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The leucine aminopeptidase of *Staphylococcus aureus* is secreted and contributes to biofilm formation

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SUMMARY

Background: Staphylococcus aureus has emerged as a major drug-resistant pathogen in hospital- and community-acquired infections. Leucine aminopeptidase (LAP) is known to be essential for survival of the bacteria; however the LAP of *S. aureus* has not been extensively characterized. In this study, we report a detailed characterization of the *S. aureus* LAP.

Methods: LAP from *S. aureus* was cloned, purified, and further biochemically characterized. The expression of LAP was analyzed by Western blotting. Growth and biofilm formation were analyzed spectrophotometrically.

Results: LAP was cloned from *S. aureus* and expressed as a 55 kDa protein, whereas the molecular weight of the native protein is approximately 600 kDa. LAP showed amidolytic activity against L-leucine *p*-nitroanilide. Optimal activity was observed at pH 8.5 and 37 °C with a V_{max} of 2500 μmol/min/mg protein. LAP enzymatic activity was inhibited by ion chelators and enhanced by divalent metal ions, specifically Ni. LAP is secreted by laboratory as well as clinical strains. Bestatin, an inhibitor of LAP, inhibits *S. aureus* growth and biofilm formation.

Conclusions: To our knowledge, this is the first detailed characterization of LAP from *S. aureus* and suggests its importance in survival and pathogenesis.

1. Introduction

Staphylococcus aureus is one of the leading causes of hospital-acquired infections and of mortality. It is one of the pathogens causing more fatalities than HIV/AIDS. The emergence of antibiotic-resistant strains and major outbreaks worldwide have alarmed the medical community.[1] The anterior nares are one of the major ecological niches of *S. aureus*. Many other sites are also colonized by *S. aureus*, such as the axillae, groin, and gastrointestinal tract. *S. aureus* infections range from minor wound infections to life-threatening conditions like endocarditis, osteomyelitis, and septic shock.[2] Therefore, it is important to understand the molecular mechanisms of staphylococcal pathogenesis and to identify new therapeutic targets/drug scaffolds for the development of effective anti-Staphylococcus agents.

Aminopeptidases play a critical role in cellular metabolism and catalyze the removal of N-terminal amino acid residues from peptides.[3] Emerging experimental evidences suggest the role for leucine aminopeptidases (LAPs) in the survival of pathogens under different conditions. The LAP from *Plasmodium vivax* catalyzes the removal of amino acids from the peptide generated in hemoglobin degradation and is utilized for the growth and development of the pathogen.[4] *Plasmodium falciparum* parasites over-expressing LAP are two-fold less susceptible to killing by bestatin (half-maximal effective concentration (EC_{50}) = 64 μM) compared to wild-type parasites (EC_{50} = 25 μM).[5] LAP is upregulated in metronidazole-resistant *Helicobacter pylori*.[6] These experimental observations strongly suggest a role for LAPs in the survival of pathogens in different extreme environments and in the acquirement of drug resistance.

LAPs and their role in the survival of Gram-negative bacteria has been reported,[7] however they are less well characterized in Gram-positive pathogens including *S. aureus*. In this study, we cloned and characterized the LAP from *S. aureus*.

2. Materials and methods

2.1. Bacteria and culture conditions

The standard laboratory strain of *S. aureus* NCTC 8325 was used in this study and was cryopreserved at −80 °C. Clinical strains of *S. aureus* (P189, P469, P768, P359, and P790) were obtained from

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the Staphylococcal Phage Typing Center, Department of Microbiology, Maulana Azad Medical College, New Delhi, India. All *S. aureus* strains were cultured in nutrient broth (Himedia, India) and incubated overnight on a shaker at 200 rpm and 37 °C. *Escherichia coli* BL21-Gold (DE3) (Stratagene) and *E. coli* DH5α (Invitrogen) were grown in Luria broth (LB) at 37 °C and supplemented with appropriate antibiotics when needed. Thioredoxin A (TrxA) antibody was provided by Dr Jan Maarten van Dij, Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, Groningen, the Netherlands.

2.2. Cloning and expression of S. aureus LAP

Using the published sequence of the *S. aureus* genome (GenBank accession number **NC_007795**), forward (5′-CGCGGATCCATGAATTTTAAATTAAAT-3′) and reverse (5′-CCGCTCGAGTTGTTGTTTTAACCATTG-3′) primers were synthesized. The PCR amplified product was cloned in pET-28a, confirmed by sequencing.

