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HAMLET is a complex of human α-lactalbumin (ALA) and oleic acid and kills several Gram-positive bacteria by a mechanism that bears resemblance to apoptosis in eukaryotic cells. To identify HAMLET's bacterial targets, here we used *Streptococcus pneumoniae* as a model organism and employed a proteomic approach that identified several potential candidates. Two of these targets were the glycolytic enzymes fructose bisphosphate aldolase (FBPA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Treatment of pneumococci with HAMLET immediately inhibited their ATP and lactate production, suggesting that HAMLET inhibits glycolysis. This observation was supported by experiments with recombinant bacterial enzymes, along with biochemical and bacterial viability assays, indicating that HAMLET's activity is partially inhibited by high glucose–mediated stimulation of glycolysis but enhanced in the presence of the glycolysis inhibitor 2-deoxyglucose. Both HAMLET and ALA bound directly to each glycolytic enzyme in solution and solid-phase assays and effectively inhibited their enzymatic activities. In contrast, oleic acid alone had little to no inhibitory activity. However, ALA alone also exhibited no bactericidal activity and did not block glycolysis in whole cells, suggesting a role for the lipid moiety in the internalization of HAMLET into the bacterial cells to reach its target(s). This was verified by inhibition of enzyme activity in whole cells after HAMLET but not ALA exposure. The results of this study suggest that part of HAMLET's antibacterial activity relates to its ability to target and inhibit glycolytic enzymes, providing an example of a natural antimicrobial agent that specifically targets glycolysis.

HAMLET[3] (Human alpha-lactalbumin made lethal to tumor cells) consists of human α-lactalbumin (ALA) in a partially unfolded state, stabilized by binding the human milk–specific fatty acid oleic acid (OA; C18:1 *cis*-9) (1). HAMLET was first purified from human milk casein and shown to kill tumor cells by induction of apoptosis, while sparing healthy cells (2, 3). Part of HAMLET's activity is related to its ability to enter cancer cells and interact with mitochondria to induce depolarization and permeability transition that leads to apoptosis execution (4, 5). Using an shRNA-based screen in tumor cells, Svanborg and collaborators (6) demonstrated that glycolytic enzymes were required for full HAMLET activity. It was therefore suggested that HAMLET interferes with glycolysis to mediate a change in cancer cell metabolism as part of its death induction.

HAMLET also kills several species of bacteria (the evolutionary predecessor to mitochondria), with its highest activity against *Streptococcus pneumoniae* (7, 8), an aerotolerant, anaerobic organism that lacks the Krebs' cycle and oxidative phosphorylation and relies primarily on glycolysis and fermentation for energy production (9). Interestingly, akin to features seen in eukaryotic cells, HAMLET-induced death in bacteria is accompanied by depolarization of the bacterial membrane that requires sodium-dependent calcium transport, cell shrinkage, DNA condensation and fragmentation, and other biochemical hallmarks of mitochondria-induced apoptosis (8, 10). These phenotypes are also present when these bacteria die from other physiological stimuli, such as starvation, and represent a novel and general bacterial death mechanism that we are currently exploring. This suggests that physiological cell death pathways in prokaryotic and eukaryotic cells may be more similar than previously considered.

Besides its direct antibacterial activity, HAMLET can also potentiate the effect of antibiotics to render bacterial species sensitive to antibiotics they are resistant to (11, 12). This effect is not limited to species sensitive to HAMLET but can also be seen for antibiotic-resistant organisms of species that are not directly killed by HAMLET (12). HAMLET's potentiating effect was shown to also require ion transport and membrane depolarization, and HAMLET treatment resulted in an increased binding/association of the antibiotics used to the bacterial population treated (12).

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This article contains Figs. S1–S4.

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[3] The abbreviations used are: HAMLET, human α-lactalbumin made lethal to tumor cells; ALA, α-lactalbumin; DLDH, dihydrolipoamide dehydrogenase; FBPA, fructose-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GK, glucokinase (hexokinase); HRP, horseradish peroxidase; OA, oleic acid; PTS, phosphotransferase; LB, Luria–Bertani; OD, optical density; THY, Todd-Hewitt media with yeast extract; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

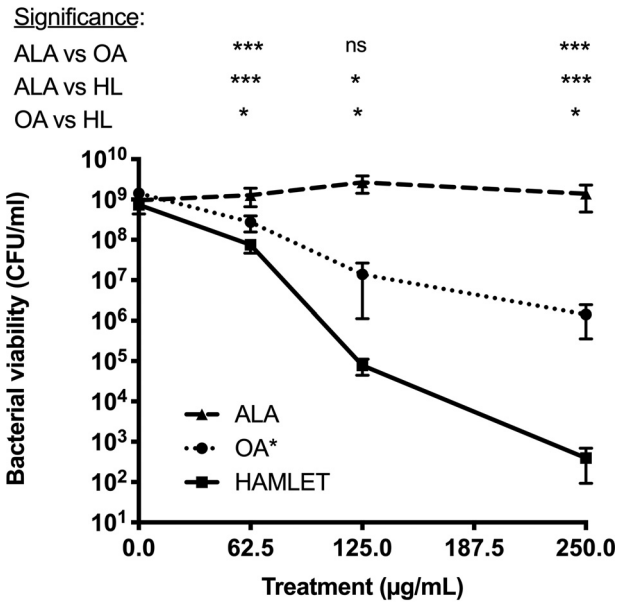

Figure 1. Bactericidal activity of HAMLET, ALA, and oleic acid. *S. pneumoniae* D39 was treated with native ALA and HAMLET at the concentrations indicated or with OA using a concentration equivalent to the concentration present in HAMLET (10%, w/w) and incubated for 1 h at 37 °C. Viable organisms were assessed after plating dilutions of each sample onto blood agar plates and enumerating cfu after overnight growth. The results represent six individual experiments and are presented as the mean with *error bars* representing the S.D. Statistical analysis was performed using Student's unpaired *t* test (*, $p < 0.05$; ***, $p < 0.001$; *ns*, nonsignificant).

In this study, we show that HAMLET disrupts glycolysis in whole pneumococci and that this disruption is partially responsible for, and clearly potentiates, HAMLET's bactericidal activity. Unlike previous studies associating the bactericidal activity of HAMLET with the OA component, this study showed that the protein component (ALA) was responsible for HAMLET's inhibitory effect on glycolysis in whole cells. We show that HAMLET binds to and inactivates two key glycolytic enzymes, fructose-bisphosphate aldolase (FBPA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both *in vitro* and *in vivo*. This inhibition results in an impaired production of ATP that augments activation of HAMLET's cell death mechanism. A detailed understanding of HAMLET's inhibition of glycolysis could provide future leads to therapeutic intervention against a broad range of bacterial species.

