Antibacterial activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis*

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Cyt1Aa is a δ -endotoxin protein that is produced by *Bacillus thuringiensis* subsp. *israelensis*. It is a membrane pore-forming toxin that is lethal to insect larvae and is broadly cytolytic to vertebrate as well as invertebrate cells. Cyt1Aa is produced as a protoxin of 27 kDa. Proteolytic activation results in a reduction of the molecular mass to approximately 23–24 kDa and a threefold increase in activity. In this research, Cyt1Aa crystals were purified from *B. thuringiensis* IPS78/11 harbouring the expression vector pHT-cyAp20. The activity of the activated form of Cyt1Aa (23–24 kDa) was examined on a pathogenic strain of the Gram-negative *Escherichia coli* and the Gram-positive species *Staphylococcus aureus*. The Cyt1Aa minimal inhibitory concentration for *E. coli* and *S. aureus* was 1.25 and 5 $\mu\text{g ml}^{-1}$, respectively. Cyt1Aa was found to be bactericidal for *E. coli*, whereas it was bacteriostatic for *S. aureus*. Furthermore, Cyt1Aa increased the lethal effect when acting in combination with antibiotics. The association of Cyt1Aa with cells of these two bacteria was demonstrated by Western blot analysis using antibodies against the whole δ -endotoxin crystal. Scanning electron microscopy displayed damage to Cyt1Aa-treated cells. Ion imbalance due to damage of the cell walls and membranes was confirmed by X-ray microanalysis. These experiments show that Cyt1Aa has an antibacterial effect on pathogenic species and demonstrate, apparently for the first time, that exogenous Cyt1Aa has a bactericidal effect upon Gram-negative bacteria.

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INTRODUCTION

The Gram-positive bacterium *Bacillus thuringiensis* subsp. *israelensis* is regarded as the most promising biological control agent against some dipteran insect larvae (Goldberg & Margalit, 1977; Margalith & Ben-Dov, 2000). During sporulation, this entomopathogenic bacterium produces a larvicidal parasporal crystalline protein (δ -endotoxin) which is composed of four main polypeptides, Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa. These belong to two structurally distinct groups: receptor-specific Cry toxins which are active against insects and non-receptor-specific Cyt toxins that lyse a broad range of eukaryotic cells (Hofte & Whiteley, 1989). The Cyt proteins do not share any sequence homology with the Cry proteins. The encoding genes are located on a large plasmid (128 kb) called pBtoxis (Ben-Dov *et al.*, 1999; Berry *et al.*, 2002). The plasmid contains two other *cyt* genes, *cyt2Ba* and *cyt1Ca*, in addition to *cyt1Aa* (Guerchicoff *et al.*, 1997; Li *et al.*, 1996; Manasherob *et al.*, 2006).

Abbreviations: MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; SEM, scanning electron microscopy; XRMA, X-ray microanalysis.

Cyt1Aa (249 amino acids; 27 kDa) is the major component (45–50%) of the δ -endotoxin and was the first cytolytic toxin to be isolated and comprehensively characterized (Bourgouin *et al.*, 1986). Its maximal toxicity is obtained upon alkaline solubilization and proteolysis from both the N- and the C-termini, which converts the protoxin into its active form (23–24 kDa). This is carried out by the insect gut proteases (Al-yahyaee & Ellar, 1995; Dai & Gill, 1993). Endogenous bacterial proteases and several other proteases such as trypsin, chymotrypsin and proteinase K can also activate Cyt1Aa (Donovan *et al.*, 1997; Nisnevitch *et al.*, 2006; Oppert, 1999). Cyt1Aa's highly hydrophobic nature enables its interaction with unsaturated membrane phospholipids such as phosphatidylcholine, sphingomyelin and cholesterol (Promdonkoy & Ellar, 2003; Thomas & Ellar, 1983). Cyt1Aa forms pores 1–2 nm in diameter in the cell membrane, leading to cell lysis (Knowles *et al.*, 1989; Knowles & Ellar, 1987). It belongs to the β -barrel subclass that enters the membrane by forming a β -barrel composed of β -sheet hairpins from each monomer (Bravo *et al.*, 2007; Parker & Feil, 2005).

