A Novel Initiation Mechanism of Death in *Streptococcus pneumoniae* Induced by the Human Milk Protein-Lipid Complex HAMLET and Activated during Physiological Death*\[^5\]*

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**Background:** Ion transport and initiation of death in bacteria is poorly understood.

**Results:** Both the human milk complex HAMLET and physiological death stimuli required sodium-dependent calcium influx and kinase activation to kill pneumococci and form optimal biofilms.

**Conclusion:** Specific ion transport during bacterial death initiation is essential.

**Significance:** The study provides novel information about ion transport in bacteria and its role in bacterial physiology.

To cause colonization or infection, most bacteria grow in biofilms where differentiation and death of subpopulations is critical for optimal survival of the whole population. However, little is known about initiation of bacterial death under physiological conditions. Membrane depolarization has been suggested, but never shown to be involved, due to the difficulty of performing such studies in bacteria and the paucity of information that exists regarding ion transport mechanisms in prokaryotes. In this study, we performed the first extensive investigation of ion transport and membrane depolarization in a bacterial system. We found that HAMLET, a human milk protein-lipid complex, kills *Streptococcus pneumoniae* (the pneumococcus) in a manner that shares features with activation of physiological death from starvation. Addition of HAMLET to pneumococci dissipated membrane polarity, but depolarization *per se* was not enough to trigger death. Rather, both HAMLET- and starvation-induced death of pneumococci specifically required a sodium-dependent calcium influx, as shown using calcium and sodium transport inhibitors. This mechanism was verified under low sodium conditions, and in the presence of ionomycin or monensin, which enhanced pneumococcal sensitivity to HAMLET- and starvation-induced death. Pneumococcal death was also inhibited by kinase inhibitors, and indicated the involvement of Ser/Thr kinases in these processes. The importance of this activation mechanism was made evident, as dysregulation and manipulation of physiological death was detrimental to biofilm formation, a hallmark of bacterial colonization. Overall, our findings provide novel information on the role of ion transport during bacterial death, with the potential to uncover future antimicrobial targets.

There is growing evidence that bacteria live in complex communities during colonization and infection (1–3). In analogy with multicellular eukaryotes, optimal function of such communities requires tight regulation of both bacterial growth and death. Although much is known about pathways of programmed cell death (PCD)\(^2\) in eukaryotic systems, little is understood about terminal differentiation and PCD in prokaryotes and how they may be related.

Two of the best-described bacterial PCD systems are the toxin-antitoxin system and autolysis-associated death mechanisms. The toxin-antitoxin system is found throughout prokaryotic species and consists of a stable toxin and its corresponding labile antitoxin. These components exist in a delicate balance to control death of the cell for altruistic purposes during various environmental conditions (4–6). Membrane-associated holin- and antiholin-like proteins have been described in *Staphylococcus aureus* and other bacterial species, and have also been shown to control cell death, autolysis, and release of genomic DNA, all of which contribute to the structural integrity of biofilms and potentially to the virulence of the organism (7, 8). Autolysis is also observed in several other bacterial species, including *Bacillus subtilis*, *Enterococcus faecalis*, and *S. pneumoniae*. This lytic activity has recently been shown to be central during events known as “cannibalism” and “fratri-cide,” where a subpopulation of immature or susceptible cells is killed by sibling cells to release DNA, cellular nutrients, and intracellular toxins (such as pneumolysin) to benefit the population during events such as sporulation, genetic transforma-

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\[^2\] The abbreviations used are: PCD, programmed cell death; AM, acetoxymethyl; MSC, mechanosensitive channel; DCB, dichlorobenzamil; HAMLET, Human α-lactalbumin Made LEthal to Tumor cells; HKPBS, high-potassium PBS preparation; RuR, ruthenium red; NCX, Na\(^+/Ca\(^{2+}\)\) exchanger; DMSO, dimethyl sulfoxide; CFU, colony-forming units.

\[^5\] This article contains supplemental Fig. S1.
HAMLET causes a rapid dissipation of the bacterial membrane

A major hurdle to elucidating the initiation events of bacterial death is the paucity of information on ion transport mechanisms and electrophysiology of the bacterial membrane (13, 14), combined with the difficulty of measuring these events in prokaryotic systems based on their less accessible cytoplasmic membranes. Radioisotopes have been used with some success (15, 16), whereas the loading of acetoxyethyl (AM) fluorescent indicator dyes has only been successful in a few bacterial species (17–19). Patch clamping is not realistic based on the small size of the bacteria and the presence of an inhibitory cell wall, however, some work with giant protoplasts has been performed with variable results (20, 21). Additionally, based on the lack of studies, few eukaryotic inhibitors have been evaluated for activity in bacterial systems, making interpretations with such inhibitors difficult, and fewer yet have been directly developed for bacterial transport systems.

During our studies of the anti-infectious protection conferred to infants by breastfeeding, we identified a milk fraction named HAMLET, for Human α-lactalbumin Made LEthal to Tumor cells, that induced apoptosis in tumor cells, but also killed a variety of bacterial species (22, 23). As the apoptotic effect was partly induced through a direct interaction of HAMLET with mitochondria (24), and as bacteria have long been postulated to be the evolutionary predecessors of mitochondria (25), we hypothesized that similarities in death activation pathways may exist. Indeed, similarities were identified and recently described in our study revealing apoptosis-like phenotypes in bacteria after exposure to HAMLET (26). After exposure, both tumor cells and bacteria showed hallmark morphological changes such as cell shrinkage, DNA condensation, and high-molecular weight DNA fragmentation (26). The bacterial effects were associated with a dissipation of the membrane potential, just as we observed in mitochondria, and required protease activation. These striking phenotypic parallels suggested that an apoptosis-like pathway of death exists in bacteria, which can be activated by HAMLET by targeting effectors that are potentially conserved in tumor cells and various bacterial species.