Results

Bactericidal activity of HAMLET, ALA, and oleic acid

HAMLET was used to treat *S. pneumoniae* D39 in a time-kill assay over 1 h. This treatment caused a concentration-dependent death of pneumococci with a 3 $\log_{10}$ reduction in bacterial viability at ~100 μg/ml (6 μM) and eradication of the inoculum reached at 250 μg/ml (15 μM). (Fig. 1, *solid line*). As HAMLET is a complex of ALA and oleic acid, we also tested each component separately. Native ALA had no bactericidal activity (Fig. 1, *dashed line*), and we know from previous experience that no death occurs even after exposure to 10,000 μg/ml (7, 10). OA, however, has been shown to have bactericidal activity by itself (13). Measurements of HAMLET/OA stoichiometry consistently show a molar ratio of ~1:5 (14, 15) with OA constituting ~9–11% of the total complex weight. To understand the role of OA in the bactericidal activity of HAMLET, we treated the bacteria with OA at the concentration present in the complex (based on a 10% (w/w) OA content). Although OA had bactericidal activity by itself, the concentration associated with various concentrations of HAMLET complex consistently resulted in significantly lower bactericidal activity than the HAMLET complex itself (Fig. 1, *dotted line*). These results suggest that although lipid is required for HAMLET's bactericidal activity, both ALA and OA are necessary and contribute to bacterial killing.

HAMLET inhibits glycolysis and ATP production in pneumococci

To investigate the role of HAMLET treatment on glycolysis activity and ATP production, D39 pneumococci were washed and resuspended in PBS without any carbon source. At $T = 0$ min, the bacteria were energized by adding 10 mM glucose, and at $T = 5$ min or $T = 10$ min, 125 μg/ml HAMLET (a concentration that resulted in a 4 $\log_{10}$ reduction in viability over 1 h) was added to subsets of energized bacteria, and lactate secretion from the cells and ATP content in the cells were measured over time (Fig. 2, *A* and *B*). HAMLET addition resulted in an immediate and drastic inhibition of both ATP and lactate production over time, resulting in significantly lower ATP accumulation and lactate production in the cells at the 30-min time point (Fig. 2).

To make sure that this was not just an effect of the bacteria dying rapidly in the presence of high concentrations of HAMLET, the cells were treated with sublethal concentrations of HAMLET (25, 37.5, and 50 μg/ml). HAMLET was added 5 min after energizing the cells with glucose, and ATP content of the bacteria was measured (Fig. 2*C*). No effect was seen for the lowest HAMLET concentration, but both 37.5 and 50 μg/ml HAMLET significantly reduced the ATP production in the cells after 30 min of incubation ($p < 0.05$ and $p < 0.01$ for 37.5 and 50 μg/ml, respectively).

Treatment with native ALA had no effect on either ATP or lactate production (Fig. 2, *A* and *B*). Similarly, the addition of 12.5 μg/ml OA (equivalent to the amount of lipid present in 125 μg/ml HAMLET that resulted in a >2 $\log_{10}$ reduction in bacterial viability) did not significantly affect ATP production in the pneumococci (Fig. 2*D*), suggesting that bacterial death induced by OA was not directly associated with inhibition of glycolysis or energy production.

Modulation of glycolysis function affects pneumococcal sensitivity to HAMLET

We next addressed the role of HAMLET on glycolysis by testing HAMLET's activity in the presence of a high concentration of glucose that would accelerate glycolysis or in the presence of 2-deoxyglucose, a glucose analog that competitively inhibits glycolysis as it cannot be hydrolyzed for energy. Bacterial cells in buffer alone (PBS) served as a control. None of the treatments had any impact on bacterial survival by themselves over the 1-h time course of the assay. HAMLET was added at increasing concentrations and incubated for 1 h at

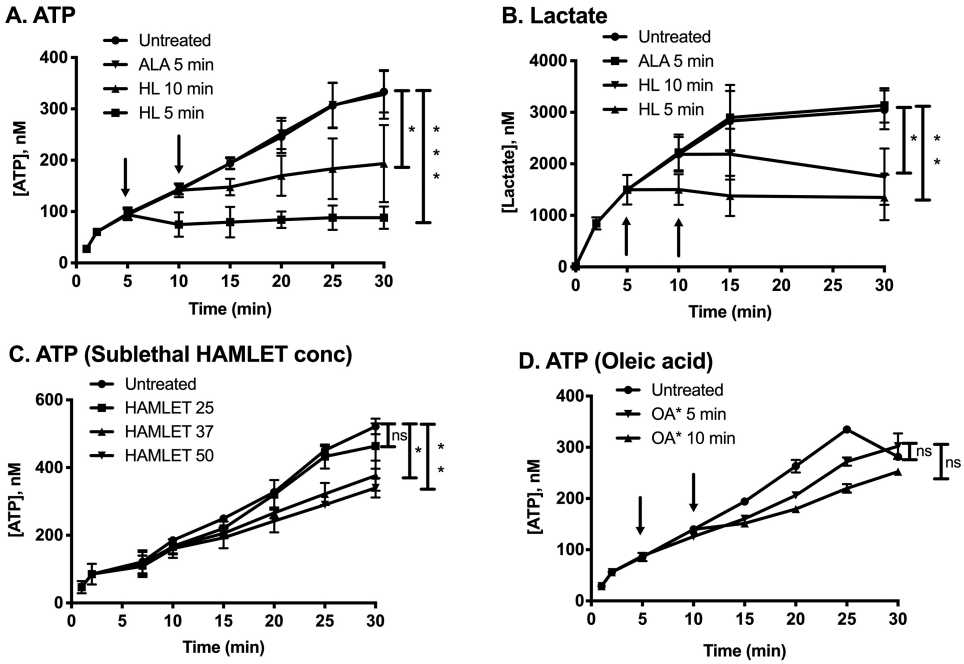

Figure 2. Inhibition of glycolysis and energy production in HAMLET-treated *S. pneumoniae*. *S. pneumoniae* D39 were energized with 10 mM glucose and treated with 125 μg/ml ALA after 5 min or 125 μg/ml HAMLET after either 5 or 10 min (see *arrows*), and intracellular ATP production (*A*) and lactate secretion (*B*) were recorded over time. *C*, D39 pneumococci were energized with 10 mM glucose and treated with sublethal concentrations of HAMLET (25, 37, and 50 μg/ml) that were added 2 min after the addition of glucose. *D*, D39 pneumococci were energized with 10 mM glucose and treated with the OA concentration present in 125 μg/ml HAMLET (12.5 μg/ml) that was added after either 5 or 10 min. All results represent three individual experiments with duplicate samples and are presented as the mean with *error bars* representing the S.D. Statistical analysis was performed using Student's unpaired *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, nonsignificant).

37 °C, and viability was monitored by viable plate counts (Fig. 3*A*). The addition of glucose in the buffer significantly reduced HAMLET-induced death at all concentrations tested, with 1.3 $\log_{10}$ less death occurring at the highest concentration compared with cells treated in the absence of carbohydrate ($p < 0.01$) and 2.7 $\log_{10}$ less death compared with cells treated with the glycolysis inhibitor 2-deoxyglucose ($p < 0.01$), indicating that modulation of glycolysis activity affects HAMLET's bactericidal activity.

