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Identification of an intracellular M17 family leucine aminopeptidase that is required for virulence in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a highly virulent bacterial pathogen capable of causing a variety of ailments throughout the human body. It is a major public health concern due to the continued emergence of highly pathogenic methicillin resistant strains (MRSA) both within hospitals and in the community. Virulence in *S. aureus* is mediated by an array of secreted and cell wall associated virulence factors, including toxins, hemolysins and proteases. In this work we identify a leucine aminopeptidase (LAP, *pepZ*) that strongly impacts the pathogenic abilities of *S. aureus*. Disruption of the *pepZ* gene in either Newman or USA300 resulted in a dramatic attenuation of virulence in both localized and systemic models of infection. LAP is required for survival inside human macrophages and gene expression analysis shows that *pepZ* expression is highest in the intracellular environment. We examine the cellular location of LAP and demonstrate that it is localized to the bacterial cytosol. These results identify for the first time an intracellular leucine aminopeptidase that influences disease causation in a Gram-positive bacterium.

Keywords

Leucine aminopeptidase; LAP; virulence; peptidase; staphylococcus

1. Introduction

Aminopeptidases constitute a diverse set of proteolytic enzymes that selectively remove amino acids from the N-terminus of proteins [1]. Traditionally the role of aminopeptidases falls into one of two general categories; nitrogen metabolism or protein activation/

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degradation [1]. In many species, in particular lactic acid bacteria, aminopeptidases degrade exogenous peptides, which are imported into the cell and subsequently used as a nitrogen source [2, 3]. Alternatively, recent work has demonstrated that aminopeptidases play crucial roles in the processing of self-derived proteins. By liberating amino acids from the N-terminus of proteins, aminopeptidases perform key steps in a variety of activation/inactivation pathways [4–8]. In recent years it has begun to emerge that aminopeptidases can also play an important role in pathogenesis. Virulence-associated aminopeptidases are typically secreted and play a role outside the cell in a variety of bacterial and protozoan pathogens, demonstrating that these enzymes possess a far more dynamic range of functions than previously thought [9–15].

Aminopeptidases are subdivided into functional categories or families based on the residues/chemical groups involved in catalysis and substrate specificity [16]. One family that has come under intensive study recently is the M17 family of metallo-peptidases. M17 family members are leucine aminopeptidases (LAPs) found throughout nature in animals, plants and microbes [17]. LAPs are highly conserved at the amino acid level and monomers (typically 53–55 kDa) assemble into a homo-hexameric enzyme. While many LAPs demonstrate a preference for catalyzing the hydrolysis of leucine residues, others exhibit a broader substrate range. Studies of M17 family members have increased in recent years due to their emergence as potential candidates for vaccine development and therapeutic intervention in a number of protozoan and parasitic diseases. Furthermore, in bacteria, even though LAPs have only been studied in a handful of species, it has begun to emerge that in many organisms, LAPs are involved in virulence. Interestingly, in a number of Gram-negative bacteria (*E. coli*, *S. typhimurium*, *V. cholera* and *P. aeruginosa*) LAPs have acquired DNA binding abilities (in addition to aminopeptidase activity), and are functional as transcriptional regulators [18–21]. While in these cases it is through DNA binding/transcriptional regulation that LAP affects virulence, this is not always the case. In the dental pathogen *Treponema denticola*, LAP is involved in the production of H₂S, which plays a role in periodontal disease [22]. In this case LAP does not bind DNA, but rather it is the aminopeptidase activity of the enzyme that modulates virulence. Despite these emerging roles for aminopeptidases a large number of them remain uncharacterized and poorly understood. This is especially true in the Gram-positive bacteria. LAPs have been identified in *Bacillus kaustophilus* and *Bacillus thuringiensis*, however their functions are not well characterized [23, 24]. Limited evidence from *Bacillus thuringiensis* suggests that LAP may play a role in activation of the Cyt1Aa toxin.

Staphylococcus aureus is a highly successful pathogen of humans. Its virulence is mediated by an array of secreted and cell wall associated factors, with secreted proteases being amongst the best-studied [25–28]. While a great deal of research has focused on extracellular proteolytic enzymes as virulence factors, relatively little is known about the role of intracellular proteases, including aminopeptidases, in this or other Gram-positive bacteria. In this work we perform a screen of intracellular proteases, and identify an M17 LAP as being required for virulence in *S. aureus*. Importantly, this is the first identification of an intracellular aminopeptidase required for virulence in a Gram-positive bacterium. Using a variety of infection models we demonstrate that strains containing a mutation in the *pepZ* gene (which encodes LAP) are severely attenuated in virulence. LAP is also required for survival inside human macrophages and gene expression analysis shows that *pepZ* expression is highest in the intracellular environment. In addition, we demonstrate that LAP is not expressed or required for growth in peptide rich media, suggesting that its function is not related to nutrition, but rather plays other important roles within the bacterial cell that contribute to pathogenesis.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All bacterial strains, plasmids and primers used in this study are listed in Table 1. Routinely, *E. coli* was grown in LB and *S. aureus* in TSB, both at 37°C with shaking. For growth assays, *S. aureus* cultures were synchronized as described previously [29]. Antibiotics were used at the following concentrations for *S. aureus*; chloramphenicol 5 µg ml⁻¹, tetracycline 5 µg ml⁻¹, erythromycin 5 µg ml⁻¹, lincomycin 25 µg ml⁻¹ and for *E. coli*; ampicillin 100 µg ml⁻¹.

