

Membrane-targeted synergistic activity of docosahexaenoic acid and lysozyme against *Pseudomonas aeruginosa*

Jose G. MARTINEZ*, Michael WALDON*, Qiyu HUANG*, Sandra ALVAREZ*, Ami OREN†, Natalie SANDOVAL*, Ming DU‡, Feimeng ZHOU‡, Alexandra ZENZ§, Karl LOHNER§, Robert DESHARNAIS* and Edith PORTER*†¹

*Department of Biological Sciences, California State University Los Angeles, 5151 State University Drive, Los Angeles, CA 90032, U.S.A., †David Geffen School of Medicine, University of California Los Angeles, 10833 Le Conte Ave, Los Angeles, CA 90095, U.S.A., ‡Department of Chemistry and Biochemistry, California State University Los Angeles, 5151 State University Drive, Los Angeles, CA 90032, U.S.A., and §Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedlstrasse 6, A-8042 Graz, Austria

Antimicrobial polypeptides, including lysozymes, have membrane perturbing activity and are well-documented effector molecules of innate immunity. In cystic fibrosis, a hereditary disease with frequent lung infection with *Pseudomonas aeruginosa*, the non-esterified fatty acid DA (docosahexaenoic acid), but not OA (oleic acid), is decreased, and DA supplementation has been shown to improve the clinical condition in these patients. We hypothesized that DA may, either alone or in conjunction with lysozyme, exert antibacterial action against *Ps. aeruginosa*. We found that DA and lysozyme synergistically inhibit the metabolic activity of *Ps. aeruginosa*, in contrast with OA. Electron microscopy and equilibrium dialysis suggest that DA accumulates in the

bacterial membrane in the presence of lysozyme. Surface plasmon resonance with live bacteria and differential scanning calorimetry studies with bacterial model membranes reveal that, initially, DA facilitates lysozyme incorporation into the membrane, which in turn allows influx of more DA, leading to bacterial cell death. The present study elucidates a molecular basis for the synergistic action of non-esterified fatty acids and antimicrobial polypeptides, which may be dysfunctional in cystic fibrosis.

Key words: antimicrobial peptide, bacterial cell wall, cystic fibrosis, innate host defence, large unilamellar vesicle (LUV), non-esterified fatty acid.

INTRODUCTION

The innate immune system is the first line of defence against invading micro-organisms. Among the key players are antimicrobial cationic peptides and proteins, which are thought to kill micro-organisms by membrane perturbation [1,2] and possibly by disruption of membrane-bound multienzyme complexes and intracellular events [3–5]. In addition, antimicrobial peptides also have immune modulatory functions that are important for innate and adaptive host defence [6]. Human lysozyme (~14.5 kDa) is one of the predominant antimicrobial polypeptides in all body fluids, reaching concentrations up to mg/ml levels [7]. It is well known for its peptidoglycan-hydrolysing activity [8], but it also exerts antibacterial activity through membrane perturbation [9].

Non-esterified fatty acids such as DA (docosahexaenoic acid) and OA (oleic acid) are also found in body fluids, including respiratory secretions, at high nanomolar concentrations [10,11]. DA (C_{22:6}) is a polyunsaturated fatty acid with documented activity against Gram-positive and Gram-negative bacteria [12]. OA (C_{18:1}) is a mono-unsaturated fatty acid. Reduced levels of DA, but increased levels of OA, have been measured in patients with cystic fibrosis, a hereditary disease affecting the cystic fibrosis transmembrane regulator protein [13]. These patients produce an altered mucus, leading to gravely delayed clearance of their airways and chronic lung infection with *Pseudomonas aeruginosa*, an opportunistic Gram-negative bacterium with characteristic phenotypic changes in cystic fibrosis patients [14]. The association between cystic fibrosis and diminished DA levels has been corroborated with an animal model [15], and dietary supplementation of DA has improved the clinical condition of cystic fibrosis patients in some studies [16].

Given that lysozyme is one of the essential antimicrobial polypeptides in the airways [7] and lysozyme levels are not reduced in cystic fibrosis patients [17], but DA levels are reduced, we hypothesized that DA may act synergistically with lysozyme, and we sought to define the contributions of DA to the effects of lysozyme on *Ps. aeruginosa* by employing metabolic assays and electron microscopy, and by performing biochemical and biophysical binding studies with live bacteria and bacterial model membranes.

EXPERIMENTAL

Lipids

OA and DA were purchased from Sigma–Aldrich and were kept at –20 °C as 20 mM stock solutions in 95 % ethanol under nitrogen. ¹⁴C-labelled DA and OA were purchased as sterile solutions in ethanol (American Radiolabeled Chemicals) and kept at –20 °C. DPPG (dipalmitoylphosphatidylglycerol) (Avanti Polar Lipids) stock solutions were prepared in chloroform/methanol (2:1, v/v).

Human lysozyme

Human lysozyme (pI 9.3) was purified from human milk (Mothers' Milk Bank, Denver, CO, U.S.A.) using a weak cationic-exchange matrix (CM MacroPrep, Bio-Rad Laboratories), equilibrated in 25 mM ammonium acetate (pH 8.25), at a slurry/milk ratio of 1:20. After overnight extraction at 4 °C, the matrix was washed with equilibration buffer three times for 5 min at room temperature (22 °C). Lysozyme was batch-eluted with first 10 % and then 5 % ethanoic (acetic) acid, at a slurry/ethanoic

Abbreviations used: CFU, colony-forming units; DA, docosahexaenoic acid; DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; LUV, large unilamellar vesicle; OA, oleic acid; SEM, scanning electron microscopy; SPR, surface plasmon resonance; TEM, transmission electron microscopy; TSB, trypticase soy broth.

