The antibacterial action of protamine: evidence for disruption of cytoplasmic membrane energization in *Salmonella typhimurium*

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Protamine is a polycationic peptide found in the nuclei of sperm of different animal species. While it has long been known to have antimicrobial properties, its mode of action has remained elusive. We have investigated the mechanism of action of protamine and established that this peptide exerts its antibacterial effect without causing cell lysis or permeabilization of the cytoplasmic membrane. Respiring cells were more susceptible than nonrespiring cells, and loss of viability could be prevented by incubation at low pH or the addition of respiratory poisons. This indicates that protamine activity is influenced by the electrical membrane potential ($\Delta\psi$): increased killing occurs at higher $\Delta\psi$ values. Protamine caused inhibition of proline uptake, rapid efflux of proline from preloaded cells, and a reduction in the cellular ATP content. Furthermore, protamine-treated cells first lost the ability to accumulate leucine and then could not carry out protein synthesis. Cumulatively, our data indicate that protamine disrupts energy transduction and nutrient uptake functions, and suggest that the cytoplasmic membrane is the target of protamine action.

**Keywords**: protamine, antimicrobial peptide, membrane potential, *Salmonella*

INTRODUCTION

Small cationic peptides with broad-spectrum antimicrobial activity are part of the innate immune response in a variety of animal species (Zasloff, 1992). Peptides of this class are often amphiphilic and generally thought to act against cell membranes (Boman, 1995). For example, defensin NP-1 (Kagan *et al.*, 1990), cecropin A (Christensen *et al.*, 1988) and magainin 1 (Duclohier *et al.*, 1989) have been shown to permeabilize artificial membranes through the formation of voltage-dependent channels. Channel formation is expected to result in leakage of cellular metabolites and the disruption of membrane-linked, energy-transducing processes (Westerhoff *et al.*, 1989). Indeed, magainins and cecropins can rapidly lyse bacterial cells. However, channel formation is not the only means by which bacterial cell death occurs (Boman, 1995) because the proline- and arginine-rich PR-39 (Boman *et al.*, 1993) and the bactenecins Bac5 and Bac7 (Skerlavaj *et al.*, 1990) are bactericidal, yet they do not cause cell lysis.

Histone-like proteins from lysosomes of mouse macrophages (Hiemstra *et al.*, 1993) and histones from rabbit leukocytes (Hirsch, 1958) exhibit bactericidal activity against both Gram-positive and Gram-negative bacteria. Likewise, protamine is a cationic peptide with broad-spectrum antimicrobial activity. Protamine is found in the nuclei of fish sperm, where it replaces histone during maturation of the spermatid (Louie & Dixon, 1974). This peptide does not possess the amphiphilic character required for channel formation – 21 of its 32 amino acids are arginine (Louie & Dixon, 1974; Kaiser & Kézdy, 1987); yet it can disrupt some membrane-associated processes. Protamine, polylysine, as well as PC-III – a low-molecular-mass cationic peptide from rabbits (Carroll & Martinez, 1981) – have been shown to inhibit specific components of the electron transport systems of mitochondria (Person & Fine, 1961; Person *et al.*, 1964; Mochan *et al.*, 1973) and bacteria (Carroll & Martinez, 1981). While the bactericidal activity of protamine is well established (Miller *et al.*, 1942; Hirsch, 1958; Johansen *et al.*, 1995; Soboh *et al.*, 1995), its specific physiological effect(s) on the cell are unknown.

Our laboratory has been interested in the antimicrobial
properties of protamine because *Salmonella typhimurium* mutants that exhibit hypersensitivity to this peptide are also attenuated for virulence in mice (Groisman et al., 1992b). For example, mutations in the transcriptional regulatory gene *phoP* (Fields et al., 1989) confer hypersensitivity to protamine and also to several channel-forming peptides including magainin 2, defensin NP-1, cecropin P1, mellitin and mastoparan (Groisman et al., 1992a; García-Véscovi et al., 1994). On the other hand, mutations in *sapJ*, *sapG*, or *sapABCD* - encoding the components of a low-affinity K⁺ uptake system (Parra-Lopez et al., 1993, 1994) - result in strains with heightened susceptibility to protamine but not to channel-forming antimicrobial peptides (Groisman et al., 1992b). This suggests that the target and/or mechanism of action of protamine is distinct from that of channel-forming peptides.

