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Cloning, expression and purification of autolysin from methicillin-resistant *Staphylococcus aureus*: potency and challenge study in Balb/c mice

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ABSTRACT

Staphylococcus aureus (MRSA) is an opportunistic pathogen which causes a variety of clinical diseases and leads to high rates of morbidity and mortality. Development of an effective vaccine appears to be a useful strategy to control the infection.

Here, the internal region of *atl* was cloned into the pET24a plasmid and expressed in *E. coli* BL21 (DE3). Cloning of *atl* was confirmed by colony-PCR, enzymatic digestion and sequencing. Protein expressed in E coli, BL21 DE3 and was confirmed with SDS-PAGE and western blot analysis. Subsequently, BALB/c mice were injected subcutaneously three times with 20 μg of the recombinant autolysin. After Bleeding, autolysin-specific total IgG antibodies and isotypes were evaluated using ELISA. Opsonophagocytic killing assay was performed and experimental challenge was done by intraperitoneal injection with sub lethal doses of MRSA in mice and also survival rate was regularly monitored.

Results showed that vaccinated mice could exhibit higher levels of autolysin-specific antibodies ($P < 0.0001$) with a predominant IgG1 response versus control group. Results from in vitro experiments indicated that *S. aureus* opsonized with immunized-mice sera displayed significantly increased phagocytic uptake and effective intracellular killing versus non-immunized mice. The number of viable bacteria in the kidney of immunized mice showed 1000 times less than the control mice; additionally, an increased survival rate was found after immunization with the candidate vaccine versus control group.

Results from this study demonstrated that the autolysin is a valuable target for the development of immunotherapeutic strategies against *S. aureus* and candidate vaccines.

1. Introduction

Hospital-acquired infections are a leading cause of morbidity and mortality worldwide (Peleg and Hooper, 2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) has been identified as one of the most frequent nosocomial pathogens, accounting for approximately 25% of the 2 million nosocomial infections ranging from mild skin to septicemia (Chatterjee and Otto, 2013; David and Daum, 2010). *S. aureus* strains have developed resistance to almost all antibiotics; however, MRSA has shown susceptibility to vancomycin, a final option to overcome infections caused by strains resistant to other antibiotics (Tarai et al., 2013; Schito, 2006; Deresinski, 2009). However, the first case of new "superbug" bacteria was reported, which is completely resistant to vancomycin (Waness, 2010). Therefore, there is an urgent need to develop an effective vaccine for the prevention of the infection (Ventola, 2015; Giersing et al., 2016).

Bacterial cell separation, in addition to the ability of bacteria to adhere to host tissues and express genes involved in the infection, is a critical event in most staphylococcal infections (Wilson et al., 2002; Bien et al., 2011; Beceiro et al., 2013). Bacterial cell separation is a dynamic event in the cell cycle, which requires the cleavage of peptidoglycan connecting recently-developed daugh-

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ter cells. In a variety of bacteria, it was demonstrated that there is a correlation between the lack of peptidoglycan hydrolase activity and a failure in cell separation (Vollmer et al., 2008; Rico-Lastres et al., 2015; Yamada et al., 1996; Oshida et al., 1998). Bacterial autolysins are potentially lethal enzymes, which can hydrolyze the peptidoglycan component of the cell wall, and involve in the separation of daughter cells after cell division (Yamada et al., 1996; Oshida et al., 1995; Houston et al., 2011; Heilmann et al., 2005). The gene encoding autolysin, *atl,* produces the autolysin protein, a unique, functional protein with both amidase and glucosaminidase activities. Autolysin undergoes proteolytic processing to generate two extracellular peptidoglycan hydrolases, endo-$^{\beta}$-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase (Oshida et al., 1995; Houston et al., 2011; Ahn and Burne, 2006; Foster, 1995). In the precursor Atl protein, the two catalytic functions (cat) are each linked to targeting repeats (R1–R3) and also connected to a propeptide and a signal peptide (Büttner et al., 2014) considered as a vaccine candidate.

Some studies have reported the presence of different autolysins in other species of staphylococci such as *S. epidermidis*, showing their important role in the colonization of host tissues, biofilm formation and excretion of cytoplasmic proteins (Heilmann et al., 2003). A wide variety of studies showed that inhibition of autolysin results in the inhibition of bacteria (Sieradzki and Tomasz, 2006). Therefore, the development of new strategies to inhibit autolysin either by drugs or immune responses seems to result in the elimination of bacteria as well as clearance of infection (Varrone et al., 2011).

Morbidity and mortality from MRSA infection may depend on the status of host immunity, especially humoral immunity, which is believed to play a significant role against staphylococcal infections (Haghighat et al., 2013; Brown et al., 2013). In this light, alternative strategies to prevent and treat multiresistant bacterial infections, such as vaccines and passive immunization, are being extensively evaluated, but none has yet been proven to be effective for use in clinical practice (Otto, 2010; Zhang et al., 2015; Roth and Machado, 2006; Jansen et al., 2013; Kurlenda and Grinholc, 2012; Garcia-Quintanilla et al., 2016).

