AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

MOLECULAR PATHOGENESIS

Arcanobacterium haemolyticum Utilizes Both Phospholipase D and Arcanolysin To Mediate Its Uptake into Nonphagocytic Cells

Patrick S. Gellings,[a] David J. McGee[a]

[a]Department of Microbiology and Immunology, Louisiana State University Health Sciences Center—Shreveport, Shreveport, Louisiana, USA

ABSTRACT *Arcanobacterium haemolyticum* is an emerging human pathogen that causes pharyngitis and wound infections. A few studies have suggested that *A. haemolyticum* is able to induce its uptake into nonphagocytic epithelial cells, but the bacterial factors associated with host cell invasion and the host cell processes involved have yet to be studied. We investigated how two *A. haemolyticum* virulence factors, arcanolysin (ALN) and phospholipase D (PLD), affect the ability of the bacteria to adhere to and subsequently invade Detroit 562 pharyngeal epithelial cells. The sphingomyelinase activity of phospholipase D was necessary to increase bacterial adherence, while the absence of a functional arcanolysin had no effect on *A. haemolyticum* adherence but did lead to a decrease in *A. haemolyticum* invasion into Detroit 562 cells. Because of the known roles of cholesterol-dependent cytolysins in disrupting calcium gradients and inducing F-actin-mediated bacterial internalization, we sought to determine whether ALN and PLD played a similar role in the ability of *A. haemolyticum* to invade nonphagocytic cells. Elimination of extracellular calcium and inhibition of the Arp2/3 complex or F-actin polymerization also caused a decrease in the ability of *A. haemolyticum* to invade Detroit 562 cells. Overall, our findings suggest that *A. haemolyticum* utilizes phospholipase D primarily for adherence and utilizes arcanolysin primarily for invasion into Detroit 562 cells in a process dependent on extracellular calcium and F-actin polymerization. Our work marks the first insight into how the individual activities of arcanolysin and phospholipase D affect *A. haemolyticum* host-pathogen interactions using the biologically relevant Detroit 562 cell line.

KEYWORDS *Arcanobacterium*, actin, calcium, cholesterol-dependent cytolysin, host cell invasion, phospholipase, toxin

Arcanobacterium haemolyticum, a Gram-positive, pleomorphic rod-shaped bacterium, is an understudied human pathogen that most commonly causes pharyngitis and wound infections in adolescents (1–4). The life cycle and pathogenesis of *A. haemolyticum* are poorly defined, but multiple studies have suggested that *A. haemolyticum* has the ability to induce its own uptake into nonphagocytic cells (5, 6). However, neither the mechanism by which this occurs nor the virulence factors of *A. haemolyticum* that are involved in this process have been well investigated. In fact, of the many putative virulence factors produced by *A. haemolyticum*, the activities of only two have begun to be examined: the cholesterol-dependent cytolysin (CDC) named arcanolysin (ALN) (7–9) and a sphingomyelin-specific phospholipase D (PLD) (6, 7, 10, 11). The amino acid sequences of both ALN and PLD contain a signal peptide, suggesting that both are secreted by *A. haemolyticum* and factors that likely affect host-pathogen interactions (8, 12).

PLD is a lipase produced and secreted by the bacteria that specifically utilizes

Citation Gellings PS, McGee DJ. 2019. *Arcanobacterium haemolyticum* utilizes both phospholipase D and arcanolysin to mediate its uptake into nonphagocytic cells. Infect Immun 87:e00832-18. https://doi.org/10.1128/IAI.00832-18.

Editor Victor J. Torres, New York University School of Medicine

Address correspondence to David J. McGee, dmcgee@lsuhsc.edu.

Received 16 November 2018
Returned for modification 13 December 2018
Accepted 4 February 2019
Accepted manuscript posted online 11 February 2019
Published 23 April 2019

sphingomyelin as a substrate, generating cyclic ceramide phosphate and choline (11). Secreted PLD increases the ability of *A. haemolyticum* to adhere to host cells while simultaneously promoting the formation of lipid rafts in host cell membranes (6). In addition, PLD appears to be necessary for internalized *A. haemolyticum* to escape from an unidentified, intracellular vesicle and enter the host cell cytoplasm and cause cell death in a necrosis-like fashion (6).

Like most CDCs, ALN requires membrane cholesterol to form the characteristic large, oligomeric pores associated with this family of pore-forming toxins (8, 13). A threonine-leucine (Thr-Leu or T-L) motif present in loop 1 of domain 4 of CDCs confers susceptibility to membrane cholesterol, and replacing one or both amino acids blocks the ability of a CDC, including ALN, to recognize cholesterol (7, 14). One of the known consequences of CDC pore formation in host cell membranes is the disruption of ion or protein gradients that are normally present in uninfected cells (15, 16). Ca^{2+} gradients, in particular, are known to be disrupted by the presence of CDCs and, in some cases, are necessary for the internalization of bacteria into nonphagocytic cells (15). Unfortunately, all prior work regarding *A. haemolyticum* host-pathogen interactions was not conducted in a biologically relevant cell line. While ALN is known to be lytic to human cells (7–9), the effect that ALN has on host-pathogen interactions is currently unknown.

Together, PLD and ALN have been reported to have a synergistic relationship, wherein the enzymatic activity of PLD promotes the binding of ALN to a host membrane, which leads to more pore formation in host membranes by ALN (7). However, neither the role of ALN nor the synergism between PLD and ALN has been examined in the context of host-pathogen interactions. Here, we examined the roles that ALN and PLD play in the initial interactions between *A. haemolyticum* and the pharyngeal epithelial Detroit 562 cell line, specifically, in the ability of the bacteria to adhere to and invade host cells. Our data suggest that ALN does not play a role in the ability of *A. haemolyticum* to adhere to host cells but is involved in the subsequent invasion step in the bacterial life cycle. In addition, our data validate the previous finding that PLD promotes *A. haemolyticum* adherence and go further by showing that the sphingomyelinase activity of PLD is necessary and, in fact, that any sphingomyelinase activity is sufficient to promote bacterial adherence to Detroit 562 cells. Finally, we present evidence that the absence of extracellular calcium decreases the ability of *A. haemolyticum* to induce its own uptake into the host cell and that F-actin polymerization is necessary for efficient bacterial uptake. Overall, this work details previously unknown activity of two *A. haemolyticum* putative virulence factors, ALN and PLD, and their role in promoting bacterial uptake (adherence plus invasion) into the nonphagocytic Detroit 562 cell line.