Treatment with bactericidal concentrations of OA was not affected by the presence of glucose or 2-deoxyglucose in the medium (Fig. 3*B*), again suggesting that OA-induced death does not act by directly regulating glycolytic activity and energy production in the pneumococcal cells.

HAMLET binds to FBPA and GAPDH

To identify potential bacterial targets responsible for HAMLET's glycolytic inhibition, we first separated bacterial lysate from strain D39 by one-dimensional SDS-PAGE, transferred it to nitrocellulose membrane, and overlaid the blot with biotinylated HAMLET (biotinylation did not affect HAMLET's bactericidal activity, as shown in Fig. S1). Several bands were identified as potential interaction partners for HAMLET (Fig. 4*A*), and the identities of the two strongest bands were determined by N-terminal sequencing using Edman degradation. Only FBPA (31.4 kDa) and GAPDH (35.9 kDa) were possible candidate proteins based on the N-terminal sequence data and on having appropriate molecular mass in the blot (Fig. 4*A*).

In a second approach, biotinylated HAMLET conjugated with NHS-LC-Diazirine, a UV-excitable cross-linker, was added to pneumococcal cell lysate. After incubation, the reactions were cross-linked by UV light, HAMLET and its bound targets were captured on streptavidin-conjugated magnetic beads, and captured proteins were identified by LC-MS/MS. Two independent assays were performed, and the results of the assay confirmed three peptides and one peptide, respectively, with perfect match for FBPA and eight peptides and five peptides, respectively, with perfect match for GAPDH in experiments 1 and 2. As the identification of captured proteins was performed by independent peptide sequencing, the identity will have >99.9% certainty if two or more peptides are identified (ProtTech). In conclusion, both assays identified two central enzymes in glycolysis (FBPA and GAPDH) as potential HAMLET targets.

To ensure a direct interaction between HAMLET and the two glycolytic proteins, pneumococcal, recombinant FBPA and GAPDH were produced and separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with biotinylated HAMLET or ALA. Interestingly, both HAMLET and ALA (which had no effect on cell viability, ATP, or lactate production) bound strongly to FBPA and GAPDH (Fig. 4*B*). Binding of GAPDH and FBPA to HAMLET was also observed in solution after capturing the His-tagged enzymes by magnetic beads (Fig. S2). To confirm that HAMLET's interaction with FBPA and GAPDH was specific, binding assays employing the glycolytic enzyme glucokinase (GK; also known as hexokinase) and dihydrolipoamide dehydrogenase (DLDH; the E3 component of 2-oxo acid dehydrogenases) were performed in solution. No binding was observed to glucokinase; however, some binding was observed to DLDH (Fig. S2).

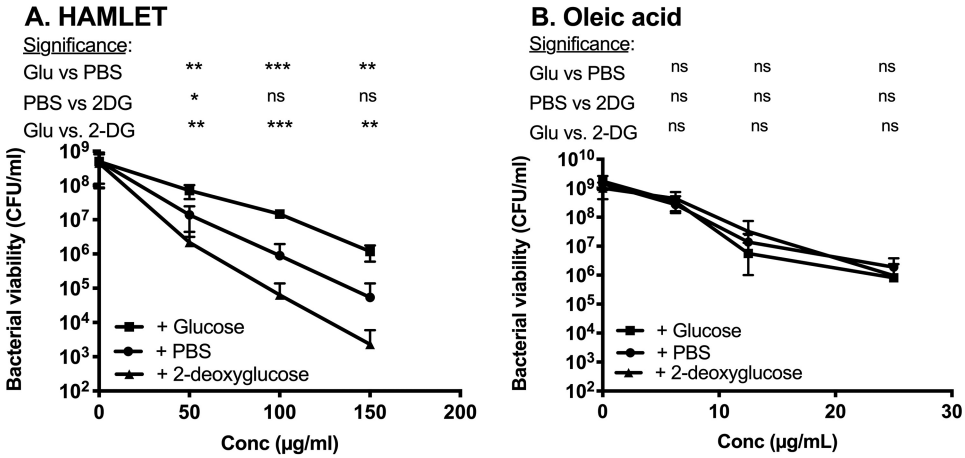

Figure 3. Modulation of glycolysis affects HAMLET sensitivity. D39 pneumococci were washed in PBS and resuspended in PBS alone or PBS with the addition of either 25 mM glucose or 25 mM 2-deoxyglucose. After 15 min, increasing concentrations of HAMLET (*A*) or oleic acid (*B*) were added to the bacterial cells, and viable organisms were determined by viable counts after 1 h. All results represent three individual experiments with duplicate samples and are presented as the mean with *error bars* representing the S.D. Statistical analysis was performed using the Student's unpaired *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, nonsignificant).

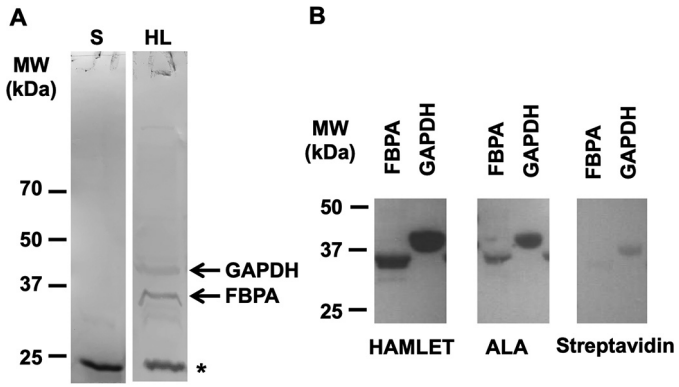

Figure 4. Binding of HAMLET to FBPA and GAPDH. *A*, D39 whole bacterial lysates were separated by SDS-PAGE and blotted to nitrocellulose membrane. The blot was blocked and incubated in the presence of buffer alone (*S*) or biotinylated HAMLET (*HL*), and after the addition of HRP-conjugated streptavidin, binding was detected in the presence of ECL reagents. The two major bands detected were cut out and identified by Edman degradation. *, presence of biotin carboxyl carrier protein (17.0 kDa), a protein that is naturally biotinylated and thus reacts directly with the HRP-conjugated streptavidin and does not represent HAMLET binding. The blots were photographed and are displayed without any image correction. *B*, pneumococcal recombinant FBPA or GAPDH were separated by SDS-PAGE, blotted to nitrocellulose membrane, and overlaid with HAMLET, ALA, or buffer alone (Streptavidin). After incubation with HRP-conjugated streptavidin, binding was detected in the presence of ECL reagents. The blots were captured by a GelDoc system set to automatic mode for optimal capture of signal in each blot image, and blots are presented without image correction.