In the current study, we focused on the initiating mechanism(s) of HAMLET- and starvation-induced bacterial death. Using the pneumococcus as a model system, we found that HAMLET causes a rapid dissipation of the bacterial membrane potential and integrity. These membrane effects and bacterial death were accompanied by a sodium-dependent influx of calcium and an efflux of potassium, and were induced by several compounds that block ion transporters, specifically those associated with calcium transport and sodium-calcium exchange activity. Additionally, we observed that kinase activation was required for HAMLET-induced death. Importantly, we observed that all reagents that interfered with the ability of HAMLET to induce pneumococcal death and autolysis also altered the natural susceptibility of the pneumococcus to starvation-induced death and autolysis, suggesting that HAMLET is uniquely able to activate cell death machinery similar to that already in place for physiological death. Finally, dysregulation of physiological death was detrimental to biofilm formation, suggesting that tight control of pneumococcal death is required for optimal colonization and infection.

**EXPERIMENTAL PROCEDURES**

**HAMLET Production**

The HAMLET batches used for these studies were generously provided by Dr. Catharina Svanborg, Lund University, Lund, Sweden. HAMLET was produced by converting native α-lactalbumin in the presence of oleic acid (C18:1) as described (27). The preparations were stored in lyophilized form, and resuspended in 1× phosphate-buffered saline (PBS, pH 7.2; Invitrogen; 155.17 mM NaCl, 2.71 mM Na2HPO4, 1.54 mM KH2PO4) to create working stocks.

**Reagents for Modulating Ion Transport**

All of the following reagents are from Sigma unless noted otherwise and the majority of these reagents have never been evaluated in a bacterial system. Solvents are noted in parentheses ("aq" indicates "aqueous").

**K⁺ Transport Reagents**—High-K⁺ PBS (HKPBS) containing 60 mM K⁺: 96.5 mM NaCl, 58.5 mM KCl, 2.7 mM Na2HPO4, 1.54 mM KH2PO4 (corresponding “regular PBS” buffer contained 58.5 mM N-methyl-D-glucamine-Cl instead of KCl); valinomycin (DMSO).

**Ca²⁺ Transport Reagents**—Ruthenium red (aq), nifedipine (DMSO), verapamil hydrochloride (aq), sodium orthovanadate (aq), and ionomycin (DMSO).

**Na⁺ Transport Reagents**—3',4'-Dichlorobenzamil hydrochloride (DMSO); amiloride hydrochloride hydrate (DMSO); low Na⁺ PBS (“PBS(25%)”) was made by substituting most NaCl with N-methyl-D-glucamine chloride (aq) (35 mM NaCl, 120 mM N-methyl-D-glucamine-Cl, 2.71 mM Na2HPO4, 1.54 mM KH2PO4); monensin sodium salt (DMSO).

**Mechanosensitive Channel Inhibitor**—Gadolinium(III) chloride hexahydrate (DMSO).

**Bacterial Strains and Growth Conditions**

We used three strains of *S. pneumoniae* in this study: D39 (wild-type) (28), D39ΔlytA (D39 lacking the major autolysin LytA) (29), and R36A (an unencapsulated derivative of the wild-type strain D39) (28). All of these strains display equal sensitivity to HAMLET. We used D39ΔlytA throughout most of the experiments to limit the autofluorescent interference gener-

**Mechanism of Apoptosis-like Death in Pneumococci**
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Assessing the Effect of HAMLET on Pneumococcal Viability

Upon reaching late log phase in growth media, the pneumococci were pelleted by centrifugation at 2,400 \( \times g \) for 10 min and washed twice by resuspension in PBS. The bacterial pellet was resuspended in PBS to half of the original volume and energized with 50 mM glucose for 15 min at 37 °C. In each well of a 96-well microtiter plate (Falcon, BD Biosciences), a 100-\( \mu \)l volume of the energized bacteria was added to 100 \( \mu \)l of PBS containing the required HAMLET concentration and/or the specific ion transport inhibitor compound, and was subsequently incubated for 1 h at 37 °C. At the end of the incubation, serial dilutions of the bacteria were made in PBS and plated onto solid media composed of Tryptic Soy Broth (Bacto, BD Diagnostics) supplemented with 5% defibrinated sheep’s blood (Bio Link Inc., Liverpool, NY) for viable counts. The concentration of HAMLET used throughout the study was that which induced between 3 and 4 log\(_{10}\) of death. Upon adding any inhibitory compounds, their effect on HAMLET-induced death was expressed as a percentage of the total logs of death observed in the presence of the sample treated with HAMLET alone. To determine the minimal inhibitory concentration of HAMLET, \( S.\ pneumoniae \) D39 was seeded at a concentration of 10\(^{6}\) colony-forming units (CFU) per ml in THY in 96-well microtiter plates in the presence of 2-fold dilutions of HAMLET and growth was determined by measuring the optical density at 600 nm, every 5 min over 18 h in a Bioteck Synergy 2 plate reader. The minimal inhibitory concentration was determined to be the lowest concentration of HAMLET where no growth was detected.

Assessing Membrane Potential and Integrity

To detect membrane potential and rupture of the pneumococcal membrane, respectively, 500 \( \mu \)M DiBAC\(_4\)(3) (bis-(1,3-dibutylbarbituric acid) trimethine oxonol; Molecular Probes, Eugene, OR) and propidium iodide (40 \( \mu \)g/ml; Sigma) were added to energized pneumococci (prepared as described above). In a 96-well plate, a 100-\( \mu \)l volume of this bacterial suspension was then added to 100 \( \mu \)l of PBS containing HAMLET and/or the specific ion transport inhibitor compound in the wells of a 96-well plate yielding a final concentration of 25 mM glucose, 250 \( \mu \)M DiBAC\(_4\)(3), 20 \( \mu \)g/ml of propidium iodide, and the specified concentrations of HAMLET and/or the inhibitor. The plate was then placed immediately into a pre-warmed (37 °C) Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) where fluorescence readings from DiBAC\(_4\)(3) (485/20 nm excitation, 528/20 nm emission) and propidium iodide (530/25 nm excitation, 590/35 nm emission) were taken every minute for 1 h. Sodium deoxycholate (Sigma) was added as a positive control for membrane depolarization and rupture. The difference in fluorescence intensity between the untreated control and the HAMLET-treated sample was calculated for the “no inhibitor” samples and for the “inhibitor” samples using the values after 60 min. The fluorescence intensity difference for the inhibitor samples was then expressed as a percentage of the intensity of the HAMLET alone (no inhibitor) sample, describing the degree of depolarization compared with the “HAMLET alone” sample (see supplemental Fig. S1).