RESULTS

The absence of ALN does not affect *A. haemolyticum* adherence but negatively impacts *A. haemolyticum* invasion of Detroit 562 cells. A Δ*aln* strain was constructed and complemented with wild-type (WT) *aln* (Δ*aln*::*aln*$^+$) as previously described (6, 7) and is isogenic to the *A. haemolyticum* ATCC 9345 strain. The adherence to and invasion of the mutant and complemented bacteria to Detroit 562 cells relative to those of the WT strain were evaluated. The absence of ALN had no significant impact on the ability of *A. haemolyticum* to adhere to the Detroit 562 cells (Fig. 1A). In contrast, the Δ*aln* strain had an ~50% defect in invasion relative to that of either the WT strain or the strain complemented with WT *aln* (Fig. 1B). In order to evaluate if the ALN pore formation activity contributed to *A. haemolyticum* invasion, the Δ*aln* strain was complemented with a plasmid encoding a mutated version of *aln* encoding the Thr-Leu mutations previously mentioned that produces a nonhemolytic ALN that fails to recognize cholesterol (Δ*aln*::mutated *aln*) (7). The presence of the mutated ALN did not affect the ability of the bacteria to adhere to the Detroit 562 cells (Fig. 1A). However, production of mutated ALN failed to restore the invasive phenotype associated with the WT strain or the strain complemented with WT *aln* (Fig. 1B). These data suggest that

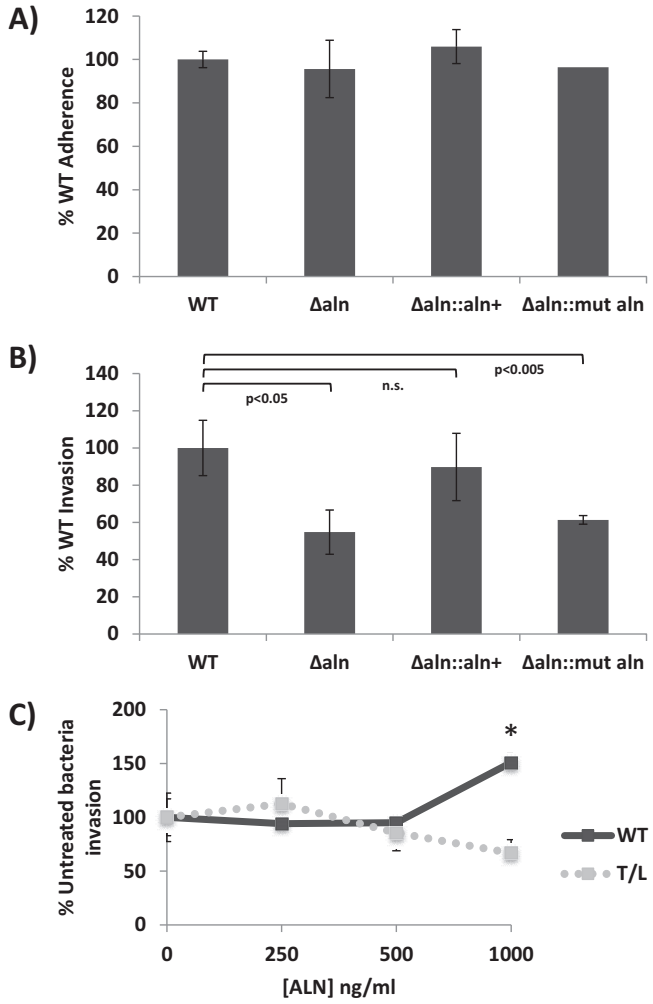

FIG 1 Loss of a functional ALN does not affect *A. haemolyticum* adherence but does negatively affect the ability of the bacterium to invade Detroit 562 cells. (A) Adherence assays were conducted with WT and *A. haemolyticum* Δ*aln* strains as well as *A. haemolyticum* Δ*aln* strains that were complemented with a plasmid encoding a WT *aln* gene (Δ*aln*::*aln*$^+$) or a mutated *aln* gene incapable of binding to cholesterol and forming pores (Δ*aln*::mut *aln*). (B) Each of the strains mentioned in the legend to panel A were also subjected to invasion assays. Strains capable of producing a functional ALN (WT and Δ*aln*::*aln*$^+$) had an invasion phenotype noticeably stronger than that of the strains that either did not produce ALN or produced a pore formation-deficient ALN (Δ*aln* or Δ*aln*::mut *aln*). (C) *A. haemolyticum* Δ*aln* was incubated with various concentrations of purified WT or mutated (T/L, $ALN_{TL557\text{-}558AA}$) purified His-ALN for 30 min prior to being added to Detroit 562 cell monolayers, followed by the remainder of the invasion assay. *, $P < 0.05$. The data shown are representative of those from three separate experiments, each of which was done in triplicate. Significance was determined using a Student's *t* test. Values are shown as percent WT adherence or invasion, with error bars representing standard deviations.

ALN pore formation contributes to the ability of the bacteria to invade Detroit 562 cells, while it does not play a role in the ability of the bacteria to adhere to the cells.

Exogenously added purified ALN restores the ability of *A. haemolyticum* Δ*aln* to invade Detroit 562 cells. It was previously shown that exogenous, purified PLD was sufficient to rescue the ability of *A. haemolyticum* Δ*pld* to adhere to epithelial cells (6). This observation suggests that *A. haemolyticum* secretes PLD into its surrounding environment to modify the host membrane to make it more amenable to bacterial adherence. Similarly, it has also been shown that the presence of listeriolysin O (LLO) can increase the invasive phenotype of a *Listeria monocytogenes* Δ*hly* strain whether it is produced by the complemented Δ*hly* bacteria or purified and exogenously added (17). Given that we see that a similar phenomenon occurs with ALN, which increases the ability of *A. haemolyticum* to invade Detroit 562 cells, we investigated if exogenous,