HAMLET specifically inhibits FBPA and GAPDH enzymatic activity

As HAMLET bound strongly to FBPA and GAPDH, two central glycolysis enzymes, we were interested in investigating whether this binding resulted in inhibition of their activity as a mechanism of blocking ATP and lactate production. Recombinant enzymes were assayed for enzymatic activity in the presence of increasing concentrations of HAMLET, ALA, and OA. IgG was used as an unrelated protein control for comparison (Fig. 5).

HAMLET and ALA both showed an effective inhibition of FBPA activity with the IC_{50} being ~2 μg/ml for both proteins and with over 75% of inhibition seen at a concentration of 8 μg/ml. The inhibitory activity of OA was negligible, with around 5% inhibition seen at the highest concentration tested, which represents 80 μg/ml HAMLET (8 μg/ml OA; Fig. 5*A*). IgG did not inhibit FBPA activity at all (Fig. 5*A*).

For GAPDH, the general inhibitory activity was somewhat lower, with around 50% inhibition seen at 6 μg/ml HAMLET and around 40% inhibition seen for ALA at the same concentration (Fig. 5*B*). OA inhibited GAPDH to a significantly lower degree (22%; $p < 0.01$ compared with HAMLET's inhibitory activity) at the concentration present in the HAMLET complex (Fig. 5*B*). Again, IgG lacked any inhibitory activity. This indicates that HAMLET and ALA show strong inhibitory activity of both enzymes, whereas OA shows only a slight inhibitory activity against GAPDH.

HAMLET did not inhibit the activity of glucokinase even at high concentrations (100 μg/ml), which was consistent with a lack of binding between the two proteins (Fig. S3*A*). Similarly, even though HAMLET bound to DLDH, this binding was not associated with any inhibitory activity even at concentrations of HAMLET of up to 100 μg/ml (Fig. S3*B*), suggesting that interaction *in vitro* does not automatically correlate with inhibitory activity.

HAMLET but not ALA can access its target enzymes in vivo

Although ALA effectively inhibited both FPBA and GAPDH activity *in vitro* (Fig. 5), it had no activity on the production of ATP and lactate when added to whole cells (Fig. 2). This suggested indirectly that HAMLET, but not ALA, may gain entry to the bacterial cells and reach the respective targets to inhibit glycolysis, similar to its ability to traverse the membrane and enter tumor cells (4).

To provide further evidence that HAMLET directly targets the glycolytic pathway intracellularly *in vivo*, we measured FBPA activity in whole-cell extracts from D39 pneumococci pretreated for 10 min with various concentrations of HAMLET and compared the activity with cells treated with ALA or OA (Fig. 6). Cells were treated with HAMLET, ALA, OA, or buffer alone for 10 min at 37 °C and were immediately washed in 4 °C PBS to remove unassociated protein or lipid,

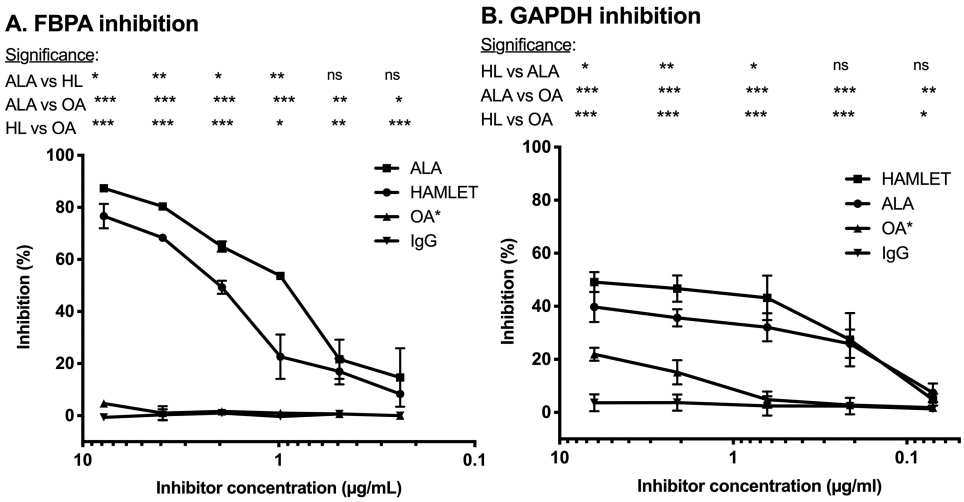

Figure 5. HAMLET and ALA inhibit FBPA and GAPDH activity. Recombinant FBPA (*A*) or GAPDH (*B*) was preincubated with buffer alone or decreasing concentrations of HAMLET, ALA, OA, or mouse IgG for 15 min. At this time, the mixtures were added to reaction buffer, the reaction was started by adding NADH (for FBPA activity) and NAD (for GAPDH activity), and activity was monitored by a decrease and increase, respectively, in the absorbance at 340 nm. The results are presented as the percentage activity compared with the buffer-alone control. All results represent three individual experiments with duplicate samples and are presented as the mean with *error bars* representing the S.D. Statistical analysis was performed using Student's unpaired *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, nonsignificant).

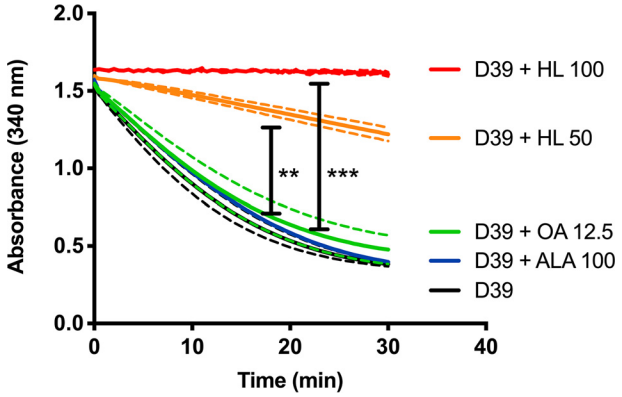

Figure 6. HAMLET inhibits FBPA activity in whole cells. D39 pneumococci were exposed to buffer alone (*D39*), ALA (100 μg/ml), HAMLET (50 or 100 μg/ml), or OA (12.5 μg/ml) for 10 min; bacterial cells were washed in precooled buffer; and pneumococcal lysates were produced at 4 °C and tested for FBPA activity presented as the breakdown of NADH at 340 nm over time. The results represent three individual experiments with duplicate samples and are presented as the mean (*center line*) with S.D. depicted as the *hatched lines surrounding* the *center line*. Statistical analysis was performed by comparing the enzymatic activity (in milliunits/mg of lysate) over the first 10 min with Student's unpaired *t* test (**, $p < 0.01$ when comparing D39 lysate with lysate in the presence of 50 μg/ml HAMLET; ***, $p < 0.001$ when comparing D39 lysate with lysate in the presence of 100 μg/ml HAMLET).

and bacterial lysates were produced. Lysates from cells treated with ALA showed no significant change in FBPA activity compared with untreated D39 pneumococci (FBPA activity of 421 and 408 milliunits/mg for lysates from ALA-treated and untreated bacteria, respectively). However, pretreatment of D39 pneumococci with HAMLET for 10 min resulted in a significant reduction in FBPA activity in treated lysates (94 and 2.1 milliunits/mg in extracts from bacteria treated with 50 and 100 μg/ml of HAMLET, respectively). Similar to its inability to inhibit FBPA activity *in vitro* (Fig. 5*A*), OA had no inhibitory activity on FBPA activity in this assay at the concentrations present in the HAMLET complex (396 milliunits/mg at 12.5 μg/ml OA). The results suggest that HAMLET can traverse the membrane and access the glycolytic machinery associated with the inner leaflet of the membrane (16), which ALA cannot access.

