed to activate *P. aeruginosa* PAO-B1 cell-free supernatants. It therefore appears that the active elastase in LB-grown culture supernatants is what activates inactive elastase in the MPPA supernatants.

To this point, these data suggest that cells grown in the presence of MPPA (80 μM) show reduced *lasB* transcription and correspondingly reduced levels of elastase accumulation. Overall this appears to result in the production of low levels of inactive elastase, which can be activated under the appropriate conditions.

LasA synthesis in the presence of MPPA

LasA, a second *P. aeruginosa* PAO1 zinc metalloprotease, is thought to be selective for Gly-Ala peptide bonds within Gly-Gly-Ala sequences in elastin (Kessler *et al*., 1997), and it is required for maximal elastase activity on elastin (Kessler *et al*., 1997). LasA synthesis is positively regulated by quorum sensing (Brint & Ohman, 1996; Passador *et al*., 1993). LasA is synthesized as a 42 kDa proenzyme that is processed extracellularly via a transient 28 kDa intermediate and a 14 kDa propeptide fragment (Kessler *et al*., 1998). The 28 kDa intermediate is then further processed to the mature 20 kDa LasA protein (Kessler *et al*., 1998). The relative amounts of intracellular and extracellular LasA protein present in 48 h LB-grown cultures of *P. aeruginosa* PAO1 and in cultures incubated in the presence of MPPA (80 μM) were determined by immunoblotting, using antibodies to LasA. Although 48 h LB-grown culture supernatants contained amounts of the 20 kDa mature LasA protein that could be diluted fourfold and still be within the detection limit for the immunoblotting procedure, the 20 kDa mature LasA protein was not found in *P. aeruginosa* PAO1 MPPA cell extracts nor in culture supernatants (Fig. 3). Neither the 42 kDa proenzyme, the 28 kDa intermediate nor the 14 kDa propeptide fragment were found in 48 h LB-grown *P. aeruginosa* PAO1 culture cell extracts or in their culture supernatants, neither were they found in the MPPA *P. aeruginosa* PAO1 culture cell extracts or in their culture supernatants (data not shown). It would appear, therefore, that in addition to inhibiting the intracellular and extracellular accumulation of elastase, MPPA also inhibited the intracellular and extracellular accumulation of the LasA protease.

Alginate synthesis and biofilm formation

P. aeruginosa PAO1 forms a thick, readily visible biofilm in the menisci of static LB-grown cultures. In the presence of 80 μM MPPA, such a biofilm is not apparent. To confirm this observation, biofilm formation was assessed after 28 and 48 h of incubation using the staining and dye-solubilization assay described by O'Toole *et al*. (1999). The results shown in Table 3 demonstrate that at 28 and 48 h post-inoculation in the presence of MPPA growth was inhibited by approximately twofold (55 and 43 % at 24 and 48 h, respectively), whereas biofilm formation was inhibited by almost 10-fold (89 % inhibition) at 28 h and fourfold (73 % inhibition) at 48 h. It is likely that the 48 h data actually underestimate the level of inhibition on biofilm formation, in as much as the heavy biofilms formed at 48 h in LB-grown cultures appeared to separate from the sides of the microtitre plates, resulting in somewhat lower levels of staining and dye recovery than were expected.

Since *P. aeruginosa* biofilms form in a matrix of alginate (Baltimore, 1993; Speert *et al*., 1987), 48 h static *P. aeruginosa* PAO1 LB-grown cultures and LB + MPPA-grown cultures were tested for alginate production. In two separate experiments, *P. aeruginosa* PAO1 LB-grown cultures produced 24·1 μg of alginate (mg cell protein)$^{-1}$ and 37·2 μg of alginate (mg cell protein)$^{-1}$, respectively, whereas in both experiments *P. aeruginosa* PAO1 grown in the presence of 80 μM MPPA produced only 1·2 μg of alginate (mg cell protein)$^{-1}$. Therefore, in addition to inhibiting the accumulation of elastase, LasA protease and pyoverdin, MPPA also inhibited the accumulation of alginate by 20- to 30-fold.