Fluorescent Detection of Ion Transport

Loading—The following protocol was developed. After reaching late log phase, R36A pneumococci were washed twice and resuspended in PBS containing 5 \( \mu \)M fluorescent indicator dye (PBFI/AM for potassium, or Fura-2/AM or Fluo-4/AM for calcium), 1× PowerLoad\(^{TM}\) (solutubilization reagent), and 1× probenecid (all from Molecular Probes). Protected from light, the pneumococci were incubated at 37 °C for 2 h to allow for loading of the dyes and cleavage of the ester, and subsequently washed twice and resuspended with PBS.

Readings—200 \( \mu \)l of the washed/loaded bacteria were added per well of a 96-well plate, which was then placed in the pre-warmed (37 °C) Biotek Synergy 2 plate reader where a baseline reading was taken by measuring the fluorescence every second for 1 min (Fura-2/AM, PBFI/AM, excited at 340/11 and 380/20, emission detected at 508/20; Fluo-4/AM, 485/20 excitation, 528/20 emission). If an inhibitor was present, it was added prior to this baseline reading. At the end of the baseline reading, HAMLET was added and fluorescence readings were immediately taken every second for another 4 min. Valinomycin and ionomycin (Sigma) were added as positive controls for K\(^+\) and Ca\(^{2+}\) transport, respectively. The degree of inhibition was expressed as a percentage of the fluorescence intensity from the sample treated with HAMLET alone (see supplemental Fig. S1 for a visual depiction of the measurements).

Radioisotope \(^{45}\)Ca\(^{2+}\) Transport Assays

D39ΔlytA was grown to log phase, washed three times, and resuspended in 1× PBS containing 0.5 mM CaCl\(_2\) (CaPBS). Alternately, for experiments with low extracellular sodium, we washed and resuspended the cells in an isosmotic buffer containing 25% of the normal PBS Na\(^+\) concentration (PBS(25%)). No glucose was added to minimize the interference created by extrusion of Ca\(^{2+}\) via ATPase pumps. \(^{45}\)CaCl\(_2\) (PerkinElmer Life Sciences) was added to the cells at a final concentration of 2.5 \( \mu \)Ci/ml, followed by inhibitor compound, with each addition given 2 min equilibration time. The untreated baseline sample was measured at this point. The sample was then divided, HAMLET was added to one of the tubes, and \(^{45}\)Ca\(^{2+}\) uptake was measured at various intervals. For each sample, 100 \( \mu \)l was dispensed onto MilliPore 0.3-\( \mu \)m PHWP filters (EMD Millipore, Billerica, MA) presoaked in the CaPBS, and immedi-
Results

Hamlet Kills S. Pneumoniae in a Dose-dependent Manner

Although Hamlet is not universally bactericidal and fails to kill many species of bacteria including S. aureus and E. coli, it exhibits significant antibacterial activity against a handful of species, with S. pneumoniae displaying the greatest susceptibility (22). All pneumococcal wild-type strains we have tested are equally sensitive to Hamlet, regardless of capsule presence or type, or absence of genes for various virulence factors, including the major autolysin (LytA) (26). As shown in Fig. 1, D39ΔlytA (an autolysin-negative pneumococcal mutant) was killed by Hamlet in a concentration-dependent manner. The minimal inhibitory concentration for Hamlet was determined to be 5–10 μg/ml, whereas ~110 μg/ml was required to kill 6 log10 pneumococci within 1 h (Fig. 1). These numbers are comparable with what is required to kill pneumococci with other antibiotics in the same time frame (our preliminary data and see Refs. 31 and 32) and may well be within a physiological range considering that the concentration of α-lactalbumin in milk is 2,000 μg/ml and oleic acid is the most prevalent fatty acid in human milk. For all experiments described throughout this study, we employed a concentration of Hamlet that induced between 3 and 4 log10 of death over a 1-h incubation period, unless otherwise indicated.

Exposure to Hamlet Leads to Depolarization and Rupture of the Pneumococcal Membrane

To measure depolarization of the bacterial membrane we tested a variety of fluorescent potential-sensitive probes, including the commonly used mitochondrial probes JC-1, TMRE, and Rhodamine 123, all of which failed to produce a recordable change in membrane potential in heat-killed or detergent-treated pneumococci compared with healthy cells (not shown). However, we did find success with bis-oxonol dyes in all our bacterial cell systems and settled on using the bis-oxonol DiBAC4(3), which enters depolarized cells and fluoresces more intensely upon interacting with intracellular components. Just as it induces depolarization of the mitochondrial membrane (33), Hamlet triggered a rapid dissipation of the pneumococcal membrane potential, which plateaued after about 6 min (Fig. 2A). Using the membrane-impermeable fluorescent dye propidium iodide, we simultaneously monitored membrane integrity and observed that, at about the same time that depolarization plateaued, rupture of the membrane began (Fig. 2B), with fluorescence rising steadily throughout the remainder of the incubation period. This suggests that Hamlet-induced depolarization and rupture of the pneumococcal membrane are independent events, with dissipation of polarity preceding rup-
Mechanism of Apoptosis-like Death in Pneumococci

![Graph A](image1)

**FIGURE 2. Disruption of pneumococcal membrane polarity and integrity.** Mid-log phase D39ΔlytA pneumococci were incubated with the fluorescent indicator dyes DiBAC(3) and propidium iodide (PI) concurrently to detect (A) membrane depolarization and (B) membrane rupture, respectively, by measuring fluorescence over time. HAMLET was added at time 0 (arrow). The detergent sodium deoxycholate (0.05%) was included as a positive control for both events. The results presented are from a representative experiment.

ture, rather than depolarization resulting from lost membrane integrity, as was seen upon treatment with the detergent sodium deoxycholate (Fig. 2).

