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Antimicrobial properties of the *Escherichia coli* R1 plasmid host killing peptide

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Abstract

The 52 amino acid host killing peptide (Hok) from the *hok/sok* post-segregational killer system of the *Escherichia coli* plasmid R1 was synthesized using Fmoc (9-fluorenylmethoxycarbonyl) chemistry, and its molecular weight was confirmed by mass spectroscopy. Hok kills cells by depolarizing the cytoplasmic membrane when it is made in the cytosol. Six microorganisms, *E. coli*, *Bacillus subtilis, Pseudomonas aeruginosa*, *P. putida*, *Salmonella typhimurium*, and *Staphylococcus aureus* were exposed to the purified peptide but showed no significant killing. However, electroporation of Hok (200 μg ml^{-1}) into *E. coli* cells showed a dramatic reduction (100 000-fold) in the number of cells transformed with plasmid DNA which indicates that the synthetic Hok peptide killed cells. Electroporation of Hok into *P. putida* was also very effective with a 500-fold reduction in electrocompetent cells (100 μg ml^{-1}). Heat shock in the presence of Hok (380 μg ml^{-1}) resulted in a 5-fold reduction in *E. coli* cells but had no effect on *B. subtilis*. In addition, three Hok fragments (Hok(1–28), Hok(31–52) and Hok(16–52)) killed cells when electroporated into *E. coli* at 200 μg ml^{-1} (over 1000-fold killing for Hok(1–28), 50-fold killing for Hok(16–52) and over 1000-fold killing for Hok(31–52)). *E. coli* cells electroporated with Hok and visualized using transmission electron microscopy showed the same morphological changes as control cells to which Hok was induced using a plasmid inside the cell.

Keywords: Hok peptide; Antimicrobial; Post-segregational killing

1. Introduction

The evolution of highly-resistant bacteria has led to a demand for new antibiotics (Andersson and Levin, 1999). Diseases induced by *Escherichia coli*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* are only treatable with one antibiotic and many new variants are resistant even to these (Alonso et al., 2001; Carbon, 2000; Vincent, 2000). Peptide antimicrobials are one new source of antibiotics; they range from 5 to 50 amino acids (aa), tend to be cationic, and many are also amphiphilic (Nissen-Meyer and Nes, 1997). They have been classified into related families based on

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their structure which includes β-stranded (defensins), α-helical (cecropins and magainins), extended helical (indolicidins), and loop structure (bactencein) (Epand and Vogel, 1999; Zhang et al., 1998). They disrupt the cell membrane either by disrupting the lipid structure or forming a pore that depolarizes the membrane (Piers et al., 1994). The host killing peptide (Hok peptide) contains 52 aa, is cationic, and resembles these peptides in size and character (Gerdes et al., 1997b; Pedersen and Gerdes 1999).

The Hok killer peptide is part of the *hok*/*sok* post-segregational killer system of the *E. coli* plasmid R1 and is a well-characterized member of the *gef*/*hok* family of killer genes (Franch et al., 1997; Gerdes et al., 1997a, 1985; Mikkelsen and Gerdes, 1997). *hok* and *sok* are transcribed from opposite strands of DNA, and *sok* RNA acts as an antisense inhibitor of *hok* mRNA translation. The *hok* gene codes for a toxic peptide that kills the cell from within by depolarizing the membrane (Pedersen and Gerdes, 1999) (functionally similar to holins (Bläsi et al., 1999; Wang et al., 2000; Young and Bläsi, 1995) which depolarize the membrane immediately before lysis by bacteriophage from inside the cell but show no homology to the *hok* gene family). Hok protein expression can take place only when antisense *sok* RNA is not present so under most conditions toxic Hok is not synthesized. Differential decay rates are responsible for cell killing as the *sok* transcript has a very short half life (less than 30 s) whereas the *hok* mRNA folds into a structure that is resistant to degradation (half life on the order of hours). Blocking transcription of *sok* leads to rapid *sok* RNA decay, *hok* mRNA processing, translation, and cell death. The evolutionary origin of Hok appears to be related to its ability to act as a T4 phage exclusion system (thereby allowing cells to behave altruistically) by killing cells before phage replication can be completed (Pecota and Wood, 1996).