¹ To whom correspondence should be addressed (email eporter@calstatela.edu).

acid ratio of 1:1 and 1:2 respectively for 30 min each at 4°C, then dialysed against 0.01 % ethanoic acid using a Spectra/Por® membrane (Spectrum Laboratories) with a molecular-mass cut-off of 12–14 kDa, concentrated and purified further by reverse-phase HPLC [10 mm × 250 mm Vydac C₈ column, TP® silica, 300 Å pore size (1 Å = 0.1 nm), 2 ml/min flow rate] using 0.1 % trifluoroacetic acid as pairing agent and an acetonitrile gradient in water: 5 % for 6 min, to 35 % for 10 min, to 38 % for 3 min, to 39 % for 16 min, to 43 % for 2 min, to 45 % for 5 min and to 60 % for 5 min. Lysozyme was adjusted to 1 mg/ml (68 µM) stocks in 0.01 % ethanoic acid according to BCA (bicinchoninic acid) protein assay (Pierce Biotechnology) using hen's-egg white lysozyme (Sigma) as standard and stored at –20°C.

Bacteria

Mid-exponential growth phase *Ps. aeruginosa* cells (original strain from Dr M.J. Welsh, University of Iowa, Iowa City, IA, U.S.A.) were prepared as described previously [18]. Briefly, one isolated colony was inoculated into 50 ml of 1 × TSB (trypticase soy broth) and, after incubation for 18–20 h at 37°C with shaking at 200 rev./min, 500 µl of the culture was transferred into 50 ml of fresh pre-warmed TSB, and incubation was continued for exactly 3 h. Thereafter, bacteria were washed and adjusted to McFarland 0.5 [equivalent to $\sim(1.0\text{--}2.0) \times 10^8$ CFU (colony-forming units)/ml] in assay buffer and kept on ice until further use (within 3 h). Assay buffer was 10 mM sodium phosphate (pH 7.3), 100 mM NaCl and 4 % TSB.

Metabolic assay

Reduction of the non-fluorescent dye resazurin to the fluorescent compound resorufin by bacterial metabolites such as NADPH was used to assess bacterial viability [19] in the presence or absence of lysozyme and/or fatty acids over a prolonged period of time. Assay buffer was supplemented with 100 µM resazurin. Samples were prepared in 100 µl volumes and contained 10^6 CFU/ml, lysozyme at 0, 62.5, 125, 250 or 500 µg/ml (0, 4.25, 8.5, 17 and 34 µM respectively), and non-esterified fatty acids at 0 and 10 µM. Incubation was in UV-sterilized Non-Binding Surface microtitre plates (Corning-Costar) in a SpectraMax GeminiEM fluorimeter (Molecular Devices) at 37°C for 20 h with intermittent shaking. Readings of relative fluorescence were taken every 10 min (λ_{ex} , 530 nm; λ_{em} , 590). The area under the resulting curve representing the accumulated total reducing activity of bacteria, a function of the individual metabolic activity and the number of viable bacteria, was calculated using SoftmaxPro 4.1 software.

Quantification of lysozyme enzymatic activity

Enzymatic activity was quantified using the method of Jenzano and Lundblad [20]. Briefly, *Micrococcus lysodeikticus*, Gram-positive cocci with a thick peptidoglycan layer and high susceptibility to the enzymatic action of lysozyme, was added at 0.5 mg/ml to 66 mM sodium phosphate buffer, pH 7.0, containing 1 % agarose, and 10 ml was poured into square plates (10 cm × 10 cm area). After solidification, holes with 3 mm diameter were punched into the agar aseptically. Lysozyme standards were prepared at 125, 250 and 500 µg/ml (8.5, 17 and 34 µM respectively) in assay buffer. Test samples consisted of one part of 250 µg/ml lysozyme (17 µM) or solvent plus one part of 20 µM fatty acid or solvent in assay buffer. Duplicate samples of 5 µl/well were pipetted, and plates were incubated for 18 h at room temperature. The resulting zones of clearing

reflecting the enzymatic activity of lysozyme were measured in mm with a ruler and converted into arbitrary units, using the equation: $\{[\text{diameter} - 3 \text{ (for the hole)}] \times 10\}$.

Electron microscopy

Reaction volumes were scaled up to obtain 10^8 CFU/ml per sample and a final sample volume of 2.38 ml. Lysozyme was tested at 500 µg/ml (34 µM) and DA at 10 µM. After 20 h of incubation, bacteria were fixed in 2 % electron-microscopy-grade glutaraldehyde (Ted Pella) for 30 min at 4°C, washed and resuspended in 1 ml of ice-cold PBS (pH 7.4). For SEM (scanning electron microscopy), 100 µl of the sample was transferred into 950 µl of PBS and deposited on to 0.1 filter discs (Millipore). The discs were washed in PBS, dehydrated with 50, 75, 95 or 100 % ethanol, then gold-coated with an EMS-76 mini-coater (Ernest Fulham). Scanning was performed using a Cambridge 360 instrument. For TEM (transmission electron microscopy), the remaining bacteria were dehydrated as above and embedded in Epon. Ultrathin sections were stained with saturated uranyl acetate for 1 h at 60°C and examined at 80 keV with a Jeol CX-100 electron microscope [21].

Equilibrium dialysis

To measure fatty acid binding to *Ps. aeruginosa* in the presence or absence of lysozyme, equilibrium dialysis was employed [22]. In this method, ligand (*cis*, non-esterified fatty acid in the present study) and target (*trans*, bacteria in the present study) are separated by a dialysis membrane that allows only the free diffusion of the ligand. If the ligand binds to the target, the free solute concentration of the ligand in the *trans*-compartment will be lowered, thus forcing additional diffusion of ligand from the *cis*-compartment. Equilibrium dialysis units were constructed with 10 kDa molecular-mass cut-off Slide-A-Lyzer Mini Dialysis units (Pierce) (upper chamber) and sterile polypropylene transfer pipettes (bottom chamber). Bacteria (target) were placed in the lower (*trans*) chamber only. Fatty acids (ligands) were placed into the upper (*cis*) chamber only. Lysozyme, when employed, was added to both chambers. The final test conditions were 10 µM radiolabelled DA and OA (0.025 µCi/µl) or solvent only, 250 µg/ml (17 µM) lysozyme or solvent only, 5.0×10^5 CFU/ml bacteria or assay buffer only (10 mM sodium phosphate, pH 7.3, 100 mM NaCl, 4 % TSB and 0.1 % BSA) with a final volume of 300 µl in each chamber. Incubation was at 37°C with constant agitation for 4 h. Before and after incubation, 50 µl aliquots were taken from both chambers of each dialysis unit and subjected to liquid-scintillation counting. In addition, 10 µl aliquots from each lower chamber were spotted on to pre-dried TSA plates and incubated at 37°C overnight to assess bacterial viability and purity.