Here, we investigate the mode of action of protamine. We demonstrate that protamine targets the cytoplasmic membrane by disrupting energization and inhibiting nutrient uptake. We also show that protamine does not permeabilize the cytoplasmic membrane of either wild-type or hypersensitive strains of *S. typhimurium*.

**METHODS**

**Bacterial strains.** All strains used in this study are derivatives of wild-type *S. typhimurium* 14028s. EG5195 (*sapG pheP*) is a protamine-hypersensitive mutant (Parra-Lopez et al., 1994). MST872 (*pro leuF* pro *lacZ::lacZ Y::A*) and MST2706 (*hisF* pro *lacZ::Tn100CdM*) were kindly provided by Stanley Maloy, University of Illinois, Urbana, USA. EG8176 (*sapG pheP F* pro *lacZ::Tn100CdM*) was constructed as follows. A P22 lysate prepared from MST2706 was used to transduce MST872 cells to chloramphenicol resistance. Transductants expressing β-galactosidase were selected on LB agar plates containing 25 µg chloramphenicol ml⁻¹ and 1 mM IPTG (Miller, 1972). To identify transductants that were *lacZ* Y*::lacZ* Y, candidates were streaked onto Lactose MacConkey indicator plates (Miller, 1972). One transductant that was Lac⁺ on X-Gal plates but Lac⁻ on MacConkey medium was designated EG8176. The generated F' transductants that were *lacZ* Y*::Tn100CdM* was introduced into E. coli MST2706 was introduced into MST872 from an overnight culture of strain EG5195. At early exponential phase, OD₅₅₀ 0.2 (about 2.5 x 10^⁶ c.f.u. ml⁻¹), [*-4,5-^3H]leucine was added to 2 µCi ml⁻¹ (74 kBq ml⁻¹). For protamine-treated cultures, protamine was added 30 s before the [*-4,5-^3H]leucine. To collect the radiolabelled protein 100 µl culture samples were transferred to Eppendorf tubes containing 1 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). The samples were vortexed, incubated for 30-40 min on ice then filtered through Whatman GF/C glass-fibre filters. The filters were washed with 5 ml 10% (w/v) TCA then dried prior to liquid scintillation counting. [*-4,5-^3H]Leucine transport assays were done in parallel with protein radiolabelling. Samples (20 µl) were filtered, at room temperature, through prewashed 0.45 µm HA Millipore filters. The filters were washed with 2 ml growth medium and then dried before liquid scintillation counting.

**Radiolabelling of protein and [*-^3H]leucine transport assays.** Minimal medium, pH 7.7, supplemented with 50 µM [*-3H]leucine was inoculated to an OD₅₅₀ of 0.1 from an overnight culture of strain EG5195. After 15 min incubation, [*-4,5-^3H]leucine was added to 2 µCi ml⁻¹ (74 kBq ml⁻¹). For protamine-treated cultures, protamine was added 30 s before the [*-4,5-^3H]leucine. To collect the radiolabelled protein 100 µl culture samples were transferred to Eppendorf tubes containing 1 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). The samples were vortexed, incubated for 30-40 min on ice then filtered through Whatman GF/C glass-fibre filters. The filters were washed with 5 ml 10% (w/v) TCA then dried prior to liquid scintillation counting. [*-4,5-^3H]Leucine transport assays were done in parallel with protein radiolabelling. Samples (20 µl) were filtered, at room temperature, through prewashed 0.45 µm HA Millipore filters. The filters were washed with 2 ml growth medium and then dried before liquid scintillation counting.

**Determination of viability by fluorescence staining.** The fluorescent nucleic acid stains were provided as a kit from Molecular Probes. The growth medium ingredients did not interfere with staining, so the strain was added directly to aliquots of cell culture. In this method, viability is based on cell membrane integrity; viable cells are those with intact cell membranes and nonviable cells are those with damaged cell membranes. Viability was determined by counting the number of green (viable) and red (nonviable) cells for a given field of view. A minimum of five fields, with >80 cells per field, were counted in each experiment.