The *hok*/*sok* locus has been shown to be very effective at stabilizing plasmids by killing plasmid-free cells that form after poor segregation (Gerdes, 1988; Gerdes et al., 1997b; Pecota et al., 1997; Wood et al., 1990; Wu et al., 1994; Wu and Wood, 1994). Hok is so toxic that two of its analogs, *relF* and *gef*, have been cloned and used for biological containment (Jensen et al., 1993; Knudsen et al., 1995; Ronchel et al., 1998). This suggests that Hok might be useful as an antimicrobial agent. The possible advantages of Hok as an antimicrobial include that this killer peptide is lethal in a wide range of bacteria (all Gram-negative bacteria tested and at least one gram-positive bacterium, *B. subtilis*) (Gerdes, 1988) so it may have broad clinical utility. Since its target is the cytoplasmic membrane, it is bactericidal. Unlike many antimicrobials made by biological organisms, there is no set of resistance genes associated with it that can spread. In addition, resistance to Hok would destabilize some clinically important antibiotic resistant plasmids (e.g. plasmids R1 and R100) which use a member of the *hok*/*sok* gene family for stabilization. Therefore, if bacteria arise that are resistant to Hok, there should be a diminished ability of the bacteria to maintain antibiotic resistance plasmids, and application of Hok could decrease resistance to other antimicrobials.

Attempts to isolate a Hok-resistant strain with a killing factor of 10^9 were unsuccessful (Poulsen et al., 1992). Two *E. coli* strains resistant to the Hok family of peptides were created by repeated induction (Poulsen et al., 1991, 1992); but, they are still sensitive to high concentrations of the peptide, and one strain was unstable (Gerdes et al., 1997a). Resistance to Hok is therefore unlikely to arise (requiring at least three mutations) (Poulsen et al., 1992) and if it does arise, it will likely be unstable. Hok-resistant bacteria will also lose the benefits of the killer system for resisting phage (Pecota and Wood, 1996).

In this paper, the Hok peptide was synthesized using organic synthesis and then tested for antimicrobial activity against several strains. Since the membrane may prevent Hok from entering the cell (Hok normally kills cells from within), two methods of disrupting the membrane were used, heat shock and electroporation. Heat shock and electroporation are well-established methods for the delivery of DNA into bacteria (Sambrook et al., 1989), but, proteins have also been electroporated into mammalian cells along with a fluorescent labeled dextran indicator (Graziadei et al., 1991). Rather than using a fluorescent indicator, plas-

mids with detectable markers (antibiotic resistance and colony color change) were used here to measure the level of competency. Three Hok fragments were also synthesized and electroporated to see if the toxicity could be better characterized to one region of the peptide. To confirm that killing was due to the peptide, treated cells were examined using an electron microscope and compared to untreated cells and cells in which Hok was induced from an internal plasmid.

2. Materials and methods

2.1. Reagents and peptide synthesis

All chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless indicated otherwise, and normal reagent grade chemicals were used unless otherwise stated. Four peptides were synthesized using automated Fmoc (9-fluorenylmethoxycarbonyl) synthesis: Hok(1–52), Hok(1–28), Hok(16–52), and Hok(31–52). Attempts at synthesizing the full-length material using *t*-butoxycaronyl chemistry were unsuccessful so the Hok(1–52) was synthesized at Alpha Diagnostics (San Antonio, TX, 65% purity by HPLC). The three Hok fragments were synthesized using a Millipore Plus Pepsynthesizer (Millipore, Bedford, MA) using Novabiochem (San Diego, CA) protected aa and activators. The fragments were purified to greater than 95% purity by HPLC (C18 Vydac column, 490E detection system, Waters, Milford, MA). Hok(1–52) was found to be 75% pure by reverse-phase HPLC. The molecular weight of each peptide was verified by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS, Perceptive Biosystems Framingham, MA, model LDE17K). The cysteine residues for the peptide fragment were not fully deprotected and still contained the Cys (position 31) acetamidomethyl (ACM) protecting group. The peptides were lyophilized and stored frozen after synthesis and purification. Small portions were also dialyzed or repeatedly lyophilized to ensure removal of organic solvents or other small byproducts. Dissolution of the lyophilized full-length and Hok(16–52) in water or water acetic acid (50%) was difficult; therefore, all the peptides were dissolved in dimethyl sulfoxide (DMSO).