LAP cloned in pET-28a was transformed in *E. coli* BL21-Gold (DE3). Inoculum (1%) from culture grown overnight was inoculated into LB, incubated at 37 °C on an orbital shaker at 200 rpm, and induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were harvested by centrifugation (7000 rpm, 10 min, 4 °C), resuspended in binding buffer A (20 mM HEPES (hydroxyethyl piperazineethanesulfonic acid) pH 7.4, 1.5 mM $MgCl_2$, 100 mM NaCl, 1% Triton, 1 mM DTT (dithiothreitol), and 1 mM PMSF (phenylmethylsulfonyl fluoride)) and lysed by sonication (Sonics & Materials, Inc.). The lysate was centrifuged (15 000 rpm, 10 min, 4 °C) and the supernatant collected. The lysate was incubated with TALON Metal Affinity Resin (Clontech Takara Bio) for 1 h at 4 °C on a Rotospin device and washed three times with buffer A to remove non-specific binding. The purity of LAP was evaluated on Coomassie blue-stained 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bead-bound LAP was eluted with 100 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C, concentrated, and the EDTA exchanged with phosphate buffered saline (PBS) using a Centricon (Millipore) device with 10-kDa cut-off. The protein concentration was determined by Bradford reagent (Bio-Rad).

2.3. Blue native-PAGE (BN-PAGE)

To determine the native molecular weight of LAP, BN-PAGE was performed as described previously,[8,9] with minor modifications. Acrylamide (50% w/v)/bis (0.5% w/v) solution was prepared that yielded highly porous gels on polymerization, for the separation of higher order protein complexes. The final concentration of the stacking gel was 4% and that of the resolving gel was 8%. Purified LAP was mixed with sample buffer, and non-gradient BN-PAGE was performed at room temperature as described previously.[8] The gel was stained with Coomassie brilliant blue G-250 (CBB G-250), followed by destaining in a solution containing 10% (v/v) methanol and 8% (v/v) acetic acid. All gel images were documented using an Epson Perfection 1670 scanner.

2.4. In-gel activity staining

Activity staining of LAP was performed as described previously,[10] with minor modifications. Briefly, purified LAP was resolved on 8% native-PAGE, as described above, and the gel washed twice with distilled water for 10 min. The gel was equilibrated twice with buffer B (Tris–HCl 50 mM, pH 8.5) and incubated in the presence of substrate (0.0015% w/v leucine *p*-nitroanilide (LpNA) solution in buffer A) at 30 °C for 10 min. Diazotization of liberated *p*-nitroaniline was performed at room temperature by immersing the gel into freshly made 0.1% $NaNO_2$, 1 M HCl for 2 min. The excess $NaNO_2$ was removed by shaking the gel in 1% (w/v) urea. The diazotized gel was then immersed in 0.025% (w/v) 1-naphthylamine solution in 22% v/v ethanol with gentle agitation until a distinct pink azo dye developed.

2.5. Enzymatic analysis of recombinant LAP

Kinetic analysis of LAP was performed as described earlier,[11] with minor modifications. The standard reaction mixture contained 50 mM Tris–HCl (pH 8.5), 1 mM $MnCl_2$, 1 μg LAP protein, and 1 mM of L-leucine *p*-nitroanilide (Sigma-Aldrich); this was incubated at 37 °C for 15 min. The progress of the reaction was observed by monitoring the optical density (OD) at 405 nm by spectrophotometry.

The substrate saturation curve of LAP was determined by incubating LAP (1 μg/ml in 50 mM Tris–HCl, and 1 mM $MnCl_2$) with increasing substrate concentrations from 0.1 mM to 4.5 mM. The Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) were calculated from the Lineweaver–Burk plot.

To determine the optimum temperature for activity, LAP (1 μg/ml in 50 mM Tris–HCl, and 1 mM $MnCl_2$) was incubated at different temperatures (15–85 °C) for 15 min before the addition of substrate (1 mM L-leucine *p*-nitroanilide).

The LAP pH profile was determined by incubating LAP (1 μg/ml, 37 °C, 15 min) in constant ionic strength acetate/2-(N-morpholino)ethanesulfonic acid (MES)/Tris buffer (AMT buffer: 50 mM acetic acid, 50 mM MES, and 100 mM Tris–HCl,[12] pH 4–11), and 1 mM $MnCl_2$ before addition of the substrate (1 mM L-leucine *p*-nitroanilide).

The metal ion dependence of LAP was investigated by assaying the activity after pre-incubation of the enzyme (1 μg/ml, 37 °C, 15 min) in 50 mM Tris–HCl pH 8.5, containing a specified metal chloride (1 mM). The substrate (1 mM L-leucine *p*-nitroanilide) was added and the reaction monitored spectrophotometrically as described above.

2.6. Generation of polyclonal antibody

Polyclonal antibody was generated in a 6-month-old healthy New Zealand white rabbit. Fifty micrograms of purified LAP with complete Freund's adjuvant (Sigma) was injected subcutaneously under the arm. The rabbit was bled and serum was collected.

2.7. Western blotting

Culture of *S. aureus* grown overnight was centrifuged at 4 °C; the bacterial pellet and supernatant (culture media) were collected. The supernatant was passed through a 0.22-μM filter to remove any bacterial cell. The pellet was lysed in PBS containing protease inhibitor (Roche), centrifuged at 14 000 rpm at 4 °C for 15 min, and the lysate collected. The protein concentration was determined using Bradford reagent (Bio-Rad). Equal amounts of proteins from the different samples were resolved on 12% SDS-PAGE and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was probed with LAP antibody (1:7000 dilution). Similarly equal amounts of supernatant and lysate were loaded onto a 12% SDS-PAGE and electroblotted onto a PVDF membrane and probed with TrxA antibody (1:5000 dilution). Clinical strains of *S. aureus* were also grown and Western blotting performed as described above.