**Outer membrane permeabilization.** The outer membrane permeability of protamine-treated cells was measured by their sensitivity to lysis by deoxycholate (Vaara, 1981). Cells were incubated with protamine for 10 min at 37 °C in MOPS buffer, pH 7.7. Following protamine treatment the cells were pelleted then resuspended in the same buffer. After measuring OD₅₅₀, deoxycholate was added to 0.5% and the suspension incubated for 10 min at 37 °C, after which time the OD₅₅₀ was again measured.
Inner membrane permeabilization. EG8176 cells were grown to exponential phase in minimal medium, pelleted, then resuspended to about $4 \times 10^6$ c.f.u. ml$^{-1}$ (OD$_{600}$ 0.04) in minimal medium, pH 7.7, lacking a nitrogen source but containing 11 mM glucose, 2 mM ONPG and 1 mM IPTG to induce expression of $\beta$-galactosidase. Hydrolysis of ONPG by $\beta$-galactosidase was monitored by measuring $A_{420}$. Total $\beta$-galactosidase activity was determined in control and protamine-treated cells permeabilized by chloroform and SDS.

Determination of ATP concentration. Intracellular ATP was extracted by a modification of the method described by Klein & Boyer (1972). EG5195 cells were grown in minimal medium, pH 7.7, with glucose as sole carbon source. Protamine was added to 15 or 30 $\mu$g ml$^{-1}$, at OD$_{600}$ 0.2 (about $2.5 \times 10^8$ c.f.u. ml$^{-1}$). Intracellular ATP was extracted from culture samples with 6% TCA and incubation on ice for 30 min. After adjusting the pH to 7.5 with NaOH the samples were microfuged for 3 min at 4°C to remove the cell debris. The ATP concentration was measured with a luminometer as bioluminescence after adding 20 $\mu$l of cell extract to 80 $\mu$l of a luciferin/luciferase mixture (Sigma). The values are presented, on an arbitrary scale of 1, as ATP concentration relative to the time zero sample.

RESULTS
Rationale

The polycationic and nonamphiphilic nature of protamine suggested that its mechanism of action might be different from that of cationic peptides that insert in membranes and form channels. Thus, we conducted experiments to assess the role of membrane potential, microbial growth and permeabilization of both inner and outer membranes in bacterial susceptibility to protamine. We used both a wild-type S. typhimurium strain and an isogenic $phoP$ sapG protamine-hypersensitive mutant (Parra-Lopez et al., 1994).

Effect of membrane potential on sensitivity to protamine

The fact that several protamine hypersusceptible mutants of Salmonella are defective for inner-membrane proteins (Parra-Lopez et al., 1993, 1994) suggested that protamine may specifically target the inner membrane of Gram-negative bacteria. We investigated whether treatments that affect the electrical membrane potential ($\Delta \psi$) altered bacterial susceptibility: we tested protamine activity at different pH$_{out}$ values and in the presence of respiratory poisons that depress $\Delta \psi$. The $\Delta \psi$ is oriented such that the interior of the cell carries a net negative charge. The $\Delta \psi$ is at a maximal (most negative) value at pH 7.5–7.8 and decreases as the pH of the medium is lowered (Kashket, 1985; Booth, 1985). The protamine sensitivity of both wild-type and mutant cells increased as the pH of the medium increased from 6.3 to 7.7 (Fig. 1). The sensitivity profile of the wild-type strain was similar to that of the hypersensitive mutant, except that more protamine was required for killing.

If the effect of pH on protamine sensitivity is due to changes in $\Delta \psi$, then other treatments that depress $\Delta \psi$ (Shioi & Taylor, 1984) should also confer increased resistance. Indeed, addition of DNP (0.3 mM) increased survival of both wild-type and mutant strains two- to fourfold (Fig. 1). Moreover, assays conducted in the presence of KCN (1 mM) increased survival of the mutant strain more than 100-fold (Fig. 2). Cumulatively, these results indicate that protamine sensitivity is influenced by...
of bacterial growth in protamine resistance
The metabolic state of the bacterial cell can influence the activity of certain antimicrobial peptides. For instance, PR-39 is more effective against growing cells whereas cecropin P1 is equally active against growing and nongrowing cells (Boman et al., 1993). To examine the effect of bacterial growth on protamine activity, we resuspended exponentially growing cells in fresh medium with or without glucose, and then added protamine. After 20 min, the percentage viability was 113 and 34% for cells incubated in the absence and presence of glucose, respectively (Fig. 3a). While this result suggests that growing cells are more susceptible to protamine than nongrowing cells, it could also be interpreted as protamine requiring an energized membrane and not growth per se. To distinguish between these two possibilities, we investigated protamine activity in cells that were incubated in the presence of an inhibitor of protein synthesis: chloramphenicol-treated cells that were energized by glucose (but otherwise inhibited for growth) were nearly as susceptible to protamine as growing cells (Fig. 3b). This indicates that an energized cytoplasmic membrane is required, and sufficient, for antibacterial activity.