2.2. Strains and plasmids

The six strains tested for Hok susceptibility were *E. coli* TG1 (Sambrook et al., 1989) (*supE hsd*Δ*5 thi* Δ*(lac-proAB)* F′ (*traD36 ProAB*$^{-}$ *lacI*^{q} *lacZ*Δ*M15*)), *Bacillus subtilis* WB600 (Wu et al., 1991) (deficient in 6 extracellular proteases), *Pseudomonas aeruginosa* JB2 (van der Woude et al., 1995), *P. putida* KT2440 (Ramos-Diaz and Ramos, 1998), *Salmonella typhimurium* Pro Peg (Neidhardt, 1996), and *Staphylococcus aureus* (Baird and Lee, 1995). *E. coli* plasmid pTKW106 (*hok*/*sok* Kan^{R} *lacI*^{q} *lacz*) is a derivative of pBR322 (Wood et al., 1990). *E. coli* plasmid pKG1010 (*tac-hok* Amp^{R}) contains hok under the control of the isopropyl-D-thiogalactopyranoside (IPTG) inducible promoter *tac* (K. Gerdes personal communication). *E. coli* plasmid pBS KS Kan TOM (Kan^{R}) is a pBluescript II KS (+) (Stratagene, La Jolla, CA) (Alting-Mees and Short, 1989) derived plasmid which expresses toluene *o*-monooxygenase constitutively and causes transformed colonies to turn brown on LB plates (Sambrook et al., 1989) (this allowed transformants to be identified without antibiotics (Canada et al., 2002)). Plasmid pMMB277 (Cm^{R}) is a broad-host range plasmid which replicates in *P. putida* (Morales et al., 1991), and plasmid pBE92 (Kan^{R}) is a derivative of *B. subtilis* expression vector pBE60 (Nagarajan et al., 1992) which can also goes in *E. coli*. All cells were grown at 37 °C.

2.3. Hok exposure experiments

The procedure described here is based on that used previously by Jayaraman et al. (1999). Ten milliliter of an overnight LB culture were centrifuged (3800 g, J2-21 rotor, Beckman Instruments, Palo Alto, CA), the cells washed with 5 ml of LB and resuspended in 1 ml of LB. Hok samples dissolved in DMSO were used directly or allowed to fold in 0.1 M Tris pH 7.5 with glutathione (2:1) for 5–24 h, then added to 250 μl of cells. The cells were incubated for 1 h at 37 °C, then diluted and

plated on LB agar. Controls lacking peptide but with the same volume of DMSO and buffer were used for comparison. A control containing kanamycin was used to test that cells were able to be killed by an antimicrobial compound. Each experiment was performed at least in triplicate.

2.4. Heat shock with hok

Two different heat shock procedures were used, a modified form of the transformation storage solution (TSS) method for *E. coli* (Chung et al., 1989) and the two-step transformation method for *B. subtilis* (Cutting and Vander Horn, 1990). For the TSS method, *E. coli* TGI overnight cells were diluted in LB 50-fold and grown to an optical density at 600 nm (OD) of approximately 0.4. Then the cells were set on ice for ~ 20 min followed by centrifugation at $2800 \times g$. The cells were resuspended in 1 ml of TSS solution for each 10 ml of cells. The pBS KS Kan TOM DNA (1 μg ml^{-1} purified using a Qiagen plasmid Midi kit) and Hok peptide (dissolved in DMSO with the same amount of DMSO added to each sample) were combined and 100 μl of cells added. The cells were heat shocked by placing them in a water bath at 42 °C for 1 min. The cells were immediately put on ice, 1 ml of LB added, and shaken for 1 h at 250 rpm (series 25 shaker, New Brunswick Scientific, Edison, NJ) at 37 °C. The cells were then diluted and plated on LB and LB kanamycin (50 μg ml^{-1}) and allowed to grow for 8–12 h. The colonies were counted and their color recorded which was used to determine the percentage of transformed cells killed at a particular peptide concentration.

For the two-step transformation method of *B. subtilis*, overnight cultures in SPI media were used to inoculate 20 ml, at an OD of 0.15–0.2; these cells were then grown to an OD of 1.0. One milliliter of cells was then added to 100 ml of SPII medium and incubated at 37 °C (250 rpm) for 90 min. The cells were harvested by centrifuging at $5000 \times g$ for 10 min, and 10 ml of the supernatant and 1 ml of glycerol were used to resuspend the cells. The cells were then aliquoted into 1.5 ml microcentrifuge tubes and frozen at −80 °C. A Hok–DNA mixture was prepared by combining 50 μl SPII–EGTA (SPII with the $CaCl_2$ replaced by 1.8 mM EGTA), 1 μg pBE92 (8 μg μl^{-1}, purified using a scaled up miniprep (Rodriguez and Tait, 1983)), and 4 μl of Hok and DMSO. The cells were quickly thawed in a 37 °C water bath, stored at room temperature, then 2 ml of SPII–EGTA were added. The Hok–DNA mixture was then added to 300 μl of the SPII–EGTA cells. After incubating at 37 °C for 20–30 min with shaking at 250 rpm, 100 μl of 10% yeast extract was added. After an additional 60 min for recovery, the cells were diluted and plated on LB and LB kanamycin (50 μg ml^{-1}) to measure the level of the transformation.