O-antigen synthesis in the presence of MPPA

P. aeruginosa strains isolated from cystic fibrosis patients produce reduced levels of exoproteases (Luzar & Montie, 1985) and O-antigen (Hancock *et al*., 1983). Since MPPA inhibited the accumulation of pyoverdin, elastase, LasA and alginate, it was of interest to determine whether growth of *P. aeruginosa* PAO1 in the presence of 80 μM MPPA had an effect on the O-serotype or on O-antigen synthesis. *P. aeruginosa* PAO1 grown in static 48 h LB cultures and in LB cultures containing 80 μM MPPA were tested with 20 polyclonal sera specific to each of the 20 international antigenic typing system (IATS) O-serotypes of *P. aeruginosa*. When grown in either the presence or the absence of MPPA, *P. aeruginosa* PAO1 serotyped as O2/O5. LPS was also purified from *P. aeruginosa* PAO1 cells grown in the presence and the absence of 80 μM MPPA. This was examined for O5 antigen by immunoblotting. No major difference was observed in the amount of O5 antigen produced by *P. aeruginosa* PAO1 when grown in the presence or in the absence of MPPA (data not shown).

Table 3. Effect of MPPA on *P. aeruginosa* PAO1 biofilm formation

All results are the mean for five replicates ± SD. Cultures were grown statically at 37 °C in 24-well microtitre plates. At the indicated times, cells were recovered and the wells were washed and stained with crystal violet.

Growth medium	Incubation time (h)	Growth (OD_{500})	Biofilm (A_{580})*
LB alone	28	1·87 ± 0·04	1·73 ± 0·36
LB + MPPA (80 μM)	28	0·84 ± 0·12	0·19 ± 0·12
LB alone	48	1·67 ± 0·13	0·45 ± 0·04
LB + MPPA (80 μM)	48	0·96 ± 0·01	0·12 ± 0·03

* Biofilm formation is expressed as the recovery (at A_{580}) of crystal violet following solubilization of the stain in 95 % ethanol.

Autoinducer synthesis in the presence of MPPA

The data presented above indicate that MPPA has a broad range of inhibitory effects relating to the production of virulence factors by *P. aeruginosa* PAO1. Since the synthesis of elastase and a number of other factors are believed to be regulated by quorum sensing, we speculated that differences in the concentration of signal molecules in the presence and the absence of MPPA could, at least in part, account for the observed differences in the extracellular accumulation of virulence factors. To examine this possibility, signal molecules were extracted at 4 h intervals between 12 and 48 h post-inoculation from 2 ml cultures of *P. aeruginosa* PAO1 grown statically in LB and in LB containing 80 μM MPPA. The concentration of the signal molecules was estimated by means of OdDHL- and BHL-specific indicator bacteria which expressed bioluminescence in a concentration-dependent manner. The data from one of two experiments performed with essentially identical results are illustrated in Fig. 4. Extracellular BHL began to accumulate between 12 and 16 h post-inoculation in LB-grown cultures, and accumulation peaked at about 20 h post-inoculation. The BHL level dropped thereafter (Fig. 4). Extracellular OdDHL began to accumulate between 16 and 20 h post-inoculation in LB-grown cultures, and its accumulation levelled off at about 28 h post-inoculation (Fig. 4). In the presence of 80 μM MPPA, the extracellular levels of both OdDHL and BHL reached LB-grown culture levels; however, the extracellular appearance of each lactone was delayed by about 8 h, relative to the LB control (Fig. 4). Therefore, enough OdDHL and BHL accumulated in *P. aeruginosa* PAO1 cultures grown in the presence of MPPA to stimulate *lasB*::*gfp* transcription. The failure of OdDHL and BHL to do so suggests either that these signal molecules are unable to re-enter *P. aeruginosa* PAO1 cells grown in the presence of 80 μM MPPA or that *lasB*::*gfp* transcription is inhibited because other regulators required for *lasB* expression are affected by MPPA.