Protamine does not cause cell lysis
We investigated whether protamine treatment resulted in bacterial cell lysis. We found that the optical density of a bacterial culture did not decrease even when challenged with bactericidal concentrations of protamine (Fig. 4). Furthermore, microscopic examination of protamine-treated cells did not indicate any obvious disruption of cell integrity. These results are in agreement with earlier studies on the antibacterial effects of protamine and histone-like compounds from mammalian tissues which suggested that these compounds do not cause cell lysis (Hirsch, 1958). However, protamine did have an effect on cell morphology: over time, protamine-treated cells adopted a swollen appearance, a feature also described for Escherichia coli cells exposed to the antibacterial peptide PR-39 (Boman et al., 1993). Like protamine, this peptide is also arginine-rich and is not known to cause cell lysis.

An examination of the killing kinetics gave the appearance that loss of viability and inhibition of cell growth were separate events: the optical density of the bacterial cultures increased for about 30 min after adding protamine, yet viability decreased one order of magnitude within 15 min of adding the peptide (Fig. 4). We investigated this apparent discrepancy and found that the increase in optical density was due, at least in part, to protamine altering the refractive property of cells, giving

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We have previously reported that incorporation of 20 mM resistance to protamine (Parra-Lopez et al., 1994) into the assay medium conferred increased bacteriostatic effect as it remained associated with the cell membrane damage and loss of viability did occur rapidly, a subset of cells remained alive with intact cell membranes but were unable to grow. On the other hand, some cell membrane damage and loss of viability did occur rapidly, a subset of cells remained alive with intact cell membranes but were unable to grow. On the other hand, the discrepancy seen between the plate count and fluorescence staining viability assays could reflect the continued action of protamine (rather than an immediate bacteriostatic effect) as it remained associated with the cell during incubation on the agar plate.

We have previously reported that incorporation of 20 mM MgSO4 into the assay medium conferred increased resistance to protamine (Parra-Lopez et al., 1994). This presumably results from competition between Mg2+ and protamine for anionic binding sites on the cell surface (Vaara, 1992). To investigate whether excess Mg2+ might displace protamine bound to bacterial surfaces thus relieving the cell of its inhibitory effect, we compared the recovery of colonies of cells exposed to protamine for 30 min and then spread onto LB-agar or LB-agar with added MgCl2 (100 mM). Plates containing Mg2+ yielded two to three times more colonies than plates without added Mg2+, indicating that the effect of protamine is, in part, reversible. A similar effect has been reported for cells of E. coli treated with PR-39 (Boman et al., 1993). While high concentrations of Cl- ions can inhibit the activity of defensin-like peptides (Smith et al., 1996), the effect of MgCl2 on protamine-treated cells was due to the Mg2+ rather than the Cl- because the same effects were observed with MgSO4.

Cumulatively, these results indicate that protamine does not cause cell lysis and that its effect can be partially reversed by the addition of Mg2+.

**Effect of protamine on cytoplasmic membrane energization**

To examine whether protamine perturbs the generation of a proton-motive force at the cytoplasmic membrane (Kashket, 1985; Berger, 1973), we measured the transport and efflux of proline in cells treated with protamine. Both wild-type and mutant Salmonella strains exhibited a marked decrease in their ability to accumulate proline when a sublethal concentration of protamine was present (Fig. 6). Furthermore, protamine (at both sublethal and lethal concentrations) caused a rapid efflux of proline from preloaded cells (Fig. 7), an effect similar to that seen with KCN, an agent that is known to perturb the proton-motive force (Berger, 1973).