2.5. Electroporation with Hok

Electrocompetent cells were made fresh for each experiment using the method of Smith and Iglewski (1989). An Electro cell manipulator 600 (Genetronics, San Diego, CA) was used with 2 mm electroporation cuvettes, 100 μl of cells, 2 μg pBS KS Kan TOM (added immediately before electroporation), and the Hok or Hok fragment (12.5–200 μg ml^{-1}) dissolved in DMSO (total volume 4 μl). As a control to make sure that protein does not inhibit transformation, two protein controls BSA (Promega, Madison, WI) and ovalbumin (Sigma-Aldrich, St Louis, MO) were also electroporated into the cells. The cells were electroporated at 12–13 kV cm^{-1}, resistance 200 Ω, capacitance 25 μF, and a time constant of 4.6–5.2 ms. The cuvette was immediately washed with LB (to make the total volume of 1 ml) and incubated for 1 h at 37 °C. The cells were then diluted using warm LB and plated (in triplicate) on LB and LB kanamycin (50 μg ml^{-1}) agar plates to get approximately 30–300 colonies per plate. Brown *E. coli* colonies on LB plates indicated transformed cells (white colonies were untransformed) which was corroborated by a comparison of the plates with and without antibiotic. The electroporation conditions for *P. putida* were the same except a 1 mm electroporation cuvette, 40 μl of cells, 1 μg plasmid pMMB277 (purified by large scale miniprep (Rodriguez and Tait, 1983)) and LB chloramphenicol (500 μg ml^{-1}) for plasmid selection) were used.

2.6. Microscopy

E. coli cells electroporated with Hok were viewed using a transmission electron microscope (Philips 300, Philips Research, Briarcliff Manor, NY) and were prepared using the electroporation procedure with three times as many cells to have adequate cells for fixing. One negative control was not electroporated and the second negative control was electroporated without Hok; each was allowed to recover for 20 min in LB (37 °C) after which they were stored on ice until fixing. Electrocompetent *E. coli* SCS1/pKG1010 cells with Hok expression induced with 5 mM IPTG for 30 min was used as a positive control for cell killing. Another positive control was electrocompetent *E. coli* BK6 pTKW 106 cells which were diluted 10-fold with LB and allowed to grow for 10 min at 37 °C followed by 20 min of Hok induction with 150 μg ml^{-1} rifampicin (rifampicin stops transcription and induces the translation of the *hok* mRNA). The use of electrocompetent cells for Hok induction was not ideal but gave an indication of how the cell morphology changes when Hok is electroporated into the cells. The samples were then put on ice until fixed. *E. coli* TG1 electroporated with Hok was treated the same way as for the electroporation experiments except the cells were only allowed to recover for 20 min and stored on ice until fixation. After recovery or induction, the cells were plated to determine the transformation efficiency and extent of killing. Standard osmium epoxy electron microscopy fixation methods were used (Ronchel et al., 1998). The fixed cells were sectioned and examined using a light microscope (toluidine blue staining) to ensure that the preparation process had worked properly. Transmission electron microscope photographs were taken at various magnifications, and cells were classified into four different categories: live (cells that were dark inside, showed no membrane damage, and less than two white spots), dying (cells that showed some damage to the membrane or were not very dark inside), containing white vacuoles (cells that had two or more white spots), and dead (cells that were clear inside or had significant damage to the membrane).

3. Results

3.1. Peptide synthesis

Fmoc synthesis gave peptides of the correct molecular weight as determined by mass spectroscopy (Table 1). The yields were estimated at 75% for the full-length peptide and 40–50% for the Hok fragments based on HPLC. Disulfide bond formation was not determined for the full-length peptide since not enough material was synthesized for folding studies. The cysteine at position 31 was not deprotected for the Hok(31–52) and Hok(16–52) to prevent disulfide formation during processing.

3.2. Hok exposure experiments

The results of exposing late-exponential phase cells to Hok(1–52) are shown in Table 2. There appeared to be some killing of *S. aureus* (57%) and *P. putida* (19%) but this is insignificant compared to when Hok is synthesized within the cell. Hence, direct, external addition of Hok, whether folded or unfolded, was unable to effectively kill the bacteria.