2.8. Effect of bestatin on LAP activity and growth of S. aureus in vitro

Bestatin is known to inhibit LAP enzymatic activity.[13] The inhibitory effect of bestatin on LAP was assayed by pre-incubation of purified enzyme (1 μg/ml, 37 °C, 15 min) with different concentrations of inhibitor in 50 mM Tris–HCl pH 8.5.

To study the effect of bestatin on *S. aureus* growth, 1% of culture grown overnight was re-inoculated in nutrient broth supplemented with increasing concentrations of bestatin and the OD_{600} monitored 3 h after addition of the inhibitor.

2.9. Analysis of S. aureus biofilm formation in the presence of bestatin

S. aureus cultures grown overnight were diluted (1:40) in tryptone soya broth (TSB) containing 0.25% glucose and inoculated in sterile culture plates. Bestatin was added to a final concentration of 100 μg/ml. Plates were incubated at 37 °C without shaking for 16 h. Plates were washed three times with sterile water, stained with 0.1% crystal violet for 1 min, rewashed with sterile water, and photographed. Crystal violet was solubilized using 30% glacial acetic acid, and relative biofilm formation was determined by monitoring OD_{562}.[14]

3. Results

3.1. Phylogenetic analysis of LAP from S. aureus

This study focused on the characterization of the LAP of *S. aureus*, an enzyme that is essential for the survival and pathogenesis of other bacteria. Analysis of the *S. aureus* NCTC 8325 genome identified only one copy of a putative LAP gene, which has thus far not been characterized. This open reading frame (ORF) is 1496 base pairs and encodes a protein of 491 amino acids. The protein sequence analysis of LAP showed characteristic similarity with members of the M17 family of proteases (Figure 1A). The alignment of the protein sequence of LAP from *S. aureus* with members of the M17 family of proteases revealed typical signature sequences and the existence of multiple conserved amino acids within predicted functional domains. The residues for metal binding (M) and catalytic activity (A) are conserved across different bacteria and higher eukaryotes (Figure 1B). The BlastP program revealed that the LAP from *S. aureus* shares 35%, 34%, and 35% sequence similarity to human, *Haemophilus influenzae*, and *E. coli* LAPs, respectively. The LAP from *S. aureus* is phylogenetically closest to that of *Rickettsia prowazekii*, divergent from other bacteria and human (Figure 1C).

3.2. Characterization of LAP from S. aureus

LAP was expressed as a 55 kDa protein when analyzed on 12% SDS-PAGE (Figure 2A). The molecular weight of the native LAP was determined by modified BN-PAGE (Figure 2B). BN-PAGE is a higher