Due to their ability to induce humoral immune responses, recombinant vaccines appear to be a suitable strategy for generating protective immune responses against MRSA (Roth and Machado, 2006; Jansen et al., 2013; Senna et al., 2003). Staphylococcal autolysins Aas from *Staphylococcus saprophyticus* and AtlC from *Staphylococcus caprae* (Allignet et al., 2002) are homologous to AtlE and Atl from *Staphylococcus aureus* (Oshida et al., 1995; Foster, 1995), respectively. This emphasizes the fact that *staphylococcus* spp. contain major antigenic types of autolysin (Pasztor et al., 2010), as an important virulence factor expressed during the infection. Because of the critical role of autolysin in cell division, a specific inhibition of autolysin activity can kill the organism (Yamada et al., 1996; Houston et al., 2011; Pasztor et al., 2010). Because of its critical functions for MRSA bacteria, we therefore hypothesized that autolysin can be considered as a potential vaccine candidate to prevent infections caused by such bacteria.

In light of this, the aim of the present study was to clone, express and purify MRSA autolysin, and evaluate the immunogenicity and protectivity of the recombinant autolysin as a vaccine candidate in BALB/c mice.

2. Materials and methods

2.1. Bacterial strains and vectors

The *S. aureus* strain COL (methicillin-resistant *S. aureus*), generously donated from Dr. Mohammad Emaneini, was grown at 37 °C on blood agar, and used for PCR amplification and challenge studies. *E. coli* strainsDH5α (Invitrogen, California, USA) and BL21 (DE3) (Novagen, Wisconsin, USA) were used for cloning and expression of the recombinant protein, respectively. *E. coli* cells harboring recombinant plasmids were grown aerobically at 37 °C in Luria-Bertani broth (Merck, Darmstadt, Germany) with or without 50 μg/ml Kanamycin (Sigma, Saint Louis, MO, USA). The plasmid pET-24a (Novagen, Wisconsin, USA) was used as an expression vector.

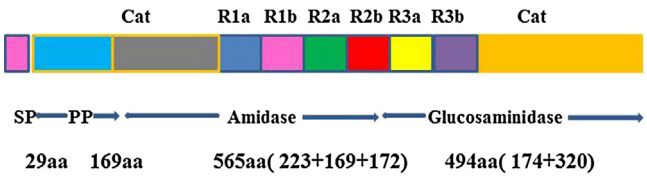

Fig. 1. Structure of autolysin. Domain arrangement of Atl with sites of post-translational cleavage indicated by *arrows*. *SP*, signal peptide; *PP*, propeptide; *cat*, catalytic domain; *R*, repeat domain.

2.2. Amplification and cloning of the atl gene

Genomic DNA of the *S. aureus* strain COL (methicillin-resistant *S. aureus*) was extracted using the bacterial genomic DNA isolation kit (Metabion, South Korea). PCR amplification yielded a 1060-bp fragment of the *atl* gene (amino acids 72-424) with *N*-acetylmuramoyl-L-alanine amidase activity (Fig. 1). Table 1 represents the specific primers used in this study. Amplifications were carried out in 25 vols containing 1× Pfu buffer with $MgCl_2$, 1 mM dNTPs (Fermentase), 10 pmoles of each primer, 1.25 U Pfu DNA polymerase (Fermentase) and 100 ng of genomic DNA. PCR conditions were as follows: one cycle of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 60 s at 58 °C, 2 min at 72 °C, and a final cycle of 10 min at 72 °C. PCR products were recovered from the gel and purified using the PCR purification kit (Roche, Germany). The purified *atl* fragment was digested by restriction enzymes, and then ligated into the pET-24a vector containing a sequence of six His residues at the C-terminus to facilitate the specific purification of the expressed protein. The recombinant vector pET24a-*atl* was transformed into the competent *E. coli* DH5α cells. The integrity of the recovered plasmid was confirmed by colony PCR, restriction endonuclease digestion and sequencing.

2.3. Protein expression

To express the recombinant plasmid, pET24a-*atl* was transformed into competent *E. coli* BL21 (DE3), and the bacteriawere grown at 37 °C in Luria-Bertani medium (LB) supplemented with kanamycin (50 mg/ml) until exponential phase (OD600 nm = 0.6). Then, isopropyl-β-D-thiogalacto pyranoside (IPTG, Fermentase) was added to a final concentration of 1 mM to induce protein expression. Samples were collected every 3 h for 12 h and analyzed by SDS-PAGE to follow the best time point of protein expression. In addition, western blot analysis was performed to confirm protein expression (Haghighat et al., 2013; Roth and Machado, 2006; Goudarzi et al., 2009).

2.4. Purification of the recombinant peptide

The pellet of bacterial cells expressing the peptide was harvested, resuspended in lysis buffer 8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris (pH 8.0) containing protease inhibitors, disrupted by sonication and then centrifuged for 20 min at 10000 rpm. After centrifugation, the recombinant peptide (with a molecular weight of approximately 43 kDa) was purified from the supernatant under denaturing conditions using Ni-nitrilotriacetic acid (Ni-NTA) affin-