Depolarization Alone and K⁺ Efflux Are Not Sufficient for Activation of HAMLET-induced Death

Information from other bacterial systems has suggested that execution of bacterial death may be linked to initial depolarization of the bacterial membrane (7, 12). However, the specificity of depolarization as a direct trigger of death and lysis in bacteria has not been studied. We therefore evaluated the role of depolarization as a trigger for HAMLET-induced death.

While investigating pneumococcal depolarization and rupture at different concentrations of HAMLET, we observed that low-grade depolarization could be induced without membrane rupture or death, suggesting that either depolarization alone was not a trigger for bacterial death or that the depolarization at low concentrations was not sufficiently strong to trigger death. Interestingly, depolarization was also induced in the HAMLET-resistant species E. coli (Fig. 3A) and S. aureus (Fig. 3B) to a level that, when induced in S. pneumoniae (Fig. 3C), resulted in about 2 log₁₀ of pneumococcal death (p = 0.0442) (Fig. 3D), providing further evidence that depolarization per se may not be the trigger of bacterial death.

To more specifically address the role of membrane depolarization in HAMLET-induced death we investigated the role of K⁺ transport. In eukaryotic cells, the efflux of K⁺ is well described as an essential event for successful execution of apoptosis (34, 35), whereas its transport, in concert with Na⁺, is also central to maintaining membrane polarity (36). As such information is available in prokaryotic systems, both of these functions were explored in relationship to HAMLET-induced bacterial death.

We first monitored intracellular K⁺ levels by developing a loading protocol for loading of AM ester fluorescent indicator dyes into pneumococci. AM-esters have only been successfully loaded into a handful of species but the loading efficiency varies. For pneumococci, we found that capsule expression interfered with loading of the dyes and that PowerLoad™ and probenecid (from Invitrogen) were required to obtain an optimal intracellular concentration of dye to make measurements possible.

We then successfully loaded the unencapsulated S. pneumoniae strain R36A with the AM-ester version of the K⁺-sensitive fluorescent indicator dye PBFI. Although the fluorescence of the bacteria from PBFI/AM remained unchanged upon treatment with PBS, the signal intensity immediately decreased following addition of HAMLET (Fig. 4A, green line), indicating a decrease in the intracellular concentration of K⁺ that is most likely due to an efflux of the ion, similar to what takes place during apoptosis of eukaryotic cells. The decrease in fluorescence leveled off approximately 4 min after addition of HAMLET, suggesting that this efflux occurred rapidly and that the lower intracellular concentration of K⁺ was sustained over time. The K⁺ efflux seen after HAMLET exposure was mirrored using the K⁺ ionophore valinomycin that allows K⁺ to immediately flow out of the cell down its concentration gradient (Fig. 4A, black line). This is the first time K⁺ transport has been directly measured in pneumococci, and contributes to the very few studies of potassium transport performed in bacterial species (37).

To determine the role of the detected K⁺ efflux in the bacterial activity of HAMLET, we directly manipulated the transmembrane K⁺ gradient by creating an extracellular high-K⁺ PBS solution (HKPBS; 60 mM extracellular K⁺) that diminishes the electrochemical potential gradient of K⁺. This solution depolarizes the bacterial membrane and significantly limits the ability of K⁺ to flow out of the cells. The degree of HAMLET-induced depolarization and death in the pneumococcus (comparing the HAMLET-treated bacteria with the untreated bacteria; see supplemental Fig. S1 for a visual depiction of these calculations) in HKPBS was no different from that observed in regular PBS (Fig. 4B). On the other hand, HKPBS significantly limited the HAMLET-induced decrease in intracellular K⁺ levels compared with the HAMLET-induced decrease observed in
bacteria bathed in PBS ($p = 0.0018$) (Fig. 4C). These results suggest that K\(^+\) efflux is not central to the bactericidal mechanism of HAMLET, and is likely more of a compensatory cellular response to repolarize the membrane. Furthermore, the failure of the HKPBS, which depolarizes the membrane, to either trigger death in pneumococci by itself or potentiate the effect of HAMLET treatment further demonstrates that depolarization per se is not sufficient to trigger pneumococcal death, and that more specific signaling events are required. As K\(^+\) transport was not involved in initiation of HAMLET-induced death, no further studies were performed to identify potential transport mechanisms.

**Mechanosensitive Channels Play a Role in HAMLET-induced Death**

During eukaryotic apoptosis, depolarization of the mitochondrial membrane causes water to fill the mitochondria, leading to swelling (33, 38). In bacteria, one of the major ion transport elements that serve to detect such swelling is the stretch-activated or mechanosensitive channel (MSC), which responds by opening and nonspecifically releasing a variety of cations (especially K\(^+\)), causing water loss that relieves the cell of the increased turgor pressure. To determine whether mechanosensitive channels might be involved in HAMLET-induced death in bacteria, we treated pneumococci with HAMLET in the presence or absence of the MSC inhibitor gadolinium and observed that, although the compound had no dramatic effect on the degree of HAMLET-induced depolarization, it did rescue a significant portion of the cells from death ($p = 0.0051$) (Fig. 4B), suggesting that MSCs are involved in facilitating the lethal effects of HAMLET. Gadolinium also blocked more than half of the K\(^+\) efflux ($p = 0.0487$) (Fig. 4C), suggesting that mechanosensitive channels are involved in that event, but failed to impact Ca\(^{2+}\) influx (see Fig. 5D, below). However, as K\(^+\) transport was unrelated to HAMLET-induced death and gadolinium did not affect Ca\(^{2+}\)-transport, the involvement of other ions in the trigger of bacterial death is suggested.