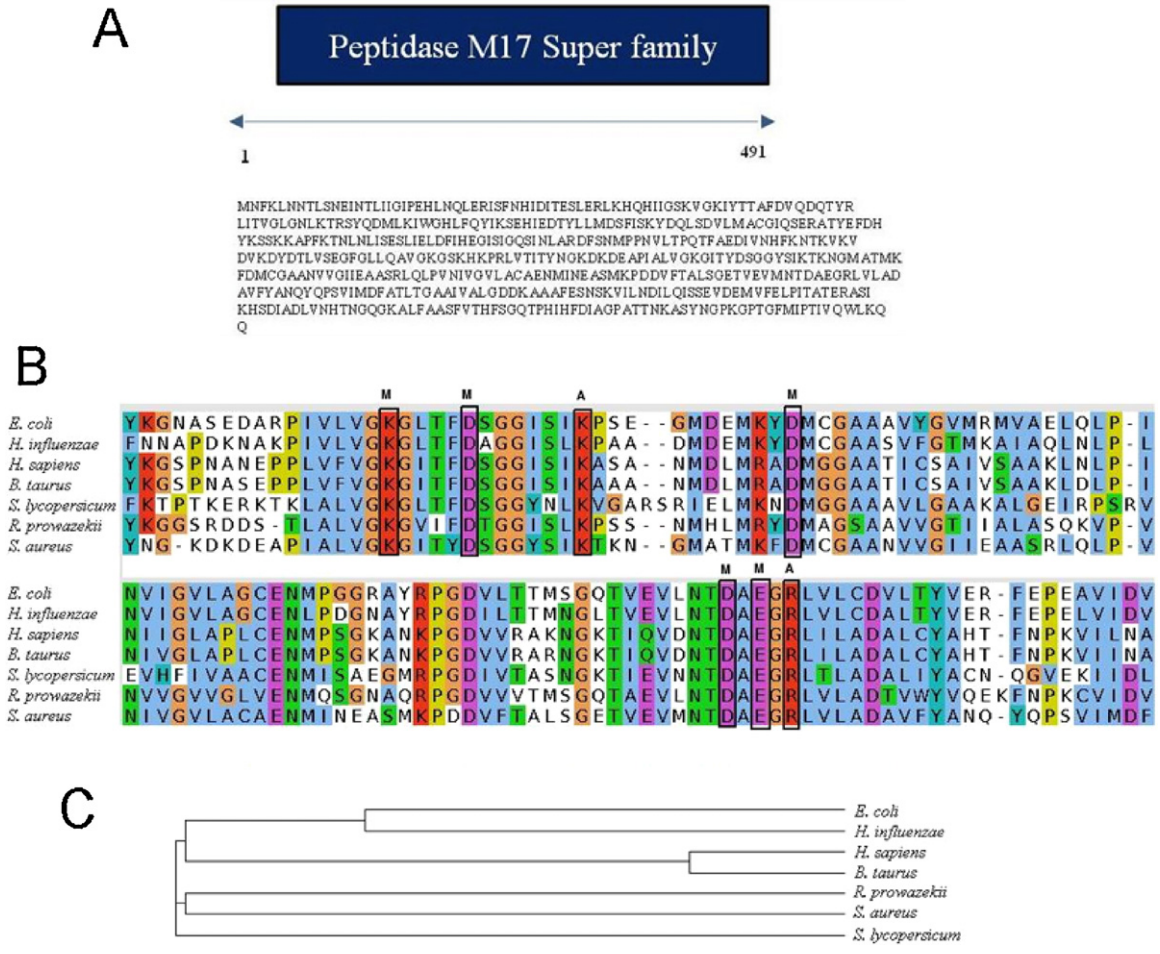

Figure 1. Domain and phylogenetic analysis of *Staphylococcus aureus* LAP. (A) Analysis of probable LAP ORF: the probable LAP sequence (**YP_499432**) from *Staphylococcus aureus* NCTC 8325 was analyzed. The MEROPS database showed that it belongs to the M17 superfamily of aminopeptidases. (B) Amino acid sequence alignment of catalytic domains of the M17 LAPs: protein sequences with the following accession numbers were analyzed: *Escherichia coli* **NP_290893**, *Haemophilus influenzae* **ZP_00154606**, *Homo sapiens* **AAD17527**, *Bos taurus* **S65367**, *Solanum lycopersicum* **AAC49456**, *Rickettsia prowazekii* **AJ235270**, and *Staphylococcus aureus* **YP_499432**. An uprooted dendrogram was prepared by comparing the full-length amino acid sequences of seven members of the family using the CLUSTAL W alignment tool. The residues essential to metal binding (M) and catalytic activity (A) are boxed. (C) Phylogenetic relationship of the M17 LAP family members: an unrooted dendrogram was prepared by comparing the full-length amino acid sequences of LAP family members using the CLUSTAL W alignment tool.

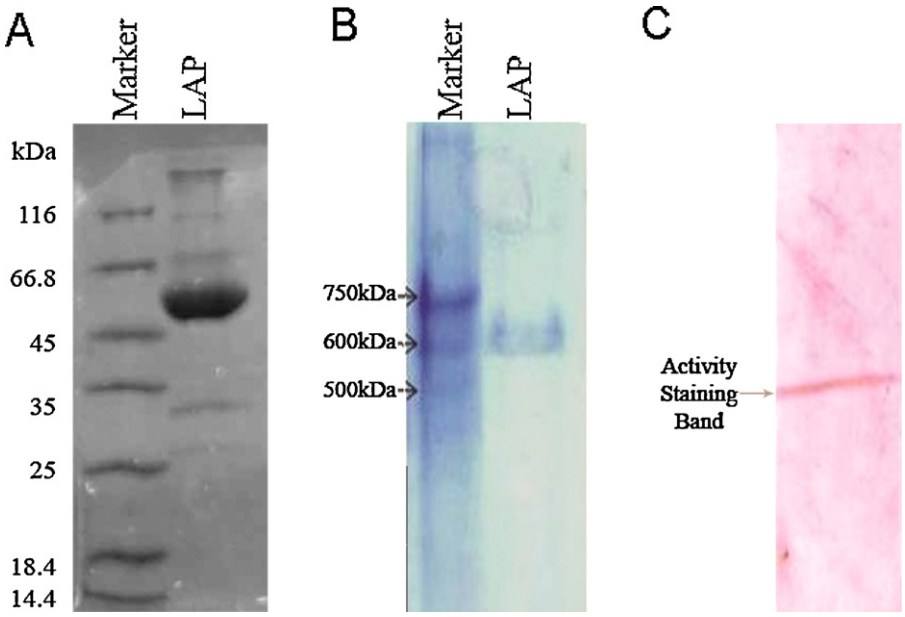

Figure 2. Analysis of *Staphylococcus aureus* LAP. (A) Over-expression of LAP: LAP cloned in pET-28a was induced by addition of IPTG (1 mM) at room temperature for 4 h. The bacterial pellet was lysed and the protein purified, resolved on 12% SDS-PAGE, and stained with Coomassie brilliant blue. (B) Native molecular weight determination: the native molecular weight of LAP was determined by resolving the purified LAP on 8% BN-PAGE at room temperature with rat liver mitochondrial respiratory chain complexes as reference molecular weight. (C) In-gel activity of LAP: the purified LAP was loaded onto 8% native PAGE and the gel was washed twice with distilled water and then with 50 mM Tris–HCl pH 8.5. The gel was incubated with 0.0015% substrate and diazotization was done as described in the Materials and methods section.

