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ORIGINAL ARTICLE

Effect of a low concentration of a cationic steroid antibiotic (CSA-13) on the formation of a biofilm by *Pseudomonas aeruginosa*

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Abstract

Aims: Cationic steroids like CSA-13 have been designed by analogy with antimicrobial cationic peptides and have bactericidal properties. The purpose of this work was to evaluate the effect of a low concentration (1 mg l^{-1}) of CSA-13 on the formation of a biofilm by eight strains of *Pseudomonas aeruginosa* (four mucoid and four nonmucoid strains) on an inert surface.
Method and Results: The biofilm formation was measured with the Crystal Violet method. CSA-13 inhibited the formation of a biofilm by three strains. The zeta potential varied among the strains. The inhibition by the cationic steroid analogue affected the populations of bacteria with the lowest zeta potential. *P. aeruginosa* bound a fluorescent, more hydrophobic analogue of CSA-13 but there was no correlation between this binding and the inhibition by CSA-13 of biofilm formation. The interaction of CSA-13 with bacteria did not modify their ability to produce rhamnolipids.
Conclusions: A low concentration of CSA-13 inhibits the formation of a biofilm by *P. aeruginosa* through electrostatic interactions and without affecting the production of rhamnolipids.
Significance and Impact of the Study: A low, nontoxic concentration of CSA-13 might be beneficial for the prevention of biofilm formation.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium in the environment, usually found in soil and water; it is also an opportunistic pathogen responsible for infections mostly in immunocompromised hosts like patients with burns, cancer or HIV (Lyczak *et al.* 2002). It is responsible for 10% of nosocomial infections (Grisaru-Soen *et al.* 2007) and is also the most common pathogen in patients with cystic fibrosis (CF) (Hassett *et al.* 2009). Recurrent infections by *P. aeruginosa* in these patients rapidly result in chronic infections. The sequelae of these incurable infections are the major cause of respiratory failure provoking the death of patients with CF (Ramsey *et al.* 1999). The chronicity of these infections has been related to the ability of *P. aeruginosa* to form a biofilm (Costerton *et al.* 1999). The formation of the biofilm modifies the expression of proteins involved in the synthesis of molecules associated with quorum sensing (Dickschat 2010). These molecules which are acylated derivatives of homoserine lactone interact and activate transcription factors in adjacent bacteria and modify their proteasome. Many of the overexpresssed proteins confer a greater resistance of the bacteria against antibiotics (Høiby *et al.* 2010). The sensitivity to antibiotics of bacteria in planktonic cultures or in biofilms is thus very different, making difficult to predict the outcome of a treatment chosen on the basis of results obtained with planktonic cultures (Tré-Hardy *et al.* 2008). Biofilms are dynamic structures secondary to the attachment of

bacteria on a surface (Monds and O'Toole 2009). The twitching motility which mostly involves type IV pili promotes the piling of bacteria on each other and the formation of a cap on top of a stalk consisting of bacterial aggregates. Within this mushroom-like structure, bacteria form a mass of interacting cells. They secrete proteins and polysaccharides which, in conjunction with DNA (Whitchurch *et al.* 2002) and cellular debris (Webb *et al.* 2003), are the matrix of the biofilm. Inside the mushroom-like structure, water-filled channels facilitate the inward transport of nutrients and contribute to the exit of waste products from the bacterial colonies (Stoodley *et al.* 2002). The expansion of the colonies inside the biofilm deteriorates local conditions and provokes the depletion of nutrients or oxygen and the accumulation of wastes (Hunt *et al.* 2004). Bacteria could separate from biofilms and return to a planktonic life. The release of bacteria promotes their dissemination and favours their attachment to multiple other locations extending the original infection. For *P. aeruginosa*, the cycle of the biofilm takes about 12 days (Webb *et al.* 2003).

The rationale for the development of new therapeutic strategies is based on two major concepts. First, the new antimicrobial agents should target simple but fundamental properties of the bacteria (like its membrane) which would render resistance much more difficult to develop. Second, the antimicrobial agents should have anti-biofilm properties. Cationic antimicrobial peptides like defensins or LL-37 share these properties: they destroy the bacterial membranes and also prevent the formation of a biofilm (Overhage *et al.* 2008). To circumvent the fragility of these peptides, synthetic analogues have been developed including the ceragenins (Epand *et al.* 2008). These molecules are derived from cholic acid to which aminoalkyl groups have been added. Previous results have demonstrated the efficacy of CSA-13 on bacteria not only in planktonic cultures but also within a biofilm (Nagant *et al.* 2010a). This drug also prevents the formation of a biofilm on tooth surface or on abiotic surfaces like implants or catheters (Isogai *et al.* 2009). Bactericidal concentrations of CSA-13 (higher than 50 mg l^{-1}) have some toxic effects on eukaryotic cells precluding its clinical use (Leszczyńska *et al.* 2011). The purpose of our work was to evaluate the effect of a low, nontoxic concentration of CSA-13 (1 mg l^{-1}) on the formation of a biofilm by eight strains of *P. aeruginosa*. The biofilm was assayed with the Crystal Violet method. To study the interaction of CSA-13 with the bacteria, we have used CSA-119, a fluorescent analogue of CSA-13. Our results show that the drug inhibited the formation of a biofilm by three of the eight strains. This inhibition was correlated not with the ability of the strains to bind the fluorescent analogue of CSA-13 but with a very low zeta potential of the bacteria. Two of the three strains sensitive to CSA-13 produced a large amount of rhamnolipids (Nagant *et al.* 2010b). Our results show that the inhibition exerted by CSA-13 on the formation of the biofilm by these strains was not coupled to the inhibition of their rhamnolipid synthesis.