It might be argued that since OdDHL and BHL appear about 8 h later in MPPA-supplemented cultures than they do in LB-grown cultures, there has not been sufficient time for the expression of *lasB* in the MPPA-supplemented cultures when assayed at 48 h post-incubation. However, when the incubation period for MPPA cultures of *P. aeruginosa* PAO1 was extended to 72 h, *lasB*::*gfp* expression remained at the same level as in the 48 h MPPA cultures, and elastase was still undetectable in the cell-free supernatants (data not shown). In addition, when, at 24 h post-inoculation, *P. aeruginosa* PAO1 cultures were centrifuged, resuspended in the 2 ml of LB in which they were grown and incubated for an additional 24 h, no effect on pyoverdin or elastase accumulation, or on *lasB*::gfp expression was observed. In contrast, centrifugation and resuspension of 24 h LB-grown cultures in LB containing 80 μM MPPA limited *lasB*::*gfp* expression and pyoverdin accumulation to 30 % and elastase accumulation to 6 % of the levels of LB cultures at 48 h (data not shown). In other words, MPPA is effective in blocking virulence factor synthesis, even when *P. aeruginosa* PAO1 has been incubated in LB for 24 h. Finally, when at 24 h post-inoculation *P. aeruginosa* PAO1 growing in MPPA culture medium was centrifuged, resuspended in 24 h MPPA culture medium to cell densities equal to those present in 48 h LB cultures and incubated for an additional 24 h, elastase still failed to accumulate, *lasB*::*gfp* expression was still inhibited and pyoverdin accumulation was still reduced (data not shown), indicating that in MPPA cultures a reduction in *P. aeruginosa* PAO1 density alone was not responsible for the lack of response to the presence of OdDHL and BHL.

RpoS plays no role in the MPPA-mediated inhibition of the accumulation of alginate, pyoverdin and elastase

RpoS, the stationary phase σ factor, regulates the expression of a number of *P. aeruginosa* PAO1 genes, the products of which confer increased tolerance to various forms of stress (Suh *et al.*, 1999). Expression of the *P. aeruginosa* PAO1 *rpoS* gene increases as cultures reach stationary phase (Whiteley *et al.*, 2000). Moreover, *P. aeruginosa* PAO1 *rpoS* mutants have been reported to accumulate increased levels of pyoverdin, pyocyanin and BHL (Suh *et al.*, 1999; Whiteley *et al.*, 2000). To