2.2. Construction of *pepZ* mutant

Mutation of the *pepZ* gene was carried out as described previously [28]. Briefly, an internal fragment of *pepZ* ranging from nucleotides 76–565 of the gene was PCR amplified using primer pair OL461/OL462. This was cloned into the suicide vector pAZ106 using restriction enzymes XbaI and BamHI (Table 1). The resulting plasmid (pLES244) was transformed into *S. aureus* strain RN4220 to generate strain LNS245, with clones selected for based on plasmid encoded erythromycin resistance, and confirmed by PCR analysis. A representative clone was used to generate an 80α phage lysate for transduction to *S. aureus* strains Newman and USA300 FPR to generate strains LNS249 and LNS885 respectively. Clones were again selected for according to the erythromycin cassette and confirmed by PCR analysis and Southern blot.

2.3. Construction of *pepZ-lacZ* reporter fusion

A *pepZ-lacZ* reporter fusion was constructed using a 1211 bp fragment containing the *pepZ* promoter region. This region was amplified by PCR using primers OL895 and OL462 (Table 1). The fragment was then cloned into the suicide vector pAZ106, upstream of the promoterless *lacZ* gene. The construct (pJED635) was confirmed in *E. coli*, electroporated into *S. aureus* strain RN4220, and subsequently transduced into the wild-type Newman and USA300 FPR strains as detailed above, to generate strains LNS647, LNS662 and LNS889.

2.4. Construction of the His tagged LAP expression plasmid

The *pepZ* gene, and upstream region containing the *pepZ* promoter, was amplified using primers OL958 and OL959 to generate fragment *pepZ*-6his. OL959 introduced 18 additional nucleotides at the 3' end of the *pepZ* gene immediately prior to the stop codon, which, when translated, result in an additional six C-terminal histidine residues with *S. aureus* codon preference. The *pepZ*-6his PCR product was cloned into the Gram-positive shuttle vector pMK4 using restriction enzymes BamHI and PstI, creating pLES734. This plasmid was then subject to DNA sequence analysis (MWG) to confirm correct construction. Plasmid pLES734 was electroporated into RN4220 and transferred into USA300 FPR as outlined above, generating strains LNS736 and LNS1143 respectively.

2.5. Co-culture experiments

Co-culture studies were performed as described by us previously [30]. Briefly, exponentially growing cultures of wild type and *pepZ* mutant strains were used to inoculate fresh TSB at a 1:1 ratio. To confirm equal quantities of the relevant strains were used to inoculate cultures, a frozen stock was made of each strain and the number of viable bacteria confirmed by cfu counts. Viability of frozen stocks was confirmed by individual inoculation and growth in TSB. Frozen stocks were used to inoculate co-culture flasks in equal 1:1 ratios. Co-cultures were grown at 37°C with shaking and monitored by viable cell counts performed daily for seven days. Viable cell counts were performed by serial dilution and plating onto both TSA, and TSA containing erythromycin (which selects for the *pepZ* mutant only). The

competitive index (CI) was calculated by dividing the number of wild-type cells by the number of *pepZ* mutant cells. The average results from three independent experiments are presented. Error bars are $\pm$ SEM between the three experimentally calculated competitive index values, and significance was determined using a Student's *t*-test, with a *p* value < 0.05 deemed significant.

2.6. Western blotting

Intracellular proteins were extracted at various time points from cultures of USA300 FPR containing plasmid pLES734. As a negative control, proteins were extracted from USA300 FPR containing the empty pMK4 vector. Equal quantities of protein were resolved using SDS-PAGE and transferred onto PVDF membrane. Following protein transfer, the membrane was probed using an anti-His monoclonal mouse antibody (Covance). Detection was performed using an anti-mouse, HRP linked secondary antibody and visualized on x-ray film.

2.7. Identification of the *pepZ* transcription start site by 5' RACE

5' RACE was carried out using the 5' Full RACE core set (Takara) according to the manufacturer's protocol, and oligonucleotide primers OL1339, OL1340, OL1341, OL1342 and OL1343. To determine the *pepZ* transcription start site individual 5' RACE products were sequenced. Twelve independent samples returned the transcription start site depicted in Fig 2B.