resolution analytical tool that is capable of separating low molecular weight proteins (kDa) and multi-protein complexes (MDa). It has recently been observed that BN-PAGE has higher resolution than gel filtration or sucrose density ultracentrifugation, and BN-PAGE has been used to resolve many higher order protein complexes in bacteria.[15,16] Using BN-PAGE, the molecular weight of LAP was found to be approximately 600 kDa, which indicates that the LAP may be a dodecamer in native form. Purified LAP was separated on native-PAGE, and incubation with the substrate resulted in the development of a pink azo dye (Figure 2C), suggesting that our purified LAP was enzymatically active.

3.3. Biochemical characterization of LAP

We carried out biochemical characterization of the purified LAP and determined several enzyme kinetics parameters. The K_m and V_{max} for LAP were determined using leucine *p*-nitroanilide as substrate at concentrations ranging from 0.1 mM to 4.5 mM. Their values were calculated respectively from the *x* and *y* intercepts of the double-reciprocal plot of substrate concentration (mM) versus LAP activity. K_m and V_{max} values were found to be 10 mM and 2500 μmol/min/mg protein, respectively (Figure 3, A and B).

Amidolytic activity against L-leucine *p*-nitroanilide was optimal at pH 8.5 and detectable at up to pH 10.5 (Figure 3C). The activities declined sharply at further alkaline pH and even under moderate acidic conditions (pH 5.0). LAP activity was determined at different temperatures in the range of 5–100 °C, and optimal activity was observed at 37 °C. The activity declined sharply at higher and lower temperatures (Figure 3D).

LAP belongs to the M17 family, which consists of metalloaminopeptidases with a broad range of preferences for metal ions. LAP showed enhanced activity in the presence of metal ions including Mn^{2+}, Co^{2+}, and Ni^{2+}. The maximum activity was observed in the presence of Ni^{2+}, followed by Mn^{2+} and Co^{2+}. The activity was inhibited by metal ion chelators such as EDTA. PMSF did not show any effect on enzymatic activity, which shows that it did not exhibit serine protease activity (Figure 3E).

3.4. LAP of S. aureus is secreted

The virulence factors of many pathogenic bacteria are secreted into the extracellular milieu and play a critical role in infection.[17,18] We investigated if LAP is secreted by *S. aureus*. The polyclonal antibody raised was specific to LAP, as it recognized a protein of 55 kDa corresponding to the LAP of *S. aureus* and showed no cross-reactivity with other proteins in the lysate.

The Western blotting of the supernatant and lysate of *S. aureus* culture grown overnight showed immunoreactivity against the protein of 55 kDa in the lysate as well as in the supernatant (culture media). The presence of significant amounts of LAP in the supernatant was surprising and suggests the possibility of bacterial lysis. To eliminate this possibility, Western blotting of a known intracellular protein – thioredoxin A (TrxA) – was performed under similar conditions. TrxA is a cytoplasmic bacterial protein that facilitates the reduction of cysteine disulfides of other cytoplasmic proteins and acts as an antioxidant. Since TrxA is normally a cytoplasmic protein, it would only be found in the extracellular milieu if the bacterial cells underwent lysis under similar culture conditions.[19] The Western blot analysis showed a protein of 10 kDa corresponding to TrxA only in the cell lysate, and no corresponding band was detected in the supernatant (culture media). This strongly suggests that LAP is secreted by a regulated process rather than by cell lysis (Figure 4A).

LAP expression was also examined in lysates and supernatants of five methicillin-sensitive clinical strains of *S. aureus* (MSSA) grown under similar conditions and processed in the same way. We clearly observed immunoreactivity against a 55 kDa protein, corresponding to LAP, in the lysates as well as in the supernatants (culture media) of the clinical strains P469, P768, P359, and P790 (Figure 4B). Interestingly a low level of LAP was observed in the lysate as well as the supernatant of one of the clinical strains, P189 (Figure 4B). It will be interesting to further compare the pathogenesis of P189 as compared to other clinical strains (P469, P768, P359, and P790).