Cyt1Aa has a synergistic action with the other *B. thuringiensis* subsp. *israelensis* toxins when used against

insect larvae (Crickmore *et al.*, 1995; Khasdan *et al.*, 2001). Purified Cyt1Aa is cytolytic to cells of dipteran origin. At concentrations that are several fold higher, it is also toxic to a broad range of vertebrate and invertebrate cells (Gill *et al.*, 1992; Koni & Ellar, 1994). We have recently demonstrated that Cyt1Aa can be specifically targeted towards multiple myeloma cancer cells which surface-express and secrete anti-myelin basic protein (MBPp) antibodies (Cohen *et al.*, 2007). This targeted delivery of Cyt1Aa has been carried out by chemical conjugation as well as by genetic fusion to the major epitope of MBPp. Both forms (chemically conjugated and genetically fused) were found to be toxic to the multiple myeloma cells.

Information on Cyt1Aa activity in prokaryotic cells is very limited. Expression of *cyt1Aa* in *Escherichia coli* (without the 'helper gene' *p20*) leads to compaction of the nucleoid, associated with loss of colony-forming ability (Douek *et al.*, 1992; Manasherob *et al.*, 2003). The effect of exogenous Cyt1Aa on Gram-negative bacteria has not been reported previously. Several δ -endotoxins from *B. thuringiensis* have antibacterial effects on Gram-positive bacteria as well as on archaea, detected by zones of inhibition on agar and morphological changes in the treated bacteria (Revina *et al.*, 2005; Yudina *et al.*, 2003, 2007). Here, we examined the effect of exogenous Cyt1Aa on both Gram-positive and Gram-negative bacteria. The results provide what appears to be the first evidence for the antibacterial effect of exogenous Cyt1Aa on Gram-negative bacteria, reflected in morphological changes, alteration in the ion balance and cell lysis. Combined treatment of Cyt1Aa and antibiotics increased the antibacterial effect several fold.

METHODS

Bacterial strains and growth conditions. *B. thuringiensis* strain IPS78/11 harbouring the pHT-*cyAp20* plasmid encoding Cyt1Aa and the 'helper protein' P20 was used for purification of the Cyt1Aa crystal (Cohen *et al.*, 2007). The culture was grown in peptone glucose sporulation medium (PGSM) with erythromycin ($10 \mu\text{g ml}^{-1}$) prepared according to Brownbridge & Margalit (1986) at 30°C for 4 days until most cells were lysed, followed by a release of spores as well as Cyt1Aa crystals into the culture medium.

Wild-type strains of *E. coli* and *Staphylococcus aureus* were obtained from Meir Hospital, Kfar Saba, Israel, and grown in nutrient broth (NB) or on NB agar plates (Difco) overnight at 37°C .

Crystal protein isolation, solubilization and activation. The sediment of *B. thuringiensis* strain IPS78/11 harbouring pHT-*cyAp20*, containing crystals, spores and cell debris, was washed twice with cold distilled water (15 000 g for 45 min) followed by isolation on a stepwise sucrose gradient: 20 %, 40 %, 60 % and 80 % (4500 g for 2 h) (Debro *et al.*, 1986). The Cyt1Aa crystals were removed from the 60 % sucrose layer, washed with distilled water and solubilized in 50 mM Na_2CO_3 , pH 10.5, at 37°C for 1 h followed by centrifugation (4500 g for 45 min). The supernatant containing solubilized Cyt1Aa was adjusted to pH 8 with 1 M Tris/HCl, pH 6.5. Solubilized Cyt1Aa was activated by digestion with 1 % proteinase K for 1 h, followed by addition of 5 mM PMSF.