2.8. Murine macrophage cell culture and infection

RAW 264.7 murine macrophage-like cells were maintained in tissue culture medium (TCM) consisting of DMEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). For infection assays cells were seeded into 6-well plates 24 h prior to use and infected at an MOI of 100. To synchronize infections, plates were centrifuged at 1000 rpm for 10 min after the addition of bacteria. Cells were subsequently incubated for 1 h at 37°C to allow phagocytosis to occur. After this time, cells were washed with PBS and extracellular bacteria killed by the addition of TCM containing 30 $\mu\text{g ml}^{-1}$ gentamicin, for 1 h. The media was then replaced with fresh TCM containing 5 $\mu\text{g ml}^{-1}$ gentamicin for the remainder of the assay. At the time-points indicated samples of intracellular bacteria were collected for β -galactosidase assay as follows: RAW 264.7 cells were washed with PBS and lysed using PBS containing 0.5% Triton X-100. Samples were withdrawn to determine bacterial numbers and the remaining bacteria pelleted by centrifugation.

2.9. β -galactosidase assays

β -galactosidase assays were performed with 4-MUG as a substrate, as described by us previously [29, 31], with the following modification. β -galactosidase activity is expressed as arbitrary units corresponding to the value of light units released divided by the bacterial cfu per sample. All experiments were carried out in triplicate. Error bars represent standard deviation. Statistical significance was determined using a Student's *t*-test, with a *p* value < 0.05 deemed significant.

2.10. Human macrophage survival assay

A human model of macrophage survival and clearance was performed as described by us previously using Newman wild-type or *pepZ* mutant cells [29, 32]. The number of viable *S. aureus* cells per well was monitored by quantitative plating at hours 0, 2, 24, and 48 post-inoculation [33]. Two independent experiments were performed each of which consisted of two technical replicates using primary macrophages from two separate human donors. The data presented is the average number of surviving bacterial cfu at each given time-point.

Error bars represent standard deviation and significance was determined using Student's *t* test ($p < 0.5$).

2.11. Septic arthritis infection model

These assays were performed with Newman wild-type and *pepZ* mutant cells as described by us previously [30, 34]. Arthritic index and histological index scores were determined as follows by an individual blinded to the identity of the experimental groups. Severity of arthritis in each limb was scored on a scale of 0 to 3 (0, no swelling or erythema; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema). Arthritic index for each animal was generated by adding together the scores for each limb of that animal. Histopathological index was generated by scoring tissue sections for synovitis and bone erosion on a scale of 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe). Addition of the scores for each limb generated the histological score for each animal.

2.12. Murine abscess infection model

Murine wound formation was measured using experimental protocols detailed by us previously [28], with the following modifications. Ten hairless, SKH-1 immunocompetent mice were inoculated subcutaneously in the right flank with 1×10^8 cfu of USA300 FPR wild-type or *pepZ* mutant cells for each strain. Infections were monitored for seven days and any abscesses formed were harvested following animal euthanasia [35, 36]. Viable *S. aureus* cells were quantified by serially diluting harvested abscess homogenates in PBS, and plating onto TSA. Data is presented as cfu per abscess, and was analyzed using a Student's *t*-test, with a *p* value < 0.05 deemed significant.

2.13. Murine sepsis infection model

Ten female six-week old CD-1 Swiss, outbred and immunocompetent mice were inoculated via tail vein injection with 1×10^7 cfu of either USA300 FPR wild-type or *pepZ* mutant cells. Mouse survival was monitored daily for seven days and the data analyzed via a Kaplan-Meier plot. Significance was determined by log rank test.

3. Results

3.1. The Leucine Aminopeptidase (LAP) is required for *S. aureus* virulence

As part of an ongoing screen to determine the role of proteases and peptidases in *S. aureus* physiology and pathogenesis, we generated a mutant in a leucine aminopeptidase (LAP, *pepZ*) and tested its ability to cause disease in a murine model of septic arthritis. Female NRMI mice, 6 to 8 weeks old, were inoculated intravenously with either *S. aureus* strain Newman or a Newman *pepZ* mutant. Infected mice were monitored continuously over a twelve-day period for outward signs of infection, followed by a histological examination and quantification of bacterial burden in the kidneys. Mice infected with the *pepZ* mutant displayed a significant reduction in weight loss (10% compared to 27% for the wild type) and severity of disease, as determined by arthritis index (Fig. 1A and B). Further evidence of the decreased severity of infection with the *pepZ* mutant was demonstrated following a histological examination of the joints of infected mice. Synovitis and bone erosion were reduced approx 4- and 6-fold respectively in the joints of *pepZ* mutant infected mice compared to wild type (Fig. 1C). Furthermore, the *pepZ* mutant demonstrated markedly reduced septic dissemination compared to the wild type (Fig. 1D). Approximately 3-fold less bacteria were recovered from the kidneys of mice infected with the *pepZ* mutant, indicating that this strain was less able to disseminate within the host. Taken together, these results demonstrate that the Newman *pepZ* mutant is strongly attenuated for virulence in a septic arthritis model of infection, suggesting that this previously uncharacterized aminopeptidase is an important virulence-affecting agent.