**Calcium Influx Is Central to the Activation of the Bactericidal Mechanism of HAMLET**

The role of Ca\(^{2+}\) in apoptotic signaling is well-established (39); Ca\(^{2+}\) overload of the mitochondrial matrix through the Ca\(^{2+}\) uniporter leads to the opening of the permeability transi-
Mechanism of Apoptosis-like Death in Pneumococci

A ~ gray line remained unchanged (~).

In the isotope experiment, we observed a brief plateau or “overshoot” of $^{45}$Ca$^{2+}$. In the isotope experiment, we observed a brief plateau or “overshoot” of $^{45}$Ca$^{2+}$). In the isotope experiment, we observed a brief plateau or “overshoot” of $^{45}$Ca$^{2+}$). In the isotope experiment, we observed a brief plateau or “overshoot” of $^{45}$Ca$^{2+}$) in the presence of HAMLET, suggesting that this transport event occurred rapidly and was sustained (Fig. 5A).

To investigate the importance of Ca$^{2+}$ influx for the bactericidal activity of HAMLET, we introduced various inhibitors of Ca$^{2+}$ transport to assess their effects on membrane depolarization, Ca$^{2+}$ transport, and death. The general Ca$^{2+}$ channel inhibitor RuR was the first inhibitor evaluated in the pneumococcal system based on its inhibitory role in eukaryote mitochondria (33). We observed that RuR almost completely blocked HAMLET-induced calcium influx, observed both by an almost complete inhibition of the rise in the 340/380 fluorescence ratio from cells pre-loaded with Fura-2 ($p = 0.0039$) and an inhibition of more than half of the $^{45}$Ca$^{2+}$ uptake ($p = 0.0083$) (Fig. 5D). RuR was specific for calcium transport as no effect on K$^{+}$ transport was seen (Fig. 4C). The inhibition of Ca$^{2+}$ influx was directly related to a significant and almost complete rescue of the bacteria from death ($p = 0.01$) (Fig. 5C).

To further verify a role for an influx of calcium in triggering HAMLET-induced death, pneumococci were treated with ionophycin, which, by itself, increased the intracellular Ca$^{2+}$ concentration (Fig. 5, A and B) without killing the bacteria, but was shown to significantly potentiate the bactericidal activity of HAMLET ($p = 0.0039$) (Fig. 5E), possibly by lowering the HAMLET-induced influx required to trigger death activation. Overall, the strong inhibitory effects of RuR and the potentiating effect of ionophycin suggest that Ca$^{2+}$ influx plays a central role in the activation of the bactericidal activity of HAMLET.

**FIGURE 4.** HAMLET induces K$^{+}$ efflux that is not central to the bactericidal activity. *A*, the fluorescence of PBFI-loaded (K$^{+}$-indicator) $S$. pneumoniae strain R36A was recorded to monitor concentrations of intracellular K$^{+}$. After baseline fluorescence was recorded for 1 min, either 10 μM valinomycin (positive control) or HAMLET was added (arrow) to the bacteria and the resulting fluorescence was recorded every second for the next 4 min. Tracings from a representative experiment are shown. B, D39ΔlytA pneumococci were incubated with HAMLET for 1 h in the presence of K$^{+}$-related transport inhibitors, while monitoring membrane depolarization (gray bars) and death (black bars), using DiBAC fluorescence and viable counts, respectively. Membrane depolarization and death in the inhibitor-treated samples are expressed as a percentage of the HAMLET-alone treated samples (HAMLET-alone value was set to 100%, represented by dotted line). C, PBFI fluorescence was monitored in R36A to measure the HAMLET-induced decrease in intracellular K$^{+}$ in the presence of ion transport inhibitors. K$^{+}$ efflux after 4 min is expressed as a percentage of that observed with HAMLET alone (dotted line, 100% K$^{+}$ efflux). The data in panels *B* and *C* represent the mean ± S.D. of three individual experiments with error bars, and significance was calculated using the paired $t$ test with a 95% confidence interval (*$ = p < 0.05$; **$ = p < 0.01$). Inhibitors: high-K$^{+}$ PBS buffer (HKPBS; 60 mM extracellular K$^{+}$), gadolinium (GAD), and RuR.
characterized and shown to extrude Ca$^{2+}$ from the cell (43, 44). However, HAMLET-induced depolarization and death were not dramatically changed in the presence of the P-type ATPase inhibitor sodium orthovanadate; if anything, death was slightly increased ($p < 0.04$) (Fig. 5C). As L-type Ca$^{2+}$ channels have been implicated in HAMLET-induced death of tumor cells (45), we also evaluated the effect of the L-type channel inhibitors nifedipine and verapamil, neither of which had a significant impact on HAMLET-induced depolarization or bacterial death (Fig. 5C).

In addition to P-type Ca$^{2+}$-ATPase transport activity, studies by Trombe (16) have revealed that Ca$^{2+}$ transport in S. pneumoniae appears to be linked to Na$^{+}$ via Na$^{+}$/Ca$^{2+}$ antiport activity, although the transporter(s) has not yet been iden-
Mechanism of Apoptosis-like Death in Pneumococci

To evaluate the potential influence of Na\(^+\)/H\(^+\) on the HAMLET-induced Ca\(^{2+}\)/H\(^+\) influx, we introduced the Na\(^+\)/H\(^+\) channel inhibitor amiloride and its derivate 3',4'-dichlorobenzamil (DCB), which, compared with amiloride, has greater inhibitory specificity for a Na\(^+\)/Ca\(^{2+}\)/H\(^+\) exchanger (NCX). As both of these compounds autofluoresce at the wavelengths used to detect Fura-2 fluorescence, we used Fluo-4/AM to measure intracellular Ca\(^{2+}\) levels, and also measured 45Ca\(^{2+}\) uptake. Both amiloride and DCB almost completely inhibited the HAMLET-induced rise in intracellular Ca\(^{2+}\), as indicated both by the low Fluo-4 fluorescence intensity (amiloride, \(p = 0.0084\); DCB, \(p = 0.003\)) (Fig. 5D) and the lack of 45Ca\(^{2+}\) uptake (amiloride, \(p = 0.0059\); DCB, \(p = 0.0024\)) (Fig. 5D). This provided the first evidence that Ca\(^{2+}\) flux is linked to Na\(^+\) following exposure of S. pneumoniae to HAMLET.