Purification of Cyt1Aa on DEAE-cellulose. Activated Cyt1Aa was purified by an anion-exchange chromatography system (Pharmacia)

on a DEAE-cellulose column ($1 \times 25 \text{ cm}$), pre-equilibrated with 50 mM Tris/HCl buffer, pH 8.5. Cyt1Aa was eluted with a 0–0.6 M NaCl gradient in the same buffer at a flow rate of 1.5 ml min^{-1} . Fractions (3 ml) were collected and monitored at 280 nm. Protein concentrations were determined by the method of Bradford (1976), with BSA as a standard.

Haemolysis assay. Rabbit red blood cells (RBC) were washed with 0.1 M PBS, pH 6.5, and suspended to 0.1–1 % (v/v) in the same buffer. Aliquots of Cyt1Aa were mixed with 1 ml suspended RBC and incubated for 20 min at 37°C . The absorbance of the released haemoglobin in the supernatant was monitored at 540 nm. Percentage haemolysis was calculated from the equation $[(A_{540} - A_{540}^{0\%}) / (A_{540}^{100\%} - A_{540}^{0\%})] \times 100 = \text{percentage haemolysis}$, where $A_{540}^{100\%}$ (100 % lysis) was obtained by incubating the RBC with water, and $A_{540}^{0\%}$ (0 % lysis) by incubating them in PBS.

SDS-PAGE and Western blot analysis. Protein homogeneity and molecular mass were determined by SDS-PAGE in 4 % stacking and 12.5 % separating polyacrylamide gels, using Dalton Mark VII-L mixtures (Sigma) as standards. The proteins in the gels were stained with Coomassie brilliant blue. Electrophoretic transfer of proteins from the gels to nitrocellulose was performed in 48 mM Tris base, 39 mM glycine, 20 % methanol and 1.3 mM SDS, pH 9.2. The membranes were blocked with 5 % skim milk (Difco), followed by incubation with polyclonal antibodies against whole *B. thuringiensis* subsp. *israelensis* crystal polypeptides [diluted 10^{-4} with Blotto (Difco)]. Cyt1Aa protein bands were visualized with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma).

Association of Cyt1Aa with bacterial cells. *E. coli* and *S. aureus* cultures, grown to the exponential phase, were divided into four tubes for each; the samples were treated with antibiotic and Cyt1Aa (less than the MIC for each species) separately and together. *E. coli* was treated with ampicillin, whereas *S. aureus* was treated with erythromycin. One portion of each bacterium remained untreated. All samples were incubated for 24 h at 37°C , followed by centrifugation. The pellet was washed thoroughly with PBS and sonicated. The cell lysates were loaded onto SDS-PAGE gels and the proteins were transferred from the gel to nitrocellulose for Western blot analysis.

Antibacterial activities. Determination of the minimal inhibitory concentration (MIC) of Cyt1Aa or antibiotics was performed in microtitre plates by twofold serial dilutions of antibacterial molecules in NB followed by addition of $0.5 \times 10^5 \text{ cells ml}^{-1}$ at the exponential phase. The microtitre plates were incubated for 24 h at 37°C and the absorbance was determined by an ELISA reader at 660 nm.

The minimal bactericidal concentration (MBC) of the antibacterial molecules was determined using the MIC and several higher concentrations. Viability was determined by counting c.f.u. on NB agar plates following appropriate dilutions, after 24 h incubation at 37°C . Determinations of the MIC and MBC of each antibiotic and Cyt1Aa were performed three times.

Scanning electron microscopy (SEM). Cultures in the exponential phase were treated with Cyt1Aa, with an antibiotic compound and with both for 24 h. The cultures were then washed and fixed with 2 % glutaraldehyde for 2 h, followed by 1 % osmium tetroxide. The cells were then dehydrated by incubation in increasing concentrations of ethanol. The specimens were gold-coated using an LKB device. Scanning was performed with a JEOL 840 scanning electron microscope at an accelerating voltage of 20 kV.