The results above suggest that *pepZ* plays an important role during infection. However, these experiments were carried out using a murine infection model, and the possibility remains that *pepZ* is not required for human disease. To test the hypothesis that *pepZ* is important during human infection we performed a survival assay using cultured primary human macrophages. Macrophages were infected with the Newman wild type and *pepZ* mutant strains, and the numbers of intracellular bacteria were determined at various time-points. We observed that the *pepZ* mutant was significantly less able to survive within human macrophages. Specifically, at 24 and 48 h post infection there were 6- and 17-fold less *pepZ* mutant bacteria surviving inside macrophages compared to the wild type (Fig. 1E). At later timepoints (72–96 h post infection) macrophages infected with wild type bacteria lysed, releasing the surviving intracellular bacteria into the growth medium where they rapidly proliferated. This phenomenon has previously been described for macrophages infected with *S. aureus* [32]. Interestingly, while lysis of macrophages infected with wild type Newman was observed, there was no such lysis of macrophages infected with the *pepZ* mutant. Instead, macrophages infected with *pepZ* mutant bacteria cleared the infection and by 72 h post infection no *pepZ* mutant bacteria were detected. This suggests that the *pepZ* mutant is more susceptible to killing by human macrophages, and strongly suggests that the virulence defect observed in the murine arthritis infection model is likely recapitulated in a human background.

3.2. *pepZ* is maximally expressed within the intracellular environment

The results above indicate that *pepZ* is required by *S. aureus* to mount successful infections. To understand better the role of LAP in the bacterial cell we investigated the expression profile of *pepZ*. Due to the strong virulence phenotype observed, we elected to perform these studies in a clinically relevant strain (USA300) alongside the laboratory strain Newman. By doing this we ensured that any observed phenotype was broadly applicable and not a unique, strain specific artifact. An examination of the chromosomal location of the *pepZ* gene reveals that there is a small intergenic region (132 bp) located between the LAP coding region and the neighboring gene (Fig. 2A). To determine if transcription of the *pepZ* gene originates from a promoter in this region, or 5' of the preceding gene (SACOL0944) we performed 5' RACE. Results show a transcription start site in the intergenic region between *pepZ* and SACOL0944 located 31 base pairs 5' of the *pepZ* translation start site (Fig. 2B). Analysis immediately 5' of this reveals a degenerate σ^A promoter, with a relatively consensus -10 site (TAcAAT) and a more corrupted -35 sequence (gTGtCA). Interestingly, the spacing between these 2 elements is an imperfect 19 nucleotides, rather than the preferred 17, suggesting a possible need for transcriptional augmentation via regulatory elements.

Having identified the location of the *pepZ* promoter, we next constructed a *pepZ-lacZ* transcriptional fusion in strains Newman and USA300. The resulting fusion strains contain an intact *pepZ* locus, and a single, chromosomally located *lacZ* gene under the control of the *pepZ* promoter. Using the *pepZ-lacZ* fusion we determined expression levels of *pepZ* during growth in TSB and milk media in strains Newman (Fig. 2C) and USA300 (Fig. 2D). Milk media was included as it is low in free amino acids, and rich in peptides, allowing us to determine if the presence of potential oligopeptide substrates impacts the expression of this aminopeptidase. Strains containing the *pepZ-lacZ* fusion were grown in TSB for 8 hours, with bacterial numbers (in cfu/ml) quantified and samples for β -galactosidase assays taken hourly (Fig. 2C and D: black squares). Results for both strains show *pepZ* expression is induced immediately upon subculture into fresh TSB, with the highest levels of expression seen at 1 h post inoculation, followed by a decline throughout exponential growth. We next sub-cultured the *pepZ-lacZ* fusion strains into milk medium after 3 h growth in TSB (Fig. 2C and D: open circles). As a control, we also, in parallel, sub-cultured each strain into fresh