SPR (surface plasmon resonance)

SPR studies were performed on a BIAcore X analyser (GE Healthcare) according to the manufacturer's recommended procedure (BIAcore X Control Software version 2.3, BIAevaluation Software version 4.1). CM5 chips (BIAcore) were activated with 60 µl of a mixture of equal volumes of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-[(3-dimethylamino)-propyl]-carbodi-imide hydrochloride (BIAcore Reagents) at 20 µl/min. Then, lysozyme (10 µM) was immobilized on to the activated chip surface (60 µl over 3 min). Unbound surfaces were blocked with 40 mM ethanolamine (50 µl over 2.5 min). Lastly, 60 µl of DA or OA (each 1 µM) or a mixture of both were injected at 20 µl/min.

L1 chips (BIAcore) were pre-conditioned with 5 μ l of 20 mM CHAPS at 5 μ l/min, washed with 100 μ l of running buffer (10 mM sodium phosphate, pH 7.3, and 100 mM NaCl) at 25 μ l/min. Immobilization of live *Ps. aeruginosa* was achieved by injecting 80 μ l of a 10^9 CFU/ml suspension in running buffer at 2 μ l/min, followed by a wash with 100 μ l of buffer at 25 μ l/min. Surface blocking was accomplished by injecting 5 μ l of 1% BSA in running buffer at 2 μ l/min. Biomolecular interaction analysis of the immobilized bacteria with lysozyme and fatty acids was conducted by injecting 30 μ l of 25 μ g/ml (1.7 μ M) lysozyme, 1 μ M fatty acid, or a mixture of both, at 2 μ l/min in running buffer supplemented with 0.25% BSA. Alternatively, sequential injections of 30 μ l of 25 μ g/ml (1.7 μ M) lysozyme and 30 μ l of 1 μ M fatty acid or vice versa were made.

Preparation of DPPG LUVs (large unilamellar vesicles)

LUVs were prepared from dry lipid films dispersed in 20 mM sodium phosphate buffer (pH 7.4) and 130 mM NaCl, hydrated above the main transition temperature and extruded by applying 15 cycles and Millipore filters of 0.1 μ M. Aliquots of the respective fatty acid or lysozyme were added, mixed for 1 min and incubated again at 45 °C for 30 min, then the other active reagent (lysozyme or fatty acid) was added, and samples were incubated for another 30 min at 45 °C. Samples were cooled to room temperature before DSC (differential scanning calorimetry).

Differential scanning calorimetry

Calorimetric experiments were performed with a MicroCal VP-DSC differential scanning calorimeter applying a scan rate of 30 °C/h for DPPG and 60 °C/h for lysozyme. Calorimetric enthalpies were calculated by integrating the peak areas after baseline adjustment and normalization with respect to solute concentration (phospholipid, 1 mg/ml, and lysozyme, 2 mg/ml) using MicroCal's Origin software. Reversibility of transitions was verified by at least two heating and cooling cycles for samples with LUVs and reproducibility by measuring at least two samples. In the absence of LUVs, samples were only heated once, owing to incomplete reversibility of the unfolding transition of lysozyme.

Data analysis

Data were calculated with Microsoft Excel, plotted with SigmaPlot version 9.0, and statistically analysed with SPSS version 15.

RESULTS AND DISCUSSION

Lysozyme inhibits the metabolic activity of *Ps. aeruginosa* in synergism with DA

Guided by the observed link between altered fatty acid profiles and chronic infectious diseases, we sought to investigate the potential of non-esterified fatty acids to act as an antimicrobial agent in conjunction with lysozyme, a predominant antimicrobial protein on mucosal surfaces. We first tested the prolonged effect of selected non-esterified fatty acids on the metabolic activity of *Ps. aeruginosa* in the presence or absence of lysozyme (Figure 1). The longer incubation time allowed the observation of potential bacterial adaptation processes, as they are likely to occur in the lungs of cystic fibrosis patients who have a severely impaired mucociliary clearance [23]. We found that lysozyme inhibited metabolic activity of *Ps. aeruginosa* and that this inhibition was

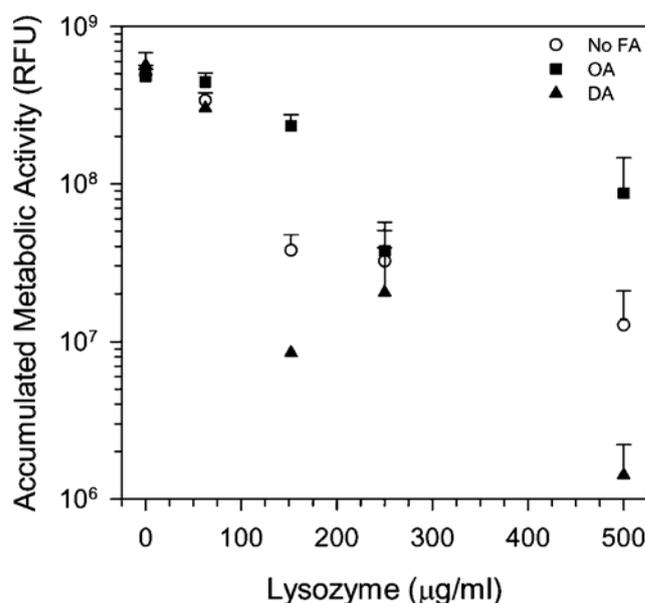


Figure 1 DA augments lysozyme-mediated metabolic inhibition of *Ps. aeruginosa*

Bacteria were incubated for 20 h with or without lysozyme (62.5, 125, 250 and 500 μ g/ml or 4.125, 8.25, 17 and 34 μ M respectively) and/or 10 μ M fatty acids (FA) in Non-Binding Surface microtitre plates, and the accumulated metabolic activity was quantified as relative fluorescence units (RFU) resulting from resazurin reduction to resorufin in the presence of bacterial metabolites. Results are the combined data of nine experiments, each conducted in duplicate and are means \pm S.E.M. (where applicable); $n = 1$ for DA + 62.5 or 125 μ g/ml lysozyme; $n \geq 4$ for all other data points. An analysis of co-variance revealed significant differences among treatment groups ($P < 0.05$).