Discussion

In this study, we show for the first time that the antimicrobial molecule, HAMLET, targets glycolysis as part of its antibacterial activity. Although this study was limited to the bacterial pathogen *S. pneumoniae*, the results are likely more generally applicable to Gram-positive bacteria. HAMLET is known to have direct bactericidal activity against several Gram-positive organisms as well as having the ability to sensitize a broader array of both Gram-positive and Gram-negative organisms to antibiotics (7, 11, 12). In all of these organisms, HAMLET treatment induces identical initial changes, such as membrane depolarization and ion transport, suggesting a conserved activation pathway. Based on this pathway conservation and the fact that the pneumococcal targets identified in this study are highly conserved between bacterial species, it is likely that the mechanisms described in this study are also present in other HAMLET-sensitive bacteria. This is interesting from several perspectives.

As HAMLET also targets glycolysis in tumor cells (but not in healthy cells (6, 17)), this suggests a conserved mechanism that induces death in such diverse cells as bacteria and eukaryotes. Although the specific glycolytic enzymes targeted by HAMLET in this study (FBPA and GAPDH) and the study by Storm *et al.* (6) (hypoxia-inducible factor 1α, hexokinase, and 6-phospho-2-fructokinase/fructose-2,6-bisphosphatase) were not identical, this merely emphasizes the complexity and potential divergence through evolution of glycolytic regulation. In eukaryotes, glucose is imported primarily through GLUT transporters, which renders the hexokinase especially prone to regulation by glucose levels in the blood in conjunction with glucose-6-phosphate levels in the cell. Also, the irreversible enzyme phospho-

fructokinase is regulated both by its substrate and cAMP levels in the cell.

In Gram-positive bacteria, regulation is somewhat different. The majority of glucose enters cells through carbohydrate phosphoenolpyruvate:phosphotransferase (PTS) systems, where glucose upon import is phosphorylated directly into glucose-6-phosphate, thus circumventing hexokinase (18, 19). Regulation of hexokinase therefore is of less importance. Instead, components of the PTS system, such as HPr, as well as metabolites, such as fructose-2,6-bisphosphate and glucose-6-phosphate, regulate catabolic activity through transcriptional regulation of genes using the major transcription factor CcpA (20). Both HAMLET targets in this study, FBPA and GAPDH, are central and essential enzymes in glycolysis in many bacterial species, including pneumococci, and at least GAPDH's function is regulated indirectly through CcpA and, at least in *Staphylococcus aureus*, through the CcpA-dependent factor GapR (21, 22). Although the regulatory framework is different in eukaryotes and bacteria, HAMLET still targets glycolysis in both systems, and it is possible that findings from the less complex bacterial system can provide leads to potential mechanisms involved also in tumor cell death, and vice versa.

Furthermore, glycolysis as a target for tumor cell death has become a topic of rising interest during the last couple of years with the renaissance of the Warburg effect and its role in cancer metabolism (23, 24). Otto Warburg described already in the 1920s that tumor cells are more glycolytically active than healthy cells and showed that tumor cells exhibited increased fermentation rather than aerobic respiration even when oxygen was present in abundance (aerobic glycolysis) (25, 26). Interference with glycolysis would provide a selective advantage in targeting tumor cells and is therefore a topic of great interest (27, 28). Inhibition of glycolysis by compounds such as 2-deoxyglucose can inhibit growth of tumor cells, and its therapeutic potential has been investigated in the treatment of certain solid tumors (29, 30). Targeting glycolysis and its central enzymes could also be effective for a multitude of bacterial species, such as *S. pneumoniae,* that produce energy primarily from glycolysis and fermentation (9, 31). Based on the structural and functional differences of glycolytic enzymes between eukaryotes and bacteria, especially with regard to FBPA (32, 33), screening for potential molecules that target the activity of bacterial FBPAs from several different species has been conducted *in vitro* (34–37), but no such efficacy tests have so far been done using whole bacteria. The known difference in structure between eukaryote and prokaryote FBPA and an evolutionary divergence in GAPDH genes between most eukaryotes and bacteria (38) suggest an explanation for the specific targeting of these enzymes by HAMLET only in bacteria. The structural motifs involved in HAMLET binding and its ensuing inhibition of enzymatic activity are not known but are worthy of future study, as they could provide clues for improved future therapeutic strategies, especially taking into account the high level of conservation of glycolytic enzymes in bacteria affected by HAMLET treatment.

Pneumococci lack the Krebs cycle and oxidative phosphorylation, and thus, the absolute majority of ATP is produced from glycolysis and fermentation of carbohydrates obtained from the harsh environment of the host (31). Engagement primarily of glycolysis is also true for many other facultative anaerobes, many of which are sensitive to HAMLET's direct bactericidal effects or its ability to potentiate the activity of antibiotics (7). These carbohydrates enter the bacterial cells through their many and effective transport systems and eventually feed into glycolysis. One of the vital roles of ATP is to maintain a polarized membrane, through ATP-driven transporters that extrude or import ions, such as sodium, potassium, hydrogen, and calcium, to retain a gradient over the membrane, resulting in the production of an optimal membrane potential and proton motive force. These gradients are then used to promote transport of substrates, volume regulation, and other aspects of cell function and integrity.

The mechanism of HAMLET-induced death of tumor cells and bacteria is not fully characterized. However, we know that HAMLET causes depolarization of the bacterial and mitochondrial membranes and that HAMLET-induced death requires an influx of calcium in both systems (5, 8, 10). By inhibiting ATP production from glycolysis, it is likely that HAMLET sensitizes the bacteria by disrupting their ability to maintain membrane polarity and specific ion gradients that, when dissipated, will activate a downstream cascade of signaling, including kinase and protease activity, that results in apoptosis-like morphological features in pneumococcal cells (10). Additionally, reduced ATP production also has the potential to both increase the membrane permeability to antibiotics as a result of dissipation of membrane polarity and may also decrease activity of drug efflux pumps. These processes, combined, could explain the increased activity of antibiotics in pneumococci as well as other bacteria treated with sublethal doses of HAMLET, leading to accumulation or increased association of antibiotics with bacterial cells (11, 12).