The importance of this Na\(^+\)-dependent Ca\(^{2+}\) influx for the bactericidal activity of HAMLET was demonstrated by the fact that the presence of amiloride caused dose-dependent inhibition, with a 1 mm concentration required for complete inhibition of depolarization (\(p = 0.0017\)) and Ca\(^{2+}\) import, as well as more than 50% log\(_{10}\) rescue of HAMLET-induced death (\(p = 0.001\)), whereas the same effect was obtained using DCB at the considerably lower concentration of 100 \(\mu\)M (depolarization, \(p = 0.0201\); death, \(p = 0.0068\)) (Fig. 5, D and F). This pattern of inhibition suggests that Ca\(^{2+}\) transport occurs through a NCX and is important for HAMLET-induced death.

To further verify the role of Na\(^+\) for Ca\(^{2+}\) influx, we monitored HAMLET-induced pneumococcal 45Ca\(^{2+}\) uptake under conditions that favor the operation of a NCX, which transports Na\(^+\) out of the pneumococcus against its electrochemical potential gradient while bringing Ca\(^{2+}\) into the cell in the direction of its substantial gradient. Using the radioisotope 45Ca\(^{2+}\), HAMLET-induced Ca\(^{2+}\) uptake in D39ΔlytA pneumococci was recorded in the presence of (B) the Na\(^+\) ionophore monensin and (C) conditions of low extracellular Na\(^+\) (PBS with 25% of standard Na\(^+\) levels; PBS(25%)); cpm from representative experiments are shown for each panel. D, after 1 h of HAMLET treatment in both of these conditions, the bacteria were serially diluted for viable counts, and the log\(_{10}\) of HAMLET-induced death were calculated. The viability data represent three individual experiments, with S.D. indicated with error bars, and significance calculated using the paired t test with a 95% confidence interval (* \(p < 0.05\); ** \(p < 0.01\)).
isoosmotic buffer containing 25% of the normal PBS Na⁺ concentration (PBS(25%)), which will facilitate Na⁺ exit via the NCX. The ⁴⁵Ca²⁺ uptake was greater in low-Na⁺ PBS compared with that observed in PBS containing a standard concentration of Na⁺ (Fig. 6C) and was associated with a significantly increased HAMLET-induced death (p = 0.0017) (Fig. 6D). Combined, these results confirm the role of Na⁺ in the bactericidal mechanism of HAMLET, and are consistent with our hypothesis that a NCX is centrally involved in this activity.

**Inhibiting Kinase Activity Limits the Lethality of HAMLET**

In eukaryotic cells, sustained elevations of intracellular Ca²⁺ are known to activate kinases, which, in turn, can trigger apoptosis (46, 47). Ca²⁺-induced kinase activation has also been recently described for conjugated linoleic acid (48), which is a fatty acid that can complex with α-lactalbumin equally as well as oleic acid and yield similar lethal effects (49). To evaluate the potential role of kinase activity in HAMLET-induced pneumococcal death, we measured the bactericidal activity of HAMLET in the presence of the pan-kinase inhibitor staurosporine, using a concentration range that has previously been reported to be effective in bacterial systems (20 μM (50)). We observed that pneumococcal viability was dramatically rescued in the presence of this inhibitor (p = 0.0017) (Fig. 7), suggesting that kinase activity is an important effector during HAMLET-induced death.

**Pneumococcal Physiological Death and Autolysis Use the Same Activation Mechanism as HAMLET-induced Death**

Previous work by Marie-Claude Trombe (51) has shown that Ca²⁺ is important for effective induction of competence and autolysis. As the results of our studies point to a central importance of Ca²⁺ and Na⁺ as well as kinase activity for the bactericidal mechanism of HAMLET, we explored the possibility that this same activation mechanism is also responsible for physiological death that results in autolysis. To do this, we took the same conditions that enhanced or inhibited HAMLET-induced death and evaluated their effect on stationary phase starvation-induced death and subsequent autolysis.

**Enhancement—**Above, we showed that the Na⁺ ionophore monensin enhanced HAMLET-induced ⁴⁵Ca²⁺ influx (Fig. 6B) and both monensin and the Ca²⁺ ionophore ionomycin enhanced death (Figs. 5E and 6D) in the pneumococcus. To determine whether monensin and ionomycin also enhance stationary-phase autolysis of the pneumococcus, we evaluated the effect of their presence during growth of wild-type D39 pneumococci, by monitoring the optical density over time. The concentrations of ionophore used did not affect growth in the logarithmic phase. The optical density tracings (Fig. 8A) demonstrate that the bacteria died and autolysed sooner and more rapidly in the presence of monensin, as well as ionomycin, suggesting that conditions that favor the influx of Ca²⁺ through a Na⁺-dependent transport mechanism to reach a triggering concentration, enhance autolysis in the pneumococcus. These results suggest that conditions that enhance the bactericidal...
activity of HAMLET also enhance starvation-induced death and autolysis in stationary phase growth of pneumococci.

**Inhibition**—As addition of the Ca$^{2+}$-transport inhibitor RuR and the Na$^{+}$-inhibitor DCB each inhibited the bactericidal activity of HAMLET, including Ca$^{2+}$ influx, depolarization, and death (Fig. 5, C, D, and F), we predicted that these inhibitors would have a similar inhibitory effect on stationary phase death and autolysis. As shown in Fig. 8B, the activation of death and autolysis was significantly delayed, causing the continual extension of the stationary phase. Similarly, pneumococci that were grown in the presence of the kinase inhibitor staurosporine, which blocked HAMLET-induced death (Fig. 7), also displayed a prolonged stationary phase, delaying the activation of autolysis (Fig. 8C). Thus, conditions that inhibit the bactericidal pathway of HAMLET-induced death also appear to inhibit pneumococcal autolysis.