3.3. Heat shock with Hok

The inability of Hok to kill cells effectively when simply added externally suggests that some type of treatment be used that would allow the peptide to reach the inner cell membrane or cytoplasm where it can fold. Heat shock is a common method used to transform cells with DNA (Sambrook et al., 1989), and it was reasoned that it would make the membrane more permeable to Hok. Since only a small percentage of the cells become competent (Table 3), it is necessary to base Hok killing only on the fraction of cells which were able to take up the Hok peptide (this assumes that DNA and the Hok peptide enter the cell with the same efficiency). The number of competent cells was estimated by having plasmid DNA present during the uptake of Hok (pBS KS Kan TOM for *E. coli* and pBE92 for *B. subtilis*).

Eighty to ninety percent of the heat shocked competent *E. coli* are killed by high concentrations

Table 1
Molecular weights determined by MALDI mass spectroscopy and peptide sequence of Hok and Hok fragments

Peptide name	Peptide sequence	Predicted MW (g mol^{-1})	MALDI MW
Hok(1–52)	MKLPRSSLVWCVLIVÇLTLLIFTYLTRKSLÇEIRYRDGHREVAAFMAYESGK	6082.34	6082.5
Hok(1–28)	MKLPRSSLVWCVLIVÇLTLLIFTYLTRK	3311.20	3311
Hok(16–52)	ÇLTLLIFTYLTRKSLÇEIRYRDGHREVAAFMAYESGK	4356.10	4884[a]
Hok(31–52)	ÇEIRYRDGHREVAAFMAYESGK	2588.90	2660.34[b]

[a] Cysteine protected with acetmidomethyl (ACM, MW 72).
[b] Alkylated with iodacetamide (MW 58.07) and second cysteine still protected with acetmidomethyl (ACM).

of Hok (Table 3). However, competent *B. subtilis* cells appear to be almost unaffected by the Hok treatment. For *E. coli*, the efficiency was not as high as would be predicted if killing was from within the cell. Since the competency level was low and there was some doubt as to how permeable the heat shock cells were, a second more efficient method, electroporation, was used.

3.4. Electroporation with Hok

Electroporation of Hok and the Hok fragments into *E. coli* and *P. putida* killed transformed cells efficiently in a concentration-dependent manner (Fig. 1). The number of transformable cells was discerned by the simultaneous electroporation of pBS KS Kan TOM (for *E. coli*) or pMMB206 (for *P. putida*). Treatment of *E. coli* with Hok(1–52) showed no effect below 12.5 μg ml^{-1} but reduced the number of live cells by 92% at 50 μg ml^{-1} and killed nearly 100% of the electrocompetent cells at 100 and 200 μg ml^{-1}. Full-length Hok was even more effective against *P. putida* since at 10 μg ml^{-1}, it reduced transformation by 89% and killed nearly 100% of the electrocompetent cells at 25, 50 and 100 μg ml^{-1}.

The Hok fragments were also able to inhibit transformation. The Hok(1–28) fragment was able to kill over 90% of the cells at concentrations above 25 μg ml^{-1}. The Hok(16–52) fragment was not as potent against *E. coli* as the concentration had to be over 100 μg ml^{-1} before 90%; however, it was very effective against *P. putida* where 99% of the cells were killed at 12.5 μg ml^{-1}. The Hok(31–52) fragment required a concentration of 100 μg ml^{-1} before 90% of the *E. coli* cells were killed and was the least effective against *P. putida* (requiring a concentration of 50 mg ml^{-1} to attain 90% killing). Therefore, all the peptides appear to be toxic with the full-length peptide and Hok(1–28) being the most toxic, and *P. putida* was more sensitive than the *E. coli*. In general 2–6 logs of killing with Hok and its fragments were obtained (killing was limited by the number of

Table 2
Effect of exposure to full-length Hok

Organism	Hok concentration (μg ml^{-1})	Solvent	Percent killed
Bacillus subtilis WB600	200	DMSO, folded in Tris (0.1 M pH 7.5)	0
E. coli TG1	0	Water, kanamycin (29 μg ml^{-1})	99.98
	200	DMSO, Tris (0.1 M pH 7.5) folded with glutathione	0
Pseudomonas putida KT2440	200	DMSO	19
Pseudomonas aeruginosa JB2	100	DMSO, folded in Tris (0.1 M pH 7.5)	0
Salmonella typhimurium Pro Peg	200	DMSO, folded in Tris (0.1 M pH 7.5)	0
Staphylococcus aureus	200	DMSO, folded in Tris (0.1 M pH 7.5)	57

Table 3
Effect of exposure to full-length Hok with heat shock

Organism	Hok concentration (μg ml^{-1})	Percent of total competent cells [a]	Percent of competent cells killed
E. coli TG1	385	0.03–0.01	80
	769		90
B. subtilis W600	50	0.001	0
	200		18

[a] Calculated by assuming all competent cells in the control are transformed with pBS KS Kan Tom.

