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Capsule polysaccharide is a bacterial decoy for antimicrobial peptides

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Antimicrobial peptides (APs) are important host weapons against infections. Nearly all APs are cationic and their microbicidal action is initiated through interactions with the anionic bacterial surface. It is known that pathogens have developed countermeasures to resist these agents by reducing the negative charge of membranes, by active efflux and by proteolytic degradation. Here we uncover a new strategy of resistance based on the neutralization of the bactericidal activity of APs by anionic bacterial capsule polysaccharide (CPS). Purified CPSs from *Klebsiella pneumoniae* K2, *Streptococcus pneumoniae* serotype 3 and *Pseudomonas aeruginosa* increased the resistance to polymyxin B of an unencapsulated *K. pneumoniae* mutant. Furthermore, these CPSs increased the MICs of polymyxin B and human neutrophil α-defensin 1 (HNP-1) for unencapsulated *K. pneumoniae*, *Escherichia coli* and *P. aeruginosa* PAO1. Polymyxin B or HNP-1 released CPS from capsulated *K. pneumoniae*, *S. pneumoniae* serotype 3 and *P. aeruginosa* overexpressing CPS. Moreover, this material also reduced the bactericidal activity of APs. We postulate that APs may trigger *in vivo* the release of CPS, which in turn will protect bacteria against APs. We found that anionic CPSs, but not cationic or uncharged ones, blocked the bactericidal activity of APs by binding them, thereby reducing the amount of peptides reaching the bacterial surface. Supporting this, polycations inhibited such interaction and the bactericidal activity was restored. We postulate that trapping of APs by anionic CPSs is an additional selective virulence trait of these molecules, which could be considered as bacterial decoys for APs.

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INTRODUCTION

Antimicrobial peptides (APs) belong to the array of host weapons against infections. There are four structural classes of APs: the disulfide-bonded β-sheet peptides, the amphipathic α-helical peptides, the extended peptides and the loop-structured peptides (Brogden, 2005; Hancock & Chapple, 1999; Nicolas & Mor, 1995). Despite their diverse size and structures, nearly all APs have a net positive charge and the three-dimensional folding results in an amphipathic structure (Brogden, 2005; Hancock & Chapple, 1999; Nicolas & Mor, 1995). The microbicidal action of APs is initiated through electrostatic interaction with the bacterial surface (Brogden, 2005; Hancock & Chapple, 1999; Nicolas & Mor, 1995; Vaara, 1992). For some peptides, lethality is linked to membrane perturbations, although there is an increasing body of evidence indicating that APs may have intracellular targets (Brogden, 2005).

Abbreviations: AP, antimicrobial peptide; CPS, capsule polysaccharide; HNP-1, human neutrophil α-defensin 1.

Bacteria have developed diverse strategies to resist APs and it is generally accepted that resistance to APs is a virulence phenotype. The strategies for resistance include the alteration of cell surface charge, the active efflux of APs by energy-driven transporters and the proteolytic degradation of APs (Nizet, 2006; Peschel, 2002). Examples of these strategies are the addition of aminoarabinose to LPS lipid A backbone (Gunn *et al.*, 1998), the efflux of APs by the MtrCDE pump from *Neisseria gonorrhoeae* (Shafer *et al.*, 1998) and the degradation of APs by the PgtE protease from *Salmonella enterica* (Guina *et al.*, 2000). Another strategy of resistance could involve trapping of APs, which would reduce the amount of peptides reaching the bacterial surface. Few examples are known of this strategy. *Staphylococcus aureus* and group A *Streptococcus* secrete proteins that complex APs, neutralizing their bactericidal activity (Frick *et al.*, 2003; Jin *et al.*, 2004).

Many pathogens express capsule polysaccharides (CPSs), which play a role in avoidance of phagocytosis and complement resistance. In previous work, we analysed

whether CPS plays a role in the resistance to APs. We showed that mutants of *Klebsiella pneumoniae* lacking CPS were more sensitive to APs than wild-type strains (Campos *et al.*, 2004). A higher susceptibility of a *Neisseria meningitidis* CPS mutant to APs was also observed (Spinosa *et al.*, 2007). Mechanistically, it has been speculated that CPS would act as a protective shield on the bacterial surface (Campos *et al.*, 2004; Spinosa *et al.*, 2007). In contrast, a *Campylobacter jejuni* mutant lacking CPS was as resistant as the wild-type to APs, whereas capsulated *Streptococcus pneumoniae* from serotypes 1, 2 and 4 were even more sensitive to APs than the isogenic CPS mutants (Beiter *et al.*, 2008; Zilbauer *et al.*, 2005). For the latter, it has been speculated that CPS might mask surface modifications implicated in the resistance to APs (Beiter *et al.*, 2008).

CPSs vary widely in their chemical composition yet most of them are anionic. Therefore, even though bacteria-bound CPS might not play any role in the resistance to APs, we hypothesized that free anionic CPSs may trap APs, preventing them from reaching membrane targets. A number of studies argue in favour of the presence of free CPS *in vivo*. For example, *K. pneumoniae* sheds an extracellular complex which contains mainly CPS (Straus *et al.*, 1985). Release of CPS from *S. pneumoniae* can be expected at least due to the induction of natural competence-dependent cell lysis (Steinmoen *et al.*, 2002, 2003). In this scenario, pathogens would use anionic CPSs as decoys to resist APs. To the best of our knowledge, this has not been proven yet.

In this study, we show that free CPSs from three important human pathogens, *K. pneumoniae*, *S. pneumoniae* and *Pseudomonas aeruginosa*, bind APs, thereby neutralizing their bactericidal activity. Moreover, APs released CPSs from the bacterial surfaces and this material acted in the same manner as purified CPSs.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain C600 is a laboratory strain used for molecular biology (Appleyard, 1954). *Klebsiella pneumoniae* 52145 is a clinical isolate (serotype O1 : K2), previously described (Nassif *et al.*, 1989); the chemical structure of its CPS has been reported (Corsaro *et al.*, 2005). *Pseudomonas aeruginosa* PAO1 and PAOMA (a *mucA* mutant derived from strain PAO1) were kindly provided by A. Oliver (Servicio de Microbiología, Hospital Son Dureta, Palma de Mallorca, Spain). Mutation in *mucA* causes alginate overexpression (Boucher *et al.*, 1997; Martin *et al.*, 1993). A high proportion of clinical isolates obtained from patients affected by chronic lung diseases harbour point mutations in *mucA* (Boucher *et al.*, 1997; Martin *et al.*, 1993). The chemical composition of alginate has been described (Sherbrock-Cox *et al.*, 1984). *Streptococcus pneumoniae* strains M24LS (expressing CPS) and M24L (isogenic mutant not expressing CPS) have been previously described (Arrecubieta *et al.*, 1994). *E. coli*, *K. pneumonia* and *P. aeruginosa* strains were grown in Luria–Bertani (LB) at 37 °C. *S. pneumoniae* strains were grown at 37 °C on blood agar plates or in Todd–Hewitt Broth supplemented with 0.5 % yeast extract as previously described (Arrecubieta *et al.*, 1994).

Reagents. Polymyxin B, human neutrophil α-defensin 1 (HNP-1), DEAE-dextran and chitosan (low molecular mass) were purchased from Sigma. Dansyl-polymyxin B was obtained from Molecular Probes. Purified CPSs from *S. pneumoniae* serotypes 3, 1 and 33F were purchased from ATCC (numbers 17-X, 25-X and 67-X respectively). The chemical composition of all known CPSs from *S. pneumoniae* has been published (Kamerling, 2000).