TSB (Fig. 2C and D: open squares). The sub-cultures were grown for an additional 5 hours and samples taken hourly for β -galactosidase assays. Interestingly, whilst sub-culturing into fresh TSB resulted in an induction of *pepZ* expression similar to that observed previously, sub-culturing into milk medium arrested expression from the *pepZ* promoter. Specifically, we observed a 3.3- and 4.3-fold decrease in *pepZ* expression in strains Newman and USA300, respectively, which rapidly fell to baseline levels. These results indicate that *pepZ* is expressed during early exponential phase growth in TSB, but is down-regulated in milk medium. The observation that *pepZ* expression is down-regulated in milk medium suggests that the role of LAP is not related to the use of exogenous peptides as a nutrition source. As such, we suggest that LAP is employed by the cell during infection for a non-nutritional function that impacts the virulence process. To test this hypothesis we used our fusion strains to determine the expression of *pepZ* inside macrophages; an *in vivo* setting where *pepZ* is required for survival (Fig. 1E). We determined a strong induction of *pepZ* in the intracellular environment in both strains tested (Fig. 2E). In strain Newman, expression of *pepZ* following 21 h inside macrophages was 18-fold higher than in TSB at 1 h. This affect was magnified using the clinical CA-MRSA strain USA300, where we observed a 76-fold up-regulation of *pepZ*. These data define that *pepZ* expression is highest during intracellular survival, strongly suggesting that its primary role is during infection.

3.3. LAP is not required for the growth of *S. aureus* in peptide-based media

Thus far we have shown that LAP inactivation results in *S. aureus* virulence defects in both human and murine systems, and that the *pepZ* gene is up-regulated during infection. Additionally, it appears that whilst transcription of this gene is strongly induced following subculture into rich media, it is shut off upon growth in peptide-rich environments. To explore the role of LAP in *S. aureus* biology and physiology we next tested growth of the mutant strain in standard laboratory media. A comparison of growth rates between both the Newman and USA300 wild type and *pepZ* mutant strains in TSB revealed no detectable differences, with similar doubling times and final yields observed (data not shown). For many lactic acid bacteria aminopeptidases are important during growth in peptide rich environments. Exogenous peptides are imported into the cell where aminopeptidases facilitate the removal of N-terminal amino acids, which are then used as a nitrogen source. To test the hypothesis that LAP is involved in the use of peptides as a nutrition source in *S. aureus*, we analyzed the ability of the *pepZ* mutants to grow in peptide rich media. Wild type and *pepZ* mutant bacteria were grown for 30 h in 10% milk medium and growth was monitored hourly by cfu/ml determination. For both backgrounds tested there was no detectable difference in growth between wild type and mutant strains (data not shown), indicating that LAP is not required for growth in a peptide rich environment. These results clearly demonstrate that there is no growth defect in the *pepZ* mutant that could account for the decrease in virulence observed, suggesting that LAP plays a non-nutritional role during the infectious process.

3.4. LAP mutants are impaired in their ability to compete with the parental strain

We next carried out competitive growth experiments (co-cultures) to assess the fitness of the LAP mutant when competing with the parental strain for nutrients. Equal quantities of wild type and *pepZ* mutant bacteria were used to inoculate the same flask of TSB and the co-cultures grown for 7 days. Each day, the number of wild type and *pepZ* mutant bacteria were determined and the ratio of wild type: *pepZ* mutant calculated (Fig. 3). Individually, each strain grows at an identical rate, however, when cultured together in the same flask, the wild type strain rapidly outgrows the *pepZ* mutant. Following 24 h incubation, the ratio of wild type: *pepZ* mutants was 0.51 and 0.47 in the Newman and USA300 strains respectively (Fig. 3). After 48 h these ratios had dropped to 0.13 and 0.08, and remained low, eventually reaching 0.03 and 0.02 on day 7. Importantly, similar growth impairments were observed for

the *pepZ* mutant when co-cultured with the parent in peptide-rich media (data not shown). As similar trends were observed in both types of media, this provides a number of key insights into the role of LAP. Firstly, it provides further support for the contention that LAP does not contribute to peptide-based nutrition in *S. aureus*, as similar defects were seen in both nutrient rich, and peptide rich environments. Secondly, although the strains display similar growth rates when grown individually, the wild-type strain does possess a growth advantage over the *pepZ* mutant that allows it to outcompete the mutant in co-culture. Finally, this observation strongly suggests that LAP functions inside the bacterial cell. If LAP were secreted by the wild type strain, and functioned externally, then the *pepZ* mutant should benefit from co-culture with the wild type strain, however the opposite is true, indicating that LAP activity is located inside the bacterial cytosol, and the absence of LAP in co-culture places the *pepZ* mutant at a physiological disadvantage.