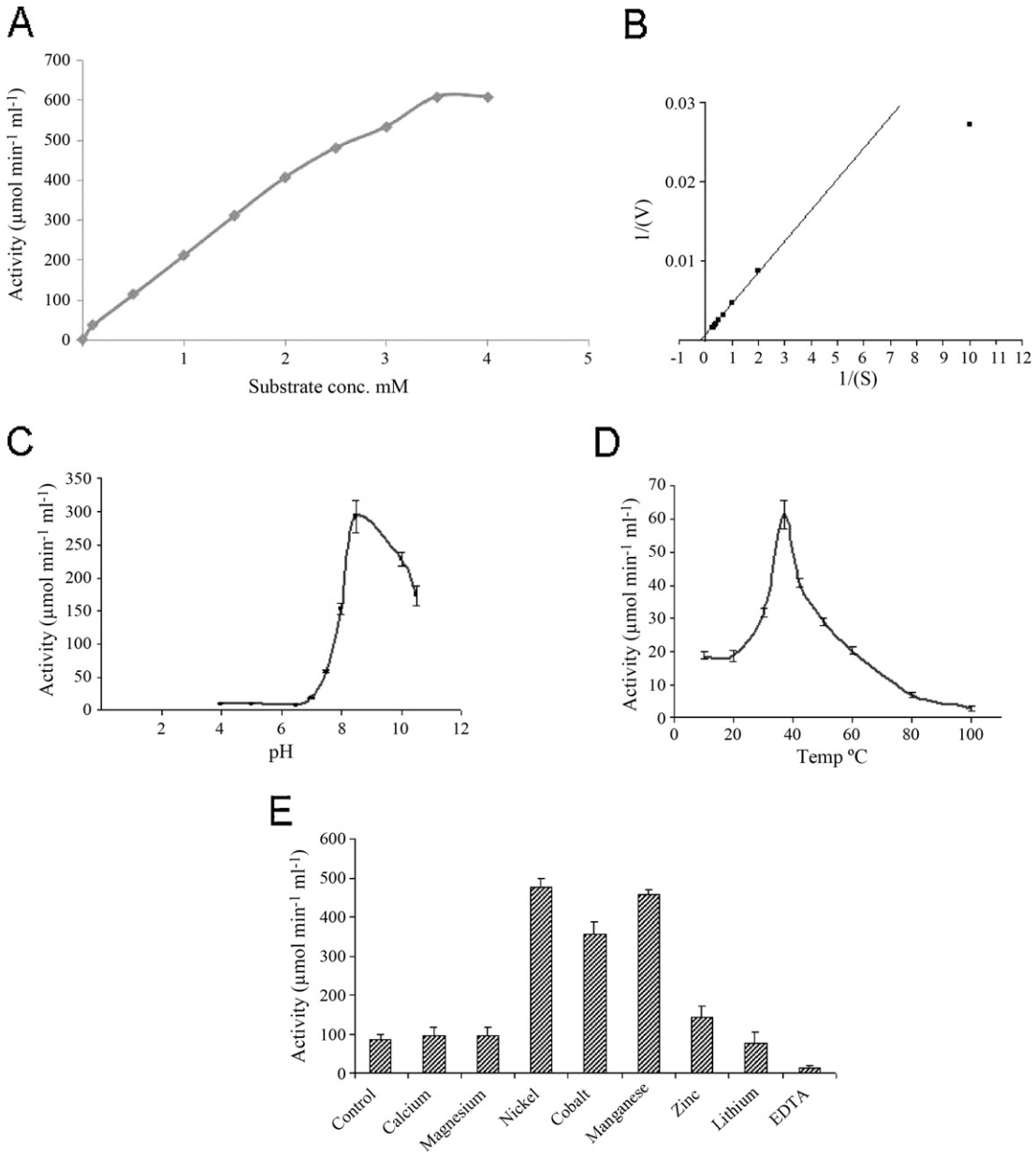

Figure 3. Biochemical characterization of LAP. (A) Substrate saturation curve of LAP: the experiment was performed with 50 mM Tris–HCl pH 8.5 and 1 mM $MnCl_2$, with substrate concentrations from 0.1 mM to 4.5 mM. (B) K_m and V_{max} of LAP: K_m and V_{max} of LAP were calculated from the intercepts of the Lineweaver–Burk plot. (C) Effects of pH on LAP activity: the experiments were performed over the pH range 4–11 in 50 mM Tris–HCl pH 8.5 with 1 mM $MnCl_2$ and 1 mM substrate. Data shown are standard error of the mean (SEM) ($n = 3$). (D) Effects of temperature on LAP activity: the experiments were carried out in Tris–HCl buffer pH 8.5 over a temperature range of 5–100 °C with 1 mM $MnCl_2$ and 1 mM substrate. Data shown are SEM ($n = 3$). (E) Effect of metal ions on LAP activity: the effect of metal ions on the LAP activity was assayed in the presence of different metal ions (1 mM concentration) in 50 mM Tris–HCl pH 8.5. Data shown are SEM ($n = 3$).

3.5. Bestatin inhibits LAP activity, growth of S. aureus, and biofilm formation

Bestatin is a potent and specific inhibitor of LAP, as described previously.[20] The enzymatic activity of purified LAP was assayed in the presence of bestatin. LAP activity decreased with increasing concentration of bestatin. The calculated half-maximal inhibitory concentration (IC_{50}) was found to be 410 nM (Figure 5A).