This study emphasized a crucial role of the protein component of the HAMLET complex. In this study, we found that the protein component *per se* is crucial for the glycolytic inhibition. Both HAMLET and native ALA effectively inhibited the activity of both FBPA and GAPDH *in vitro*, whereas OA had no activity. However, in most mechanistic studies performed to date, we and others have shown that the oleic acid component alone has the ability to induce bactericidal activity, just like HAMLET, albeit at a higher concentration than found within the HAMLET complex (see Fig. 1 for death induction and Refs. 39 and 40). A better understanding of the role of the lipid and protein components of the HAMLET complex during bacterial death or for increased activity during combination treatment with antibiotics is therefore of great interest. The prior studies indicate that HAMLET provides a way to effectively solubilize and/or present the lipid to targets in the bacterial cell, most likely the bacterial membrane. This effect is not confined to oleic acid, as it has been shown that active HAMLET complex can be made with other long-chained, unsaturated fatty acids in the *cis*-configuration, such as vaccenic (C18:1 *cis*-11) linoleic (C18:2 *cis*-9, 12), and linolenic acid (C18:3 *cis*-9, 12, 15) (41). These fatty acids, as well as other shorter-chained fatty acids, have long been known to have general bacteriostatic or bactericidal activity in and of themselves against several bacterial

species (42), albeit at higher concentration than found in the HAMLET complex.

At the same time, HAMLET's effect on cell membrane polarity and ion transport is reminiscent of other antimicrobial peptides, such as defensins, lantibiotics, and some antibiotics of the lipopeptide and glycopeptide classes. These agents associate directly with the bacterial membrane to induce lysis, and some peptides have been suggested to translocate into the cytoplasm and have intracellular targets (43, 44). However, they also target cell wall and peptidoglycan biosynthesis through direct inhibition of biosynthetic enzymes or binding to lipid II or bactoprenol-coupled cell wall precursors (45–48). The identified cell wall targets of these antimicrobial peptides, such as MraY, MurM, PBP2, LytR, and others, are conserved in pneumococci (49, 50), but their role in HAMLET-induced death is not known. Preliminary studies in our laboratory have shown an initial interaction of HAMLET with the pneumococcal cell wall that appears to be required for subsequent downstream effects. The role of these interactions both in HAMLET's bactericidal activity and in HAMLET translocation into the bacterial cell will be of great interest for future studies.

As ALA had no activity when added exogenously, this suggested that only when ALA is bound to OA in the correct conformation does it have activity, which is most likely correlated with its ability to associate with membranes (51) that promote internalization into the bacteria, similar to some other antimicrobial peptides (43). This is very similar to the internalization of HAMLET into tumor cells (4), where HAMLET is found to co-localize primarily with mitochondria. The efficiency of internalization could not be determined in this study, as intracellular visualization of HAMLET could not be observed directly due to membrane association. Still, considering that the IC_{50} *in vitro* of both FBPA and GAPDH was ~1–2 μg/ml (60–120 nM), which was 50–100 times lower than the concentration added exogenously, this provides a physiological possibility that inhibitory amounts of HAMLET will reach its targets. Additionally, the inhibitory effect of HAMLET was stronger when HAMLET was added exogenously to pneumococci than when added in combination with each enzyme alone *in vitro*. It has been shown by MS analysis after in-solution digestion that the glycolytic enzymes in pneumococci bind to each other and are clustered together in a complex associated with the pneumococcal membrane (52, 53). Thus, the binding of HAMLET simultaneously to both FBPA and GAPDH present in a glycolytic complex inside the bacterial cell may lead to more effective inhibition of glycolysis than the interaction with either enzyme alone *in vitro*.

As both FBPA and GAPDH are essential proteins in pneumococci (54, 55) as well as other organisms (56–59), we were not able to produce mutants lacking one or the other protein to better understand the mechanistic aspects of HAMLET's interaction with either enzyme inside the cells and the impact of that interaction on glycolysis inhibition. As we have also been unable over the years to produce HAMLET-resistant bacterial mutants in the laboratory, this approach to gain a better understanding of the mechanisms involved in glycolysis inhibition was also not possible.

In conclusion, this study shows that HAMLET's bactericidal activity is partly dependent on inhibition of glycolysis and ATP production, similar to what has been described in tumor cells. HAMLET inhibits ATP production from glycolysis by entering the bacterial cell and interacting with and inhibiting the activity of the glycolytic enzymes FBPA and GAPDH. HAMLET's inhibitory effect on glycolysis in whole cells is associated with the ALA component of the complex, as OA had no effect on either ATP production in whole cells or glycolysis enzyme inhibition either *in vitro* or in whole cells. This is different from most other studies that have identified oleic acid as the cytotoxic component of the complex. Still, the lipid component of the complex was required for its inhibitory activity in whole bacterial cells, emphasizing the role of both ALA and oleic acid in the bactericidal mechanism. Agents that inhibit glycolysis and other energy-producing systems in bacterial cells represent a novel class of antimicrobial agents in this era of rapidly increasing antibiotic resistance development. Importantly, they offer novel and promising therapeutic potential against targets where there are no existing resistant mechanisms.

Experimental procedures

Reagents

Protein markers were from Thermo Fisher Scientific, and enhanced chemiluminescence substrate was from GE Healthcare. Plasmid purification reagents, expression vectors, nickel-nitrilotriacetic acid–agarose, and anti-RGS-His antibody were from Qiagen (Valencia, CA). Anti-ALA antibody was from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated anti-IgG antibody and magnetic Dynabeads conjugated with streptavidin or histidine were from Invitrogen, Inc. Bacto-Todd Hewitt medium and Bacto-yeast extract were from BD Biosciences. Remaining reagents were from Sigma.

HAMLET was produced as described (1) by complexing EDTA-treated ALA, purified from human milk, with oleic acid on an anion-exchange matrix (see Fig. S4 for the staining pattern of HAMLET and ALA after separation by SDS-PAGE). Lipid content of the HAMLET batch used was tested with the free fatty acid quantitation kit (Sigma), resulting in a stoichiometry of 6.1 molecules of oleic acid per molecule of ALA, representing 10.8% of the complex (w/w).

Bacterial strains

The bacterial strains, plasmids, and primers used for the study are listed in Table 1. *S. pneumoniae* strain D39 (60) was used throughout the study. Pneumococci were stored at −80 °C in the presence of 15% glycerol and grown at 37 °C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or on tryptic soy agar supplemented with 5% sheep blood, as appropriate.

Escherichia coli XL1-Blue (Stratagene/Agilent Technologies, Santa Clara, CA) was used for cloning of recombinant protein constructs, and *E. coli* M15 (Qiagen, Valencia, CA) was used to express recombinant proteins. Ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added to Luria–Bertani (LB) growth medium and LB agar plates to provide required selection, when appropriate.