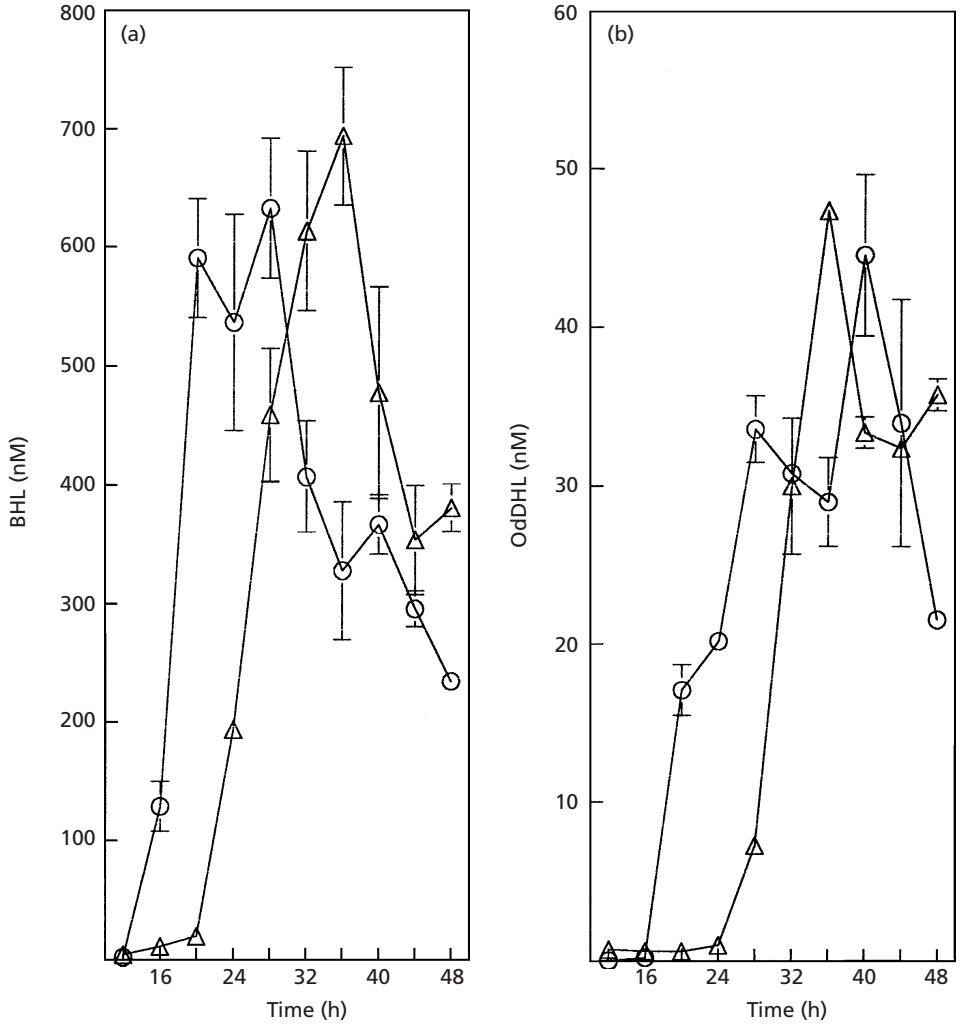


Fig. 4. Kinetics of synthesis of BHL (a) and OdDHL (b) in *P. aeruginosa* PAO1 LB-grown cultures (○) and in *P. aeruginosa* PAO1 cultures grown in LB containing 80 μM MPPA (△). Data are presented as the mean ± SD for three separate cultures.

determine whether the expression of *rpoS* was involved in the MPPA-induced inhibition of virulence factor production, *P. aeruginosa* PAO-MW20, a *P. aeruginosa* PAO1 *rpoS* mutant, was grown in 2 ml static cultures for 48 h and the effects of 80 μM MPPA on growth and on alginate, pyoverdin and elastase production were determined. Although PAO-MW20 appeared to be less susceptible to the growth-inhibiting effects of MPPA (LB, $OD_{500} = 1{\cdot}50 \pm 0{\cdot}08$, $n = 5$; MPPA, $OD_{500} = 1{\cdot}33 \pm 0{\cdot}05$, $n = 5$), pyoverdin production was inhibited by about 65% (LB, $A_{380} = 3{\cdot}12 \pm 0{\cdot}22$, $n = 5$; MPPA, $A_{380} = 1{\cdot}07 \pm 0{\cdot}08$, $n = 5$), alginate production was reduced by more than 10-fold [LB, 44·9 (experiment 1) and 68·3 (experiment 2); MPPA, 0·59 (experiment 1) and 6·1 (experiment 2); results expressed as μg alginate (mg cell protein)$^{-1}$] and elastase was essentially completely inhibited (LB diameter of zone of casein hydrolysis, $22{\cdot}5 \pm 0{\cdot}39$ mm, $n = 5$; MPPA, no detectable activity, $n = 5$; MPPA upon activation, about 2% of LB activity, i.e. $9{\cdot}06 \pm 0{\cdot}05$ mm, $n = 5$). While the data suggest a role for *rpoS* expression in limiting the growth of *P. aeruginosa* PAO1 in the presence of MPPA, it would appear that *rpoS* expression plays no role in the MPPA-mediated inhibition of pyoverdin, elastase and alginate accumulation.

Effect of the addition of calcium, iron, magnesium and zinc to LB containing MPPA

MPPA binds divalent cations in LB (Krogfelt *et al.*, 2000). LB contains 10·4 μM zinc (see Methods), 7·2 μM iron (see Methods), 263 μM calcium (Krogfelt *et al.*, 2000) and 225 μM magnesium (Krogfelt *et al.*, 2000). In the presence of 80 μM MPPA, the zinc concentration in LB is reduced to 5·7 μM (see Methods), the calcium concentration is reduced to 167 μM (Krogfelt *et al.*, 2000), the magnesium concentration is reduced to 128 μM (Krogfelt *et al.*, 2000) and the iron concentration is reduced to 3·6 μM (see Methods). In the presence of 80 μM MPPA, the transcription of *lasB*::*gfp*, as measured by GFP synthesis, was reduced to about 38% of the LB culture control level when corrected for growth,

Table 4. The effect of the addition of calcium (100 μM), iron (10 μM), magnesium (100 μM) and zinc (100 μM) on *P. aeruginosa* PAO1(pMRP17R⁻) growth, pyoverdin production, *lasB*::*gfp* expression and elastase activity in the presence of MPPA (80 μM)

Cultures (2 ml) were grown statically at 37 °C. Values of 100 % for growth, pyoverdin production and elastase activity are essentially as in the legend to Table 2, and the 100 % value for *lasB*::*gfp* expression (fluorescence at 515 nm) is 291.