The fact that protamine de-energizes the bacterial membrane suggested that it may also alter the cellular ATP concentration. For cells growing aerobically on glucose, ATP can be synthesized via substrate-level phosphorylation or via oxidative phosphorylation, a process dependent on electron flow and subsequent generation of a proton-motive force. Hence, if protamine disrupts the ability to generate a proton-motive force the cellular ATP

![Fig. 5. Effect of protamine on the refractive property of cells. EG5195 cells were grown in minimal medium at 37°C to exponential phase, pelleted, then resuspended to about 4 x 10^7 c.f.u. ml^{-1} in minimal medium, pH 7.7, containing glucose but lacking a nitrogen source. After 10-15 min protamine was added (arrow). ○, Untreated cells; ●, 3 μg protamine ml^{-1}; ■, 6 μg protamine ml^{-1}. The results are representative of three similar, independent experiments.](image1)

![Fig. 6. Effect of protamine on [14C]proline accumulation. Exponential-phase cells (about 2.5 x 10^9 c.f.u. ml^{-1}) growing at 37°C in minimal glucose medium, pH 7.7, were treated with chloramphenicol 15 min prior to adding protamine and [14C]proline. In each case, protamine was added to a sublethal concentration 30 s before the addition of [14C]proline. ○, ●, ○, ○, 1402B's without and with 4 μg protamine ml^{-1}, respectively; □, EG5195 without and with 1 μg protamine ml^{-1}, respectively; △, EG5195 treated with 2 mM KCN. Values, except for KCN, are the average of three trials.](image2)
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Effect of protamine on [14C]proline-preloaded cells.
EG5195 cells were prepared as described in the legend to Fig. 4 but with protamine added after a steady state of [14C]proline accumulation had been reached. The arrow shows the time of addition of protamine or KCN. , 1 and 15 µg protamine ml⁻¹, respectively; △, 2 mM KCN. The results shown are representative of three independent experiments.

Effect of protamine on [14C]proline-preloaded cells.

Fig. 9. Effect of protamine on [3H]leucine transport (a), (c) and protein synthesis (b), (d). EG5195 cells were grown at 37 °C in minimal medium, pH 7.7, containing glucose or DL-lactate. At exponential phase (about 2.5 x 10⁸ c.f.u. ml⁻¹) protamine was added to 15 µg ml⁻¹ and [3H]leucine added 30 s later. ○, ○, without protamine; ●, ●, with protamine; (a), (b), effect on transport and protein synthesis in cells grown on glucose; (c), (d), cells grown on DL-lactate. Values are the mean of three trials for glucose-grown cells and two trials for lactate-grown cells.

Effect of protamine on cellular ATP content.
EG5195 cells were grown at 37 °C in minimal medium, pH 7.7, containing 0.2% glucose. At early-exponential phase (about 2.5 x 10⁸ c.f.u. ml⁻¹) protamine was added to 15 or 30 µg ml⁻¹. Intracellular ATP was extracted by treatment with TCA and the ATP concentration measured with a luminometer as bioluminescence (see Methods). ATP concentrations are presented as values relative to the time zero sample. ○, Untreated cells; ▲, ■, 15 and 30 µg protamine ml⁻¹, respectively. The results are representative of two, independent experiments.

Effect of protamine on [3H]leucine transport and protein synthesis

To investigate whether protamine altered bacterial protein synthesis, we measured leucine uptake and its incorporation into proteins. Within 2 min of adding protamine, cells growing on glucose as the sole carbon source ceased to accumulate leucine. However, protein synthesis continued normally for about 7 min (Fig. 9a, b). The temporal separation between cessation of transport and protein synthesis was more apparent in cells growing on lactate: protein synthesis continued for 10 min after leucine uptake had stopped (Fig. 9c, d). This provides additional evidence that the inner membrane is the target of protamine action. Protein synthesis in the absence of leucine uptake might have been sustained by the leucine that had been accumulated prior to inhibition of uptake. Finally, protamine did not cause the accumulation of mistranslated proteins because SDS-PAGE of proteins isolated from protamine-treated EG5195 cells showed distinct banding (data not shown) and lacked the 'smearing' characteristic of cells killed by streptomycin (Busse et al., 1992).

Protamine does not permeabilize the inner membrane

Because protamine affected inner membrane functions, we investigated its ability to permeabilize it. We measured hydrolysis of the chromogenic substrate ONPG mediated by the cytoplasmic enzyme β-galactosidase (Skerlavaj et...
Protamine permeabilizes the outer membrane

Agents that disrupt the permeability barrier of the outer membrane render the cell more susceptible to detergents and hydrophobic antibiotics (Vaara, 1981, 1992). We tested the effect of protamine on outer membrane permeability by measuring susceptibility to the lytic effects of a detergent: protamine-treated cells were rendered susceptible to deoxycholate (Fig. 11). However, the antibacterial effect of protamine cannot be accounted for by its effect on the outer membrane because deoxycholate caused cell lysis even at sublethal concentrations of protamine.