***K. pneumoniae* 52145-Δ*wca*$_{K2}$ mutant construction.** To obtain a *K. pneumoniae* 52145 mutant lacking CPS, a chromosomal in-frame *wca*$_{K2}$ deletion was generated using a previously described strategy (Link *et al.*, 1997). Primers for *manC* mutant construction were designed from the known *K. pneumoniae* K2 gene cluster sequence (Arakawa *et al.*, 1995). Primer pairs ManCa (5′-CGC*GGATCC*-GCATTGCTAACCTCCTGCT-3′; *Bam*HI site is in italic)/ManCb (5′-CCCATCCACTAAACTTAAACACAGACGGAGGAACTGTTTC-3′) and ManCc (5′-TGTTTAAGTTTAGTGGATGGGATCCAGTC-GGGGTCGTAC-3′)/ManCd (5′-CGC*GGATCC*CGGGAACTGGTT-CTCCTC-3′; *Bam*HI site is in italic) were used in two sets of asymmetrical PCRs to amplify DNA fragments of 624 (ab) and 529 (cd) bp, respectively. DNA fragments ManCa-b and ManCc-d were annealed at their overlapping region (underlined nucleotides in primers ManCb and ManCc) and amplified by PCR as a single fragment (1132 bp), using primers ManCa and ManCd. The fusion product was purified, *Bam*HI-digested and ligated into *Bam*HI-digested and phosphatase-treated pKO3 vector (Link *et al.*, 1997) to give pKO3Δwca$_{K2}$. This plasmid was electroporated into *K. pneumoniae* 52145 and a non-polar *manC* mutant was obtained as previously described (Regue *et al.*, 2005). The mutation was confirmed by sequencing of the whole construct in an amplified PCR product. Mutant 52145-Δ*wca*$_{K2}$ was completely resistant to the CPS-specific phage ϕ2 and their cells did not react with anti-K2 specific antibodies. In addition, the presence of cell-bound CPS was observed in the wild-type strain but not in mutant 52145-Δ*wca*$_{K2}$ by electron microscopy (data not shown).

CPS purification. CPS from *K. pneumoniae* 52145 was obtained using the hot phenol-water method (Campos *et al.*, 2004; Westphal & Jann, 1963). CPS from *P. aeruginosa* PAOMA mutant, also known as alginate, was extracted exactly as previously described (Cobb *et al.*, 2004; Ma *et al.*, 1998). Polysaccharides were dissolved in distilled water, dialysed against water and freeze-dried. To purify these preparations, they were dispersed (10 mg ml^{-1}) in 0.8 % NaCl/0.05 % NaN_3/0.1 M Tris/HCl (pH 7) and digested with nucleases (50 mg ml^{-1} of DNase II type V and RNase A; Sigma) for 18 h at 37 °C. Proteinase K was added (50 μg ml^{-1}; Merck), and the mixture was incubated for 1 h at 55 °C and for 24 h at room temperature. The proteinase K digestion was repeated twice and the polysaccharides were precipitated by adding 5 vols methanol plus 1 % (v/v) of a saturated solution of sodium acetate in methanol. After incubation for 24 h at −20 °C, the pellet was recovered by centrifugation, and dissolved in distilled water. The LPS was removed by ultracentrifugation (105 000 ***g***, 16 h, 4 °C). The enzymic treatment and ultracentrifugation steps were repeated another time. Finally, samples were freeze-dried.

CPS was quantified by determining the concentration of uronic acid in the samples, using a modified carbazole assay (Bitter & Muir, 1962), exactly as described by Rahn & Whitfield (2003). Protein content in this preparation was quantified using the Coomassie protein assay (Pierce) referenced against a BSA standard. LPS presence was determined by measuring the 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) content by the thiobarbituric acid method modified to correct interference due to deoxysugars (Díaz-Aparicio *et al.*, 1993). Protein content was less than 0.5 % and Kdo content was less than 0.1 %.

AP resistance assay. Bacteria were grown at 37 °C in 5 ml LB, harvested (5000 ***g***, 15 min, 5 °C) in the exponential phase of growth (OD_{600} 0.6) and washed three times with PBS. A suspension containing approximately 1×10^5 c.f.u. ml^{-1} was prepared in 10 mM PBS (pH 6.5), 1 % Tryptone Soya Broth (TSB; Oxoid), 100 mM NaCl. Aliquots (5 µl) of this suspension were mixed in Eppendorf tubes with various concentrations of AP or AP and CPSs. In all cases the final volume was 30 µl. After 1 h incubation, the contents of the Eppendorf tubes were plated on LB agar. Colony counts were determined and results were expressed as percentages of the colony count of bacteria not exposed to antibacterial agents. The 50 % inhibitory concentration of AP (IC_{50}) was defined as the concentration producing a 50 % reduction in the colony counts compared with bacteria not exposed to the antibacterial agent. All experiments were done with duplicate samples on four independent occasions.

Radial diffusion assay. To assess the sensitivity to APs, the previously described radial diffusion assay was also used (Bengoechea *et al.*, 2004; Lehrer *et al.*, 1991). Briefly, an underlay gel that contained 1 % (w/v) agarose (SeaKem LE agarose, FMC), 2 mM HEPES (pH 7.2), and 0.3 mg TSB powder per ml was equilibrated at 50 °C, and inoculated with the different bacteria to a final concentration of 5×10^5 c.f.u. per ml of molten gel. This gel was poured into standard square Petri dishes ($10 \times 10 \times 1.5$ cm), and after solidification, small wells of 15 µl capacity were carved. Aliquots of 10 µl of APs or APs plus CPSs were added to the wells and the plates were incubated for 3 h at 37 °C. After that, a 30 ml overlay gel composed of 1 % agarose and 6 % TSB powder in water was poured on top of the previous one and the plates were incubated at 37 °C. After 18 h, the diameters of the inhibition haloes were measured to the nearest 1 mm, and after subtracting the diameter of the well, were expressed in inhibition units (10 units=1 mm). The minimal inhibitory concentration (MIC) was estimated by performing linear regression analysis (units vs $\log_{10}$ concentration) and determining the *x*-axis intercepts (Bengoechea *et al.*, 2004; Lehrer *et al.*, 1991). All the experiments were run in quadruplicate on three independent occasions.

Binding of APs to CPSs. In Eppendorf tubes, different amounts of CPSs (final concentrations ranging from 10 to 0.01 µg ml^{-1}) were prepared in 5 mM HEPES (pH 7.5) and mixed with polymyxin B (final concentration 16 µg ml^{-1}) or HNP-1 (final concentration 100 µg ml^{-1}) to get a final volume of 50 µl. When the effect of the polycations DEAE-dextran or chitosan was tested, the amount of CPS used was 1 µg ml^{-1}, the concentrations of both polycations were 0.5 µg ml^{-1}, and the concentrations of polymyxin B and HNP-1 were the same as before. Suspensions were incubated for 30 min at room temperature (22–25 °C). The suspensions were centrifuged (12 000 ***g***, 10 min), the supernatant centrifuged two more times under the same conditions, and the unbound AP measured in a bioassay (see radial diffusion assay above), using *E. coli* C600 as indicator bacteria. The amount of AP was estimated from the diameter of the inhibition halo by comparison with AP standards on the same plate. Results were expressed as percentages of bound peptide. All measurements were done in triplicate on three separate occasions.