Materials and methods

Bacterial strains

Clinical isolates from sputum of patients with CF from the Erasme Hospital of Brussels (*P. aeruginosa* PYO1 and PYO2), reference strains *P. aeruginosa* ATCC 15692 PAO1, ATCC 9027 and ATCC 15442 and respiratory tract clinical isolates from patients with CF from the University Hospital of Gent (*P. aeruginosa* MC75-450457, MC099-450467 and MC093-450507), were tested. Four of these eight strains had a mucoid phenotype (PYO2, MC75-450457, MC099-450467 and MC093-450507). The strains were stored at −20°C in glycerol. Before use, the strains were spread onto Mueller–Hinton solid medium and incubated at 35°C for 24 h. After culture, identification of the strains was confirmed by applying the API 20 NE system (BioMérieux, Marcy-l'Etoile, France). Bacteria were plated not more than three times onto Mueller–Hinton solid medium. Colonies were then transferred in sterile brain heart infusion medium (BHI) and incubated under constant shaking (150 rev min^{-1}) at 35°C in an orbital shaking incubator (Gallenkamp Orbital Incubator; Sanyo, Pocklington, UK). After overnight incubation, the culture was adjusted to a final optical density at 600 nm of 1·00 ± 0·05 by adding sterile BHI medium. The initial bacterial suspension (IBS) was obtained by a 250-fold dilution of the bacterial suspension.

Study of the formation of a biofilm with the Crystal Violet staining method

The wells of a 96-well microplate were inoculated with 200 μl IBS and 20 μl of CSA-13 at 11 mg l^{-1} and incubated at 35°C in a humid atmosphere. A control well was inoculated with sterile BHI medium. Each strain was evaluated in triplicate. Medium was removed from the wells which were washed three times with sterile distilled water. The wells were air-dried for 45 min, and the adherent cells were stained with a 0·1% Crystal Violet solution. After 45 min, the Crystal Violet in excess was removed and the wells were washed five times with 300 μl sterile distilled water. The dye was dissolved with a 33% acetic acid solution, and the absorbance of each well was read at 540 nm in a microplate reader (Synergy HT, BioTek). The results were expressed as variation of OD_{540} (OD_{540}

sample – OD_{540} control). The experiment was repeated at least three times, for each strain and incubation time.

Effect of CSA-13 on the production of rhamnolipids

The amount of rhamnolipids (3-deoxyhexose) produced by three strains of *P. aeruginosa* was estimated by the amount of rhamnose in the culture medium. The sugar was assayed with a colorimetric test (Wilhelm *et al.* 2007). Cells were grown for 48 h at 30°C in 20 ml of a PPGAS medium, adjusted to an initial optical density (580 nm) of 0·05, in the presence or in the absence of a CSA-13 solution at a 1 mg l^{-1} final concentration. The flasks were constantly shaken at 120 rev min^{-1} to stimulate rhamnolipid production. At the end of the incubation, the bacterial suspension was centrifuged and 300 μl of the supernatant was extracted twice with 600 μl diethylether. The extracts were pooled and evaporated to dryness. The residues were then dissolved in 100 μl distilled water and mixed with 100 μl 1·6% orcinol and 800 μl 60% sulfuric acid. After incubation at 80°C for 30 min, the absorbance was measured at 421 nm using a Biotek multi-detection microplate reader. The assay was performed in triplicate, and the results were compared with a standard curve established with known concentrations of rhamnose.