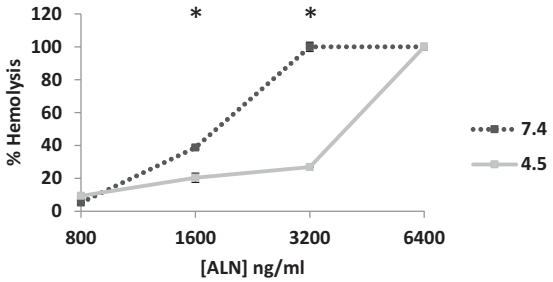

FIG 2 Purified ALN is more hemolytic at neutral pH than at acidic pH. Various concentrations of purified His-ALN were incubated in buffer C at either pH 7.4 or pH 4.5 for 30 min at 24°C and then added to 4% human erythrocytes suspended in identical buffer at 37°C for an additional 30 min. The remainder of the hemolysis assay was carried out as described in Materials and Methods. Significance was determined using a Student's *t* test. The results are representative of those from three separate experiments, with error bars representing standard deviations. *, $P < 0.05$.

purified ALN can rescue the invasion defect with *A. haemolyticum* Δ*aln* and whether ALN pore formation is a requirement. Using an anti-ALN antibody, our laboratory previously showed that ALN can be detected both in *A. haemolyticum* culture supernatants and in whole-cell extracts (data not shown). This observation, coupled with the N-terminal signal peptide present in the ALN amino acid sequence, suggests that ALN is secreted by *A. haemolyticum* into its surrounding environment. Equal amounts of purified WT ALN or T-L-mutated ALN were added to *A. haemolyticum* Δ*aln* before being used to infect Detroit 562 cells in a gentamicin (Gm) protection assay. Remarkably, exogenous ALN increased the invasive phenotype of *A. haemolyticum* Δ*aln* relative to that of *A. haemolyticum* Δ*aln* alone (Fig. 1C). Interestingly, the addition of the T-L-mutated ALN to *A. haemolyticum* Δ*aln* failed to promote increased bacterial invasion, suggesting that the ALN pore formation phenotype is necessary to promote bacterial invasion into Detroit 562 cells (Fig. 1C). We determined that none of the concentrations of purified ALN used in our experiments affected overall cell viability relative to the viability of buffer-treated cells using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell viability assay (data not shown). Therefore, our data suggest that *A. haemolyticum* secretes ALN into its environment, at least in part, to promote its uptake into nonphagocytic cells.

Purified ALN is more active at a neutral pH than at an acidic pH. Certain CDCs, including inerolysin (INY) and listeriolysin O (LLO), have been reported to be more active at acidic pH levels, while other CDCs, including perfringolysin O (PFO), have an optimal cytolytic activity level at neutral pH (18). The pH at which a CDC is most active can give a clue as to which part of the bacterial life cycle that a CDC is needed. As an example, LLO is utilized by *L. monocytogenes* to escape a maturing endosome to avoid bacterial degradation (19). As the endosome matures, the pH of the endosome becomes more acidic, increasing the pore formation activity of LLO. Unlike LLO, INY, and PFO, the optimal pH of ALN has not yet been reported. We analyzed the hemolytic activity of ALN in environments with pH levels of 4.5 to represent an acidic environment and pH 7.4 to represent a neutral environment. Using identical concentrations of ALN, hemolysis data revealed that ALN at a pH of 7.4 lysed 100% of erythrocytes, while ALN at a pH of 4.5 lysed only 26.8% of erythrocytes (Fig. 2). A concentration of 2,310 ng/ml of ALN was necessary to cause 50% hemolysis at pH 7.4, whereas a concentration of 3,697 ng/ml of ALN was necessary to cause 50% hemolysis at pH 4.5. Similar to PFO, ALN pore formation appears to be more active at neutral pH levels than at acidic pH levels, and therefore, ALN has the potential to form pores when secreted by *A. haemolyticum* in a neutral, extracellular environment.

PLD enzymatic activity is necessary to promote *A. haemolyticum* adherence to Detroit 562 cells. Previous studies determined that the absence of PLD decreased the ability of *A. haemolyticum* to adhere to HeLa cells *in vitro* (6). However, whether the enzymatic activity of PLD was required for full adherence was not explored further. We

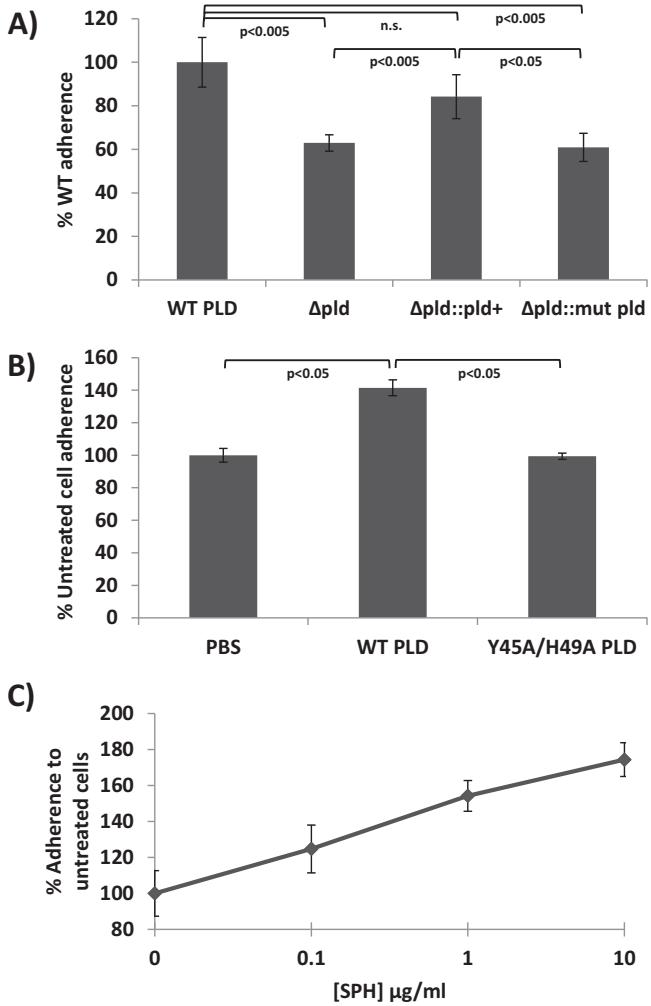

FIG 3 PLD enzymatic activity is necessary to promote *A. haemolyticum* adherence to Detroit 562 cells. (A) Adherence assays were conducted with WT or *A. haemolyticum* Δ*pld* strains as well as *A. haemolyticum* Δ*pld* strains that were complemented with either WT *pld* (Δ*pld*::*pld*$^{+}$) or *pld* with the Y45A/H49A mutations to eliminate sphingomyelinase activity [Δ*pld*::*pld* (Y45A/H49A)]. n.s., not significant. (B) Adherence assays were conducted by treating Detroit 562 cells with 500 ng/ml purified WT PLD or Y45A/H49A PLD for 30 min, followed by infection with *A. haemolyticum* Δ*pld*. (C) Detroit 562 cells were similarly treated with various concentrations of *B. anthracis* SPH, followed by an adherence assay, as described in the legend to panel A. Adherence is shown as a percentage of the WT or untreated cell adherence. Significance was determined using a Student's *t* test. Representative data from three separate experiments are shown. Error bars represent standard deviations.