CPS released by APs. Bacterial strains were grown at 37 °C in 5 ml of LB (*K. pneumoniae* 52145 and *P. aeruginosa mucA*) or Todd–Hewitt Broth supplemented with 0.5 % yeast extract (*S. pneumoniae*). At the exponential phase of growth (OD_{600} 0.6) bacterial cells were harvested (5000 ***g***, 15 min, 5 °C), washed twice with PBS and resuspended in 5 ml PBS. Either polymyxin B or HNP-1 was added to the suspensions, which were incubated for 15 min at 37 °C. Control experiments revealed that this treatment caused neither bacterial lysis nor decrease in viability. A 2 ml sample from this suspension was centrifuged (4000 ***g***, 20 min, 22 °C) and the upper 1.5 ml of the supernatant was processed to determine the amount of CPS present. Aliquots of these supernatants were plated and no c.f.u. were recovered. The hot phenol-water method followed by the modified carbazole method was used to purify and quantify the CPS present in the supernatants (Rahn & Whitfield, 2003). Neither polymyxin B nor HNP-1 affects the quantification of CPS. Results were expressed as amount of CPS released (in µg) per 10^5 c.f.u.. All measurements were done in duplicate on three separate occasions.

Dansyl-polymyxin B binding studies. CPS suspensions (final concentration 10 µg ml^{-1}) were prepared in 5 mM HEPES (pH 7.5) and 1 ml of this suspension was transferred to a 1 cm fluorimetric cuvette to which dansyl-polymyxin B (final concentration 0.5 µM) was added. Fluorescence spectra of dansyl-polymyxin B were recorded at room temperature from 400 to 600 nm at an excitation wavelength of 340 nm with a spectrofluorophotomoter (RF-5301PC Shimadzu). The slit width was 3 nm. Results were expressed as arbitrary units of fluorescence. Measurements were recorded as ASCII files and exported to a personal computer for plotting. All measurements were done in duplicate on three separate occasions.

Statistical methods. Statistical analyses were performed using the analysis of variance (ANOVA) or the two-sample *t* test or, when the requirements were not met, by the Mann–Whitney U test. $P<0.05$ was considered statistically significant. These analyses were performed using Prism4 for PC (GraphPad Software).

RESULTS

Free CPSs increase bacterial resistance to APs

To investigate whether free CPS may protect against APs, we asked whether purified CPS could increase the resistance to APs of a *K. pneumoniae* CPS mutant which is sensitive to APs (Campos *et al.*, 2004). We and others have used polymyxin B as a model AP since resistance to this agent reflects well the resistance to mammalian APs and correlates with virulence (Groisman, 1994; Gutsmann *et al.*, 2005; Nizet, 2006; Wiese *et al.*, 2003). Survival assays, shown in Fig. 1, demonstrated that purified CPS from wild-type *K. pneumoniae* strain 52145 increased the resistance to polymyxin B of strain 52145-Δwca_{K2} 3-fold (IC_{50} 1 µg ml^{-1} vs 3 µg ml^{-1}). We sought to determine whether CPSs from other pathogens also increase the resistance to APs of the mutant. Either CPS from a *S. pneumoniae* serotype 3 strain or CPS from *P. aeruginosa*, also known as alginate, increased the resistance of 52145-Δwca_{K2} to polymyxin B (Fig. 1) (IC_{50} values also increased 3-fold).

To further study the ability of CPSs to increase the resistance to APs, we determined the MICs of polymyxin B and human neutrophil α-defensin-1 (HNP-1) for three unencapsulated strains, namely *K. pneumoniae* 52145-Δwca_{K2}, *E. coli* C600 and *P. aeruginosa* PAO1, in the presence or absence of CPSs (Table 1). CPS from *K. pneumoniae* increased the MICs of polymyxin B and HNP-1 for the three bacterial species (Table 1). The effect was more dramatic for HNP-1 than for polymyxin B (Table 1). Similar experiments were conducted using CPSs from *S.*

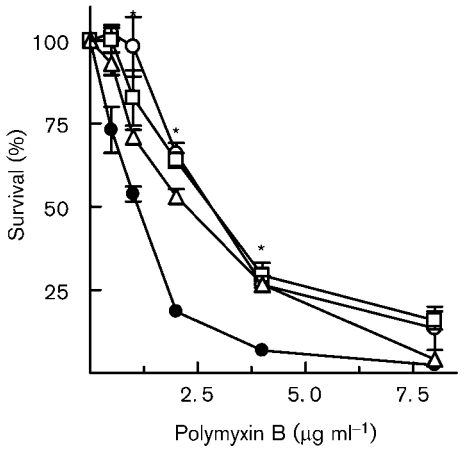

Fig. 1. Purified CPSs increase survival in the presence of polymyxin B. The survival of *K. pneumoniae* 52145-Δ*wca*$_{K2}$ in the presence of polymyxin B was determined in the absence (●) or presence of 10 μg ml^{-1} of CPSs from *K. pneumoniae* (○), *S. pneumoniae* (△) or *P. aeruginosa* (□). Each point represents the mean and standard deviation (not shown where smaller than symbol) of eight samples from four independently grown batches of bacteria; significant survival differences ($P<0.05$) between bacteria incubated with and without CPSs are indicated by asterisks.

pneumoniae serotype 3 and *P. aeruginosa* PAOMA. Both polysaccharides also increased the MICs of polymyxin B and HNP-1 for the three bacterial species tested (Table 1).

Collectively, our data indicate that purified CPSs increased the resistance to APs of different bacterial species. Since the structures of polymyxin B and HNP-1 are different, the effect of CPS does not appear to depend on the AP tested. The fact that CPSs of different bacterial origin similarly increased the resistance to APs suggests that the molecular mechanism(s) underlying this effect would be common to all of them.

APs release CPS from the bacterial surface

Considering that *K. pneumoniae*, *S. pneumoniae* and *P. aeruginosa* will encounter APs *in vivo*, we asked whether APs may release CPS from the bacterial surface which, in turn, might increase the resistance to APs. For this set of experiments we used wild-type *K. pneumoniae* strain 52145, wild-type *S. pneumoniae* strain M24LS (Arrecubieta *et al.*, 1994) and *P. aeruginosa* PAOMA mutant. The results demonstrated that in the absence of APs, all three strains released CPS to the medium (Table 2). The amount of CPS released was increased upon incubation of the pathogens with either polymyxin B or HNP-1 (Table 2). HNP-1 at 1 μg ml^{-1} caused release of significantly more CPS than HNP-1 at 0.1 μg ml^{-1} (Table 2). Control experiments revealed that treatment with APs caused neither bacterial lysis nor viability loss (data not shown). It is of note that polymyxin B also released CPS from UV-killed bacteria (3 min exposition to 1 J; after this treatment no c.f.u. were recovered). The amount of CPS (in μg per 10^5 c.f.u.) released by polymyxin B from UV-killed *K. pneumoniae*, *S. pneumoniae* and *P. aeruginosa* was 335, 1597 and 452 respectively. These amounts of CPSs were similar to those released by polymyxin B from live bacteria (Table 2).