significantly enhanced when DA was present and was decreased in the presence of OA ($P < 0.001$ for lysozyme concentration and $P < 0.05$ for the effects of fatty acids in an analysis of co-variance). Synergism between non-esterified fatty acids and antimicrobial peptides has been reported previously [24], and *in vitro* acylation has been shown to increase the antibacterial activity of antimicrobial peptide derivatives of lactoferricin [25] and dermaseptin [26], as well as lipopeptides [27].

Lysozyme is bimodal, and the observed augmentation of the lysozyme effect could be the result of either enhanced peptidoglycan-hydrolysing activity or increased disruption of the bacterial membrane. We therefore tested the enzymatic activity of lysozyme in the presence of fatty acids and performed electron microscopy to examine alterations in the bacterial membrane structure.

Fatty acids do not affect the enzymatic activity of lysozyme

There was no significant change of the enzymatic activity of lysozyme in the presence of DA or OA or when tested at 250 μ g/ml lysozyme (17 μ M) and 10 μ M fatty acid (results not shown).

Electron microscopy suggests accumulation of DA in the disrupted bacterial cell membrane in the presence of lysozyme

In SEM (Figures 2A–2D), *Ps. aeruginosa* appeared after a 20 h incubation in assay buffer as short rods (Figure 2A). After treatment with lysozyme, the cells were aggregated and had formed spheroplasts (Figure 2B). Bacteria treated with DA alone were elongated (Figure 2C). After incubation with DA and

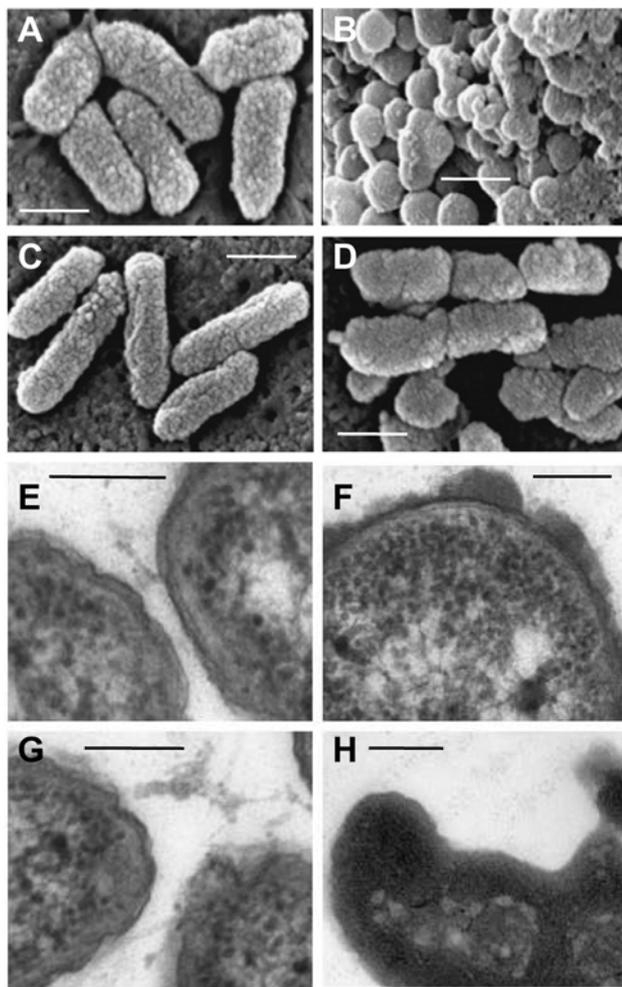


Figure 2 DA increases ultrastructural damage induced by lysozyme in *Ps. aeruginosa*

Bacteria were incubated for 20 h with or without lysozyme and/or DA and subjected to SEM (A–D) and TEM (E–H). (A, E) Control bacteria; (B, F), incubation with 500 μg/ml (34 μM) lysozyme only; (C, G) incubation with 10 μM DA only; (D, H) incubation with lysozyme and DA. Scale bar, 1 μm.

lysozyme, spheroplasts and shortened rods were observed, and bacterial surfaces appeared densely packed (Figure 2D). In TEM (Figures 2E–2H), control bacteria (Figure 2E) showed an intact cell membrane (consisting of the outer membrane, peptidoglycan and inner membrane) and intact cytoplasmic material. The three layers of the bacterial cell wall treated with lysozyme (Figure 2F) could not be differentiated and showed depositions on the outer surface and loss of cytoplasmic material. The ultrastructure of bacteria treated with DA alone was comparable with the structure of control bacteria (Figure 2G). In bacteria treated with both DA and lysozyme (Figure 2H), the cell wall was thickened and amorphous, and the cytoplasm appeared very condensed and displaced. The appearance of electron-dense material within the bacterial cell wall in the presence of lysozyme and DA is consistent with accumulation of DA in the bacterial cell wall in the presence of lysozyme, possibly facilitated by lysozyme-initiated membrane lesions. Cell wall thickening after treatment with antimicrobial agents has been also reported for *Prototheca zopfii* in response to silver nitrate [28]. To substantiate further the binding of DA to the bacterial surface in the presence of lysozyme, we performed equilibrium dialysis and SPR.