previously determined two combined amino acid substitutions within PLD (Y45A and H49A) that abolished the sphingomyelinase activity of the enzyme (7). We engineered both mutations into *pld* in the complementing plasmid (pBJ61) and transformed the Δ*pld* strain of *A. haemolyticum*, creating a strain of *A. haemolyticum* that produces an enzymatically dead PLD (Δ*pld*::mutated *pld*) to determine the effect that sphingomyelinase activity has on *A. haemolyticum* adherence. As previously reported (6), the Δ*pld* strain adherence was only 60% of the that of WT *A. haemolyticum*, with the strain complemented with WT *pld* adhering near WT levels (84%) (Fig. 3A). However, the Δ*pld*::mutated *pld A. haemolyticum* strain adhered at 61% of WT levels (Fig. 3A).

We also evaluated if treating Detroit 562 cells with exogenous, purified PLD could increase *A. haemolyticum* adherence. Detroit 562 cells were pretreated with equal amounts of WT PLD or Y45A/H49A PLD for 30 min, followed by infection with *A. haemolyticum* Δ*pld*. *A. haemolyticum* adherence to cells treated with WT PLD reached 141%, while adherence to cells treated with Y45A/H49A PLD reached 99% when

normalized to the level for cells treated with buffer (Fig. 3B). To verify that the loss of enzymatic activity of PLD and not a different mitigating factor was responsible for promoting *A. haemolyticum* adherence, we tested a different version of purified PLD previously constructed (7), P43A PLD, which retains approximately 60% of the enzymatic activity of WT PLD (7). Purified exogenous P43A PLD increased *A. haemolyticum* Δ*pld* adherence to Detroit 562 cells relative to that of Y45A/H49A PLD but not to an extent as great as that to WT PLD-treated cells (data not shown). Overall, these data suggest that the enzymatic activity of PLD is necessary to promote *A. haemolyticum* adherence to Detroit 562 cells.

Sphingomyelinase C activity is also sufficient to increase *A. haemolyticum* adherence to Detroit 562 cells. We previously reported on the ability of *A. haemolyticum* PLD to promote the cytotoxicity of ALN in Detroit 562 cells (7). In addition, we also showed that a sphingomyelinase (SPH) from another bacterial species, *Bacillus anthracis*, also promoted ALN-mediated cytotoxicity (7). The elimination of the sphingomyelin-mediated sequestering of cholesterol by SPH or PLD was sufficient to increase ALN-mediated hemolysis, even though the two enzymes generate distinct ceramide products (7), suggesting that removal of the choline head of sphingomyelin was more important than the presence of the product of the enzymatic reaction. Given the impact that PLD sphingomyelinase activity has on *A. haemolyticum* adherence, we investigated if the enzymatic activity of *B. anthracis* SPH could also confer an increase in *A. haemolyticum* Δ*pld* adherence to Detroit 562 cells. Detroit 562 cells were treated with various concentrations of SPH for 30 min, followed by the addition of *A. haemolyticum* Δ*pld*. As the concentration of SPH increased, so, too, did *A. haemolyticum* adherence to Detroit 562 cells (Fig. 3C). This observation suggests that the increase in *A. haemolyticum* adherence is not due to the generation of a specific lipid product but, rather, is due to targeting of the sphingomyelin choline head for removal in the outer leaflet of the plasma membrane and an increase in ALN pore formation.

***A. haemolyticum* invasion of Detroit 562 cells is dependent on extracellular calcium levels.** It has been previously reported that a sudden influx of calcium is sufficient to cause F-actin polymerization at the plasma membrane via protein kinase C activation, which converts Rac1-GDP to Rac1-GTP, ultimately culminating in Arp2/3-mediated F-actin polymerization (15–17). Therefore, we hypothesized that the absence of environmental calcium would decrease *A. haemolyticum* invasion into Detroit 562 cells. Two different media were used: calcium-rich medium (M1 medium) and calcium-depleted medium (M2 medium). Incubating *A. haemolyticum* cells in either M1 or M2 medium alone for the duration of the invasion assay (6 h) did not affect their viability (data not shown). Detroit 562 cells were cultured using M1 or M2 medium and subsequently infected with *A. haemolyticum* suspended in the identical medium. When normalized to *A. haemolyticum* invasion in M1 medium, bacterial invasion in M2 medium reached only 20% (Fig. 4). As a control, exogenous $CaCl_2$ was added to the M2 medium at various concentrations to evaluate if the introduction of small amounts of calcium was sufficient to restore *A. haemolyticum* invasion. As expected, increasing the concentration of $CaCl_2$ in M2 medium was sufficient to restore *A. haemolyticum* invasion to the levels observed with M1 medium, suggesting that environmental calcium is a necessary component in *A. haemolyticum* invasion of Detroit 562 cells (Fig. 4).

Actin polymerization inhibition blocks effective *A. haemolyticum* invasion into Detroit 562 cells. Because of the necessity of environmental calcium for *A. haemolyticum* invasion, we hypothesized that an influx of calcium mediated by *A. haemolyticum* resulted in F-actin polymerization and subsequent internalization of *A. haemolyticum*. To challenge our hypothesis, we carried out invasion assays using WT *A. haemolyticum*, Detroit 562 cells, the F-actin polymerization inhibitor cytochalasin D, and the Arp2/3 inhibitor CK-636. Cytochalasin D was incubated with Detroit 562 cells at a concentration of 10 μM, followed by infection with *A. haemolyticum*. The presence of cytochalasin D significantly decreased the ability of *A. haemolyticum* to invade the Detroit 562 cells (Fig. 5, top). This observation suggests that the ability of *A. haemo-*