Next, we tested whether the released material would increase the resistance to APs, as we have previously shown for purified CPS. Naturally released CPS material from each pathogen or polymyxin B-extracted CPS was adjusted to get a final concentration of 0.1 μg CPS ml^{-1} and the effect on the MIC of polymyxin B to *E. coli* C600 was tested. The MIC in the absence of CPSs was 0.8 ± 0.2 μg ml^{-1} whereas the MIC in the presence of naturally released CPSs from *K. pneumoniae*, *S. pneumoniae* and *P. aeruginosa* increased to 3.0 ± 0.2, 2.8 ± 0.1 and 2.8 ± 0.2 μg ml^{-1} respectively ($P<0.05$ between MIC in the presence of a given CPS and MIC in the absence of polysaccharides). The MIC of polymyxin B to *E. coli* C600 in the presence of polymyxin B-extracted CPSs from *K.*

Table 1. Effect of CPSs on the MICs of polymyxin B and HNP-1 for *K. pneumoniae* 52145-Δ*wca*$_{K2}$, *E. coli* C600 and *P. aeruginosa* PAO1

Bacterial strain	AP	MIC (μg ml^{-1})								
		No CPS	CPS *K. pneumoniae* (μg ml^{-1})			CPS *S. pneumoniae* (μg ml^{-1})			CPS *P. aeruginosa* (μg ml^{-1})		
			10	1	0.1	10	1	0.1	10	1	0.1
52145-Δ*wca*$_{K2}$	Polymyxin B	1.7 ± 0.1	5.5 ± 0.7*	4.8 ± 0.8*	4.1 ± 0.8*	3.5 ± 1.0 *	2.2 ± 0.8	2.3 ± 0.4	4.3 ± 0.4*	1.8 ± 0.5	1.7 ± 0.2
	HNP-1	10.2 ± 3.3	>50*	>50*	18.0 ± 0.2*	>50*	>50*	13.2 ± 1.6*	>50*	>50*	10.4 ± 0.7
C600	Polymyxin B	0.9 ± 0.3	3.5 ± 0.2*	3.1 ± 0.3*	2.9 ± 0.3*	3.7 ± 0.7*	3.4 ± 0.9*	2.9 ± 0.1*	3.8 ± 0.8*	3.0 ± 0.1*	2.6 ± 0.1*
	HNP-1	1.2 ± 0.4	>50*	21.8 ± 6.8*	6.2 ± 1.0*	>50*	30.1 ± 2.0*	8.5 ± 0.8*	>50*	29.5 ± 2.7*	8.9 ± 0.7*
PAO1	Polymyxin B	0.7 ± 0.1	2.0 ± 0.1*	1.5 ± 0.1*	0.9 ± 0.6	2.0 ± 0.2*	2.0 ± 0.8*	1.2 ± 1.0	3.2 ± 0.5*	2.5 ± 0.5*	1.9 ± 1.0
	HNP-1	9.7 ± 1.7	>50*	>50*	17.0 ± 1.0*	>50*	>50*	11.6 ± 1.3*	>50*	>50*	10.0 ± 0.1

*MIC significantly different ($P<0.05$) from MIC in the absence of a given CPS.

Table 2. Amount of CPS released from *K. pneumoniae* 52145, *S. pneumoniae* M24LS and *P. aeruginosa* PAO1 *mucA* by either polymyxin B or HNP-1

Strain	Amount of CPS released (μg CPS per 10^5 c.f.u.)* by:			
	No treatment	Polymyxin B	HNP-1	
		0.1 μg ml^{-1}	0.1 μg ml^{-1}	1 μg ml^{-1}
K. pneumoniae 52145	219±10	308±5†	256±4†	301±8†
S. pneumoniae M24LS	1341±40	1630±25†	1490±30†	1797±51†
P. aeruginosa PAO1 *mucA*	401±8	453±11†	436±9†	540±12†

*Data are means±SD derived from three independent experiments run in duplicate.
†Significantly different ($P<0.05$) from the amount of CPS released to the culture supernatant in the absence of APs.

pneumoniae, *S. pneumoniae* or *P. aeruginosa* increased to 3.1±0.2, 3.0±0.1 and 2.9±0.2 μg ml^{-1} respectively ($P<0.05$ between MIC in the presence of a given CPS and MIC in the absence of polysaccharides). To analyse the possible contribution of CPS co-extracted material to the MIC increase, polymyxin B extracts were obtained from unencapsulated bacteria, *K. pneumoniae* 52145-Δwca_{K2}, *P. aeruginosa* PAO1 and *S. pneumoniae* M24L. However, these materials did not change the MIC of polymyxin B to *E. coli* C600 (data not shown).

Together these data demonstrate that APs release CPS from the surface of three different pathogens which, in turn, reduces the bactericidal activity of APs as shown by a 3-fold increase in the MIC. Naturally released CPS from the three pathogens also neutralize the bactericidal activity of APs.

CPSs bind APs

To explain how CPSs neutralize the bactericidal activity of APs, we hypothesized that CPSs might bind APs, thereby reducing the amount of peptides reaching the bacterial surface. This hypothesis was based on the fact that nearly all APs are cationic whereas the CPSs tested are anionic (Corsaro *et al.*, 2005; Kamerling, 2000; Sherbrock-Cox *et al.*, 1984) and hence an electrostatic interaction between them could be expected. To explore this, firstly we determined whether CPSs would bind polymyxin B and HNP-1. By using a bioassay, we showed that CPSs from *K. pneumoniae*, *S. pneumoniae* serotype 3 and *P. aeruginosa* bound polymyxin B (Fig. 2a) and HNP-1 (Fig. 2b). Secondly, we evaluated the binding of dansyl-polymyxin B to CPSs. This compound has been extensively used to study the binding of APs to polysaccharides, proteins and intact bacteria (Bengoechea *et al.*, 1998; Campos *et al.*, 2006; Moore *et al.*, 1986). The dansylated peptide exhibits low fluorescent yield when diluted into aqueous buffer; however, the fluorescent yield increases, with the maximum fluorescence shifting to a lower wavelength, in a hydrophobic environment (Moore *et al.*, 1986; Newton, 1955). Therefore there is a correlation between fluorescence and the amount of dansyl-polymyxin B bound (Bengoechea *et al.*, 1998; Campos *et al.*, 2006; Moore *et al.*, 1986). The results shown in Fig. 2(c–e) demonstrate that dansyl-polymyxin B bound to CPSs from *K. pneumoniae* (Fig. 2c), *S. pneumoniae* serotype 3 (Fig. 2d) and *P. aeruginosa* (Fig. 2e). In all cases, unlabelled polymyxin B outcompeted dansyl-polymyxin B, showing that binding was specific and not due to non-specific features of the dansyl group (data not shown).

Taken together, our data indicate that CPSs bind APs and this is dependent neither on the bacterial origin of the polysaccharide nor on the AP tested. Given the cationic nature of APs and the anionic nature of the polysaccharides studied, it is reasonable to assume an electrostatic interaction between CPSs and APs. Then we reasoned than non-anionic CPSs should not bind APs and hence not neutralize their bactericidal activity. To test this, we asked whether CPS from *S. pneumoniae* serotypes 1 (cationic CPS) or 33F (no net charge CPS) (Kamerling, 2000) would bind dansyl-polymyxin B. The results shown in Fig. 3 demonstrate that these CPSs did not bind dansyl-polymyxin B, because there was no change in the fluorescence yield. Due to the lack of binding of APs to both CPSs we hypothesized that these CPSs would not change the MIC of APs for a sensitive strain such as *E. coli* C600. Indeed, the MIC of polymyxin B for *E. coli* C600 in the absence of CPSs (0.9±0.3 μg ml^{-1}) was not significantly different ($P>0.05$) from those in the presence of 10 μg ml^{-1} of either CPS from serotype 1 (1.1±0.3 μg ml^{-1}) or CPS from serotype 33F (1.2±0.6 μg ml^{-1}).