**Inhibition of Biofilm Formation**

We have recently shown that pneumococci form complex biofilms during colonization of the murine nasopharynx and that the ability of strains to form biofilms on epithelial cells, but not on abiotic surfaces, is directly associated with their ability to cause nasopharyngeal colonization in vivo (30). As colonization and infection through biofilm formation in vivo is thought to require a tight regulation of death and survival of the bacteria (1), we sought to determine how modulation of cell death affects biofilm formation on epithelial cells. D39 bacteria were seeded on top of NCI-H292 bronchial carcinoma cells in the presence or absence of RuR and DCB, and biofilm formation over 48 h was recorded as the total biomass. Even though these inhibitors caused prolonged survival of planktonic pneumococci in stationary phase (Fig. 7B), the pneumococci formed less structured biofilms of significantly lower biomass in the presence of these inhibitors (RuR, $p = 0.0171$; DCB, $p = 0.0078$) (Fig. 9). The significance of these findings are, first, that we have identified components of a novel bactericidal activation mechanism induced by HAMLET that is also used by pneumococci during physiological death, and second, that a tight regulation of the execution of this death pathway by physiological stimuli is required for optimal biofilm formation and, by extension, the potential ability of bacteria to cause colonization and infection in vivo.

**DISCUSSION**

This study characterized the activation mechanism of pneumococcal death induced by HAMLET, a human milk protein-lipid complex, and comprehensively demonstrated the importance of specific ion transport mechanisms and kinase activation in triggering bacterial death (see Fig. 10 for a summary of our findings). These mechanistic features also appeared to be the same or overlapping with the mechanism used to trigger physiological death from starvation of the bacteria in stationary phase cultures, and constitutes the first study to address the activation events responsible for initiating death in bacteria from natural stimuli. The importance of this death mechanism was further verified when its modulation resulted in significantly decreased biofilm formation, suggesting an important role for a tight regulation of bacterial growth and death during colonization and infection.

It is well-documented that breastfeeding protects infants against infections, including those of the upper respiratory tract, where S. pneumoniae is a major pathogen (52, 53). During our studies of this protective activity we identified HAMLET, a complex of human $\alpha$-lactalbumin in a partially unfolded state that is stabilized with the human milk-specific fatty acids oleic and linoleic acid that constitutes $\sim75\%$ of the fatty acid content in human milk (27, 54). Active complex can be isolated from human milk, that in itself has antibacterial activity (23, 54), but the specific concentration of HAMLET present in human milk is difficult to quantify as milk contains other antibacterial components besides HAMLET, as the purification process has the potential to induce HAMLET complex formation, and as we lack specific tools to differentiate the bactericidal conformation of $\alpha$-lactalbumin from the natively folded version. Still, the concentrations required to kill pneumococci (5–100 $\mu$g/ml) lie well within the physiological range, as the concentration of $\alpha$-lactalbumin is especially high in human milk (2,000 $\mu$g/ml).

Our earlier studies have shown that HAMLET binds to the pneumococcal surface and induces death with morphological changes that are similar to those observed during eukaryote apoptosis (26). In this study, we characterized the initiation events leading to HAMLET-induced bacterial death, with a focus on membrane-associated events leading to depolarization and ion transport events that we already know are involved in HAMLET-induced tumor cell apoptosis involving mitochondria (33).

Membrane polarity and ion transport have been comprehensively studied in eukaryotic systems and are centrally responsible for various cellular processes and events, including apoptosis (55). Furthermore, methodology to study these events is well established. However, this is not true for prokaryotic systems.
There is a significant paucity in the literature of studies concerning ion transport in bacteria and its role in cellular signaling (13), especially as it concerns bacterial death. With the exception of the studies by Trombe and co-workers (16, 51) of the role of calcium in pneumococcal autolysis, and previous work demonstrating that, in a few bacterial systems, various ionophores can enhance autolysis (12, 56), the role of ion transport in bacterial death has not been studied and no studies are available describing the specific ion transport events and signals required for autolysis activation under natural circumstances. Similarly, virtually nothing is known about the specific ion fluxes involved in the activation of bacterial death. Much of this can be attributed to logistical challenges, as the smaller size of bacteria is incompatible with standard methods of measurement (i.e., patch clamping) and makes ion transport studies in prokaryotes very difficult, which, we speculate, accounts for the lack of literature describing bacterial ion transport. The central novelty of this study, besides its findings, lies in the utilization and establishment of methodology to comprehensively study these events in Gram-positive organisms, providing the most comprehensive evaluation and characterization of membrane polarity and ion transport in a prokaryotic system to date.

Our results demonstrated that HAMLET associates with the bacterial surface and triggers dissipation of membrane polarity, with a subsequent loss of membrane integrity and eventual rupture. Some groups have speculated that alterations in membrane energy or polarity are involved in activating bacterial PCD, including pneumococcal autolysis (12, 57), but no specific data supporting this has been provided. However, several of our results suggested that depolarization in and of itself is not sufficient to activate HAMLET-induced death. First, high potassium in the extracellular environment (HKPBS), which caused membrane depolarization by itself, did not induce pneumococcal death, nor did it potentiate the bactericidal activity of HAMLET. Second, HAMLET effectively depolarized the membrane of S. aureus and E. coli, two species that are not killed by HAMLET; when the same degree of depolarization was induced in S. pneumoniae, it caused about 2 log10 of death. This suggested that membrane depolarization during HAMLET-induced death of pneumococci is an event that is parallel or secondary to specific ion transport events involved in triggering bacterial death.