LB plus	Growth (% of LB)*	Pyoverdin (% of LB per cell)†‡	*lasB*::*gfp* (% of LB per cell)§‡	Elastase activity (% of LB per cell)‖‡
No additions	100 ± 5·56¶	100 ± 10·7¶	100 ± 18·1	100 ± 3·75¶
MPPA	60·0 ± 7·80¶	55·2 ± 19·6¶	37·5 ± 16·9¶	0¶
MPPA + Ca^{2+} + Fe^{2+} + Mg^{2+} + Zn^{2+}	110 ± 4·46#	73·9 ± 6·8#	91·1 ± 23·5#	111 ± 14·7#

* OD_{500} of 48 h cultures.

† A_{380} of 48 h cultures.

‡ % of LB per cell was calculated as a percentage of the LB raw data value divided by the fractional growth relative to LB.

§ Fluorescence (at 515 nm).

‖ Casein hydrolysis on casein agar plates.

¶ $n = 13$.

$n = 6$.

pyoverdin accumulation was reduced to 55 % of the LB level and elastase was undetectable (Table 4). When LB containing 80 μM MPPA was supplemented with ferrous sulfate (10 μM), calcium chloride (100 μM), magnesium chloride (100 μM) and zinc chloride (100 μM), *P. aeruginosa* PAO1 growth levels returned to LB levels, pyoverdin accumulated at about 75 % of the LB levels, *lasB*::*gfp* expression was about 90 % of the LB levels and the extracellular accumulation of active elastase returned to the LB levels (Table 4). It therefore appears that the effects of MPPA on *P. aeruginosa* PAO1 can be reversed to a large extent by supplementing the medium with divalent cations.

Effect of EDTA on *P. aeruginosa* PAO1 growth, and on pyoverdin and elastase synthesis

Since EDTA chelates divalent cations and the effects of MPPA on the extracellular accumulation of pyoverdin and elastase appeared to be due, at least in part, to MPPA binding divalent cations, EDTA was tested for its ability to inhibit *lasB*::*gfp* expression and the accumulation of pyoverdin and elastase. *P. aeruginosa* PAO1 failed to accumulate active extracellular elastase in the presence of between 32 and 64 μM EDTA, depending on the experiment (data not shown). To ensure the inhibition of the extracellular accumulation of elastase, EDTA was used at a concentration of 125 μM in succeeding experiments. EDTA decreased *P. aeruginosa* PAO1 growth by about 15 % and *lasB*::*gfp* expression by about fourfold, and it completely prevented the accumulation of active elastase (Table 5). Immunoblot analysis showed that the elastase protein was present in EDTA supernatants at levels 5–10 % those of the LB supernatants (data not shown). In contrast to MPPA, however, EDTA did not inhibit pyoverdin production and, in fact, caused about a 30 % increase in its production ($P < 0{\cdot}05$) (Table 5). The addition of magnesium (125 μM), calcium (125 μM), zinc (125 μM) and iron (10 μM) to the growth medium reversed the effect of the presence of EDTA on growth, much of its effect on *lasB*::*gfp* expression, and allowed the accumulation of extracellular elastase (Table 5). Therefore, EDTA appears to mimic the effect of MPPA on *lasB*::*gfp* transcription and on the accumulation of extracellular elastase, but unlike MPPA, it does not inhibit the accumulation of extracellular pyoverdin.