3.5. LAP is localized to the bacterial cytosol

Given the strong virulence defect present in LAP mutants, we considered it important to determine if this was mediated via an important intracellular function, or if the enzyme is perhaps secreted, and functions as a classical virulence determinant, as with other *S. aureus* proteases [28]. The results from the co-culture experiments appear to suggest that LAP is located intracellularly. Typically, aminopeptidases that are secreted function in a monomeric form, while intracellular aminopeptidases, including M17 family members, tend to function as higher order multimers [1]. An examination of the LAP crystal structure (PDB ID: 3KZW) shows that it crystallizes as a 12-subunit homodimer, and that this is the active form of the enzyme, again suggesting intracellular localization. To conclusively determine the cellular location of LAP we generated a plasmid encoded, histidine tagged form of the enzyme in the USA300 background, driven from its own promoter. Western immunoblots using an anti-his antibody and intracellular protein samples identified LAP in the intracellular fraction, confirming it is located within the bacterial cell (Fig. 4). Importantly, LAP-his was not detected in western blots using the empty vector control. In addition, LAP was not identified in western blots using secreted protein fractions (data not shown). This result strongly suggests that LAP is predominantly located inside the cell, and that its virulence impacting function is not in the extracellular milieu.

3.6. LAP is required for localized and systemic CA-MRSA infections

Our initial finding that *pepZ* impacts virulence was performed using the laboratory strain Newman. To confirm that LAP is a global affector of virulence in *S. aureus*, we performed additional and corroboratory analyses in the clinically relevant USA300 background. Two different models of infection were employed, representing both localized and systemic infection types. A murine abscess model was used to explore localized infection, while a murine sepsis model was used for systemic studies. In the abscess model, mice were infected subcutaneously and abscess formation monitored over a seven-day period. Lesions of similar size were produced by wild type and *pepZ* mutant strains, however the number of *pepZ* mutant bacteria recovered from abscesses was 2.3-fold less than the wild type (Fig. 5A). This result confirms the virulence defect of the *pepZ* mutant and demonstrates that while infection with the mutant does produce abscesses, the number of mutant bacteria recovered per abscess is significantly lower ($p = 0.029$) than the wild type. Further clear confirmation of the *pepZ* virulence defect in the USA300 strain came using a sepsis model of infection. Within 24 h of inoculation all mice infected with the wild type strain had died. In contrast, only 2 of the 10 mice infected with the *pepZ* mutant died (Fig. 5B), with the remaining 8 mice surviving the 7-day infection period ($p = 1.3 \times 10^{-5}$). These data clearly show a severe attenuation in virulence for LAP inactivated strains in the clinical USA300 background, confirming previous data in the laboratory strain Newman. Together these results demonstrate that, regardless of background, infection models, or species tested, LAP

is required for full virulence, revealing for the first time that this previously uncharacterized, intracellular aminopeptidase, is a critical *S. aureus* virulence effecting entity.

4. Discussion

Traditionally Gram-positive bacterial aminopeptidases have been studied for their role in amino acid metabolism, catabolizing imported peptides, which are used as a nitrogen source for the cell [2, 3]. Recently however, the importance of these enzymes has been emphasized as it has emerged that aminopeptidases play a crucial role in pathogenesis. Of particular interest is the M17 family of metallo-peptidases. M17 family members secreted by a number of protozoan and parasitic species are currently under intense investigation as targets for therapy and preventive medicine [12, 14, 37]. M17 family members have also been investigated in bacteria, in particular in Gram-negative bacteria, where they are involved in virulence [18, 19, 38]. Unusually, in these cases the M17 aminopeptidases function inside the bacterial cell, in contrast to other virulence-associated aminopeptidases where the enzyme is secreted and is functional extracellularly.

In this work we identify, for the first time, an M17 family member required for virulence in a Gram-positive bacteria. Moreover it is the first demonstration of an intracellular aminopeptidase that affects virulence in a Gram-positive bacterium. LAP is highly conserved and is present in all *S. aureus* genomes currently available in public databases. In both the Newman and USA300 backgrounds, disruption of *pepZ*, the gene encoding LAP, lead to a dramatic reduction in severity of disease in both a systemic model of infection and a localized abscess model. Furthermore, the ability of the *pepZ* mutant bacteria to survive inside human macrophages was significantly decreased, suggesting that this virulence defect would be recapitulated during infection of humans.

To begin to understand the role of LAP in the cell we analyzed growth of the *pepZ* mutant and expression of the *pepZ* gene. If LAP is involved in peptide nutrition we hypothesized that (i) the *pepZ* mutant would have a growth defect and (ii) *pepZ* expression would be induced in peptide based medium. The *pepZ* mutant demonstrated no growth defect in a peptide rich environment and gene expression analysis showed that the *pepZ* gene is not expressed in this media. These results strongly suggest that in contrast to aminopeptidases in Lactococci, the primary function of LAP is not to catabolize exogenous peptides. Expression of the *pepZ* gene was observed during early exponential phase growth in TSB, however, highest levels of *pepZ* expression were seen when the bacteria were in the intracellular environment. Together, these results suggest that *pepZ* expression is induced *in vivo* and is required for the bacteria to mount successful infections.