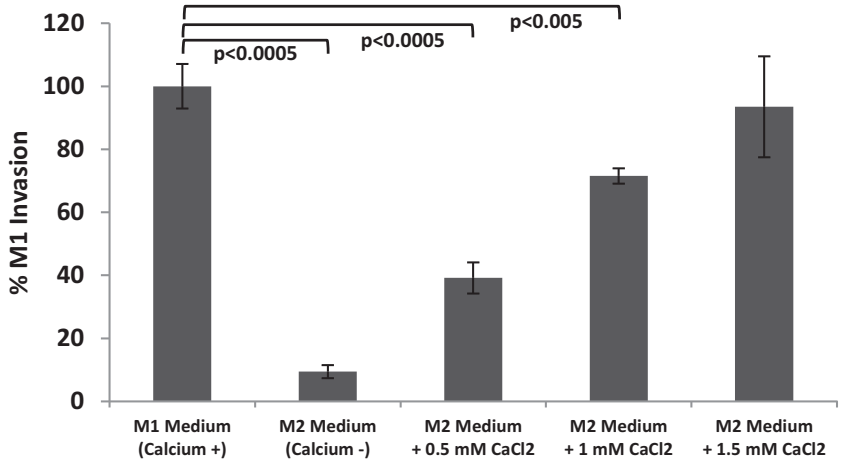

FIG 4 The presence of calcium in the medium is necessary for *A. haemolyticum* invasion of Detroit 562 cells. Invasion assays were conducted in calcium-competent medium (M1 medium), calcium-absent medium (M2 medium), or calcium-absent medium supplemented with $CaCl_2$. No difference in *A. haemolyticum* viability was observed between M1 medium and M2 medium. Adherence is shown as a percentage of *A. haemolyticum* adherence in assays conducted in M1 medium. Significance was determined using a Student's *t* test. Representative data from three separate experiments are shown. Error bars represent standard deviations.

lyticum to invade the nonphagocytic Detroit 562 cell line is mediated, at least in part, by host F-actin polymerization. In addition to directly inhibiting F-actin polymerization with cytochalasin D, we used CK-636 to block Arp2/3 activity and prevent F-actin polymerization. As hypothesized, the presence of the Arp2/3 inhibitor decreased the number of *A. haemolyticum* colonies recovered after the invasion assay to 33% of the bacteria recovered from untreated Detroit 562 cells (Fig. 5, bottom). The presence of neither inhibitor affected the ability of any *A. haemolyticum* strain to adhere to Detroit 562 cells (data not shown). The results from these two experiments suggest that *A.*

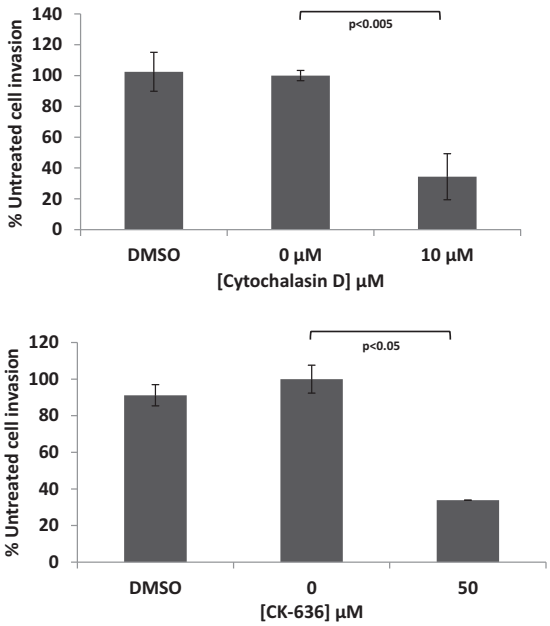

FIG 5 Inhibition of F-actin polymerization decreases *A. haemolyticum* invasion into Detroit 562 cells. Detroit 562 cells were treated with either 10 μM cytochalasin D (top) or 50 μM CK-636 (bottom) for 60 min, followed by the invasion assay with WT *A. haemolyticum* at an MOI of 20:1. Values are shown as percent invasion of untreated cells and are representative of those from three separate experiments. Significance was determined using a Student's *t* test. Error bars represent standard deviations.

haemolyticum invasion into Detroit 562 cells is dependent upon Arp2/3-mediated F-actin polymerization.

DISCUSSION

Arcanobacterium haemolyticum remains an understudied human pathogen, despite having a number of putative virulence factors and an unknown life cycle. An important observation regarding the treatment of *A. haemolyticum* infections was made when researchers noted that the bacteria are resistant to penicillin in the presence of the nonphagocytic HEp-2 cell line (5). This observation led to the hypothesis that *A. haemolyticum* is able to mediate its uptake into the HEp-2 cells, shielding it from the penicillin treatment and leading to survival and reproduction of the bacteria. Years later, a study was conducted that supported the idea that *A. haemolyticum* can invade nonphagocytic cells and that PLD is used to modify the host membrane to increase bacterial adherence to the host plasma membrane, allowing for subsequent invasion (6). Since these initial discoveries, however, efforts have not yet been taken to identify the cellular processes that *A. haemolyticum* exploits to mediate its uptake into the host cell, nor have any other bacterial factors involved in this process been identified. We sought to clarify the activity of PLD necessary to increase *A. haemolyticum* adherence and identify other factors involved in invasion of nonphagocytic cells.

One *A. haemolyticum* virulence factor that has been the focus of some investigation, arcanolysin (ALN), is a member of the pore-forming toxin family known as the cholesterol-dependent cytolysins (CDCs) (8). CDCs form unusually large pores (~250 Å) within eukaryotic cell membranes that are sufficient for disrupting a multitude of concentration gradients (13). Bacterial virulence factors that disrupt concentration gradients, specifically, calcium gradients, have been shown to contribute to the ability of many bacteria to modify host cell physiology, including the ability of *Clostridium perfringens* to induce apoptosis or necrosis (20), lysosomal exocytosis by *Photobacterium damselae* (21), chemokine production by *Streptococcus pneumoniae* (22) and *Escherichia coli* (23), and manipulating nonphagocytic cells to internalize the bacteria via Arp2/3-mediated actin polymerization by *Listeria monocytogenes* (15–17). The composition of a host cell membrane can impact the ability of CDCs to bind to membranes and subsequently form pores. As an example, sphingomyelin is known to form transient hydrogen bonds with cholesterol, creating an umbrella-like covering over cholesterol and preventing extracellular factors, like cholesterol-dependent cytolysins, from interacting with cholesterol (24, 25). In the case of the *A. haemolyticum* pore-forming toxin ALN, treating host membranes with the *A. haemolyticum* PLD sphingomyelinase increases the hemolytic and cytolytic activity of ALN, which suggests an increase in pore formation by ALN (7). In addition, treating host membranes with a sphingomyelinase C enzyme, thus cleaving sphingomyelin in a different location, generating a different ceramide product, but still eliminating the cholesterol sequestration by sphingomyelin, was still sufficient to increase ALN-mediated hemolysis (7). Coupling the ability of CDCs to disrupt calcium gradients in the context of bacterial invasion and the synergy between PLD and ALN in pore formation, we hypothesized that *A. haemolyticum* utilizes both PLD and ALN to mediate interactions with nonphagocytic eukaryotic cells.