Effect of MPPA on the cellular and extracellular protein content of *P. aeruginosa* PAO1

Experiments were performed to determine whether the growth of *P. aeruginosa* PAO1 in the presence of MPPA resulted in an excessive release of cellular proteins into the culture supernatant between 24 and 48 h of growth relative to the LB-grown cultures, as might be expected if, for example, MPPA induced cell lysis. In LB-grown cultures, the cellular protein content increased from about 170 μg (ml culture)$^{-1}$ to 220 μg (ml culture)$^{-1}$ between 24 and 30 h of incubation and remained constant thereafter. Between 24 and 36 h of incubation, the extracellular protein content for the LB-grown cultures increased from about 25 % of the cellular protein level to about 50 % of the cellular protein level, and it was still at this level after 48 h of incubation. This result was not unexpected, since *P. aeruginosa* PAO1 secretes numerous proteins involved in its pathogenesis (Howe & Iglewski *et al.*, 1984; Nicas *et al.*, 1985; Preston *et al.*, 1997; Woods *et al.*, 1982; Vasil *et al.*, 1993) as well as membrane vesicles containing virulence factors (Kadurugamuwa & Beveridge, 1997). Between 24 and 36 h of incubation in the presence of 80 μM MPPA, the cellular protein content remained constant at about 90 μg (ml culture)$^{-1}$; between 36 and 48 h of incubation

Table 5. The effect of calcium (100 μM), iron (10 μM), magnesium (100 μM) and zinc (100 μM) on *P. aeruginosa* PAO1(pMRP17R⁻) growth, pyoverdin production, *lasB*::*gfp* expression and elastase activity in the presence of EDTA (125 μM)

Cultures (2 ml) were grown statically at 37 °C. Values of 100 % for growth, pyoverdin production and elastase activity are essentially as in the legend to Table 2, and the 100 % value for *lasB*::*gfp* expression (fluorescence at 515 nm) is 291.

LB plus	Growth (% of LB)*	Pyoverdin (% of LB per cell)†‡	*lasB*::*gfp* (% of LB per cell)§‡	Elastase activity (% of LB per cell)‖‡
No additions	100 ± 4·52¶	100 ± 6·06¶	100 ± 5·04	100 ± 30·5¶
EDTA	84·5 ± 7·44¶	127 ± 14·9¶	27·1 ± 10·9¶	0¶
EDTA + Ca^{2+} + Fe^{2+} + Mg^{2+} + Zn^{2+}	128 ± 6·03¶	82·6 ± 11·6¶	61·0 ± 7·13¶	103 ± 19·2¶

* OD_{500} of 48 h cultures.

† A_{380} of 48 h cultures.

‡ % of LB per cell was calculated as a percentage of the LB raw data value divided by the fractional growth relative to LB.

§ Fluorescence (at 515 nm).

‖ Casein hydrolysis on casein agar plates.

¶ $n = 6$.

the cellular protein content increased by about 40 %, to 130 μg (ml culture)$^{-1}$, i.e. to about 60 % of the 48 h LB-grown culture cellular protein content. Between 24 and 36 h of incubation, the extracellular protein content in the MPPA cultures increased from a negligible amount to about 22 % of the cellular protein level, and remained at about this level 36 and 48 h post-inoculation. Therefore, relative to cellular protein, *P. aeruginosa* PAO1 cells grown in LB released twice the amount of extracellular proteins as cells grown in the presence of MPPA, contrary to what would be expected if cells grown in the presence of MPPA were leaking proteins or were lysing.

DISCUSSION

In the present investigation we have shown that the lysophospholipid MPPA exerts a broad range of inhibitory effects on the production of factors associated with virulence in *P. aeruginosa* PAO1, when present at a concentration of 80 μM in the growth medium. The concentration of lysophosphatidic acid in bovine foetal calf serum has been reported to be 20 μM (Ediger & Toews, 2001); therefore, the inhibitory effects observed in this study are at concentrations of MPPA only two- to fourfold higher than normal physiological concentrations. The effects of this concentration of MPPA include the inhibition of biofilm formation and inhibition of the extracellular accumulation of elastase, LasA protease, pyoverdin and alginate. In the case of elastase, its inhibition appears to be due to a decrease in *lasB* transcription (about a threefold decrease when a correction is made for differences in growth between the LB and MPPA cultures; Fig. 1 and Table 4), which is accompanied by a decrease in the amount of cellular and extracellular elastase protein that is accumulated (about a 10-fold decrease; Figs 1 and 2, and Table 4).