Synthesis of CSA-119, a fluorescent analogue of CSA-13

Tris-Boc CSA-13 (15 mg, 0·015 mmol) was dissolved in dichloromethane (1 ml), and BODIPY-succinimidyl ester [5 mg, 0·013 mmol (Invitrogen, Groningen, Netherlands)] was added. The mixture was stirred for 12 h. Column chromatography (SiO_2; 3% methanol in dichloromethane) yielded the conjugate as a dark glass (12 mg, 73·8% yield). ^{1}HNMR ($CDCl_3$, 500 MHz) δ 7·182 (s, 1H), 6·882 (s, 1H), 6·324 (s, 1H), 6·099 (s, 1H), 3·580–3·667 (m, 2H), 3·465–3·507 (m, 2H), 3·067–3·343 (m, 14H), 2·730–2·754 (t, J = 6Hz, 2H), 2·554 (s, 3H), 2·247 (s, 3H), 2·009–2·181 (m, 3H), 1·137–1·853 (m, 69H), 0·837–1·062 (m, 12H), 0·647 (s, 3H); HRMS (ESI) calculated for $C_{70}H_{117}BF_2N_6O_{10}$ $[M+NH_4]^+$: 1250·8892, found: 1250·8736. The conjugate was dissolved in dichloromethane (1 ml), the solution was cooled to 0° C, and trifluoroacetic acid (0·1 ml) was added. The mixture was stirred for 12 h. CSA-119 was isolated after column chromatography (SiO_2; 10% methanol in dichloromethane) as a dark oil (5 mg, 55% yield). ^{1}HNMR ($CDCl_3$, 500 MHz) δ 7·184 (s, 1H), 6·885 (s, 1H), 6·326 (s, 1H), 6·101 (s, 1H), 3·490–3·734 (m, 4H), 3·180–3·395 (m, 14H), 2·747 (t, J = 5·8 Hz, 2H), 2·554 (s, 3H), 2·247 (s, 3H), 2·009–2·181 (m, 3H), 1·170–1·882 (m, 42H), 0·836–1·044 (m, 12H), 0·641 (s, 3H); HRMS (ESI) calculated for $C_{55}H_{97}BF_2N_7O_4$ $[M+NH_4]^+$: 968·7633, found: 968·7756 (Fig. 1).

Kinetic study of the interaction of CSA-119 with the eight strains of *Pseudomonas aeruginosa*

Bacteria on agar plates, incubated overnight at 35°C, were suspended in PBS, and the final optical density (at 600 nm) was adjusted at 0·1 ± 0·05. Two hundred microlitres of the bacterial suspensions was transferred to the wells of a 96-well plate. The plate was read in a microplate reader. The light emitted at 495 nm after excitation at 345 nm was measured every 40 s. Three minutes after the start of the assay, various concentrations of CSA-119 (10 μl volume) were added to the wells and the fluorescence measured for 12 min. The microplate was shaken before each measurement, and each condition of CSA-119 was tested in triplicate. Photobleaching of the fluorescent probe was estimated in wells containing PBS but no bacteria. Results were corrected for this photobleaching and were expressed as the variation of the fluorescence with time.

Measure of the zeta potential of *Pseudomonas aeruginosa*

The zeta potential measurements on *P. aeruginosa* were taken at 25°C on a Malvern Nanosizer ZS (Malvern, Worcestershire, UK), which is based on electrophoretic light scattering. Attenuation selection, voltage selection and measurement duration were set on automatic. The quality of the measurements was evaluated by inspection of the phase plot. Overnight cultures of bacteria were resuspended at a 0·1 absorbance (450 nm) in 10 mmol l^{-1} phosphate buffer (pH 7·0). The bacterial suspension was transferred in folded capillary cell for zeta potential

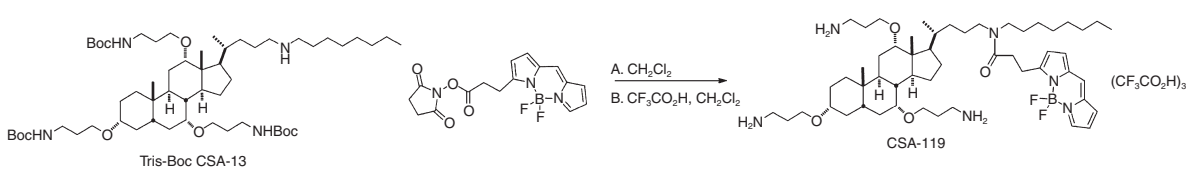

Figure 1 Synthesis of CSA-119 from CSA-13. CSA-119 was synthesized from CSA-13 according to this reaction as described in Materials and methods.

measurement (Zetasizer Nano series; Malvern) and maintained at 25°C. The assay was performed three times with each strain at two different dilutions of the bacteria, and three different batches of each strain were tested. Polystyrene nanospheres (−68 mV; Duke Scientific Corp, Palo Alto, CA, USA) were used to verify the performance of the instrument.

Statistical analysis

The results were compared using the Mann–Whitney nonparametric test (*$P < 0{\cdot}05$).