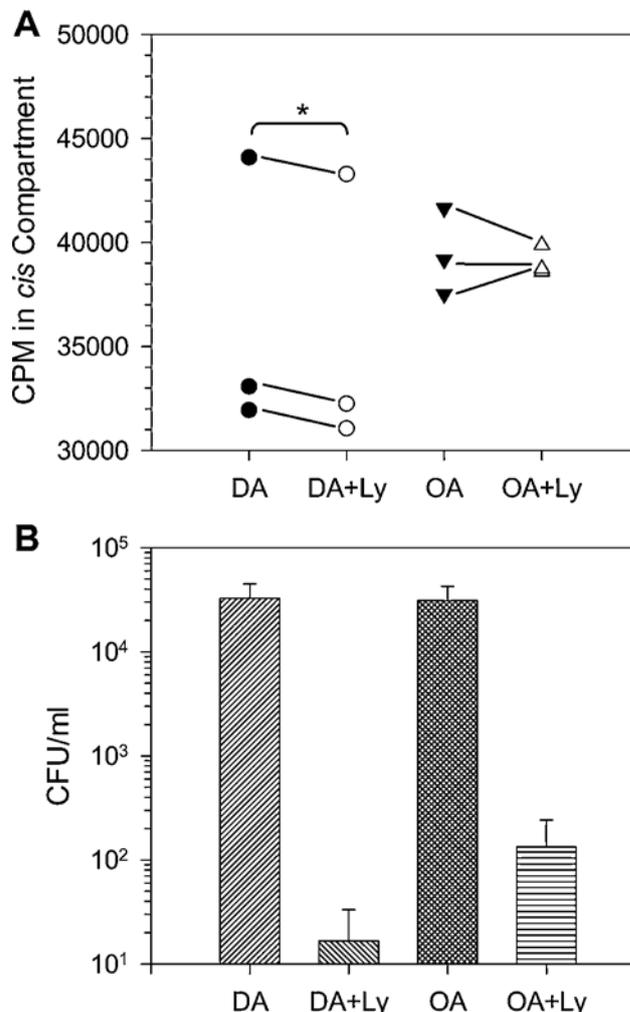


Figure 3 Equilibrium dialysis with fatty acids and/or lysozyme and whole live bacteria suggests that DA, but not OA, accumulates in the bacterial cell wall in the presence of lysozyme

Ps. aeruginosa was placed in the *trans*-compartment of an equilibrium dialysis unit, and radiolabelled fatty acid (DA and OA) was added to the *cis*-compartment separated by a 10 kDa molecular-mass cut-off membrane. After 4 h of incubation with (added to both compartments) and without lysozyme (Ly), a 50 μl aliquot was removed from the *cis*-compartment and subjected to liquid-scintillation counting (A), and 10 μl was removed from the *trans*-compartment for determination of activity, measured in CFU (B). Results in (A) are means of duplicate measurements for three different experiments. Lines connect the data points within one experiment. Accelerated diffusion with decreased residual radioactivity is consistent with fatty acid binding to bacteria. **P* < 0.001 using a Student's paired *t* test. Results in (B) are means ± S.E.M. for three independent experiments.

Equilibrium dialysis is consistent with increased binding of DA to the bacterial cell membrane when lysozyme is present

Initial studies of the equilibrium dialysis kinetics established that, in our experimental set up, the optimal equilibrium dialysis time was 4 h (results not shown). We observed a significantly accelerated loss of DA from the *cis*-compartment when lysozyme was present (Figure 3A), consistent with binding of DA to bacteria in the presence of lysozyme. On average, the c.p.m. were reduced in the *cis*-compartment by 837.0 ± 38.6 (mean ± S.D.; *n* = 3; *P* < 0.001 using a paired Student's *t* test), which translates to 1.83×10^7 more molecules of DA bound per bacterium in the presence of lysozyme. In contrast, the diffusion rate of OA, which acted antagonistically with lysozyme in the metabolic assay, was

not significantly influenced by lysozyme. CFU assays conducted in parallel (Figure 3B) showed that the number of viable bacteria was reduced when lysozyme was added to DA compared with bacteria incubated with DA alone, ruling out an accelerated diffusion of the fatty acid owing to consumption by proliferating bacteria. The effect of lysozyme appears to be weakened for OA, although there was no statistically significant difference between the means for DA + lysozyme and OA + lysozyme using a Tukey post-hoc comparison in a one-factor ANOVA. However, CFU/ml were determined after 4 h only, whereas the metabolic activity of bacteria treated with lysozyme with or without fatty acids were assessed after 20 h. This implies that DA-mediated changes may require a longer time to develop fully. These binding studies validate that the ultrastructural changes observed in electron microscopy studies reflect accumulation of DA in the bacterial membrane in the presence of lysozyme.

DA does not interact with lysozyme or affect lysozyme thermal stability

To test whether the differences in the synergistic activity observed between DA and OA are due to differing effects on lysozyme structure, we performed SPR and calorimetric studies (Figure 4). There was no binding of the fatty acids to lysozyme when applied individually or when applied together (Figure 4A). Calorimetric experiments on the thermal unfolding of lysozyme in the presence and absence of OA and DA respectively showed that both fatty acids do not affect the thermal stability of lysozyme, when added at concentrations at or below their critical micelle concentration (Figure 4B). This indicates that the fatty acids do not bind to lysozyme, confirming the observation from SPR experiments. Hence, we conclude that the synergistic effects of DA and lysozyme result from direct action on the bacterial membrane.