Previous studies have shown that a variety of ions are involved in regulating virulence factor gene expression and in the efficient production and processing of virulence factors in *P. aeruginosa* (Brumlick & Storey, 1992; Cox, 1993; Hofte *et al.*, 1993; Olson & Ohman, 1992; Rossbach *et al.*, 2000; Vasil & Ochsner, 1999). In addition, MPPA has been shown to bind divalent cations (Krogfelt *et al.*, 2000). In LB containing 80 μM MPPA, the calcium, iron, magnesium and zinc concentrations are reduced by 50–60 % relative to the concentrations of the same divalent cations in LB (see Methods and Krogfelt *et al.*, 2000). Replenishing MPPA cultures with magnesium, calcium, iron and zinc restored their growth, their *lasB*::*gfp* expression and their extracellular accumulation of elastase and pyoverdin to levels close to those of the LB-grown cultures (Table 4), suggesting that the effects of MPPA are due, at least in part, to its ability to bind divalent cations.

EDTA (125 μM) also inhibited *lasB*::*gfp* expression and the accumulation of extracellular elastase (Table 5); however, unlike MPPA, EDTA stimulated rather than inhibited the extracellular accumulation of pyoverdin. EDTA chelates zinc about 10^2-fold better than it does iron (Fe^{2+}), about 10^6-fold better than it does calcium and about 10^8-fold better than it does magnesium (Hodgins, 1961). Therefore, at an EDTA concentration of 125 μM it would be expected that all of the zinc (10·4 μM) and iron (7·2 μM), about 45 % of the calcium (117 μM) and none of the magnesium would be chelated. It might therefore be that the inhibition of pyoverdin accumulation in the presence of MPPA, but not in the presence of EDTA, is due to the differences in the cation concentrations in the two culture media. A detailed study of the effect of individual divalent cation additions to LB containing either MPPA or EDTA on the accumulation of *P. aeruginosa* PAO1 extracellular virulence factors will be the subject of a future communication.

While it appears that divalent cations can reverse or compensate for the inhibitory effects of MPPA, since

lysophosphatidic acid is known to insert into membranes (Christiansen & Carlsen, 1983), the possibility that MPPA may inhibit the accumulation of extracellular virulence factors by physically disrupting the *P. aeruginosa* PAO1 membrane structure cannot be excluded. Such membrane disruption could affect cellular secretory processes in general or alter the response of the bacterium to environmental signals.

The transcription of *lasB* is positively regulated by RpoS, and by the *lasR lasI* and *rhlR rhlI* quorum-sensing systems at high cell densities (Brint & Ohman, 1996; Gambello & Iglewski, 1991; Passador *et al*., 1993; Pearson *et al*., 1994; Suh *et al*., 1999; Whiteley *et al*., 1999, 2000). However, the inhibition of *lasB* expression by MPPA can not be explained by the small reduction in *P. aeruginosa* PAO1 growth in the presence of MPPA, since increasing the cell density did not reverse the effects of MPPA. Neither can it be explained by the amounts of OdDHL and BHL synthesized, since, in the presence of MPPA, OdDHL and BHL accumulation in culture supernatants was normal (Fig. 4). It is possible, however, that in the presence of MPPA, the signal molecules were unable to re-enter *P. aeruginosa* PAO1. Although there is, as yet, no evidence to support this hypothesis, if true, OdDHL and BHL would not be able to interact with their cognate R-proteins. As a consequence, the signals could fail to fully activate the transcription of their target genes. Experiments designed to test this hypothesis are presently under way. Similarly, it appears that the effect of MPPA on the production of *P. aeruginosa* PAO1 virulence factors does not involve *rpoS* expression, since in PAO-MW20 cultures incubated in the presence of MPPA, the levels of pyoverdin, elastase and alginate production were all inhibited to the same extent as in *P. aeruginosa* PAO1 cultures.