DISCUSSION

We have examined the mechanism of action of protamine, a proline- and arginine-rich bactericidal peptide. Protamine inhibited several metabolic processes that rely on a functional cytoplasmic membrane without causing cell lysis or altering the permeability of the inner membrane. Protamine activity required an energized membrane but not growing cells since loss of viability occurred even in the presence of chloramphenicol. Furthermore, protamine activity was influenced by the magnitude of the electrical membrane potential and its antibacterial effect could be diminished by incubation at low pH or in the presence of respiratory poisons – treatments that lower membrane potential. Given the numerous metabolic activities associated with the cytoplasmic membrane, it is not surprising to find that protamine, like other antimicrobial peptides, targets the inner membrane.

On the basis of its polycationic, nonamphiphilic nature, protamine should be prevented from inserting into the hydrophobic interior of the cytoplasmic membrane (Louie & Dixon, 1974; Kaiser & Kézdy, 1987). This implies that the mode of action of protamine is distinct from that described for channel-forming antimicrobial peptides which cause leakage of cytoplasmic metabolites and membrane de-energization. Protamine inhibited macro-molecular synthesis (Fig. 9) in the absence of cell lysis, which is reminiscent of the effects described for the pig antimicrobial peptide PR-39 (Boman et al., 1993). The inability of protamine and PR-39 to lyse cells may be related to common structural features: these peptides are of similar size, highly cationic, as well as proline- and arginine-rich (Louie & Dixon, 1974; Agerberth et al., 1991). These peptides may interact with the negative charges on the phospholipid headgroups and proteins located at the membrane surface. The partial rescue of protamine-treated bacteria that results from the addition of Mg²⁺ may perhaps reflect a displacement of protamine from anionic sites at the inner membrane surface. Protamine treatment reduced the cellular ATP content and inhibited amino acid transport (Figs 6–8), suggesting an impairment in the ability to generate a proton motive force (Maloney, 1987). Protamine and PR-39 could target membrane proteins other than those associated with respiration, such as those involved with nutrient transport or biosynthetic systems. Because the peptide/protein interaction would be driven by electrostatic forces the...
target protein need only possess the appropriate anionic binding site(s).

Protamine disrupted the permeability of the outer membrane (Fig. 11). However, several lines of evidence argue against this effect being responsible for its antibacterial action. First, disruption of outer membrane permeability was seen at subinhibitory concentrations of protamine. Second, treatments that specifically changed the electrical potential or energization of the inner membrane altered against this effect being responsible for its antibacterial potential or energization of the inner membrane altered antimicrobial activity plays an important role in the innate immunity of a wide variety of animal species ranging from insects to mammals (Zasloff, 1992; Boman, 1995). Certain bacterial pathogens have evolved mechanisms to resist killing by these peptides (Groisman, 1994), and mutations that result in peptide susceptibility lead to attenuation for bacterial pathogens have evolved mechanisms to resist killing by these peptides (Groisman, 1994), and mutations that result in peptide susceptibility lead to attenuation for virulence. For example, 11 out of 12 protamine-sensitive mutants of S. typhimurium had median lethal doses for mice that were >1000-fold higher than that of the peptide-resistant, wild-type strain (Groisman et al., 1992b). While some of these mutants displayed hypersusceptibility to several antimicrobial peptides, others were defective only in their ability to resist killing by protamine. Further characterization of these mutants as well as the identification of mutants that are hyper-resistant to protamine may help to elucidate the particular targets of protamine action in the inner membrane.

Finally, the production of small cationic peptides with antimicrobial activity plays an important role in the innate immunity of a wide variety of animal species ranging from insects to mammals (Zasloff, 1992; Boman, 1995). Certain bacterial pathogens have evolved mechanisms to resist killing by these peptides (Groisman, 1994), and mutations that result in peptide susceptibility lead to attenuation for virulence. For example, 11 out of 12 protamine-sensitive mutants of S. typhimurium had median lethal doses for mice that were >1000-fold higher than that of the peptide-resistant, wild-type strain (Groisman et al., 1992b). While some of these mutants displayed hypersusceptibility to several antimicrobial peptides, others were defective only in their ability to resist killing by protamine. Further characterization of these mutants as well as the identification of mutants that are hyper-resistant to protamine may help to elucidate the particular targets of protamine action in the inner membrane.

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