X-ray microanalysis (XRMA). Cultures in the exponential phase were treated with Cyt1Aa, with an antibiotic compound and with

both for 24 h. The cultures were then washed twice with 0.1 M ammonium acetate and resuspended in 30 μ l ammonium acetate. Each suspension (20 μ l) was attached to an aluminium grid, air-dried at room temperature for at least 24 h and then coated with a layer of carbon. XRMA was performed using an eXL Link X-ray system attached to a JEOL 840 scanning electron microscope. Each spectrum was determined with approximately 10^6 cells. The background level was the same during all measurements.

MALDI-TOF mass spectrometric analysis. Intact molecular mass measurement and peptide mass mapping were performed on a Bruker Reflex III matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer (Bruker) equipped with delayed ion extraction, a reflector and a 337 nm nitrogen laser. Each mass spectrum was generated from accumulated data of 200 laser shots. Both external and nearby calibration for proteins were achieved using BSA and myoglobin proteins, obtained from Sigma.

RESULTS AND DISCUSSION

Expression, purification and activation of Cyt1Aa

The gene *cyt1Aa* was cloned for expression with *p20*, which encodes the 'helper protein' P20 (Adams *et al.*, 1989; Wu & Federici, 1993) in the acrystalliferous strain IPS78/11. These cells produce crystals composed solely of Cyt1Aa, thus facilitating its purification. The crystals were released from the lysed cells, separated from the spores and cell debris, then solubilized and proteolytically activated by proteinase K. The activated Cyt1Aa was loaded on a DEAE-cellulose column, and eluted using a gradient of NaCl. Fractions (28–34) displaying high haemolytic activity against red blood cells were collected for further investigation (Fig. 1a). SDS-PAGE analysis (Fig. 1b) demonstrated the specific cleavage (lane 2) of the solubilized Cyt1Aa (lane 3) to a polypeptide of 23 397 Da as deduced by MALDI-TOF mass spectrometric analysis.

Inhibitory and bactericidal concentrations of Cyt1Aa

The minimal concentration of Cyt1Aa that inhibits bacterial cell growth (MIC) was determined for both species in NB. For *S. aureus*, it was 5 μ g ml⁻¹, fourfold higher than that for *E. coli* (1.25 μ g ml⁻¹; Table 1a, b). To exclude the possibility that the higher MIC for *S. aureus* resulted from Cyt1Aa degradation by extracellular protease(s), Cyt1Aa haemolytic activity was examined by incubation with the supernatant of an overnight culture of *S. aureus* for 2 h. The haemolytic activity of Cyt1Aa (0.5 μ g ml⁻¹) was the same (50 %) as after pre-incubation with NB only. Thus, Cyt1Aa may be less effective against *S. aureus* than against *E. coli* because of a difference in cell wall structure and not as a result of Cyt1Aa degradation by *S. aureus* extra-cellular protease(s). The MIC of Cyt1Aa for *E. coli* in Davis' minimal medium was double that in NB, 2.5 μ g ml⁻¹. The phenomenon that antibacterial agents are growth-rate-dependent and nutrition-dependent has been described previously (Brown *et al.*, 1990; Hadas *et al.*, 1995).

The minimal bactericidal concentration (MBC) of Cyt1Aa that inhibits colony-forming ability was performed at concentrations above the MIC to test whether the growth inhibition is associated with a bactericidal effect. *E. coli* lost colony-forming ability at the same Cyt1Aa concentration as the MIC (1.25 μ g ml⁻¹), whereas *S. aureus* lost its colony-forming ability at a concentration eightfold higher than the MIC (40 μ g ml⁻¹). Cyt1Aa can therefore be regarded as a bactericidal agent against *E. coli* and a bacteriostatic agent against *S. aureus*. The higher efficacy of Cyt1Aa against the Gram-negative *E. coli* than against the Gram-positive *S. aureus* can be explained by Cyt1Aa's mode of action: membrane perforation and cell lysis

Fig. 1. (a) Purification of the active Cyt1Aa fragment (23 kDa) by chromatography on a DEAE cellulose column (1 × 25 cm, 0–0.6 M NaCl gradient in 0.5 M Tris/HCl, pH 8.5). ■, A_{280} ; ▲, Cyt1Aa haemolytic activity; dashed line, NaCl concentration. (b) SDS-PAGE (12.5 %) of solubilized Cyt1Aa (lane 3); the proteolytically cleaved form of Cyt1Aa after purification on a DEAE cellulose column (lane 2), and molecular mass standards (kDa; lane 1).