SPR binding studies suggest that DA inserts into the bacterial membrane before lysozyme

We utilized SPR to analyse the interaction of lysozyme with the bacterial surface in the presence and absence of fatty acid. SPR is a highly sensitive and rapid technique for *in situ* surface interaction analysis. It has been used previously to study interaction of antimicrobial peptides with isolated bacterial lipids or bacterial model membranes [29,30]. Protocols for whole live bacteria in SPR have been described previously for carboxyl-methylated dextran chips, which possess a hydrophilic surface [31]. However, *Ps. aeruginosa* could not be immobilized to this surface, since the required buffers had a negative impact on its viability (results not shown) and immobilization of lysozyme would probably adversely interfere with required molecule flexibility for embedding into the bacterial cell membrane [2]. We therefore developed a new protocol for whole live bacteria using L1 (lipid-covered gold) chips that offer a hydrophobic surface, allowing non-covalent attachment [32]. Our immobilization protocol produced response units between 573 and 2944 (mean \pm S.D. = 1026.71 ± 620.63 ; $n = 14$), which was in the range of data published previously [33]. When DA and OA were applied simultaneously with lysozyme, we did not observe a statistically significant increase of response units for lysozyme and fatty acid compared with lysozyme or fatty acid alone (Figure 5A). We therefore reasoned that lysozyme and DA should exert their synergistic activity by sequential damage. To discern whether lysozyme or DA inflicts the initial lesion in the bacterial membrane, we conducted SPR with lysozyme and

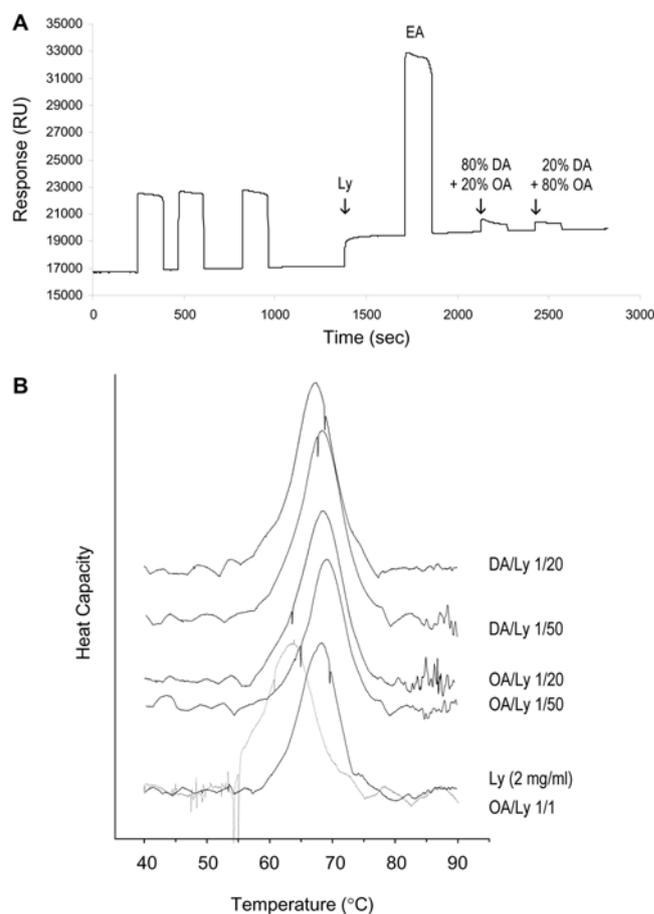


Figure 4 Synergistic effects of lysozyme and DA do not arise from molecular interaction between lysozyme and DA

Molecular interaction analyses based on SPR and DSC. (A) Representative SPR sensogram showing lysozyme immobilization on a CM5 chip, blockage by ethanolamine (EA), and the injection of $1 \mu\text{M}$ fatty acid. The arrows indicate injections of the species, and the ratios of OA/DA are shown next to the respective injection peaks. (B) Thermograms of lysozyme (Ly; 2 mg/ml) with and without DA or OA in buffer (10 mM sodium phosphate, pH 7.4, and 100 mM NaCl) at molar ratios of fatty acid/lysozyme of 1:50 [below CMC (critical micelle concentration)], 1:20 (at CMC) and 1:1 (above CMC, with visible micelle formation). Experiments were performed at a scan rate of $60^\circ\text{C}/\text{h}$. RU, response units.

DA injected consecutively (Figure 5B). We found that the SPR response was greater if DA was allowed to flow across the bacterial surface first, implying that DA facilitates lysozyme binding to the bacterial surface ($P < 0.05$ for DA then lysozyme compared with lysozyme then DA, and DA then lysozyme compared with buffer for Tukey post-hoc comparisons in a one-factor ANOVA). The opposite trend was observed for OA injection that was followed by lysozyme injection ($P < 0.05$ for OA then lysozyme compared with lysozyme then OA, and for OA then lysozyme compared with buffer for Tukey post-hoc comparisons in a one-factor ANOVA). However, similar SPR responses were observed for DA and OA when lysozyme was injected first (Figure 5C). *Ps. aeruginosa* has been shown to convert OA into DOD [7,10-dihydroxy-8(*E*)-octadecenoic acid], which can act as a surfactant to lower surface tension [34]. Such activity triggered by the additional presence of lysozyme may have possibly led to removal of bacteria from the surface, thus accounting for the observed decrease in the SPR response when OA injection preceded the lysozyme injection. Alternatively, OA might have been rapidly internalized, altering