Results

Effect of CSA-13 on the formation of a biofilm by *Pseudomonas aeruginosa*

The formation of a biofilm by the eight strains was evaluated with the Crystal Violet method after 24 and 48 h. The results obtained with nonmucoid and mucoid strains were shown in Fig. 2. As previously reported (Nagant *et al.* 2010b), the ability to form a biofilm was very variable among the eight strains. The PYO1 and the ATCC 9027 strains did not form a significant biofilm even after 48 h; the biofilm formed by the MC099-450467 strain was measurable only after 48 h. The PAO1, the PYO2, the MC093-450507 and the ATCC 15442 strains formed after 24 h, an important biofilm which was only slightly increased for the next 24 h (Fig. 2). The PAO1 strain, one of the two nonmucoid strains producing a strong biofilm, was significantly affected by CSA-13 (Fig. 2). The drug decreased the absorbance from $2{\cdot}54 \pm 0{\cdot}46$ ($n = 7$) to $1{\cdot}66 \pm 0{\cdot}20$ ($P = 0{\cdot}019$; $n = 9$) after 24 h and from $2{\cdot}98 \pm 0{\cdot}47$ to $1{\cdot}53 \pm 0{\cdot}45$ ($P = 0{\cdot}027$, $n = 8$) after 48 h. Among the four mucoid strains, CSA-13 significantly inhibited the formation of a biofilm by the MC75-450457 and the MC099-450467 and had no apparent effect on the MC093-450507 and the PYO2 strains. After 48 h, the drug decreased the absorbance from $1{\cdot}91 \pm 0{\cdot}48$ ($n = 9$) to $0{\cdot}86 \pm 0{\cdot}33$ ($n = 8$; $P = 0{\cdot}038$) for the MC75-450457 strain and from $1{\cdot}87 \pm 0{\cdot}65$ ($n = 11$) to $0{\cdot}26 \pm 0{\cdot}10$ ($n = 9$; $P = 0{\cdot}031$) for the MC099-450467 strain.

Interaction of *Pseudomonas aeruginosa* with CSA-119, a fluorescent derivative of CSA-13

In a following experiment, the interaction between CSA-13 and the bacteria was tested using CSA-119, an analogue of CSA-13 tagged with a fluorescent probe (Ding *et al.* 2004a). The fluorescence of the BODIPY group linked to the steroid increases in a lipophilic environment. The transfer of the fluorophore-appended CSA-119 from the buffer to the bacterial membrane provokes an increase in the light emitted at 500 nm after excitation at 345 nm. This is illustrated in the upper panel of Fig. 3. Incubation of *P. aeruginosa* of the PA01 strain with various concentrations of CSA-119 provoked a time-dependent increase in the fluorescence. The rate of this increase was affected by the concentration of the probe used in the assay (from 1·2 to 6 mg l^{-1}). Bacterial suspensions of the eight strains were tested for 12 min in the presence of 2·4 μmol l^{-1} CSA-119. As shown in the lower panel of Fig. 3, the fluorescence of CSA-119 varied according to the bacterial strain tested (PAO1 > MC75-450457 > ATCC 9027 > ATCC 15442 > PYO1 > MC099-450467 > MC093-450507 > PYO2). Taking the fluorescence in the

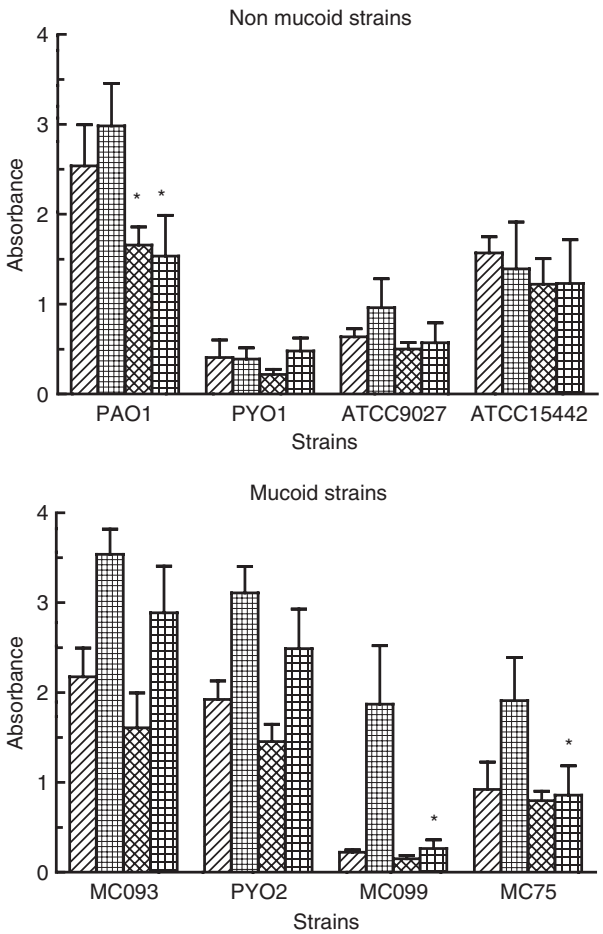

Figure 2 Study with the Crystal Violet staining method of the formation of a biofilm. The formation of a biofilm by four nonmucoid strains (upper panel) or by four mucoid strains (lower panel) was tested with the Crystal Violet method at 24 and 48 h. Results were expressed as absorbance units. They are the means ± SEM of 6–9 experiments. (▨) Control 24 h; (▦) Control 48 h; (▩) CSA-13 24 h; (▦) CSA-13 48 h.