Using the biologically relevant Detroit 562 cell line, we detailed for the first time the role that ALN plays in the ability of *A. haemolyticum* to invade nonphagocytic host cells. We observed that the loss of ALN has no measurable impact on the ability of the bacteria to adhere to host cells but does negatively impact the invasion ability of *A. haemolyticum*. Our laboratory constructed *A. haemolyticum* Δ*aln* strains complemented with mutated *aln* to show that the ability of ALN to recognize and bind to cholesterol is necessary for the toxin to increase the invasive phenotype of the bacteria, which suggests that ALN pore formation is necessary to increase *A. haemolyticum* invasion into Detroit 562 cells. A site-directed mutant complementation strategy using Y45A/H49A PLD was similarly used to determine that the sphingomyelinase D activity of PLD is necessary to promote bacterial adherence. Interestingly, it was determined that the

sphingomyelinase C activity of the SPH enzyme from *Bacillus anthracis* is also sufficient to increase bacterial adherence to Detroit 562 cells. When invasion assays were conducted in the absence of calcium in the medium, the ability of *A. haemolyticum* to induce its uptake into Detroit 562 was significantly decreased. However, when exogenous $CaCl_2$ was titrated into the calcium-depleted medium, bacterial invasion also increased. Finally, when F-actin polymerization inhibitors were included in invasion assays, *A. haemolyticum* invasion significantly decreased as well, suggesting that F-actin polymerization is necessary for successful invasion into Detroit 562 cells.

We have demonstrated a role for both ALN and PLD in the interactions of *A. haemolyticum* with Detroit 562 cells, with PLD primarily being used for adherence and ALN primarily being used for invasion. What happens next during the host-pathogen interaction is essentially unknown. A single study showed that, after entry into a HeLa cell, *A. haemolyticum* initially resides in an undefined vesicle before escaping into the cytoplasm in a PLD-dependent manner (6). However, the identity of the vesicle and the manner by which PLD mediates the escape of *A. haemolyticum* into the cytoplasm are unknown. Given the substrate specificity of PLD and the identity of other bacterium-containing vesicles, it is tempting to speculate that *A. haemolyticum* initially resides in an early endosome and that the synergistic pore formation by PLD and ALN is necessary to disrupt the membrane integrity of the endosome, leading to bacterial escape into the host cytoplasm. However, our work supports a model in which *A. haemolyticum* is able to induce its own uptake into nonphagocytic cells, which potentially confers an advantage for the bacteria to avoid clearance by select antibiotics or the immune system.

MATERIALS AND METHODS

Cell culture techniques. Detroit 562 cells (ATCC CCL-138) were cultured using Ham's F-12 medium supplemented with 10% newborn calf serum (Gibco, Grand Island, NY, USA) in either Corning T25 or Corning T75 cell culture flasks at 37°C in a 5% CO_2 humidified incubator. A total of 30 μg/ml streptomycin and 50 μg/ml penicillin were added to prevent contamination. Prior to any experiment, Detroit 562 cells were washed with 1× phosphate-buffered saline (PBS) a total of three times to dilute and remove antibiotics present during normal culturing.

Bacteria and growth conditions. *A. haemolyticum* was cultured in Todd-Hewitt broth supplemented with 10% fetal bovine serum overnight at 37°C while being shaken at 240 rpm in an Infors HT Minitron incubator, with 25 μg/ml kanamycin (Kn), 5 μg/ml chloramphenicol (Cm), or 10 μg/ml erythromycin (Er) being added when appropriate. *A. haemolyticum* was grown on *Campylobacter* agar plates supplemented with 5% blood from healthy, human donors at 37°C, with identical concentrations of antibiotics being added when appropriate.

Protein purification. WT and mutated PLD, ALN, and SPH were purified as previously described (7). Briefly, *E. coli* DH5α cells containing pTrcHis*aln*, pTrcHis*pld*, pET-15b-antB (26), or a mutated derivative were grown in 500 ml of Luria-Bertani broth at 37°C with agitation until the cultures reached an optical density at 600 nm (OD_{600}) of 0.6, at which point the cultures were spiked with isopropyl-β-D-1-thiogalactopyranoside (IPTG; final concentration, 2.5 mM) and allowed to incubate for an additional 4 h. The bacteria were centrifuged at 6,700 × *g* for 15 min at 4°C, the supernatants were discarded, and the bacterial pellets were stored at −20°C overnight. The bacterial pellets were thawed and suspended in 10 ml of wash/lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, pH 8.0) and lysed via a French press three times at 16,000 lb/in^2. To avoid protein degradation, the remainder of the purification protocol was conducted in a 4°C cold room. Cell lysates were centrifuged at 9,600 × *g* for 10 min, and the supernatants were transferred to a fresh 50-ml conical tube and allowed to incubate with Ni-nitrilotriacetic acid resin (Qiagen, Carlsbad, CA, USA) at a 2:1 ratio overnight with gentile agitation. The supernatant-slurry mix was transferred to a 5-ml polypropylene column and subsequently washed 10 times with ~5 ml of wash/lysis buffer and eluted in 1-ml quantities using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The presence of protein was confirmed using SDS-PAGE gels and Coomassie blue staining, and the proteins were dialyzed in 1× PBS using Slide-A-Lyzer cassettes (Thermo Fisher Scientific, Waltham, MA, USA) overnight. The protein concentrations were calculated using a bicinchoninic acid assay (Pierce, Rockford, IL, USA), and protein was stored at −20°C in 50% glycerol solution.