Polycations restore the bactericidal activity of APs in the presence of CPSs

Considering that anionic CPSs bind APs electrostatically we postulated that a polycationic polysaccharide would inhibit such interaction. Furthermore, we expected that the bactericidal activity of APs would be restored. To explore these hypotheses, firstly we examined whether the polycation DEAE-dextran would reduce the binding of APs to CPSs from *K. pneumoniae*, *S. pneumoniae* serotype 3 and *P. aeruginosa*. The data shown in Fig. 4 indicate that indeed

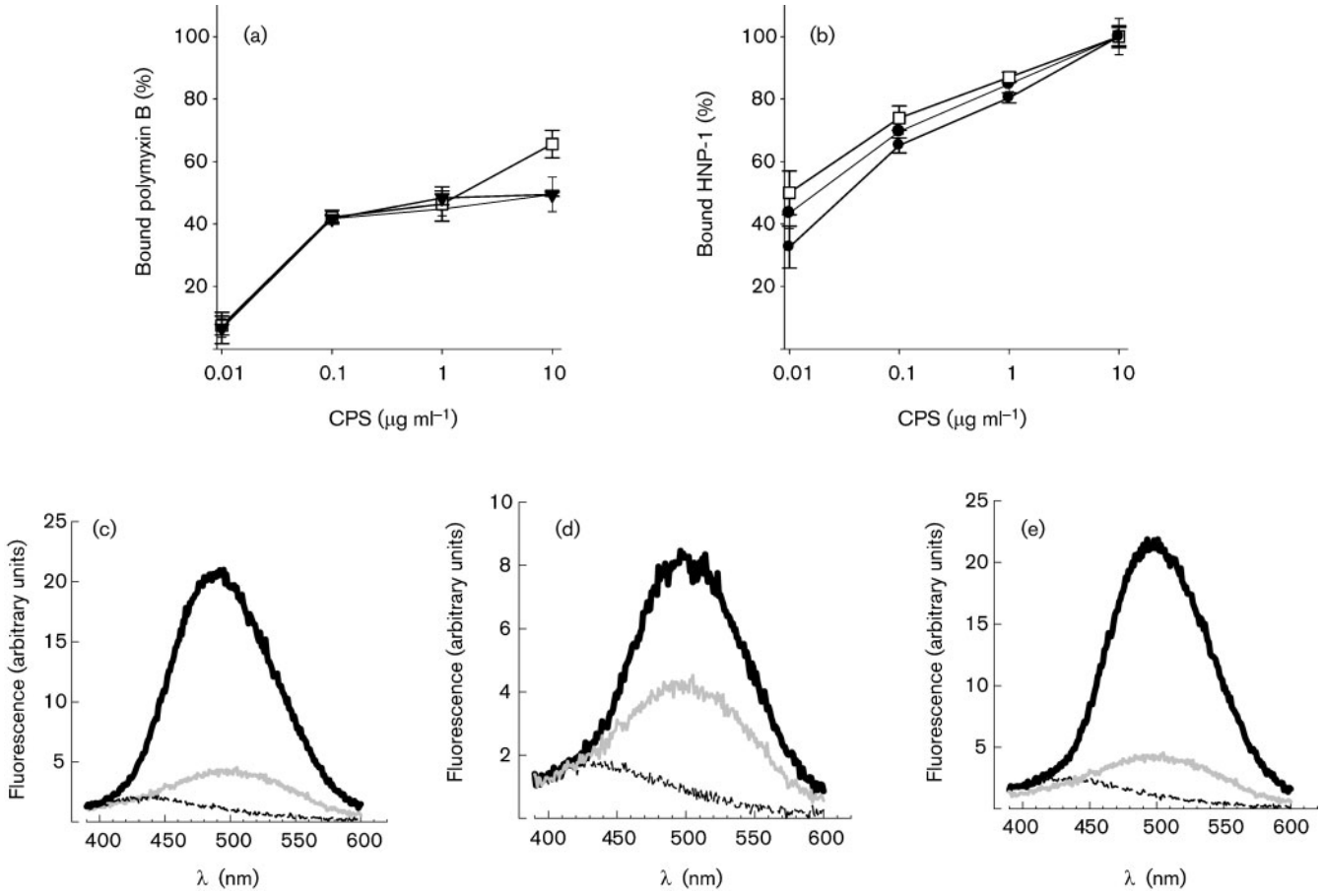

Fig. 2. CPSs bind APs. (a, b) Percentage of polymyxin B (a) and HNP-1 (b) bound to different concentrations of CPSs from *K. pneumoniae* (●), *S. pneumoniae* (▼) and *P. aeruginosa* (□). Each point in panels (a) and (b) represents the mean and standard deviation of six samples from three independent experiments. (c–e) Binding of dansyl-polymyxin B to CPS from *K. pneumoniae* (c), *S. pneumoniae* (d) and *P. aeruginosa* (e). In panels (c)–(e) the dashed line shows the fluorescence of CPS alone, the grey line shows the fluorescence of 0.5 μM dansyl-polymyxin B and the black line shows the fluorescence of 0.5 μM dansyl-polymyxin B in the presence of CPS. The data in panels (c)–(e) are representative of three separate independent experiments (coefficient of variation was less than 5 %).

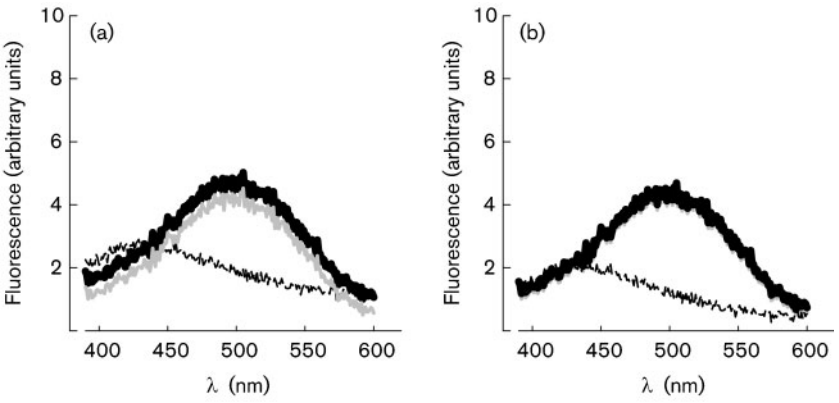

Fig. 3. Non-anionic CPSs from *S. pneumoniae* do not bind APs. (a) Binding of dansyl-polymyxin B to CPS from *S. pneumoniae* serotype 1. (b) Binding of dansyl-polymyxin B to CPS from *S. pneumoniae* serotype 33F. In both panels, the dashed line shows the fluorescence of CPSs alone, the grey line shows the fluorescence of 0.5 μM dansyl-polymyxin B and the black line shows the fluorescence of 0.5 μM dansyl-polymyxin B in the presence of CPS. Data shown are representative of three separate independent experiments (coefficient of variation was less than 5 %).