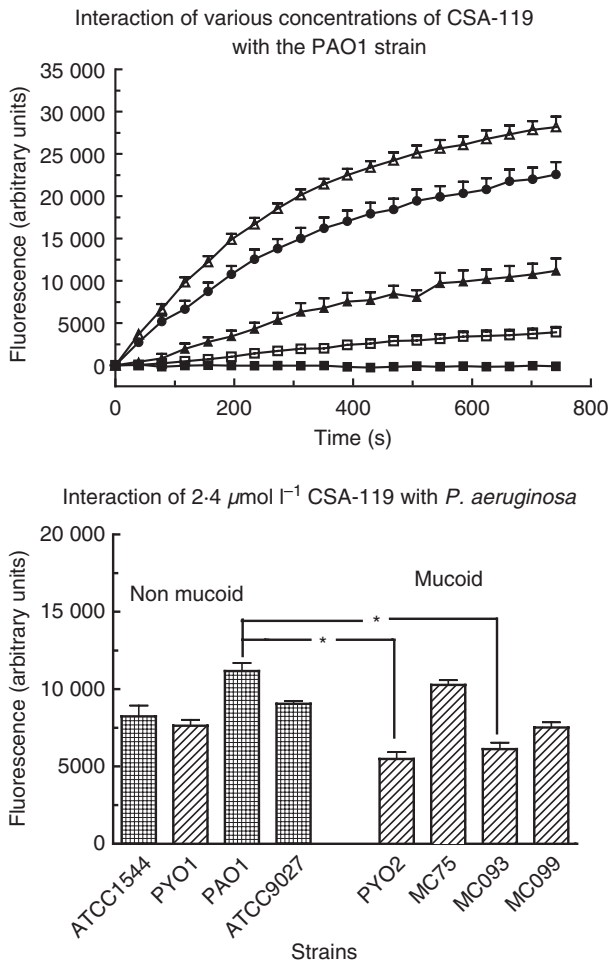

Figure 3 Kinetic of the interaction between CSA-119 and the PAO1 strain. Upper panel: Bacteria of the PAO1 strain were incubated for various times with five concentrations of CSA-119 in PBS in 96-well plates. The fluorescence of each well was measured every 40 s. To evaluate the photobleaching, some control wells contained the probe and PBS but no bacteria. Results were calculated by subtracting for each time and each concentration of CSA-119 the variation of the fluorescence in the control wells from the variation of the fluorescence measured in the presence of bacteria. They are the means ± SEM of three experiments. Lower panel: Bacteria of the eight strains were incubated for 12 min with 2·4 μmol l^{-1} CSA-119. Results were calculated as described for the upper panel. Results are the means ± SEM of three experiments. *$P < 0{\cdot}05$. (■) 0 μmol l^{-1}; (□) 1·2 μmol l^{-1}; (▲) 2·4 μmol l^{-1}; (●) 4·8 μmol l^{-1}; (△) 6 μmol l^{-1}; (▦) Reference strains; (▨) Clinical strains.

presence of the PAO1 strain as the reference, the decrease was significant only for the MC093-450507 ($P = 0{\cdot}0152$) and the PYO2 ($P = 0{\cdot}0104$) strains. The decrease in the fluorescence associated with the MC093-450507 ($P = 0{\cdot}040$) and the PYO2 ($P = 0{\cdot}0055$) strains was also significant when compared to the fluorescence associated with the MC75-450457 mucoid strain. These results suggested that the MC093-450507 and the PYO2 strains had a lower affinity for the probe.

Zeta potential of the eight strains.

The distribution of the zeta potentials showed distinct patterns among the eight strains. The distribution was unimodal for the ATCC 15442 and the MC099-450467 strains (Fig. 4), and the peak was narrow as evidenced by the rather small dispersion of the results for each measurement. The zeta potential of the two strains diverged: it averaged -30·9 ± 0·7 mV ($n = 6$) for the ATCC 15442 and −53·1 ± 1·2 mV ($n = 6$) for the MC099-450467 strain (Fig. 4). This difference was significant ($P = 0{\cdot}0022$). The dispersion of the potentials was more important for four strains (MC75-450457, MC093-450507, PAO1 and ATCC 9027). This dispersion was best explained by the appearance of a second peak leading to a clearly bimodal distribution for the PAO1 strain (−56·7 ± 1·1 mV, 17·9 ± 2·8% for the first peak and −25·6 ± 0·9 mV, 80·4 ± 2·8% for the second peak, Fig. 4). The mean zeta potential of these four strains ranged from −49·7 ± 0·9 mV ($n = 6$) for the MC093-450507 to −30·8 ± 0·3 mV ($n = 6$) for the PAO1 strain. The PYO1 and PYO2 strains had mean zeta potentials in the −20 mV range. These two strains were characterized by a very wide dispersion of the potentials ranging from +15 to −65 mV (Fig. 4). The zeta potential averaged −29·5 ± 2·5 mV for the four nonmucoid strains and −40·4 ± 7·3 mV for the four mucoid strains. The difference between these two potentials was not significant ($P = 0{\cdot}34$). Among the four mucoid strains, three strains had very low zeta potentials (MC75-450457, MC099-450467 and MC093-450507) (Fig. 5). There was no significant difference in the mean zeta potential ($P = 0{\cdot}79$) between the three reference strains (ATCC 15442, ATCC 9027 and PAO1: −32·09 ± 0·48 mV) and the five clinical strains (PYO1, PYO2, MC75-450457, MC099-450467 and MC093-450507: −36·7 ± 7·8 mV).