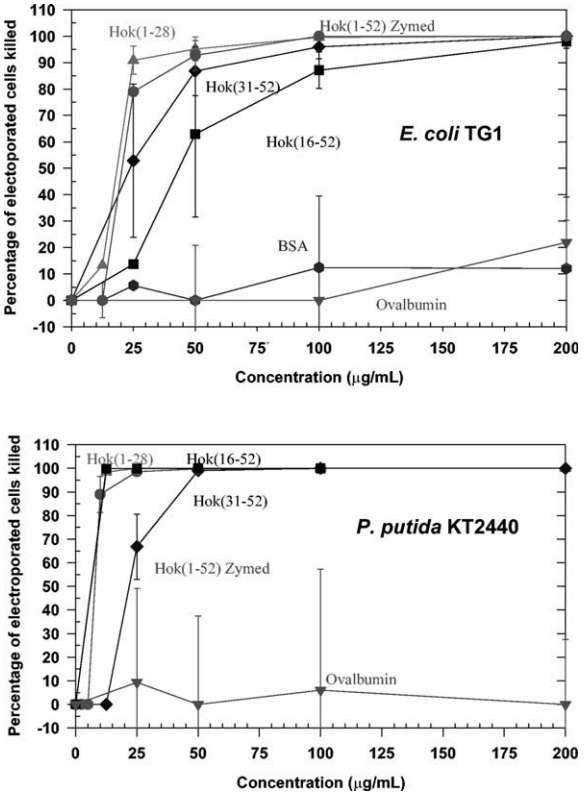

Fig. 1. Concentration-dependent killing of electrocompetent cells by Hok peptides. Hok(1–52) ●, Hok(16–52) ■, Hok(1–28) ▲, Hok(31–52) ◆, ovalbumin ▼, and BSA (bovine serum albumen) % ⬢ in *E. coli* TG1 (panel a) and *P. putida* (panel b). Standard deviations indicated.

cells which were successfully transformed under these conditions). The two control proteins (ovalbumin and BSA) showed insignificant killing regardless of concentration. The fraction of transformable cells was 0.038 ± 0.023 for *E. coli* and 0.00092 ± 0.00074 for *P. putida*.

Additional dialysis and lyophilizations of the Hok fragments had no effect on the dose response during electroporation (data not shown); therefore; killing was not due to the presence of residual chemicals from synthesis. No appreciable amount of ionic species was present since the electroporation time constants were consistent and the same as the no peptide control.

3.5. Transmission electron microscopy

Examination of the sectioned cells using a light microscope showed that they were properly fixed and no distinguishable features were evident except for a greater background of unstained cells in the electroporated samples. The electron microscope examination showed four classes of cells: live, dead, containing white vacuoles, and dying (as defined in the methods secured) (Table 4, Fig. 2). As expected the percentage of dead cells increased for all four treated samples as compared to the no treatment control; therefore, electroporation killed ~40% of the total cells. The two positive controls in which *hok* was induced also showed significant killing. The images of cells in which Hok was induced from plasmid pKG1010 using IPTG show that ~18% of the cells appear dead which is much lower than indicated by the plate counts which showed that eventually 99.3% of the cells were nonviable. This suggests that 81% of the cells were in the process of dying. The images of the positive control cells in which Hok was induced by stopping transcription of *sok* using rifampicin show that ~11% of the cells appeared dead which was again much lower than indicated by plate counts (99.9% of the cells were nonviable). This suggests that 88% of the cells were in the

Table 4
Classification of cells examined under the electron microscope at 16 100 × magnification