Bacterial adherence and invasion assays. Detroit 562 cells were seeded to 24-well Nunclon Delta Surface cell culture plates and allowed to grow to approximately 90% confluence (approximately 24 to 48 h) in Ham's F-12 medium supplemented with 10% fetal bovine serum and antibiotics. Detroit 562 cells were washed three times with 1× PBS to remove antibiotics and any cell debris before the start of the assay. *A. haemolyticum* strains were grown overnight and allowed to reach mid-log phase, prior to being washed three times with 1× PBS and added to Detroit 562 cells at a multiplicity of infection (MOI) of 10:1 (adherence assay) or 20:1 (invasion assay). For the adherence assay, *A. haemolyticum* was allowed to adhere to Detroit 562 cells for 2 h, and the cells were washed three times with 1× PBS. Bacteria were

harvested by lysing the Detroit 562 cells using 1% Triton X-100, serially diluted in Todd-Hewitt broth, and plated in triplicate onto *Campylobacter* agar plates supplemented with 5% human blood. The plates were incubated at 37°C for 2 days, and the colonies were subsequently counted. For invasion assays, *A. haemolyticum* was allowed to adhere to and invade Detroit 562 cells for 3 h, followed by three washes with 1× PBS and an additional 2 h of incubation with Ham's F-12 medium with 200 μg/ml gentamicin (Gm). The cells were then washed with 1× PBS three times and then harvested and plated as described above for the adherence assay protocol.

In the case of the invasion assays conducted in medium with or without calcium present, M1 medium (containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.36 mM K_2HPO_4, 0.44 mM KH_2PO_4, 5.5 mM D-glucose, and 4.2 mM $NaHCO_3$) was brought to a pH of 7.4 and filter sterilized (0.22-μm-pore-size cellulose acetate low-protein-binding membrane; Corning) as previously reported (27). Calcium-depleted medium (M2 medium) contained the same components as M1 medium except the $CaCl_2$ and was similarly brought to a pH of 7.4 and filter sterilized.

Cytochalasin D (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and added to Detroit 562 cells at a 10 μM concentration 1 h prior to the addition of bacteria at 37°C. Similarly, CK-636 was also dissolved in DMSO and added to Detroit 562 cells at a 50 μM concentration 1 h prior to the addition of bacteria at 37°C. For each experiment, a solvent-only control was used to verify that the volume of DMSO used in each experiment did not adversely affect the bacteria or the Detroit 562 cells.

Hemolysis assay. Hemolysis assays were conducted with human blood that was obtained from normal, healthy donors. The LSU Health Sciences Center—Shreveport Institutional Review Board and Health Insurance Portability and Accountability Act guidelines were followed for isolation of human blood from human subjects. Blood was suspended in buffer C (35 mM sodium phosphate, 125 mM sodium chloride) at pH 4.5 or 7.4 at a 2% concentration for 30 min at 23°C. At the same time, various concentrations of ALN were incubated in either buffer C at pH 4.5 or buffer C at pH 7.4 for 30 min at 23°C, at which point equal parts blood and ALN were incubated in a 96-well round-bottom plate for 30 min at 37°C. Following the incubation, 96-well plates were centrifuged at 4,200 × *g* for 10 min at 15°C to pellet intact red blood cells, and the supernatants were transferred to a flat-bottom, 96-well plate. OD_{415} readings were recorded using a FLUOstar Omega plate reader, and percent hemolysis was calculated using a buffer C negative control and 2% Triton X-100 positive control.

Construction of mutants and complementation. The *A. haemolyticum* Δ*pld*::mutated *pld* and Δ*aln*::mutated *aln* strains were constructed by mutating *pld* in pBJ61 or *aln* in pJGS182*aln*, respectively, using a QuikChange XL site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies, Cedar Creek, TX, USA). PCR products originating from pBJ61 or pJGS182*aln* from the mutagenesis reaction and primers used previously (7) were incubated with DpnI to eliminate parental strands, and the remaining DNA was transformed into heat-shocked *E. coli* Top10F′ cells. Transformants were allowed to grow overnight on Luria-Bertani agar plates supplemented with 200 μg/ml erythromycin or 20 μg/ml chloramphenicol when appropriate, and the presence of the correct mutation was confirmed by sequencing (Arizona State University DNA Sequencing Lab, Tempe, AZ, USA). The confirmed plasmids were electroporated into *A. haemolyticum* Δ*aln* or Δ*pld*, conferring either erythromycin resistance and mutated ALN production or chloramphenicol resistance and mutated PLD production, respectively.

Statistics. Three independent experiments were performed in triplicate for the results presented in each figure. *P* values were calculated using a two-tailed Student's *t* test, and a *P* value of less than 0.05 was considered significant.

ACKNOWLEDGMENTS

We thank Adam J. Ratner (NYU Langone Health) for helpful discussions, Haley S. Ruther-Norwood (Cedars-Sinai Medical Center) for construction of the *A. haemolyticum* Δ*aln* strain, the Andrew D. Yurochko laboratory for providing human erythrocytes, Stephen Leppla for generously providing the pET-15b-antB plasmid used in purifying *B. anthracis* SPH, and Fatima Iqbal for technical assistance.

We declare no conflicts of interest.

P.S.G. and D.J.M. conceptualized the study, P.S.G. established the methodology, P.S.G. performed the investigation, P.S.G. and D.J.M. performed the formal analysis, D.J.M. provided resources, D.J.M. supervised the study, P.S.G. and D.J.M. visualized the study, P.S.G. and D.J.M. acquired funding, P.S.G. prepared the original draft, and P.S.G. and D.J.M. performed review and editing.

REFERENCES

1. Linder R. 1997. *Rhodococcus equi* and *Arcanobacterium haemolyticum*: two "coryneform" bacteria increasingly recognized as agents of human infection. Emerg Infect Dis 3:145–153. https://doi.org/10.3201/eid0302.970207.
2. Banck G, Nyman M. 1986. Tonsillitis and rash associated with *Corynebacterium haemolyticum*. J Infect Dis 154:1037–1040. https://doi.org/10.1093/infdis/154.6.1037.
3. Mackenzie A, Fuite LA, Chan FT, King J, Allen U, MacDonald N, Diaz-Mitoma F. 1995. Incidence and pathogenicity of *Arcanobacterium haemolyticum* during a 2-year study in Ottawa. Clin Infect Dis 21:177–181. https://doi.org/10.1093/clinids/21.1.177.
4. Miller RA, Brancato F, Holmes KK. 1986. *Corynebacterium hemolyticum* as a cause of pharyngitis and scarlatiniform rash in young adults. Ann