Effect of CSA-13 on the production of rhamnolipids

Rhamnolipids are surfactants which might contribute to the formation of a biofilm (Wilhelm *et al.* 2007). We have previously reported that five of the eight *P. aeruginosa* strains tested in the present work produced rhamnolipids (Nagant *et al.* 2010b). No rhamnose was detected in the supernatant of the MC099-450467, ATCC 15442 and ATCC 9027 strains. We thus tested the effect of a treatment with CSA-13 on the PAO1 and the MC75-450457 strains, which produce rhamnolipids and which were sensitive to the drug, and on the MC093-450507 strain, which is the best producer of rhamnolipids (Fig. 6) (Nagant *et al.* 2010b). The cells were incubated for 2 days

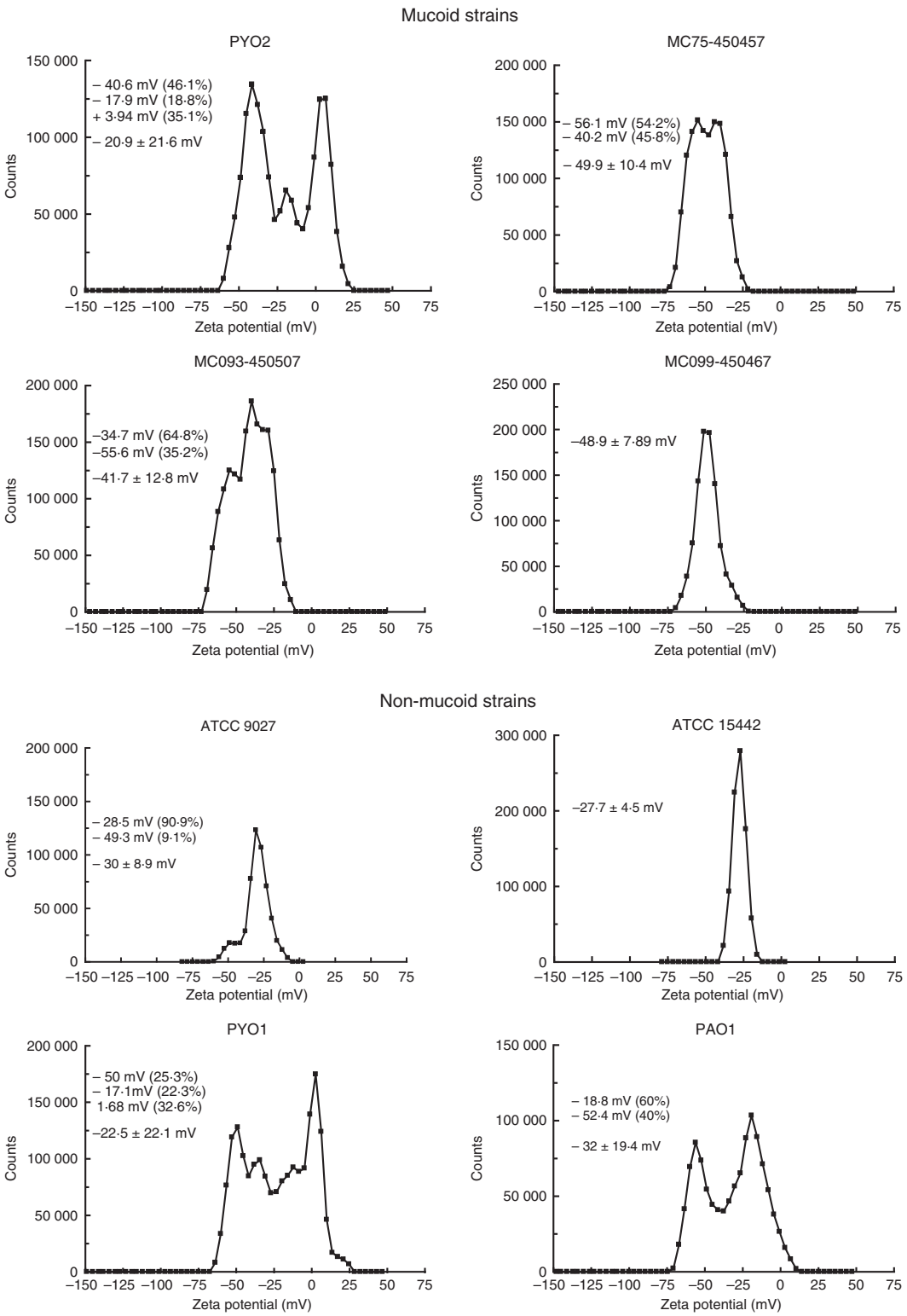

Figure 4 Distribution of the zeta potentials of the eight strains of *P. aeruginosa*. The distribution of zeta potential measurements of the eight strains of *P. aeruginosa* suspended in 10 mmol l^{-1} phosphate buffer (pH 7·0) using the Zetasizer Malvern Nano ZS. Each trace is representative of 18 traces. The analysis of the trace has been reported (mean ± SD of the zeta potential of the whole population and eventually of significant subpopulations with the respective percentage ± SD of these subpopulations).