Table 1
Strains and primers used in this study

	Description	Reference
Strains		
D39	Pneumococcal serotype 2 WT strain	Ref. 60
XL-1 Blue	*E. coli* strain used for cloning	Stratagene
M15	*E. coli* strains used for recombinant protein expression	Qiagen
Plasmids		
pQE30	*E. coli* expression vector	Qiagen Inc.
pSH-rDLDH	pQE30 vector containing fullength *dldh* sequence	Ref. 62
pHH-rFba	pQE30:: XbaI-HindIII Fba-pQE-F/R PCR product	This study
pHH-rGapA	pQE30::BamHI-HindIII Gap-pQE-F/R PCR product	This study
pHH-rGki	pQE30::BamHI-PstII Gki-pQE-F/R PCR product	This study
Primers		
Fba-pQE-F	TGCTCTAGAGGGTGCTGCTAAATACA	This study
Fba-pQE-R	CCCAAGCTTGTGTTAACGTTAACTTTGG	This study
Gap-pQE-F	CGCGGATCCGTAGTTAAAGTTGGTATTAACG	This study
Gap-pQE-R	CCCAAGCTTTTATTTAGCAATTTTTGC	This study
Gki-pQE-F	CGCGGATCCATGAGTCAAAAGATTATTGG	This study
Gki-pQE-R	CCCCTGCAGTTATTGCAATACAAGTGATG	This study

Bacterial viability testing

Pneumococci were grown to an optical density ($OD_{600\ nm}$) of 0.6, pelleted, and washed twice in PBS, pH 7.2, by centrifugation at 8,000 × *g* for 2 min. Bacteria were resuspended in PBS and preincubated with buffer alone, glucose, or 2-deoxyglucose for 15 min prior to the addition of HAMLET, ALA, or oleic acid. HAMLET and ALA are soluble in buffer up to concentrations of 50 mg/ml, whereas solubility of OA is limited in water. To improve the solubility of OA, OA was first dissolved in ethanol at a concentration of 10 mg/ml, and this stock solution was used to further dilute OA in PBS for experiments. After 1 h, 10-fold dilutions of bacterial suspension were plated on blood agar plates. Bacterial viability was measured by plate counts from plates grown overnight at 37 °C and measured as cfu/ml.

Expression of recombinant proteins

Recombinant DLDH was produced from M15 *E. coli* harboring the pQE30 plasmid containing the full-length *dldh* sequence (SPD_1026 in the D39 genome (61)), as described (62). The *fba* (SPD_0526), *gapA* (SPD_1823), and *gki* (SPD_0580) genes were amplified by PCR using primer pairs Fba-pQE-F/Fba-pQE-R, Gap-pQE-F/Gap-pQE-R, or Gki-pQE-F/Gki-pQE-R, respectively (Table 1) and chromosomal DNA from *S. pneumoniae* D39 (27) as template. The amplified fragments were cloned into the pQE30 expression vector and transformed into XL-1 Blue cells. Clones carrying the insert of interest were selected on LB agar containing ampicillin (100 μg/ml) and verified by restriction digestion and sequencing of purified plasmid. Verified plasmids were then transformed into *E. coli* M15 cells for protein expression.

Overnight cultures of M15 cells carrying each of the four enzymes were used to seed fresh cultures, and the cultures were grown shaking at 37 °C to an $OD_{600\ nm}$ of 0.5. Expression of plasmid-borne protein sequences was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h, and the overexpressed proteins were purified from bacterial lysates by affinity chromatography using nickel-nitrilotriacetic acid–agarose (Qiagen) according to the manufacturer's instructions. The proteins were analyzed by SDS-PAGE (see Fig. S4) and Western blotting and were quantified using the BioTek Synergy 2 plate reader with the Take3 microdrop addition (Biotek, Winooski, VT).

Binding assays and Western blotting

For binding assays in solution, recombinant His-tagged enzymes were produced as described above, and 3.25 μg of rFBPA and 6.5 μg of the remaining three enzymes was incubated with 5 μg of HAMLET in a total volume of 0.5 ml. After a 30-min incubation at room temperature, 20 μl of His-coupled magnetic beads (Dynabeads, Invitrogen) were added, the beads were collected by a magnet and washed five times in PBS, and beads were boiled and supernatant was run by gel electrophoresis and blotted as below.

Protein sources (1–4 μg of purified enzymes or 10 μg of bacterial lysate, as determined by the Bradford assay) were run on 4–12% BisTris gels (Invitrogen) under denaturing conditions, and the gels were electroblotted to a nitrocellulose membrane (GE Healthcare) using transfer buffer (Invitrogen) for 1 h. Blotted membranes were blocked in PBS containing 0.05% Tween-20 (PBS-T) with 2.5% skim milk for 1 h at room temperature and washed three times with PBS-T (PBS containing 0.2% Tween 20). To detect HAMLET binding in solution, membranes were incubated both with anti-ALA antibody (1:500 dilution) to detect the capture of HAMLET by the recombinant proteins and with anti-His antibody (1:1,000 dilution) to ensure the capture of the recombinant protein by the beads. The blots were further incubated with rabbit-anti-mouse IgG (His blot) or goat-anti-rabbit IgG (ALA blot) antibody conjugated with HRP for detection. Detection of bands on each blot was done using a GelDoc EZ system (Bio-Rad) with the Image Lab 6 software, set to automatic mode for optimal capture of signal in each blot image.

To determine binding on solid phase, membranes were incubated with biotinylated HAMLET or ALA (5 μg/ml), washed in PBS-T, and further incubated with streptavidin conjugated with HRP (1:5,000 dilution in PBS-T) for 1 h at room temperature. After washing, the membrane was developed as described above.

Capture compound MS

HAMLET (1 mg in a 200-μl volume) was sequentially labeled with NHS-LC-Diazirine, a UV-excitable cross-linker, and amine-reactive biotin from the EZ-link Sulfo-NHS-LC biotinylation kit (both from Pierce/Thermo Scientific) according to the manufacturer's instructions. After quenching with 10 mM Tris-HCl, pH 8, to terminate the reaction, HAMLET carrying biotin and cross-linker was purified over a Zeba desalting column, and the final protein concentration was determined using a BioTek Synergy 2 plate reader with the Take3 microdrop addition (Biotek).

D39 pneumococci (10 ml) were grown in THY to an $OD_{600\ nm}$ of 0.6, and the bacteria were pelleted by centrifugation at 1,500 $\times$ *g* for 10 min, washed once in PBS, and resuspended in 900 μl of PBS. Bacteria were lysed by the addition of 100 μl of 4% sodium deoxycholate and incubation for 5 min at room temperature, and the resulting lysate was purified through a Zeba desalting column (Thermo Fisher Scientific). The protein concentration of the final eluate was determined as above.