M17 LAPs in bacteria are most commonly found in the bacterial cytosol, however aminopeptidases from other families are often secreted and are functional extracellularly [1]. Due to the strong virulence phenotype associated with the *pepZ* mutant, and the fact that *S. aureus* secretes a number of protease virulence factors, we investigated the subcellular localization of LAP. Intracellular bacterial LAPs generally function as homo-hexameric enzymes while extracellular LAPs are functional as monomers [1]. An examination of the crystal structure of *S. aureus* LAP, present in the Protein Data Base, shows it crystallized as a 12-subunit complex, most likely representing two hexamers. This, coupled with the fact that LAP lacks a recognized signal sequence, suggests that it is an intracellular enzyme. Confirmation of the intracellular localization of LAP comes from our co-culture and western blotting data indicating that LAP, as with M17 family members in the Gram-negative bacteria, functions within the bacterial cell and it is not a secreted virulence factor. This finding is the first demonstration that an intracellular aminopeptidase can impact the virulence of a Gram-positive bacterium.

The exact cellular function of LAP remains unclear, and is the subject of ongoing research in our laboratory. In a number of Gram-negative pathogens, LAP homologues bind DNA and alter gene expression. Sequence alignment of LAP with these homologues shows that the residues involved in DNA binding are not present in *S. aureus* LAP (data not shown), thus it is unlikely that LAP is involved in DNA binding and gene regulation. In *Treponema denticola* LAP is involved in the three-step enzymatic process that converts glutathione into the volatile sulfur compound H₂S [22]. This highly toxic gas is found in periodontal pockets where it is implicated in tissue destruction [39]. In *S. aureus*, we propose a similar mechanism of action is occurring, in that LAP, rather than directly processing secreted virulence affecting proteins, is perhaps functioning in the bioactivation/inactivation of key protein targets within the cell. In such a scenario, these factors would not be involved directly in virulence (e.g. a toxin) as any N-terminal processing would affect their signal sequence, and subsequent secretion. Moreover, LAP may be cleaving and either activating or inactivating important proteins that impact cellular fitness (metabolic pathways, cell wall biosynthesis, cell signaling etc). Such proteolysis would produce a fitness advantages for the cell, that, when absent upon loss of LAP, leads to impaired survival, particularly within the harsh environment of the host. As such, this would lead to the impaired virulence observed with our multiple *pepZ* mutant studies documented herein. Determining the substrate specificity for this enzyme, and identifying target proteins within the bacterial cytosol, will help elucidate its role, and how it contributes to virulence.

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Figure 1. LAP is required for virulence in mice and survival within human macrophages

S. aureus wild type strain Newman and a Newman *pepZ*⁻ mutant were used to inoculate 10 mice each. **A.** Infected mice were monitored for weight loss over a twelve-day period. Percentage weight change for wild-type (□) and mutant (○) infected mice is plotted with statistical significance indicated by * and ** for p values >0.05 and >0.01 respectively. **B.** At 5 and 12 days post inoculation wild-type (shaded) and mutant (unshaded) infected mice limbs were observed and scored for clinical symptoms of arthritis resulting in an arthritic index score. Black lines indicate average scores, and p values for statistical significance are shown. **C.** Histological examination of mice 12 days post-inoculation for wild-type (●) and mutant (○) infected mice. Black lines indicate average index scores, and p values for statistical significance are shown. **D.** Bacterial dissemination to the kidneys for wild-type (shaded) and mutant (unshaded) infected was determined. Black lines indicate average loads, and p values for statistical significance are shown. **E.** A human infection model of clearance and survival was performed using primary cultured human macrophages and the

Newman wild-type (black) and *pepZ* mutant (grey) strains. Error bars represent standard deviation. Significance (*) was determined by *t* test ($p < 0.05$).

Figure 2. Transcription profiling of the *pepZ* gene

A. The *pepZ* locus (to scale). **B.** Identification of the *pepZ* transcription start site by 5' RACE. Shown in bold and underscore are the translation start site (ATG), the putative ribosome binding site (GGAGG), the +1 site (G), and putative -10 (TACAAT) and -35 (gTGTCA) sequences for σ^A recognition. **C** and **D.** Analysis of *pepZ-lacZ* transcription fusion in Newman (**C**) and USA300 FPR (**D**). Wild type strains containing the *pepZ-lacZ* fusion were grown in TSB for 8 hours and samples taken for β -galactisodase assays at each hour (■). Following 3 h growth, bacteria were sub-cultured into fresh TSB (□) and 10% milk medium (○), with samples taken hourly for a further 5 hours. **E.** Analysis of *pepZ-lacZ* expression inside macrophages. Cultured RAW264.7 cells were infected with Newman (grey) and USA300 FPR *pepZ-lacZ* (black) fusion strains, with samples taken at the time-points indicated for β -galactisodase assays. For comparison the maximum levels of *pepZ-lacZ* expression in each strain in TSB is also shown. β -galactisodase assays were performed in triplicate. Error bars represent standard deviation. Statistical significance was determined by *t*-test (* = $p < 0.05$).