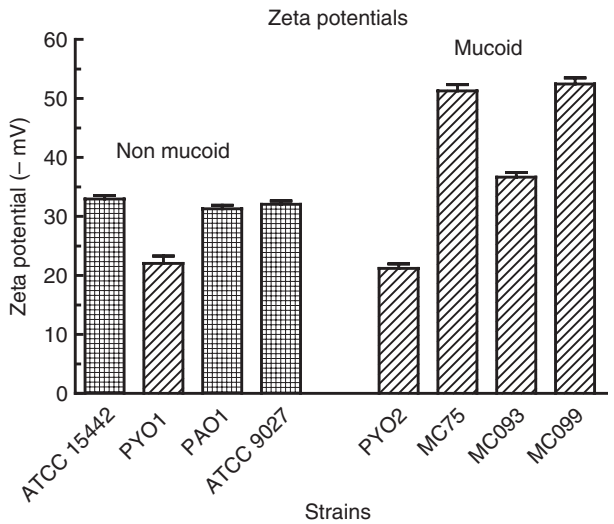

Figure 5 Zeta potential of the eight strains of *P. aeruginosa*. The mean zeta potential measurements of the eight strains of *P. aeruginosa* suspended in 10 mmol l^{-1} phosphate buffer (pH 7·0) using the Zetasizer Malvern Nano ZS. Results are the means ± SEM of six experiments. (▦) Reference strains; (▨) Clinical strains.

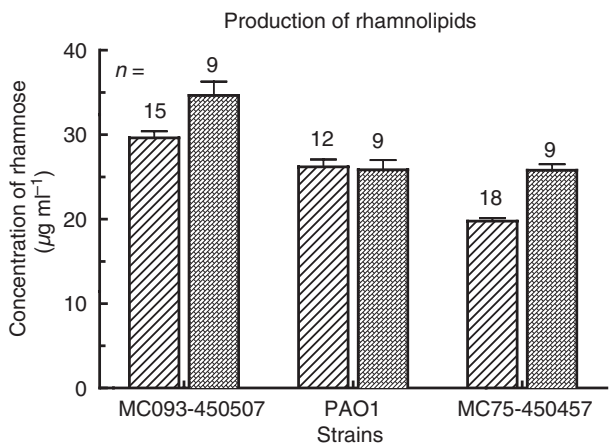

Figure 6 Production of rhamnose by three strains of *P. aeruginosa*. Bacteria of the MC75-450457, MC093-450507 and PAO1 strains were incubated for 2 days in the absence or the presence of 1 mg l^{-1} CSA-13. The production of rhamnose by the three strains was measured by the orcinol-sulfuric acid method. Results are expressed as μg rhamnose ml^{-1} culture broth. They are the means ± SEM of 9-18 experiments. (▨) Cont; (▩) + CSA-13.

in the absence or the presence of 1 mg l^{-1} CSA-13, and the culture medium was collected. After extraction of the lipids, the amount of rhamnolipids in the medium was estimated by measuring the concentration of rhamnose. In these experiments, the MC093-450507, MC75-450457 and PAO1 strains produced, respectively, 29·6 ± 3·1 (n = 15), 19·8 ± 1·5 (n = 18) and 26·2 ± 3·1 (n = 12) μg ml^{-1} rhamnose. The treatment of the three strains with CSA-13 had no significant effect on the production of rhamnose by either of the strains.