Strain	Treatment	Live (%)	Dying (%)	Dead (%)	Cells with white vacuoles (%)	Total cells counted
E. coli TG1	No electroporation, no Hok	83	8.6	6.7	1.9	105
E. coli TG1	Electroporation, no Hok	37	17	45	0.4	1722
E. coli TG1	Electroporation with Hok (200 μg ml^{-1})	30	8.9	58	2.5	1445
E. coli SCS1/ pKG1010	No electroporation, IPTG induced	53	5.1	17.6	24.4	393
E. coli BK6/ pTKW106	No electroporation, Rif induced	70	6.9	11.2	12.0	393

process of dying from either Hok or rifampicin. Many of the dying cells (24% for the IPTG-treated cells and 12% for the rifampicin-treated cells) contain significantly more vacuoles than the untreated control (Table 4, Fig. 2); therefore, it was concluded that the white vacuoles are formed by Hok in cells that have not yet leaked enough cytosolic material to become ghost cells (transparent cells without well-defined inner and outer membranes (Ronchel et al., 1998)). This is in agreement with the proposed mechanism of killing (leakage of the membrane) by the Hok peptide. The Hok electroporated sample shows 6-fold greater cells with white vacuoles than the electroporated control. Due to the large number of cells killed by electroporation, a better comparison is obtained by calculating the percentages with respect to the number of live cells. This calculation shows that 6.4% of the Hok-treated cells have white vacuoles as compared to 1.1% for the electroporated control and 2.3% for the nonelectroporated control. Plating suggests that ~5.4% of the total of transformed and nontransformed cells were killed by Hok which agrees closely with the microscope results. These results suggest that the ghost morphology attributed to Hok killing does not appear until later when the membrane starts to deteriorate. One later stage cell (*E. coli* SCS1 pKG1010 IPTG induced) is shown in Fig. 2f, and it looks similar to the electron microscope images of *gef* killed cells reported for *P. putida* (Ronchel et al., 1998). It appears that the Hok induces the formation of vacuoles before killing and forming ghost cells (a result not reported previously). The membrane damage caused by electroporation is not readily distinguishable from that caused by Hok.

4. Discussion

The Hok peptide was successfully synthesized and showed significant antimicrobial activity if it was introduced inside the cells. Heat shock appeared successful for getting the peptide into *E. coli* but not for *B. subtilis* (*hok*/*sok* has been reported to work in *B. subtilis* (Molin et al., 1987) so it is most likely the peptide did not enter the cell). There is evidence that heat shock transformation of *B. subtilis* may not require that the membrane be permeabilized but rather stimulates a protein to actively take up the DNA (Provvedi and Dubnau, 1999). Electroporation was more effective for introducing the Hok peptide and DNA into the bacteria since electrocompetent *E. coli* and *P. putida* were killed successfully. The presence of protein alone cannot be the cause of the cell death since both BSA and ovalbumin had little affect (Fig. 1).

The number of transformed cells was reduced dramatically upon electroporation of Hok due to cell killing. This is supported by the microscopic examination of cells where a higher number of white vacuoles were present in the Hok treated