An earlier report strongly suggested that the LAP family of proteases may be important for survival of the pathogen during infection.[21] In the current study, the growth of *S. aureus* was inhibited in the presence of bestatin and decreased in a dose-dependent manner (Figure 5B). This strongly suggests that the activity of LAP may be essential for *S. aureus* survival.

Biofilm formation is an important microbial survival strategy, since bacteria encased in biofilm have increased resistance to antibiotics as well as immune clearance.[22] We therefore examined whether bestatin had an effect on biofilm formation in *S. aureus*. The presence of bestatin significantly inhibited biofilm formation (Figure 5, C and D).

4. Discussion

Virulence factors produced by *S. aureus* include large families of secreted exotoxins that facilitate disease progression and many secreted proteases that may help them to catabolize exogenous proteins and promote the survival of bacteria under extreme conditions.[23] LAPs are known to be important for bacterial physiology and have not yet been characterized in many

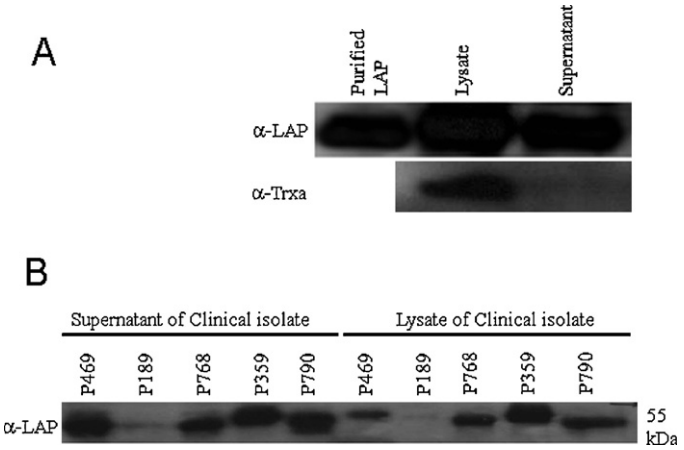

Figure 4. Extracellular secretion of LAP. (A) Detection of LAP in the supernatant of *Staphylococcus aureus*: purified LAP, supernatant (culture media), and lysate of *S. aureus* was resolved on 12% SDS-PAGE, transferred to a PVDF membrane, and blotted with LAP antibody. For control, the same amount of lysate and supernatant of *S. aureus*, from the same sample was resolved on 12% SDS-PAGE and blotted with TrxA antibody. (B) Extracellular secretion of LAP from pathogenic strains: supernatants (culture media) and lysates of clinical *S. aureus* were resolved on 12% SDS-PAGE, transferred to a PVDF membrane, and blotted with LAP antibody.

Gram-positive bacteria, including *S. aureus*. To our knowledge, we have characterized LAP from *S. aureus* for the first time; this LAP is secreted into the extracellular milieu and is important for bacterial growth and biofilm formation.

The ORF encoding the *S. aureus* LAP contains the characteristic catalytic signature sequence of the M17 family of proteases from other organisms.[24] This protein exhibited catalytic and metal binding properties, which are conserved across different species. Phylogenetic analysis clearly demonstrated that the *S. aureus* LAP is most closely related to that of *R. prowazekii*. Interestingly many pathogenic mechanisms of Rickettsia are similar to those of *S. aureus*.[25] However, the role of LAP in host–pathogen interactions in Rickettsia and other bacterial pathogens has yet to be demonstrated.

The LAPs from bacteria and other species have been demonstrated to form hexameric complexes;[26] however evidence from the current study suggests that the LAP from *S. aureus* may exist as a dodecamer or forms higher molecular order structures. This needs to be further validated by other chromatographic techniques. Analysis of the *S. aureus* genome suggests that LAP is present as a single copy, and thus may exhibit a broad substrate spectrum to ensure adequate release of amino acids from polypeptides to maintain the life cycle in different stressed conditions. Therefore, the aminopeptidase activity against other substrates (Met, Arg, Ala, Ile, Val, Phe, Gly, and Tyr) should be studied further.

LAPs belong to the M17 family of metalloproteases, which mainly prefer Zn(II),[27] and other aminopeptidases are dependent upon Mn(II),[28] Fe(II),[29] and Zn(II).[30] In the present study we observed that the *S. aureus* LAP exhibited maximum activity in the presence of Ni(II), indicating that it has a preference for metals distinct from those of other M17 proteases.