Intern Med 105:867–872. https://doi.org/10.7326/0003-4819-105-6-867.
5. Osterlund A. 1995. Are penicillin treatment failures in *Arcanobacterium haemolyticum* pharyngotonsillitis caused by intracellularly residing bacteria? Scand J Infect Dis 27:131–134. https://doi.org/10.3109/00365549509018992.
6. Lucas EA, Billington SJ, Carlson P, McGee DJ, Jost BH. 2010. Phospholipase D promotes *Arcanobacterium haemolyticum* adhesion via lipid raft remodeling and host cell death following bacterial invasion. BMC Microbiol 10:270. https://doi.org/10.1186/1471-2180-10-270.
7. Gellings PS, McGee DJ. 2018. *Arcanobacterium haemolyticum* phospholipase D enzymatic activity promotes the hemolytic activity of the cholesterol-dependent cytolysin arcanolysin. Toxins (Basel) 10:E213. https://doi.org/10.3390/toxins10060213.
8. Jost BH, Lucas EA, Billington SJ, Ratner AJ, McGee DJ. 2011. Arcanolysin is a cholesterol-dependent cytolysin of the human pathogen *Arcanobacterium haemolyticum*. BMC Microbiol 11:239. https://doi.org/10.1186/1471-2180-11-239.
9. Ruther HS, Phillips K, Ross D, Crawford A, Weidner MP, Sammra O, Lämmler C, McGee DJ. 2015. Smooth and rough biotypes of *Arcanobacterium haemolyticum* can be genetically distinguished at the arcanolysin locus. PLoS One 10:e0137346. https://doi.org/10.1371/journal.pone.0137346.
10. Selvy PE, Lavieri RR, Lindsley CW, Brown HA. 2011. Phospholipase D: enzymology, functionality, and chemical modulation. Chem Rev 111:6064–6119. https://doi.org/10.1021/cr200296t.
11. Lajoie DM, Cordes MHJ. 2015. Spider, bacterial and fungal phospholipase D toxins make cyclic phosphate products. Toxicon 108:176–180. https://doi.org/10.1016/j.toxicon.2015.10.008.
12. Cuevas WA, Songer JG. 1993. *Arcanobacterium haemolyticum* phospholipase D is genetically and functionally similar to *Corynebacterium pseudotuberculosis* phospholipase D. Infect Immun 61:4310–4316.
13. Tweten RK. 2005. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. Infect Immun 73:6199–6209. https://doi.org/10.1128/IAI.73.10.6199-6209.2005.
14. Farrand AJ, LaChapelle S, Hotze EM, Johnson AE, Tweten RK. 2010. Only two amino acids are essential for cytolytic toxin recognition of cholesterol at the membrane surface. Proc Natl Acad Sci U S A 107:4341–4346. https://doi.org/10.1073/pnas.0911581107.
15. Dramsi S, Cossart P. 2003. Listeriolysin O-mediated calcium influx potentiates entry of *Listeria monocytogenes* into the human Hep-2 epithelial cell line. Infect Immun 71:3614–3618. https://doi.org/10.1128/IAI.71.6.3614-3618.2003.
16. Lam JGT, Vadia S, Pathak-Sharma S, McLaughlin E, Zhang X, Swanson J, Seveau S. 2018. Host cell perforation by listeriolysin O (LLO) activates a Ca^{2+}-dependent cPKC/Rac1/Arp2/3 signaling pathway that promotes *Listeria monocytogenes* internalization independently of membrane resealing. Mol Biol Cell 29:270–284. https://doi.org/10.1091/mbc.E17-09-0561.
17. Vadia S, Arnett E, Haghighat A-C, Wilson-Kubalek EM, Tweten RK, Seveau S. 2011. The pore-forming toxin listeriolysin O mediates a novel entry pathway of *L. monocytogenes* into human hepatocytes. PLoS Pathog 7:e1002356. https://doi.org/10.1371/journal.ppat.1002356.
18. Rampersaud R, Lewis EL, LaRocca TJ, Ratner AJ. 2018. Environmental pH modulates inerolysin activity via post-binding blockade. Sci Rep 8:1542. https://doi.org/10.1038/s41598-018-19994-8.
19. Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Berche P. 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect Immun 57:3629–3636.
20. Chakrabarti G, McClane BA. 2005. The importance of calcium influx, calpain and calmodulin for the activation of CaCo-2 cell death pathways by *Clostridium perfringens* enterotoxin. Cell Microbiol 7:129–146. https://doi.org/10.1111/j.1462-5822.2004.00442.x.
21. von Hoven G, Rivas AJ, Neukirch C, Meyenburg M, Qin Q, Parekh S, Hellmann N, Husmann M. 2017. Repair of a bacterial small β-barrel toxin pore depends on channel width. mBio 8:e02083-16. https://doi.org/10.1128/mBio.02083-16.
22. Fickl H, Cockeran R, Steel HC, Feldman C, Cowan G, Mitchell TJ, Anderson R. 2005. Pneumolysin-mediated activation of NFkappaB in human neutrophils is antagonized by docosahexaenoic acid. Clin Exp Immunol 140:274–281. https://doi.org/10.1111/j.1365-2249.2005.02757.x.
23. Uhlén P, Laestadius A, Jahnukainen T, Söderblom T, Bäckhed F, Celsi G, Brismar H, Normark S, Aperia A, Richter-Dahlfors A. 2000. Alpha-haemolysin of uropathogenic *E. coli* induces Ca^{2+} oscillations in renal epithelial cells. Nature 405:694–697. https://doi.org/10.1038/35015091.
24. Fantini J, Barrantes FJ. 2013. How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains. Front Physiol 4:31. https://doi.org/10.3389/fphys.2013.00031.
25. Das A, Brown MS, Anderson DD, Goldstein JL, Radhakrishnan A. 2014. Three pools of plasma membrane cholesterol and their relation to cholesterol homeostasis. Elife 3:02882. https://doi.org/10.7554/eLife.02882.
26. Pomerantsev AP, Kalnin KV, Osorio M, Leppla SH. 2003. Phosphatidylcholine-specific phospholipase C and sphingomyelinase activities in bacteria of the *Bacillus cereus* group. Infect Immun 71:6591–6606. https://doi.org/10.1128/IAI.71.11.6591-6606.2003.
27. Vadia S, Seveau S. 2014. Fluxes of Ca^{2+} and K^{+} are required for the listeriolysin O-dependent internalization pathway of *Listeria monocytogenes*. Infect Immun 82:1084–1091. https://doi.org/10.1128/IAI.01067-13.