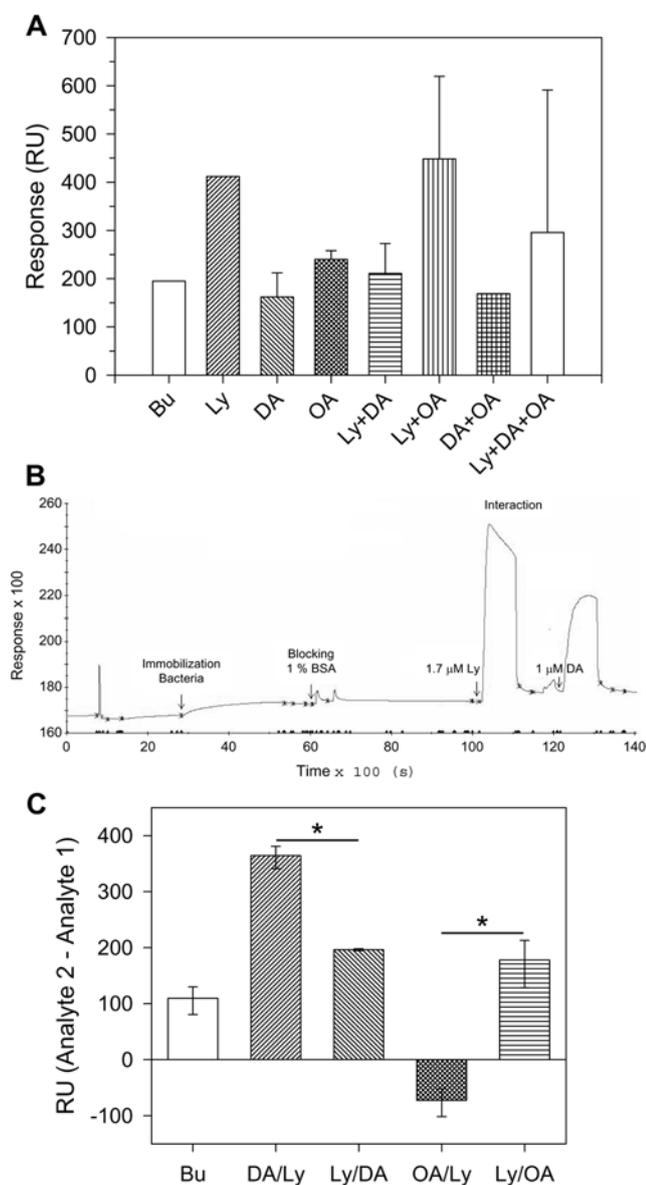


Figure 5 SPR binding studies suggest that DA inserts into the bacterial membrane before lysozyme

(A) L1 chips pre-immobilized with *Ps. aeruginosa* ($\sim 10^8$ bacteria) were treated with fatty acids and lysozyme (Ly) alone or in combination. Results are the combined data of five experiments and are means \pm S.E.M. (where applicable); $n=3$ for lysozyme + DA and lysozyme + OA; $n=2$ for all others. Bu, buffer control. There is no statistically significant difference between lysozyme in combination with fatty acid and lysozyme or fatty acid alone. (B) A representative SPR sensogram showing the immobilization of *Ps. aeruginosa* ($\sim 10^8$ bacteria), the blockage of active surface area by BSA, the treatment of $1.7 \mu\text{M}$ lysozyme and the final injection of $1 \mu\text{M}$ DA. The arrows represent the injections of these species. (C) Changes in SPR response upon exposure of DA followed by lysozyme (DA/Ly), lysozyme followed by DA (Ly/DA), OA followed by lysozyme (OA/Ly), and lysozyme followed by OA (Ly/OA). Bu, buffer control; RU, response units. The difference in response units at the end of each injection was calculated, and results are means \pm S.E.M. for two experiments. $*P < 0.02$ for DA/Ly compared with Ly/DA and $P < 0.01$ for OA/Ly compared with Ly/OA for Tukey post-hoc comparisons in a one-factor ANOVA.

the bacterial and chip surface substantially, thereby lowering the SPR response.

These data suggest that, in contrast with our original hypothesis, DA induces the initial lesion for the subsequent synergistic action of DA and lysozyme.

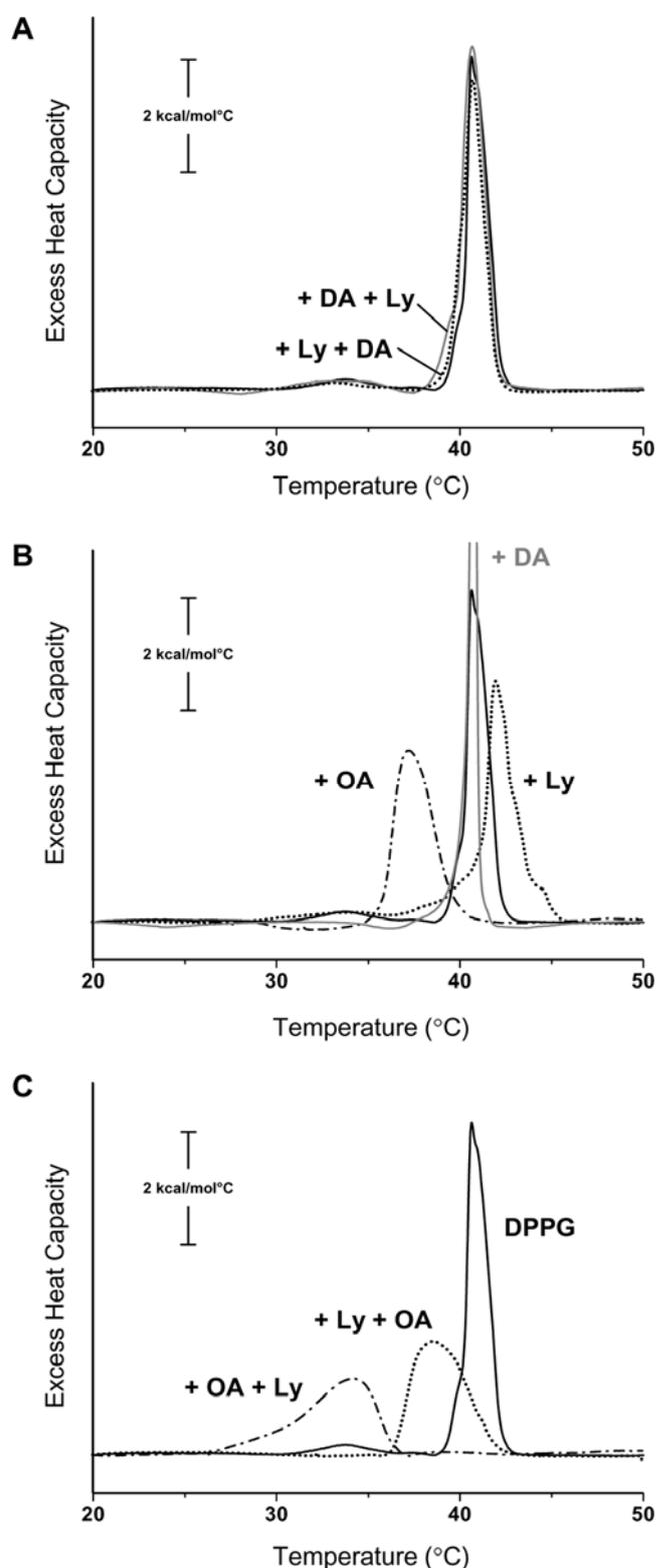


Figure 6 Calorimetric studies with bacterial model membranes support binding studies with live bacteria