Discussion

In this work, we studied the effect of CSA-13 on the formation of a biofilm by eight strains of *P. aeruginosa* using the Crystal Violet method. This method is a classical method based on the staining by a dye of the sessile bacteria and the extracellular matrix layered at the bottom of the wells of a microplate (Stepanovic *et al.* 2000). The formation of the biofilm is estimated by the measurement of the absorbance of the dye retained in the well after several washing steps. A low concentration of CSA-13 (1 mg l^{-1}) inhibited the biofilm formation of a nonmucoid strain, the ATCC PAO1 strain and of two mucoid strains, the MC75-450457 and the MC099-450467 strains. The bactericidal properties of ceragenins are usually explained by an interaction of the drugs with the bacterial membrane (Epand *et al.* 2010). LPS are components of the membrane of Gram-negative bacteria specially targeted by ceragenins (Ding *et al.* 2004b). As LPS are major contributors of the membrane potential of Gram-negative bacteria (Buchanan *et al.* 2009), we estimated the charges on the membranes of the bacteria by measuring the zeta potential of the eight strains (Wilson *et al.* 2001). This potential was variable between the strains and sometimes even within strains as already reported (Habash *et al.* 1997). In some strains, the distribution of the zeta potentials was bimodal (van der Mei and Busscher 2001). According to Habash *et al.* (1997), metabolically active bacteria have a larger subpopulation of bacteria with more negative zeta potential but Soni *et al.* (2008) reported that the growth conditions and the states of the bacteria did not affect the zeta potential of the bacteria. Flemming *et al.* (1998) observed that the long-chain B-band polysaccharide of LPS increased the zeta potential by masking the charge of the core region from bulk solution. The zeta potential of our four mucoid strains was not significantly different from the potential of the nonmucoid strains. In fact, three mucoid strains (MC75-450457, MC099-450467 and MC093-450507) had the lowest zeta potential but the fourth mucoid strain, the PYO2 strain, had a much higher zeta potential. Herzberg *et al.* (Herzberg *et al.* 2009) compared the potential of a strain and of its mucoid variant and noted that the mucoid variant had a lower zeta potential. The two strains with the lowest zeta potential (MC75-450457 and MC099-450467) were sensitive to inhibition by CSA-13. The third strain which was sensitive to the drug (PAO1) had a higher potential suggesting that there was no correlation between the charges on the membrane and the interaction with

the cationic steroid. But as pointed out by van der Mei and Busscher (2001), the heterogeneity within a strain might lead to erroneous conclusions. The PAO1 strain was clearly heterogeneous with a bimodal distribution of zeta potentials. One of these 2 subpopulations had a potential averaging −56 mV, which is one of the lowest potentials of the subpopulations of the eight strains. It can thus be concluded that the entire populations or a subpopulation of the three strains sensitive to CSA-13 had a zeta potential lower than −50 mV establishing a correlation between sensitivity to the drug and electrostatic interactions between the bacteria and the drug. This is in agreement with the results of Bruinsma *et al.* (2006) who reported that the sensitivity of *P. aeruginosa* to a polyquaternium-1 lens care solution was related to the charges of the bacteria: more negatively charged bacteria were more susceptible to the solution.

In a next attempt to correlate sensitivity to CSA-13 with properties of the bacterial membrane, the interaction with bacteria of CSA-119, a fluorescent analogue of CSA-13, was investigated. The binding of the analogue was dose dependent and rapid. It bound to the eight strains. The PAO1 and the MC75-450457 strains, which were among the three strains sensitive to CSA-13 with the Crystal Violet method and which had a very low mean zeta potential for the whole population or for a subpopulation, were the most efficient binding strains. There was no correlation between sensitivity to CSA-13 and zeta potential on the one hand and CSA-119 binding on the MC099-450467 on the other hand. This strain did not bind CSA-119 very well and yet was sensitive to CSA-13 and had a homogeneous very negative zeta potential. This is best explained by the properties of the BODIPY group, which has been added to CSA-13. The hydrophobicity of this group is at the basis of its measurement (Hu *et al.* 2009) and probably contributes to the interaction of the fluorescent probe with bacteria. These hydrophobic interactions might explain the unexpected high interaction of CSA-119 with some strains and the lack of correlation with their membrane potential.

We had previously reported that the production of rhamnolipids by the three strains sensitive to CSA-13 was very different: the PAO1 and MC75-450457 strains were among the strains which produced a large amount of rhamnolipids. No rhamnolipids could be detected in the culture medium of MC099-450467 (Nagant *et al.* 2010b). In the present work, we observed that the synthesis of rhamnolipids was not affected by long-term exposure of the bacteria to CSA-13. We can thus conclude that the operon rhlAB which regulates rhamnolipid synthesis and which is controlled by quorum sensing (Lequette and Greenberg 2005) was not the intracellular target mediating the inhibition of biofilm formation by CSA-13.

In this work, we have shown that a very low, noncytotoxic (Leszczyńska *et al.* 2011) concentration of CSA-13 (10 times lower than the MIC) inhibited the formation of a biofilm by three of the eight strains tested (the PAO1, MC75-450457 and MC099-450467 strains). In a previous work, we had shown that at much higher concentrations (from 20 to 200 $\mu g\ ml^{-1}$), CSA-13 totally eradicated a 24-h-preformed biofilm on six of the eight strains tested. Six strains were evaluated in mature biofilms, and all of them responded to the compound with increasing concentrations or duration of exposure of the biofilms to the drug. It can thus be concluded from the results of these two studies that six of the eight strains were sensitive to CSA-13 either at the initial stages of the formation of the biofilm (this study) or after completion of the biofilm (our previous study). These results thus confirmed the great potentiality as therapeutic agents against all stages of biofilm formation and development of Janus-like structure of drugs as CSA-13 with a cationic face favouring their interaction with negatively charged membranes and their hydrophobic face which contributes to the perturbation of these membranes.

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Transparency declarations

The authors have no conflict of interest to declare.

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