Table 1. MIC and MBC values ($\mu\text{g ml}^{-1}$) of antibacterial agents for *E. coli* and *S. aureus*

For the combinations with Cyt1Aa (right-hand side), the Cyt1Aa was used at a concentration fourfold lower than its MIC for each strain. Cyt, Cyt1Aa; Amp, ampicillin; Ery, erythromycin; PolyB, polymyxin B; Kan, kanamycin.

	Cyt	Amp	Ery	PolyB	Kan	Amp	Ery	PolyB	Kan
(a) <i>E. coli</i>									
Cyt1Aa ($0.315 \mu\text{g ml}^{-1}$)		–	–	–	–	+	+	+	+
MIC	1.25	6.25	100	2.5	2.5	1.56	50	1.25	1.25
MBC	1.25	6.25	800	2.5	2.5	1.56	400	1.25	1.25
Type of action*	C	C	S	C	C	C	S	C	C
(b) <i>S. aureus</i>									
Cyt1Aa ($1.25 \mu\text{g ml}^{-1}$)		–	–	–	–	+	+	+	+
MIC	5	10	5	20	0.32	5	1.25	5	0.16
MBC	40	10	40	20	0.37	5	10	5	0.16
Type of action*	S	C	S	C	C	C	S	C	C

*C, bactericidal; S, bacteriostatic.

(Knowles *et al.*, 1989; Knowles & Ellar, 1987; Chow *et al.*, 1989; Manceva *et al.*, 2004, 2005). Cyt1Aa may bind to the outer membrane of Gram-negative cells and easily penetrate the cytoplasmic membrane, whereas in Gram-positive cells, it must cross the massive peptidoglycan layer before reaching the cytoplasmic membrane.

An antibacterial effect of *B. thuringiensis* subsp. *israelensis* δ -endotoxins has been demonstrated on the Gram-positive bacterium *Micrococcus luteus* by the agar diffusion and MIC methods (Yudina *et al.*, 2003). Those authors found that increasing the CytA concentration from 6.7 to $106 \mu\text{g ml}^{-1}$, for example, led to growth-suppression zones 12.2 and 22 mm wide, respectively. MIC values of CytA (28 kDa), Cry11A (70 kDa) and activated Cry4B (50 kDa) were 6.7, 6.7, and $33.4 \mu\text{g ml}^{-1}$, respectively.

Combined treatment with Cyt1Aa and antibiotics

To test the possibility that Cyt1Aa can enhance the antibacterial effect of antibiotics and thus lower the concentrations that are necessary to eradicate bacteria, the MIC values of several antibiotics were determined for both *E. coli* and *S. aureus* (Table 1). For *E. coli*, the values for ampicillin, kanamycin and polymyxin B were 6.25, 2.5 and $2.5 \mu\text{g ml}^{-1}$, respectively (Table 1a). The very high MIC of erythromycin ($100 \mu\text{g ml}^{-1}$) is consistent with low penetration of erythromycin into Gram-negative bacteria through their cell wall to inhibit ribosomal activity. The MICs of ampicillin, kanamycin, polymyxin B and erythromycin for *S. aureus* were 10, 0.32, 20 and $5 \mu\text{g ml}^{-1}$, respectively (Table 1b).