Thermograms of DPPG LUVs with and without DA or OA in the presence or absence of lysozyme (Ly) added in the order indicated. DPPG was hydrated in buffer (10 mM sodium phosphate, pH 7.3, and 100 mM NaCl) above the main transition temperature for 1 h. Fatty acids and/or human lysozyme were added to the lipid at a molar ratio of 500:1. Experiments were performed at a scan rate of $30^\circ\text{C}/\text{h}$. Shown are representative thermograms of two experiments measured in duplicate. $1 \text{ kcal} = 4.184 \text{ kJ}$.

Table 1 Thermodynamic parameters of differential scanning calorimetric studies

The molar ratio of DPPG to additives was 500:1. 1 kcal=4.184 kJ. T_{pre} , pre-transition temperature; $\Delta T_{1/2}$, transition width at half peak; ΔH_{pre} , enthalpy of pre-transition; T_m , main transition temperature; ΔH_m , enthalpy of main transition.

Sample	T_{pre} (°C)	$\Delta T_{1/2}$ (°C)	ΔH_{pre} (kcal/mol per °C)	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH_m (kcal/mol per °C)
DPPG	33.8	3.4	1.05	40.7	1.3	12.8
DPPG + lysozyme	34.6	6.6	1.7	42.0	1.6	15.0
DPPG + DA	–	–	–	40.8	0.35	13.5
DPPG + DA then lysozyme	34.0	5.0	1.2	40.7	1.4	14.9
DPPG + lysozyme then DA	33.3	3.8	0.93	40.7	1.4	12.3
DPPG + lysozyme + DA	32.8	1.2	0.4	40.5	0.5	8.7
DPPG + OA	–	–	–	37.3	2.4	11.8
DPPG + OA then lysozyme	–	–	–	34.2	4.3	10.0
DPPG + lysozyme then OA	–	–	–	38.6	3.5	10.9
DPPG + lysozyme + OA	34.1	1.4	0.5	40.1	0.9	9.6

Calorimetric studies with DPPG LUVs confirm distinct membrane interaction of DA and OA

In order to gain further information on the membrane interaction of DA and OA in the presence or absence of lysozyme, we conducted calorimetric studies with bacterial model membranes using unilamellar liposomes composed of DPPG. Figure 6 shows representative thermograms of liposomes treated with OA, DA and lysozyme in various combinations using a molar ratio of DPPG/additive of 500:1. Table 1 summarizes the results from such thermodynamic measurements. Adding lysozyme to DPPG liposomes resulted in a shift of both pre- and main transition and a minor increase of enthalpy. This suggests that the protein binds to the negatively charged liposomal surface, leading to charge compensation. Whereas OA drastically lowered and markedly broadened the main transition, DA induced a highly co-operative main transition without significantly affecting the transition temperature. It is likely that the conformation of OA bent by the single *cis* double bond significantly perturbs the hydrocarbon chain packing of DPPG that contains saturated palmitoyl residues. On the other hand, DA rather adopts a linear conformation because of its alternating six *cis* double bonds, resulting in more homogeneous lipid packing, as is evident from the strong decrease of the transition half-width. Incubating DPPG liposomes with lysozyme first and then DA, or vice versa, resulted in thermograms very similar to that of pure DPPG. This observation is consistent with the observed increase of the SPR response under both conditions. Incubation of DPPG liposomes with lysozyme first and followed by OA resulted in thermodynamic characteristics similar to that of the DPPG/OA mixture. The broadening and shift to higher temperatures are indicative of additional surface-bound lysozyme, in accordance with the increase of signal observed by SPR. Adding OA before lysozyme resulted in a strong destabilization of the DPPG gel phase (decrease of main transition by more than 6°C). The decrease of enthalpy for this sample can be indicative of the formation of small micellar particles, which would be in line with the decrease of SPR signal. Formation of membrane vesicles, which is increased by exposure to polycationic antibiotics, has been well described for *Ps. aeruginosa* [35]. OA may similarly increase membrane vesicle formation, thus removing lysozyme and leading to its observed antagonistic activity. In contrast, and as shown previously by SPR, the obvious differences between DA and OA treatments were lost when the fatty acids and lysozyme were added together to DPPG liposomes (Table 1). Overall, the DSC data demonstrate different effects on the bilayer organization of DA and OA in the presence and absence of lysozyme, and are in agreement with the results obtained by the techniques described in the previous paragraphs.

In conclusion, we have shown that DA and lysozyme synergistically inhibit the metabolic activity of *Ps. aeruginosa*. Our work provides novel insight into the mechanism of this activity. Electron microscopy revealed an ultrastructural damage of bacterial membranes that is consistent with the incorporation of DA in the presence of lysozyme, and equilibrium dialysis confirmed increased binding of DA to *Ps. aeruginosa* in the presence of lysozyme. Unexpectedly, SPR and calorimetric studies both suggest that DA sets the initial lesion during early interaction with the bacterial membrane. Thus the non-esterified fatty acid DA facilitates lysozyme-mediated disruption of the bacterial membrane, which in turn allows influx of more DA into the membrane. The present study provides a molecular basis for the clinically observed association of DA deficiency and lung infection with *Ps. aeruginosa* in cystic fibrosis patients. The described mechanism may extend to other host-derived fatty acids and antimicrobial proteins and may lead to the development of novel antibiotics. This finding is highly significant, considering that the increasing antibiotic resistance of microbes causing infectious diseases poses escalating threats to public health.

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