Figure 3. Competitive growth index for wild type strains versus *pepZ*⁻ mutants

Flasks of TSB were inoculated with equal amounts of wild type and *pepZ*⁻ mutant and allowed to grow for 7 days. Each day the number of wild type and *pepZ*⁻ mutant bacteria were determined and the competitive index calculated. Error bars represent the standard error between three replicate experiments. Significance was determined by *t*-test (*=*p*<0.05)

Figure 4. Western blot analysis of LAP to determine subcellular localization

Strain USA300 FPR containing a plasmid encoded, his-tagged LAP (lanes 1, 3 and 5) or an empty vector control (lanes 2, 4 and 6) were grown in TSB for the times specified. Samples were taken for western immunoblot analysis using an anti-his antibody.

Figure 5. LAP is required for the full virulence of CA-MRSA

A. Abscess model of infection. The USA300 FPR wild-type and *pepZ*⁻ mutant were used to subcutaneously inoculate 10 mice each, which were monitored for 7 days, with abscesses harvested and bacterial loads determined. The average cfu/abscess is indicated by a bold marker, significance (*) was determined using a Student's *t*-test (p=0.029). **B.** Sepsis model of infection. USA300 FPR wild-type (■) and *pepZ*⁻ mutant (○) bacteria were used to intravenously inoculate 10 mice each via tail vein injection. Data is represented as percent survival over time, significance (*) was determined via a log rank test (p=1.3×10⁻⁵).

Table 1

Strains, plasmids, and primers

Name	Characteristics	Source
<i>S. aureus</i>		
RN4220	Restriction-deficient transformation recipient	Lab stocks
Newman	Wild type laboratory strain	Lab stocks
USA300-FPR	Sequenced USA300-FPR MRSA isolate cured of pUSA300-FPR-MRSA	[40] M. Smeltzer
LNS245	RN4220 <i>pepZ</i> ::pAZ106 <i>pepZ</i> ⁻	This work
LNS249	Newman <i>pepZ</i> ::pAZ106 <i>pepZ</i> ⁻	This work
LNS647	RN4220 <i>pepZ-lacZ pepZ</i> ⁺	This work
LNS662	Newman <i>pepZ-lacZ pepZ</i> ⁺	This work
LNS736	RN4220 pLES734 <i>pepZ</i> ⁺	This work
LNS803	RN4220 pMK4	This work
LNS885	USA300 FPR <i>pepZ</i> ::pAZ106 <i>pepZ</i> ⁻	This work
LNS889	USA300 FPR <i>pepZ-lacZ pepZ</i> ⁺	This work
LNS1143	USA300 FPR pLES734 <i>pepZ</i> ⁺	This work
LNS1207	USA300 FPR pMK4	This work
<i>E. coli</i>		
Dh5α	Routine cloning strain	Lab stocks
LNS224	Dh5α pLES224	This work
LNS635	Dh5α pJED635	This work
LNS734	Dh5α pLES734	This work
Plasmids		
pAZ106	Promoterless <i>lacZ</i> suicide vector <i>erm</i> ^R	[41]
pMK4	Shuttle vector <i>cm</i> ^R	[42]
pLES224	pAZ106 containing 489 bp <i>pepZ</i> fragment	This work
pJED635	pAZ106 containing 1.2 kb <i>pepZ</i> promoter fragment	This work
pLES734	pMK4 containing 1.8 kb <i>pepZ</i> -6his fragment	This work
Primers		
OL461	5'-ATGTCTAGACAGTTAGAGCGCATTAGT-3'	
OL462	5'-ATGGGATCCCATTTCGGTGGCATATTAC-3'	
OL895	5'-ATGTCTAGAGCTACACCAATCGTTGCTTG-3'	
OL462	5'-ATGGGATCCCATTTCGGTGGCATATTAC-3'	
OL958	5'-ATGGGATCCGGTTCACATGACGGTGTAGG-3'	
OL959	5'-ATGCTGCAGTTAATGATGATGATGATGTTGTTGTTTAACCATTGTAC-3'	
OL1339	5'-CGGTGCCTTCTTACTTG-3'	
OL1340	5'-CAACATATCTTGATAACTACG-3'	
OL1341	5'-CTTCCCAACTTTACTACC-3'	
OL1342	5'-CAGAACACATTGAAGATACG-3'	
OL1343	5'-CATGCGGTATTCAAAGTGAG-3'	
OL1539	5'-GTATTTATTTCGTTGCTTAGTGTG-3'	