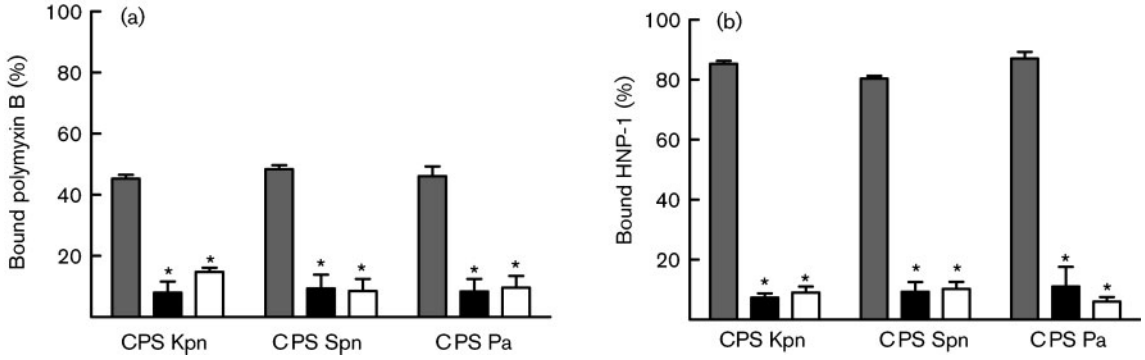

Fig. 4. Polycations reduce the binding of APs to CPSs. (a) A 0.5 μg ml^{-1} concentration of either DEAE-dextran (black bars) or chitosan (white bars) reduced the percentage of polymyxin B (initial concentration 16 μg ml^{-1}) bound to CPSs (1 μg ml^{-1}) from *K. pneumoniae* (CPS Kpn); *S. pneumoniae* (CPS Spn) and *P. aeruginosa* (CPS Pa). Grey bars show the percentage of polymyxin B bound to each CPS in the absence of polycations. (b) A 0.5 μg ml^{-1} concentration of either DEAE-dextran (black bars) or chitosan (white bars) reduced the percentage of HNP-1 (initial concentration 100 μg ml^{-1}) bound to CPSs (1 μg ml^{-1}) from *K. pneumoniae* (CPS Kpn), *S. pneumoniae* (CPS Spn) and *P. aeruginosa* (CPS Pa). Grey bars show the percentage of HNP-1 bound to each CPS in the absence of polycations. In both panels, error bars represent the standard deviation of three independent experiments; asterisks indicate a significant difference ($P<0.05$) in the percentage of AP bound by a given CPS in the presence versus the absence of polycations.

DEAE-dextran significantly reduced the binding of polymyxin B (Fig. 4a) and HNP-1 (Fig. 4b) to the three CPSs. To rule out specific features of DEAE-dextran, we tested whether the polycation chitosan would behave as DEAE-dextran. Chitosan also reduced the binding of polymyxin B and HNP-1 to the polysaccharides (Fig. 4a, b).

Secondly, we asked whether both polycations would block the neutralizing effect of CPS on AP activity. Either DEAE-dextran or chitosan completely restored the MICs of polymyxin B and HNP-1 for *K. pneumoniae* 52145-Δwca_{K2} in the presence of CPSs from *K. pneumoniae*, *S. pneumoniae* serotype 3 and *P. aeruginosa* (Table 3). Both polycations also abrogated the CPS-dependent MIC increase of polymyxin B and HNP-1 for *E. coli* C600 and *P. aeruginosa* PAO1 (Table 3). The MICs of either DEAE-dextran or chitosan for 52145-Δwca_{K2}, *E. coli* C600 and *P. aeruginosa* PAO1 were higher than 500 μg ml^{-1}. In addition, there was no synergy between polycations and APs because the MICs of polymyxin B and HNP-1 to 52145-Δwca_{K2}, C600 or PAO1 in the presence or absence of either DEAE-dextran or chitosan were not significantly different (Table 3).

DISCUSSION

In this work, we have uncovered a new strategy of resistance to APs based on the binding of APs by free anionic CPSs. This neutralizes the bactericidal activity of APs, thereby enhancing the resistance of sensitive bacteria to APs. We have also shown that CPS released from the bacterial surface, both naturally and APs-extracted, neutralized the killing ability of APs. This behaviour is reminiscent of that of chemokine decoy receptors (Mantovani *et al.*, 2006). These are molecules expressed by different viruses and parasites which act as molecular traps for the chemokines to prevent cell activation (Mantovani *et al.*, 2006). Therefore, taking into account the findings reported here, we would like to put forward the concept that anionic CPSs could be considered bacterial decoys for APs. Supporting the notion that this could be a general theme for pathogens expressing anionic CPSs, in this work we have shown that CPSs from three different pathogens, *K. pneumoniae*, *S. pneumoniae* and *P. aeruginosa*, acted in the same manner.

The best-characterized bacterial strategies of resistance to APs are based on the alteration of cell surface charge by, for example, addition of aminoarabinose to LPS lipid A moiety or D-alanylation of surface-exposed lipoteichoic acids (Nizet, 2006; Peschel, 2002). However, given the cationic nature of APs, another way to neutralize them could be the exploitation of negatively charged molecules. To the best of our knowledge, free anionic CPSs are the first bacterial molecules shown to be examples of this strategy.

What is the relative impact of this mechanism of resistance to APs in comparison to others published in the literature? In the present study, we have shown that CPSs increased the IC_{50} values of polymyxin B for an unencapsulated mutant of *K. pneumoniae* 3-fold (Fig. 1). For *P. aeruginosa* and *Salmonalla typhimurium,* there are studies showing increases of the IC_{50} of polymyxin B of 6 and 10-fold respectively (Gunn & Miller, 1996; Moskowitz *et al.*, 2004). However, it is worth noting that the *Pseudomonas* and *Salmonella* strains used in those studies are not wild-type

Table 3. Polycations DEAE-dextran (Dextran) and chitosan restore the MICs of polymyxin B and HNP-1 for *K. pneumoniae* 52145-Δwca_{K2}, *E. coli* C600 and *P. aeruginosa* PAO1 in the presence of CPSs

Bacterial strain	AP	MIC (μg ml^{-1})									
		No CPS	Polycation*		CPS *K. pneumoniae*†		CPS *S. pneumoniae*†			CPS *P. aeruginosa*†		
			Dextran	Chitosan	Alone	Dextran	Chitosan	Alone	Dextran	Chitosan	Alone	Dextran	Chitosan
52145-Δwca_{K2}	Polymyxin B	1.7 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	5.4 ± 0.6‡	1.8 ± 0.2§	2.0 ± 0.9§	3.5 ± 1.0‡	1.7 ± 0.2§	1.7 ± 0.5§	4.1 ± 0.4‡	1.9 ± 0.3§	2.1 ± 0.2§
	HNP-1	10.2 ± 3.3	10.0 ± 1.5	9.5 ± 2.0	>50‡	11.0 ± 0.9§	10.3 ± 0.9§	>50‡	12.0 ± 0.2§	13.2 ± 1.6§	>50‡	11.0 ± 0.9§	10.4 ± 0.7§
C600	Polymyxin B	1.6 ± 0.6	1.5 ± 0.5	1.3 ± 0.6	3.7 ± 0.4‡	1.6 ± 0.4§	1.5 ± 0.3§	3.7 ± 0.7‡	1.6 ± 0.2§	1.5 ± 0.4§	3.8 ± 0.8‡	1.6 ± 0.2§	1.9 ± 0.1§
	HNP-1	1.6 ± 0.4	1.6 ± 0.3	1.4 ± 0.9	>50‡	1.3 ± 1.0§	1.9 ± 0.5§	>50‡	1.9 ± 1.0§	2.4 ± 0.5§	>50‡	2.8 ± 1.0§	2.0 ± 0.5§
PAO1	Polymyxin B	0.7 ± 0.1	0.7 ± 0.2	0.8 ± 0.1	2.0 ± 0.1‡	0.6 ± 0.1§	0.8 ± 0.1§	2.0 ± 0.2‡	0.5 ± 0.1§	0.7 ± 0.1§	3.2 ± 0.5‡	1.0 ± 0.2§	1.9 ± 0.1§
	HNP-1	9.7 ± 1.7	9.7 ± 1.7	8.9 ± 1.6	>50‡	10.0 ± 1.1§	9.4 ± 0.6§	>50‡	10.0 ± 0.1§	11.6 ± 1.3§	>50‡	9.4 ± 0.6§	10.0 ± 0.1§

*The amount of either DEAE-dextran or chistosan used was 0.5 μg ml^{-1}.
†The amount of CPSs used was 10 μg ml^{-1}.
‡MIC significantly different ($P<0.05$) from MIC in the absence of a given CPS.
§MIC significantly different ($P<0.05$) from MIC in the presence of a given CPS.

strains but mutants overexpressing several loci involved in AP resistance.