Bestatin is a dipeptide analogue that has been identified as an inhibitor of LAP.[31] Bestatin inhibited LAP enzymatic activity as well as *S. aureus* growth, suggesting that LAP activity may be important

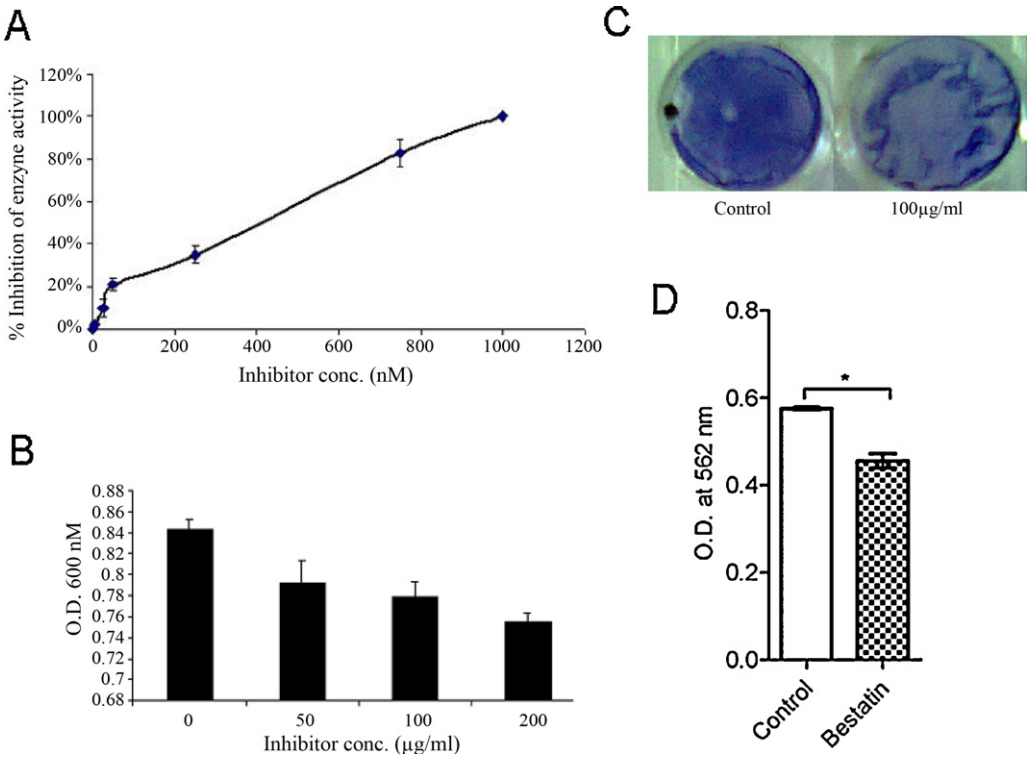

Figure 5. Bestatin inhibits LAP activity, growth of *Staphylococcus aureus*, and biofilm formation. (A) Bestatin inhibits the enzymatic activity of LAP: the experiments were carried out by pre-incubation of LAP (1 μg/ml) with different concentrations of bestatin in Tris–HCl buffer (pH 8.5) and further with 1 mM $MnCl_2$ and 1 mM substrate at 37 °C. Data shown are standard error of the mean (SEM) ($n = 3$). (B) Bestatin inhibits the growth of *S. aureus* in vitro: *S. aureus* was incubated with or without different concentrations of bestatin at 37 °C, and growth was monitored by measuring OD_{600} 3 h after addition of the inhibitor. Data shown are SEM ($n = 3$). (C) Bestatin inhibits the biofilm formation of *S. aureus*: culture of *S. aureus* grown overnight was diluted in TSB (1:40 dilution) containing 0.25% glucose and inoculated in 24-well plates. Bestatin was added at the time of inoculation at a concentration of 100 μg/ml and incubated at 37 °C for 16 h. Biofilm was stained with 0.1% crystal violet and photographed. (D) Quantification of biofilm formation: biofilm was stained with 0.1% crystal violet for 1 min and washed three times with sterile water. Crystal violet was solubilized with 30% glacial acetic acid and the OD measured at 562 nM. Data shown are SEM ($n = 3$).

for bacterial metabolism and physiology. Interestingly in some pathogens like Fasicola, LAP has been observed to be secreted and found to be important for evasion from the host immune response.[21] Similarly secreted LAP from *Legionella pneumophila* may be important for infectivity in multiple hosts, including humans.[32] In consonance with earlier observations,[21] we found that LAP was predominantly secreted in the extracellular milieu from *S. aureus* NCTC 8325, as well as in clinical strains of *S. aureus* (MSSA). This strongly suggests that LAP may be important not only for bacterial physiology but also for pathogenesis. This is an important finding and needs further study to establish the role of LAP in the pathogenesis of *S. aureus*.

The development of persistent infection is mainly attributed to biofilm formation, which is implicated in many diseases like cystic fibrosis, chronic rhinosinusitis, and pneumonia.[33] Data from the current study strongly suggest that secreted LAP may have a role in the process of *S. aureus* biofilm formation, which may be one of the important mechanisms in the development of resistance to antibiotics. It has also been demonstrated previously that biofilms are more resistant to antibiotics as compared to their planktonic counterparts.[34] The inhibition of biofilm formation suggests that bestatin could be a quorum sensing inhibitor for *S. aureus*, however this requires further experimental evidence. Bestatin may be an interesting scaffold on which to design novel inhibitors of *S. aureus*, and thus may be a helpful tool in the development of an effective strategy to combat this pathogen.

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Conflict of interest: No conflict of interest to declare.

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