Bacterial lysate (1 mg in 100 μl) was incubated with tagged HAMLET carrying cross-linker and biotin (12.5 μg in 50 μl) for 30 min on ice. Proteins in the vicinity of HAMLET were cross-linked by exposing the sample to UV light (365 nm) for 10 min, and the reaction was incubated with 50 μl of Streptavidin-coupled magnetic beads (Dynabeads, Invitrogen), collected by a magnet, and washed five times in PBS. Captured proteins were identified by nano-LC-MS (Prot Tech, Norrisville, PA).

Enzyme assays

For ATP and lactate production, bacteria were grown in THY to an $OD_{600\ nm}$ of 0.6, pelleted, washed twice in PBS by centrifugation at 8,000 $\times$ *g* for 2 min, and resuspended in PBS to the original volume. After treatment, bacterial ATP production was determined using the ATP determination kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions with slight modification. To determine intracellular ATP concentrations, 10 μl of bacteria were added to 90 μl of reaction buffer containing 0.5% Triton X-100 required to lyse the bacteria, and the luciferin conversion was measured by chemiluminescence as a measure of ATP in the sample using a Synergy 2 plate reader (Biotek). Triton X-100 was used to lyse bacteria, as both SDS and deoxycholate inhibited the luciferase in the assay as has been described previously (63).

Lactate produced by the bacteria was determined essentially as described (64) by measuring the production of NADH in Reaction 1.

$$NAD^+ + \text{lactate} \leftrightarrow NADH + H^+ + \text{pyruvate}$$

Reaction 1

Bacteria grown as described above were treated, 250 μl of bacteria were pelleted per sample, and the supernatant containing lactate was immediately saved at −20 °C until further analysis. To determine lactate, 100 μl of sample was mixed with 200 μl of reaction buffer (100 mM potassium phosphate (pH 9), 320 mM phenyl hydrazine, 2.4 mM NAD^+, and 2 units/ml L-lactate dehydrogenase), and the absorbance of formed NADH was measured at 340 nm in a Synergy 2 plate reader (Biotek). The concentration of lactate was determined from a standard curve of known concentrations of sodium lactate determined on the same plate.

FBPA (EC 4.1.2.13) activity was determined as described (65) by measuring the breakdown of NADH in the linked reaction presented in Reactions 2–4, where FBPA catalyzes Reaction 2, and Reactions 3 and 4 are catalyzed by triose-phosphate isomerase (EC 5.3.1.1) and α-glycerophosphate dehydrogenase (EC 1.1.1.8), respectively.

$$\text{D-Fructose1,6-phosphate} \leftrightarrow \text{glycerone phosphate} + \text{D-glyceraldehyde3-phosphate}$$

$$\text{D-Glyceraldehyde3-phosphate} \leftrightarrow \text{glycerone phosphate}$$

$$\text{Glycerone phosphate} + NADH + H^+ \leftrightarrow \textit{sn}\text{-glycerol3-phosphate} + NAD^+$$

Reactions 2–4

Recombinant FBPA was mixed with reaction buffer (50 mM Tris-HCl (pH 8), 100 mM KAc, 0.4 mM NADH, 5 mM fructose 1,6-phosphate, 0.6 units each of triose-phosphate isomerase and α-glycerophosphate dehydrogenase). The activity was measured as a decrease in absorbance at 340 nm in a Synergy 2 plate reader (Biotek) and quantified in units (conversion of substrate in μmol min^{-1}) per mg of enzyme using an extinction coefficient of 6.22 mM^{-1} cm^{-1} of NADH at 340 nm. For inhibition studies, recombinant FBPA was preincubated with various concentrations of HAMLET or other potential inhibitor compounds for 15 min prior to initiating the reaction.

GAPDH (EC 1.2.1.12) activity was measured according to Cori *et al.* (66) by measuring the production of NADH in Reaction 5.

$$\text{D-Glyceraldehyde3-phosphate} + NAD^+ + P_i \leftrightarrow \text{3-phospho-D-glyceroyl phosphate} + NADH + H^+$$

Reaction 5

Recombinant GAPDH was mixed with reaction buffer (15 mM potassium pyrophosphate and 30 mM sodium arsenate set to pH 8.5 with 10% phosphoric acid, 1.25 mM NAD^+, 3.3 mM DTT, and 1 mM D-glyceraldehyde-3 phosphate). Free D-glyceraldehyde 3-phosphate was prepared from a diethyl acetate barium salt, where the diethyl acetate barium was cleaved off on a DOWEX resin as described by the manufacturer (Sigma, product G5376). The activity was measured as an increase in absorbance at 340 nm in a Synergy 2 plate reader (Biotek), and activity was quantified in units/mg of enzyme using an extinction coefficient of 6.22 mM^{-1} cm^{-1} of NADH at 340 nm. For inhibition studies, recombinant GAPDH was preincubated with various concentrations of HAMLET or other potential inhibitor compounds for 15 min prior to starting the reaction.

GK (EC 2.7.1.1) was determined by measuring the production of NADH in the linked reaction presented in Reactions 6 and 7, where glucokinase catalyzes Reaction 6 and Reaction 7 is catalyzed by glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (67).

$$\text{D-Glucose} + \text{ATP} \leftrightarrow \text{D-glucose6-phosphate} + \text{ADP}$$

$$\text{D-Glucose6-phosphate} + \text{NAD}^+ \leftrightarrow$$

$$\text{6-phospho-D-glucono-1,5-lactone} + \text{NADH} + \text{H}^+$$

Reactions 6 and 7

Recombinant GK was mixed with reaction buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$, 20 mM NAD^+, 1 mM D-glucose, 1 mM ATP, and 10 units/ml glucose-6-phosphate dehydrogenase). The activity was measured as an increase in absorbance at 340 nm in a Synergy 2 plate reader (Biotek), and activity was determined in units/mg of enzyme using the extinction coefficient of 6.22 mM^{-1} cm^{-1} of NADH at 340 nm. For inhibition studies, recombinant GK was preincubated with various concentrations of HAMLET or other inhibitors for 15 min prior to starting the reaction.

Recombinant DLDH activity was measured as the oxidation of NADH in the presence of 6,8-thioctic acid amide (lipoamide), visualized as decreased absorbance at 365 nm according to Reaction 8 as described (62). Activity was determined in units/mg of enzyme using the extinction coefficient of 3.29 mM^{-1} cm^{-1} of NADH at 365 nm. For inhibition studies, recombinant DLDH was preincubated with various concentrations of HAMLET or other inhibitors for 15 min prior to starting the reaction.

$$\text{NADH} + \text{H}^+ + \text{lipoamide} \leftrightarrow \text{NAD}^+ + \text{dihydrolipoamide}$$

Reaction 8

Statistical analysis

Group comparisons were examined with the unpaired Student's *t* test using Prism version 8 software (GraphPad Software, Inc., La Jolla, CA).

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