On the other hand, it has been estimated that the extracellular concentration of HNP-1 after neutrophil degranulation is in the 1–10 μg ml^{-1} range (Ganz, 1987; Selsted & Ouellette, 1995) which is close to the MIC for *K. pneumoniae*, *E. coli* and *P. aeruginosa* (see Table 1 and Takemura *et al.*, 1996). Our results demonstrate that CPSs increased the MIC of HNP-1 for the three bacterial species at least 5-fold (up to 50 μg ml^{-1}; see Table 1). To the best of our knowledge, there is no report of a bacterial system increasing the MIC of HNP-1 for a sensitive strain to such an extent.

An important question is whether *in vivo* there would be enough free CPS to neutralize the bactericidal action of APs. Evidence suggests that *in vivo* there is CPS that is not bound to the bacterial surface in the case of *K. pneumoniae* and *S. pneumoniae* (Hardy *et al.*, 2000; Steinmoen *et al.*, 2002, 2003; Straus *et al.*, 1985; Ventura *et al.*, 2006). A prominent feature of *P. aeruginosa* isolated from patients with chronic lung infections is the conversion to alginate-overproducing strains (Poschet *et al.*, 2001). Furthermore, alginate is detected in the sputum of patients and also in the lungs of infected mice (Bragonzi *et al.*, 2005). Although it is difficult to estimate the number of bacteria in the lungs of infected patients with either *Klebsiella*, *Streptococcus* or *Pseudomonas*, perusal of the clinical guidelines indicates that in those patients diagnosed with pneumonia at least 10^4 c.f.u. per ml of bronchoalveolar lavage can be obtained. According to the data shown in Table 2, it is likely that 10^4 c.f.u. will release enough CPS to achieve protection against APs. Nevertheless, we have shown that even a sublethal concentration of HNP-1 released CPS from the bacterial surface. We postulate that APs may trigger *in vivo* the release of material which in turn will protect bacteria against APs. In fact, it is reported that activated neutrophils may release at least 1 μg ml^{-1} of HNP-1 (Ganz, 1987; Selsted & Ouellette, 1995), indicating that *in vivo* there would be enough AP to release a significant amount of CPS. Mechanistically, it is well known that APs release LPS from the surface by removing stabilizing divalent cations from their LPS-binding sites (Vaara, 1992). Likewise we speculate that APs would release CPS from the surface by disturbing cation-dependent ionic bridges between adjacent molecules of CPS. Interestingly, it has been shown that CPS from *K. pneumoniae* is associated with the cell surface by an ionic interaction between CPS and the LPS core region (Fresno *et al.*, 2006). This interaction is stabilized by divalent cations (Fresno *et al.*, 2006). In this context, it is not surprising that polymyxin B released more CPS from the bacterial surface than HNP-1 (Table 2) because polymyxin B is considered the most potent AP disturbing cation-dependent ionic bridges between LPS molecules (Vaara, 1992).

We and others have suggested that surface-bound CPS may act as a protective shield on the bacterial surface against APs (Campos *et al.*, 2004; Spinosa *et al.*, 2007). In this

study, we demonstrate that the decoy action of CPSs contributes significantly to the resistance against APs for pathogens expressing anionic CPSs. The protective action of CPS will also allow the pathogen to activate other countermeasures against APs such as alteration of surface charge, which is a time-consuming process. This is of relevance because, as discussed before, the concentrations of defensins present in tissues are close to the MIC for several bacteria.

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REFERENCES

Appleyard, R. K. (1954). Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. *Genetics* **39**, 440–452.

Arakawa, Y., Wacharotayankun, R., Nagatsuka, T., Ito, H., Kato, N. & Ohta, M. (1995). Genomic organization of the *Klebsiella pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *J Bacteriol* **177**, 1788–1796.

Arrecubieta, C., Lopez, R. & Garcia, E. (1994). Molecular characterization of *cap3A*, a gene from the operon required for the synthesis of the capsule of *Streptococcus pneumoniae* type 3: sequencing of mutations responsible for the unencapsulated phenotype and localization of the capsular cluster on the pneumococcal chromosome. *J Bacteriol* **176**, 6375–6383.

Beiter, K., Wartha, F., Hurwitz, R., Normark, S., Zychlinsky, A. & Henriques-Normark, B. (2008). The capsule sensitizes *Streptococcus pneumoniae* to neutrophil alpha-defensins HNP 1–3. *Infect Immun* **76**, 3710–3716.

Bengoechea, J. A., Lindner, B., Seydel, U., Díaz, R. & Moriyón, I. (1998). *Yersinia pseudotuberculosis* and *Yersinia pestis* are more resistant to bactericidal cationic peptides than *Yersinia enterocolitica*. *Microbiology* **144**, 1509–1515.

Bengoechea, J. A., Najdenski, H. & Skurnik, M. (2004). Lipopolysaccharide O antigen status of *Yersinia enterocolitica* O:8 is essential for virulence and absence of O antigen affects the expression of other *Yersinia* virulence factors. *Mol Microbiol* **52**, 451–469.

Bitter, T. & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Anal Biochem* **4**, 330–334.

Boucher, J. C., Yu, H., Mudd, M. H. & Deretic, V. (1997). Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect Immun* **65**, 3838–3846.

Bragonzi, A., Worlitzsch, D., Pier, G. B., Timpert, P., Ulrich, M., Hentzer, M., Andersen, J. B., Givskov, M., Conese, M. & Doring, G. (2005). Nonmucoid *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. *J Infect Dis* **192**, 410–419.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**, 238–250.

Campos, M. A., Vargas, M. A., Regueiro, V., Llompart, C. M., Alberti, S. & Bengoechea, J. A. (2004). Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect Immun* **72**, 7107–7114.

Campos, M. A., Morey, P. & Bengoechea, J. A. (2006). Quinolones sensitize gram-negative bacteria to antimicrobial peptides. *Antimicrob Agents Chemother* **50**, 2361–2367.

Cobb, L. M., Mychaleckyj, J. C., Wozniak, D. J. & Lopez-Boado, Y. S. (2004). *Pseudomonas aeruginosa* flagellin and alginate elicit very distinct gene expression patterns in airway epithelial cells: implications for cystic fibrosis disease. *J Immunol* **173**, 5659–5670.

Corsaro, M. M., De, C. C., Naldi, T., Parrilli, M., Tomas, J. M. & Regue, M. (2005). ^{1}H and ^{13}C NMR characterization and secondary structure of the K2 polysaccharide of *Klebsiella pneumoniae* strain 52145. *Carbohydr Res* **340**, 2212–2217.

Díaz-Aparicio, E., Aragón, V., Marín, C., Alonso, B., Font, M., Moreno, E., Pérez-Ortiz, S., Blasco, J. M., Diaz, R. & Moriyón, I. (1993). Comparative analysis of *Brucella s*erotype A and M and *Yersinia enterocolitica* O:9 polysaccharides for serological diagnosis of brucellosis in cattle, sheep, and goats. *J Clin Microbiol* **31**, 3136–3141.