At the present time, it is unclear as to why the elastase protein accumulates to a level of only about 10 % of that seen in LB in the presence of MPPA (Fig. 2) when the *lasB*::*gfp* fusion is expressed at a level of 38 % of that observed in LB (Table 4). However, it should be noted that the *lasB*::*gfp* fusion used here is a transcriptional fusion that may not accurately reflect the degree to which the rate of transcription of the wild-type *lasB* gene in the presence of MPPA is reduced relative to its absence or the rate at which it is translated. Nevertheless, the data show that the *lasB* promoter is about threefold less active when *P. aeruginosa* PAO1 is grown in the presence of MPPA than when it is grown in LB only.

Our interest in MPPA originated from the finding that it enhances the activity of β-lactam antibiotics against *P. aeruginosa* PAO1 and against *P. aeruginosa* strains isolated from cystic fibrosis patients (Krogfelt *et al*., 2000). MPPA is a member of a class of phospholipids called lysophosphatidic acids, which contain one fatty acid esterified to the C1 atom of the glycerol moiety, but which are lacking the fatty acid normally esterified to the C2 atom of the glycerol moiety. Lysophosphatidic acids are believed to be generated from the phosphatidic acid present in the inner leaflet of damaged cell membranes (Fourcade *et al*., 1998) by secretory phospholipase A2, which accumulates in inflammatory exudates (Paya *et al*., 1996). Lysophosphatidic acids have recently been implicated in blocking neutrophil recruitment to damaged lung tissue (Abraham *et al*., 1995) and in inhibiting the metabolic burst of human neutrophils (Chettibi *et al*., 1994). MPPA may therefore be viewed as a natural anti-inflammatory agent.

It has been suggested that during the initial stages of infection with *P. aeruginosa*, antibodies to O-antigen, extracellular proteases and other virulence factors are synthesized, and these then interact with their cognate antigens to form immune complexes in the lungs of cystic fibrosis patients (Kronborg, 1995). The immune complexes, in turn, attract polymorphonuclear leukocytes (PMNLs) (Kronborg, 1995). The PMNLs then release elastase, cathepsin, oxygen radicals, etc., which leads to the initial inflammation and tissue damage (Kronborg, 1995; Kronborg *et al*., 1992). Later on, when chronic infection sets in and *P. aeruginosa* is protected against phagocytosis by biofilm formation, PMNLs continue to infiltrate the lungs and, in attempting to eradicate the infection, cause further inflammation and tissue damage (Kronborg, 1995; Kronborg *et al*., 1992). Therefore, in infected cystic fibrosis patients, when the lung is relatively healthy, it appears likely that *P. aeruginosa* would synthesize high levels of pyoverdin, elastase and LasA protease, thereby initiating inflammation and tissue damage. Extensive tissue damage would lead to increased levels of lysophosphatidic acids in inflammatory exudates which might lower the local divalent cation concentrations. Low divalent cation concentrations could then result in inhibition of the extracellular accumulation of *P. aeruginosa* pyoverdin, elastase and the LasA protease, in the inhibition of the further recruitment of PMNLs to the site of infection and in the prevention of the metabolic burst of those PMNLs that are already present. When the tissue is repaired and the lysophosphatidic acid concentration is reduced, the synthesis of pyoverdin, elastase and the LasA protease would resume, PMNLs would be recruited and the cycle would repeat. If this scenario is correct, it may be that treating cystic fibrosis patients with MPPA will limit the deleterious effects of chronic infection by reducing inflammation and by limiting the extracellular accumulation of *P. aeruginosa* pyoverdin, elastase and the LasA protease. In addition, since MPPA inhibits the accumulation of alginate, it might reduce *P. aeruginosa* biofilm formation in the lung, making the infection more amenable to antibiotic treatment. Furthermore, if high enough concentrations of MPPA can be achieved in the lungs, its ability to enhance the activities of β-lactam antibiotics (Krogfelt *et al*., 2000) might help to limit infection. However, it should be noted that one limiting factor in the use of MPPA in the lungs may be that the concentration of calcium in the sputum of cystic fibrosis patients is 0·7 mM, about 2·5-fold higher than in LB, and the sputum concentration of magnesium is 1·2 mM, about 5·3-fold higher than in LB (Halmerbauer *et al*., 2000). Animal experiments, pre-

sently in progress, should tell us whether MPPA shows clinical promise.

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