To test whether Cyt1Aa can contribute to the antibacterial activity of the above-tested antibiotics, Cyt1Aa was added, at a concentration fourfold lower than its MIC for each species, to wells containing serial dilutions of each drug, followed by addition of the bacteria. Addition of Cyt1Aa led to a two- to fourfold decrease in the antibiotics' MICs

(right side of Table 1). It is likely that Cyt1Aa lowered (fourfold) the ampicillin MIC for *E. coli* through partial disruption of the outer membrane, enabling better penetration of the antibiotic into the periplasmic space and resulting in a greater inhibition of peptidoglycan synthesis. In *S. aureus*, Cyt1Aa only halved the MIC of ampicillin, perhaps because the multi-layered peptidoglycan diminished the penetration of Cyt1Aa. The combination with Cyt1Aa resulted in a fourfold decreased MIC of erythromycin for *S. aureus*. We suggest that even a small injury to the bacterial cytoplasmic membrane caused by

Fig. 2. Western blot analysis of *E. coli* cultures treated with Cyt1Aa and ampicillin together and each treatment alone. Lanes: 1, molecular mass standards (kDa); 2, untreated cells; 3, cells treated with ampicillin; 4, cells treated with ampicillin together with Cyt1Aa; 5, cells treated with Cyt1Aa alone. Protein bands were visualized by antibodies to *B. thuringiensis* subsp. *israelensis* crystals.

Fig. 3. SEM of *E. coli*. (a) Untreated *E. coli* cells; (b–d) cells treated with ampicillin (b), Cyt1Aa (c) and a combination of Cyt1Aa and ampicillin (d). In (b–d) the cells were treated at a concentration fourfold less than the MIC of each component. (e) *E. coli* cells treated with Cyt1Aa at the MIC. Bars: 2 μm (a–d); 5 μm (e).

Cyt1Aa in *S. aureus* contributes to the penetration of erythromycin and increases its activity, i.e. binding to the 50S ribosome and blocking protein synthesis. The small (twofold) decrease in MIC obtained by adding Cyt1Aa to erythromycin in *E. coli* is surprising because the toxin was anticipated to disrupt the membrane, thus enhancing penetration of the drug.

An MBC analysis was performed for both species, to determine whether the combined effect is bactericidal (no growth is detected at the MIC of the antibacterial agent) or bacteriostatic (no growth is detected at fourfold or higher concentrations). Table 1 demonstrates that the combined treatment of Cyt1Aa with either ampicillin, kanamycin or polymyxin B was bactericidal for *E. coli*, as was each of these agents alone. *S. aureus* cells were also treated with the combination of Cyt1Aa and the bactericidal antibiotics ampicillin, kanamycin or polymyxin B. These combinations were found to have a bactericidal effect on *S. aureus*, although Cyt1Aa alone has only a bacteriostatic effect on

this bacterium. The combined treatment of erythromycin and Cyt1Aa was bacteriostatic for *S. aureus* as well as for *E. coli*. This bacteriostatic effect for *S. aureus* may be derived from the bacteriostatic effect of each of the drugs alone. However, this combination was also bacteriostatic for *E. coli* even though Cyt1Aa alone is bactericidal for *E. coli*. We assume that because erythromycin molecules hardly penetrate the outer membrane of *E. coli*, the high erythromycin concentration ($50 \mu\text{g ml}^{-1}$) screens the surface of the Gram-negative bacterium and impairs the binding of Cyt1Aa molecules to the outer membrane.

Association of Cyt1Aa with bacterial cells

The association of Cyt1Aa with the bacterial cells was demonstrated with antibodies against *B. thuringiensis* subsp. *israelensis* crystals. The bacterial cells were treated (at a concentration lower than the MIC) with Cyt1Aa and ampicillin (*E. coli*) or erythromycin (*S. aureus*), separately

and together. All samples were incubated for 24 h at 37 °C followed by centrifugation and sonication of the pellets. The cell lysates were run on SDS-PAGE and the proteins from the gel were transferred to nitrocellulose for Western blot analysis. Antibodies against the crystals reacted with the *E. coli* lysate that was treated with Cyt1Aa (Fig. 2, lane 5), more so when the cells were treated with a combination of Cyt1Aa and antibiotics (lane 4). Lysates of the untreated cells and of the cells that were treated with antibiotics alone did not react with the antibodies (lanes 2 and 3, respectively). Similar results were obtained with the erythromycin-treated *S. aureus* (data not shown). These results lead to the conclusion that Cyt1Aa associates with cells of both species, and that its association increases when the cells are co-treated with antibiotics. We assume that the antibiotics weaken the bacterial cells, leading to better association of Cyt1Aa with the cell membrane.