Fresno, S., Jimenez, N., Izquierdo, L., Merino, S., Corsaro, M. M., De, C. C., Parrilli, M., Naldi, T., Regue, M. & Tomas, J. M. (2006). The ionic interaction of *Klebsiella pneumoniae* K2 capsule and core lipopolysaccharide. *Microbiology* **152**, 1807–1818.

Frick, I. M., Akesson, P., Rasmussen, M., Schmidtchen, A. & Bjorck, L. (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J Biol Chem* **278**, 16561–16566.

Ganz, T. (1987). Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect Immun* **55**, 568–571.

Groisman, E. A. (1994). How bacteria resist killing by host-defence peptides. *Trends Microbiol* **2**, 444–449.

Guina, T., Yi, E. C., Wang, H., Hackett, M. & Miller, S. I. (2000). A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar Typhimurium promotes resistance to α-helical antimicrobial peptides. *J Bacteriol* **182**, 4077–4086.

Gunn, J. S. & Miller, S. I. (1996). Pho-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. *J Bacteriol* **178**, 6857–6864.

Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27**, 1171–1182.

Gutsmann, T., Hagge, S. O., David, A., Roes, S., Bohling, A., Hammer, M. U. & Seydel, U. (2005). Lipid-mediated resistance of Gram-negative bacteria against various pore-forming antimicrobial peptides. *J Endotoxin Res* **11**, 167–173.

Hancock, R. E. W. & Chapple, D. S. (1999). Peptide antibiotics. *Antimicrob Agents Chemother* **43**, 1317–1323.

Hardy, G. G., Caimano, M. J. & Yother, J. (2000). Capsule biosynthesis and basic metabolism in *Streptococcus pneumoniae* are linked through the cellular phosphoglucomutase. *J Bacteriol* **182**, 1854–1863.

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J. & Tarkowski, A. (2004). *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J Immunol* **172**, 1169–1176.

Kamerling, J. P. (2000). Pneumococcal polysaccharides: a chemical view. In *Streptococcus Pneumoniae: Molecular Biology and Mechanisms of Disease*, pp. 81–114. Edited by A. Tomasz. Larchmont, NY: Mary Ann Liebert.

Lehrer, R. I., Rosenman, M., Harwig, S. S., Jackson, R. & Eisenhauer, P. (1991). Ultrasensitive assays for endogenous antimicrobial polypeptides. *J Immunol Methods* **137**, 167–173.

Link, A. J., Phillips, D. & Church, G. M. (1997). Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179**, 6228–6237.

Ma, S., Selvaraj, U., Ohman, D. E., Quarless, R., Hassett, D. J. & Wozniak, D. J. (1998). Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa. J Bacteriol* **180**, 956–968.

Mantovani, A., Bonecchi, R. & Locati, M. (2006). Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol* **6**, 907–918.

Martin, D. W., Schurr, M. J., Mudd, M. H., Govan, J. R., Holloway, B. W. & Deretic, V. (1993). Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc Natl Acad Sci U S A* **90**, 8377–8381.

Moore, R. A., Bates, N. C. & Hancock, R. E. (1986). Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrob Agents Chemother* **29**, 496–500.

Moskowitz, S. M., Ernst, R. K. & Miller, S. I. (2004). PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J Bacteriol* **186**, 575–579.

Nassif, X., Fournier, J. M., Arondel, J. & Sansonetti, P. J. (1989). Mucoid phenotype of *Klebsiella pneumoniae* is a plasmid-encoded virulence factor. *Infect Immun* **57**, 546–552.

Newton, B. A. (1955). A fluorescent derivative of polymyxin: its preparation and use in studying the site of action of the antibiotic. *J Gen Microbiol* **12**, 226–236.

Nicolas, P. & Mor, A. (1995). Peptides as weapons against microorganisms in the chemical defence system of vertebrates. *Annu Rev Microbiol* **49**, 277–304.

Nizet, V. (2006). Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr Issues Mol Biol* **8**, 11–26.

Peschel, A. (2002). How do bacteria resist human antimicrobial peptides? *Trends Microbiol* **10**, 179–186.

Poschet, J. F., Boucher, J. C., Firoved, A. M. & Deretic, V. (2001). Conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Methods Enzymol* **336**, 65–76.

Rahn, A. & Whitfield, C. (2003). Transcriptional organization and regulation of the *Escherichia coli* K30 group 1 capsule biosynthesis (*cps*) gene cluster. *Mol Microbiol* **47**, 1045–1060.

Regue, M., Izquierdo, L., Fresno, S., Jimenez, N., Pique, N., Corsaro, M. M., Parrilli, M., Naldi, T., Merino, S. & Tomas, J. M. (2005). The incorporation of glucosamine into enterobacterial core lipopolysaccharide: two enzymatic steps are required. *J Biol Chem* **280**, 36648–36656.

Selsted, M. E. & Ouellette, A. J. (1995). Defensins in granules of phagocytic and non-phagocytic cells. *Trends Cell Biol* **5**, 114–119.

Shafer, W. M., Qu, X., Waring, A. J. & Lehrer, R. I. (1998). Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. *Proc Natl Acad Sci U S A* **95**, 1829–1833.

Sherbrock-Cox, V., Russell, N. J. & Gacesa, P. (1984). The purification and chemical characterisation of the alginate present in extracellular material produced by mucoid strains of *Pseudomonas aeruginosa. Carbohydr Res* **135**, 147–154.

Spinosa, M. R., Progida, C., Tala, A., Cogli, L., Alifano, P. & Bucci, C. (2007). The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. *Infect Immun* **75**, 3594–3603.

Steinmoen, H., Knutsen, E. & Havarstein, L. S. (2002). Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. *Proc Natl Acad Sci U S A* **99**, 7681–7686.

Steinmoen, H., Teigen, A. & Havarstein, L. S. (2003). Competence-induced cells of *Streptococcus pneumoniae* lyse competence-deficient cells of the same strain during cocultivation. *J Bacteriol* **185**, 7176–7183.

Straus, D. C., Atkisson, D. L. & Garner, C. W. (1985). Importance of a lipopolysaccharide-containing extracellular toxic complex in infections produced by *Klebsiella pneumoniae. Infect Immun* **50**, 787–795.

Takemura, H., Kaku, M., Kohno, S., Hirakata, Y., Tanaka, H., Yoshida, R., Tomono, K., Koga, H., Wada, A. & other authors (1996). Evaluation of susceptibility of gram-positive and -negative bacteria to human defensins by using radial diffusion assay. *Antimicrob Agents Chemother* **40**, 2280–2284.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol Rev* **56**, 395–411.

Ventura, C. L., Cartee, R. T., Forsee, W. T. & Yother, J. (2006). Control of capsular polysaccharide chain length by UDP-sugar substrate concentrations in *Streptococcus pneumoniae. Mol Microbiol* **61**, 723–733.

Westphal, O. & Jann, K. (1963). Bacterial lipopolysaccharides extraction with phenol-water and further applications of the procedure. *Methods Carbohydr Chem* **5**, 83–91.

Wiese, A., Gutsmann, T. & Seydel, U. (2003). Towards antibacterial strategies: studies on the mechanisms of interaction between antibacterial peptides and model membranes. *J Endotoxin Res* **9**, 67–84.

Zilbauer, M., Dorrell, N., Boughan, P. K., Harris, A., Wren, B. W., Klein, N. J. & Bajaj-Elliott, M. (2005). Intestinal innate immunity to *Campylobacter jejuni* results in induction of bactericidal human beta-defensins 2 and 3. *Infect Immun* **73**, 7281–7289.

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