Besides the role of potassium in maintaining membrane polarity, its efflux is an essential aspect of apoptosis activation in higher eukaryotes and some fungi, and is required for caspase and nuclease activation during the execution phase of cell death (35, 58, 59). In this study, we showed for the first time that potassium is transported out of a bacterial cell when induced to die, but also showed that this efflux was not required for HAMLET to induce pneumococcal death. This suggests that cell death regulation performed through the cytoplasmic concentration of potassium ions is potentially confined to eukaryotes and appeared at a later stage in evolution. Although the mechanism of potassium efflux in HAMLET-treated pneumococci is...
Mechanism of Apoptosis-like Death in Pneumococci

not fully clear, MSCs played a significant role due to the ability of gadolinium to inhibit a large portion of this efflux. Using gadolinium as an inhibitor, MSCs also appeared to play some role in HAMLET-induced death, which was independent of potassium. It can be speculated that MSCs could potentially transport other ions over the membrane that facilitate HAMLET-induced death or that gadolinium inhibits other transport mechanisms of importance for HAMLET-induced death. This will be investigated in more detail in the future.

As HAMLET causes a calcium-dependent depolarization of the mitochondrial membrane (33), and given the well-established role of calcium as an important signaling molecule involved in a wide variety of cellular processes (60, 61), we investigated the role of calcium in HAMLET-induced pneumococcal death. HAMLET caused a rapid and sustained increase in the intracellular concentration of calcium. Inhibition of this calcium influx by the calcium channel inhibitor RuR rescued the pneumococci from HAMLET-induced death. The potent inhibition of calcium transport and death seen also by the sodium transport inhibitors amiloride and DCB, combined with increased calcium influx and death associated with a decreased sodium gradient, strongly indicated a role for sodium in facilitating calcium influx, potentially through a sodium-calcium exchanger. An interesting parallel is that mitochondria express a NCX that is important for mitochondrial Ca$^{2+}$ regulation during apoptosis (40). Sodium-calcium exchange activity has been suggested in earlier studies of the pneumococcus as the primary transport mechanism responsible for calcium uptake in the pneumococcus, with a direct link to optimal activation of competence and autolysis (16, 51, 62). Intriguingly, sodium-dependent calcium influx was also required for activation of pneumococcal death in stationary phase, as RuR and DCB both prolonged the duration of pneumococcal survival and integrity during this phase, whereas monensin and ionomycin (which facilitated HAMLET-induced death) reduced earlier death in stationary phase growth without affecting growth in the logarithmic phase.

The specific transporter involved is still unidentified. No sodium/calcium motif transporters are annotated in any pneumococcal genome and attempts to isolate the transporter by co-immunoprecipitations with HAMLET have failed. We have also produced mutants resistant to high concentrations of DCB and sequenced them (data not shown). However, all mutants had point mutations in the gene annotated as cardiolipin synthetase, which likely results in pleiotropic membrane effects that indirectly affect transporter activity and HAMLET accessibility; no mutations were found in open reading frames that contain transmembrane domains.

Calcium is a major signaling ion in many eukaryotic pathways that often targets downstream kinases (46, 47). As a recent example, sodium-calcium exchange activity was shown to activate mitogen-activated protein kinases (MAPK), including p38 kinases, in a calcium-dependent manner, leading to elevation of reactive oxygen species and subsequent apoptotic death (63). Calcium-induced kinase activation has also recently been described for conjugated linoleic acid (48), a fatty acid that can associate with α-lactalbumin equally well as oleic acid with similar physiological effects (49). The fact that staurosporine, a kinase inhibitor, blocked HAMLET-induced death and also displayed the ability to extend stationary phase during normal growth of D39 bacteria, provides an additional link between physiological death and HAMLET-induced death. We have identified a potential staurosporine-sensitive kinase and are currently investigating its role in the bactericidal mechanism of HAMLET.

As death is tightly regulated in bacterial communities during colonization or infection, our recent findings that pneumococci colonizing the murine nasopharyngeal tissue in vivo or epithelial cells in vitro grow in organized biofilms (30), combined with the potential overlap in molecules involved in HAMLET-induced death and physiological death, led us to predict that modulating components involved in the activation pathway of HAMLET would affect biofilm formation in vitro. As expected, we found that increasing the survival of the bacteria by inhibiting calcium influx to delay autolysis activation caused a significant decrease in biofilm biomass in vitro. This supports the postulated idea that bacteria that live in complex communities (e.g. biofilms) during colonization and infection require tight regulation of both bacterial survival and death for optimal function. It also supports the importance of the PCD systems that have been identified in bacteria and have been tied to a variety of important functions (5, 64, 65), including biofilm integrity. However, our studies are the first to mechanistically address the initiation of bacterial death.

HAMLET was recently shown to cause phenotypic changes in both S. pneumoniae and Haemophilus influenzae similar to those observed during eukaryotic apoptosis. Both bacterial species displayed cell shrinkage, DNA condensation, and high molecular weight fragmentation in association with HAMLET-induced death, and, at least for pneumococci, this was partially dependent on serine protease activity (26). We also have evidence that these organisms, as well as the HAMLET-insensitive species S. aureus and E. coli, all respond to starvation-induced death with apoptosis-like DNA fragmentation. Thus, even though HAMLET per se cannot trigger death in all bacteria, it is possible that the elements that make up the core pathway of death are conserved throughout a wider pool of bacterial species, but that the components responsible for activation of this pathway are presented with varying degrees of accessibility to HAMLET, resulting in varying degrees of bactericidal activation and corresponding susceptibility.

This concept is supported by the identification of apoptosis-like features of death seen in all organisms tested, and supports the widely accepted endosymbiotic theory that links mitochondria with bacteria. Furthermore, as HAMLET also specifically triggers apoptosis in tumor cells, we may speculate on the potential overlap in death pathways that exist between these two systems, with a more primitive, evolutionarily conserved PCD pathway that can be activated in bacteria.

Overall, the results of our present study provide a comprehensive framework for future investigation of the ion transport targets that are central to the activity of HAMLET and/or to activation of natural physiological bacterial death. Further elu-

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3 L. R. Marks, E. A. Clementi, and A. P. Hakansson, unpublished observations.
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