Morphological effects

E. coli cells were examined by SEM after treatment with Cyt1Aa and ampicillin, separately and together (Fig. 3). Treatment with Cyt1Aa (at the MIC) or with the combination (at concentrations lower than the MIC of each component) caused damage that appeared to be bactericidal (Fig. 3e and d, respectively). About 50 % of the cells were totally broken and the remainder exhibited roughness of the cell wall (Fig. 3d), whereas untreated cells looked normal (Fig. 3a). Cells that were treated with Cyt1Aa (Fig. 3c) or ampicillin (Fig. 3b) (less than the MIC of each) displayed only minor morphological damage.

The observed morphological effects of Cyt1Aa on the cells are similar to those shown for Cry toxins produced by *B. thuringiensis* subspecies *israelensis*, *kurstaki*, *galleriae* and *tenebrionis*. These Cry toxins led to significant damage to *Micrococcus luteus* (a Gram-positive bacterium), *Clostridium butyricum* and *C. acetobutylicum* (Gram-positive anaerobic species), as well as *Methanosarcina barkeri* (an archaeon) (Yudina *et al.*, 2003, 2007).

X-ray microanalysis

Membrane damage was also demonstrated by XRMA (Fig. 4). A combined treatment of Cyt1Aa and ampicillin resulted in significant changes in the ion composition of both *E. coli* and *S. aureus* (about 60 % compared to untreated cells). The same results were obtained when the cells were treated with Cyt1Aa (at the MIC) (data not shown). When treated with Cyt1Aa or ampicillin (lower than the MIC), the changes in ion composition were only about 10 % compared to untreated cells. The combined bactericidal treatment of *E. coli* with Cyt1Aa and ampicillin resulted in the release of sulfur from the bacterial cell, indicating a decrease in protein content. An efflux of phosphorus was also observed, demonstrating that the cells became empty of their content. The influx of sodium, potassium and calcium ions into the bacterial cell occurred

Fig. 4. X-ray elemental spectra of *E. coli* (a) and *S. aureus* (b). Untreated cells served as control (red lines). The blue lines show spectra for *E. coli* cells that were treated with Cyt1Aa and ampicillin and for *S. aureus* cells that were treated with Cyt1Aa and erythromycin.

in parallel, indicating that the ion pumps were not functioning and there was free influx into the cells (Fig. 4a). The XRMA spectra for *S. aureus* treated by the combined bacteriostatic treatment of Cyt1Aa and erythromycin exhibited a different picture from that for *E. coli*. The sulfur and phosphorus contents did not change. However, the potassium ion concentration decreased, indicating some damage to the membrane pumps (Fig. 4b). Untreated *E. coli* and *S. aureus* cells exhibited normal XRMA spectra with a high peak of phosphate and a very low peak of sodium. The XRMA results are in complete agreement with the above morphological changes.

Concluding remarks

In the current study, we focused on the effect of exogenous Cyt1Aa on pathogenic Gram-negative and Gram-positive bacteria. In both cases Cyt1Aa had antibacterial effects that were emphasized by its MIC and MBC values, association with the bacterial cells, morphological damage and ion imbalance. The significance of this research is the

antibacterial effect of exogenous Cyt1Aa on Gram-negative bacteria, since the outer membrane usually affords these bacteria resistance to antibacterial molecules by excluding them or reducing their penetration into the cells. Furthermore, we demonstrated that a combination of low amounts of Cyt1Aa and antibiotics is more effective against bacteria than each of the agents alone.

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