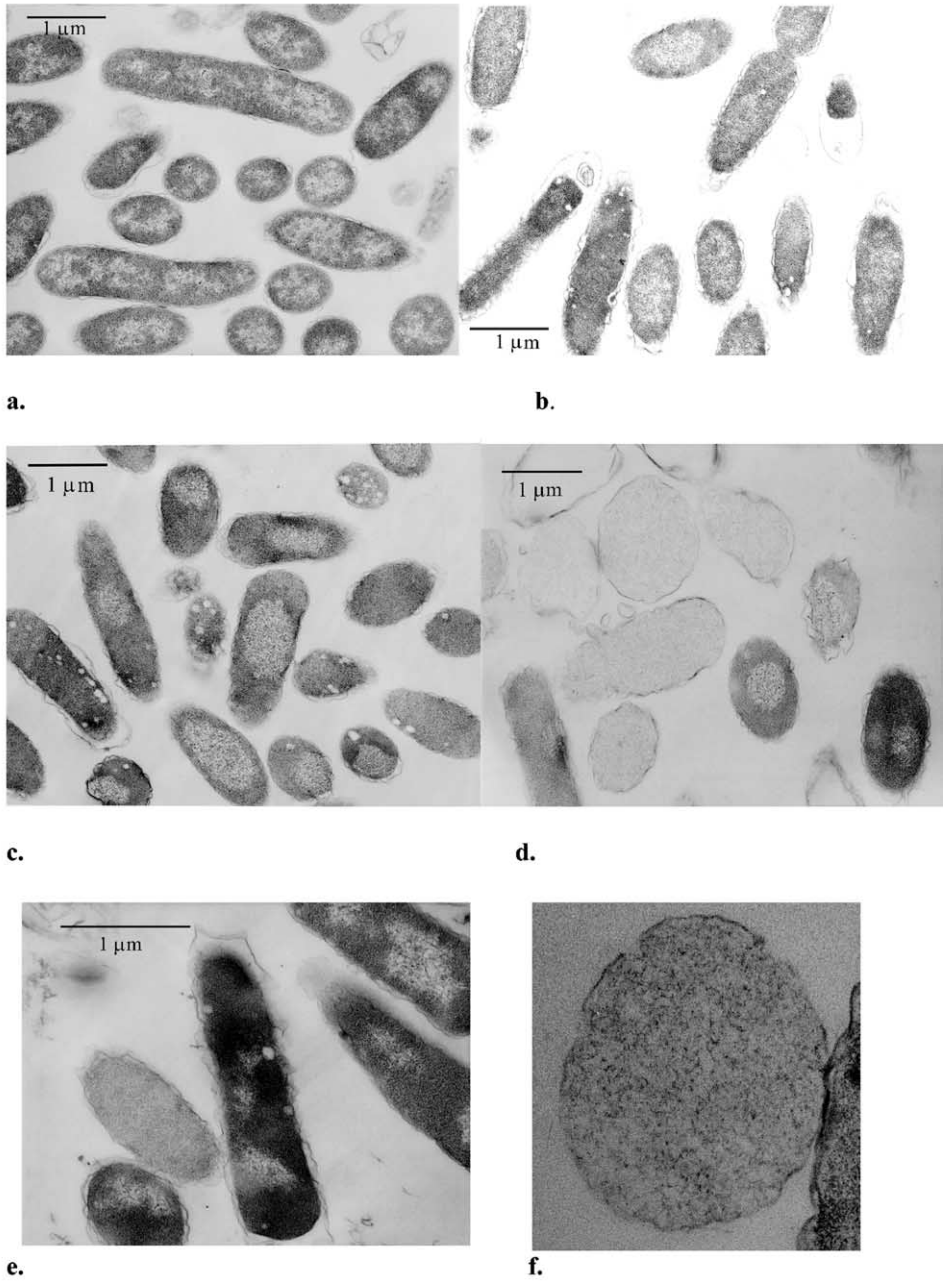

Fig. 2. Killing of *E. cells* by full-length Hok viewed by transmission electron microscopy (16 100 × magnification). (a) *E. coli* TG1 with no electroporation (control), (b) *E. coli* BK6 pTKW106 (contains hok^+/sok^+) with no electroporation but Hok killing induced with 150 μg ml^{-1} of rifampicin, (c) *E. coli* SCS1 pKG1010 (hok^+ under the control of the *tac* promoter) with no electroporation but induced with 5 mM IPTG, (d) *E. coli* TG1 with electroporation with DMSO, (e) *E. coli* TG1 with electroporation of 200 μg ml^{-1} Hok in DMSO, (f) example of an older *E. coli* SCS1 pKG1010 IPTG-induced cell that has been killed by Hok.

cells. It may be argued that the reduction in cell viability was due to plasmid DNA damage; but, this is highly unlikely since the plasmids were only exposed to Hok for a short time before transformation, and it is known that lightly damaged, nicked or relaxed DNA can frequently be repaired by the cell (transformation efficiency is over 1000-fold greater with slightly damaged DNA (Kimoto and Taketo, 1996)). Further, the protein controls (BSA and albumin) did not affect electroporation. Hok is positively charged so it may be argued that Hok could bind to the DNA and prevent it from fitting though the pore in the cell. This scenario is unlikely since Hok is hydrophobic, does not bind DNA in vivo, and is small compared to other components that have been transformed into mammalian cells (Graziadei et al., 1991). Another possibility is that some contaminant in the Hok material killed the cell. Most of the organic chemicals used for synthesis and cleavage from the resin are volatile and small in molecular weight. The cleaning process after synthesis removes most of these chemicals, and dialysis and repeated lyophilization had no affect on the toxicity of the peptide fragments. In addition, if a significant amount of ionic material (e.g. trifluoroacetic acid) were present, the time constant during electroporation would have been different from the control. Peptide contaminants should be similar to the Hok and have the same antimicrobial properties as Hok. It is therefore concluded that Hok is able to kill cells when it is delivered inside the cell.

One of the major difficulties for any antimicrobial is delivery. Hok is large and unlikely to be able to get though the bacterial membrane without help (Helander and Tiina, 2000); however, encapsulating Hok in liposomes might be successful as a delivery mechanism. In this way Hok could be perhaps used as a topical antibiotic or one targeted for oral biofilms (Jones et al., 1993). The fact that Hok is a peptide may make it susceptible to proteolytic attack but should facilitate its removal after it is no longer needed. This also allows combinatorial DNA mutagenesis techniques to be used to enhance the effectiveness of Hok (Piers et al., 1993).

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