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Expression of Sortase, a Transpeptidase for Cell Wall Sorting Reaction, from *Staphylococcus aureus* ATCC 6538p in *Escherichia coli*

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Abstract This paper describes the development of an enzymatic assay system for the identification of specific inhibitors of sortase, a transpeptidase that cleaves surface proteins of Gram-positive bacteria, from *Staphylococcus aureus* ATCC 6538p for antibacterial drug discovery. The coding region of the enzyme was amplified with the exception of the N-terminal membrane anchor sequence, cloned into a vector providing His-Patch-thioredoxin-tag at the N-terminus, expressed in *Escherichia coli*, and purified by metal chelate affinity chromatography. The enzyme activity was determined by quantifying increased fluorescence intensity upon cleavage of synthetic Dabcyl-QALPETGEE-Edans peptide. The results suggest that the developed *in vitro* assay system can be used in the search for sortase inhibitors in a short period of time.

Key words: *Staphylococcus aureus*, sortase gene, expression, *in vitro* assay

Gram-positive pathogenic bacteria display proteins on their surface that may interact with host cells and tissues and play a role in virulence [1, 2, 6]. In the case of *Staphylococcus aureus* protein A, immunoglobulins are captured on the microbial surface and camouflage bacteria during the invasion of host tissues [7, 18]. Protein A is cleaved by a transpeptidase, sortase, between threonine and glycine residues of a conserved LPXTG motif (where X is any amino acid). The carboxyl group of threonine is amide-linked to the amino group of the pentaglycine cross-bridge of the peptidoglycan. This reaction, called cell wall sorting, is likely to occur in most Gram-positive bacteria [10, 17]. Schneewind and colleagues [5, 11, 20] have brought to light definitive evidence that sortase plays a key role in the display of surface proteins in *S. aureus* and the virulence of this important human pathogen. Thus, sortase appears to be a very promising target for identifying inhibitors that could be of general use in therapeutics against Gram-positive bacteria [3, 4, 15]. To develop a screening system on the basis of the enzymatic reaction, it is necessary to isolate larger amounts of sortase. In this work, a sortase gene from *S. aureus* ATCC 6538p was cloned and expressed in *E. coli*. The availability of purified sortase for the cleavage of LPXTG peptide was investigated using a synthetic fluorophore.

Staphylococcus aureus ATCC 6538p was the source of the sortase gene (*srtA*, surface protein sorting A). *Escherichia coli* TOP10 [F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] and pBAD/Thio-TOPO vector (Invitrogen, The Netherlands) was used for all the cloning steps and the protein expression. Both the *S. aureus* and the *E. coli* strains were grown in LB medium (1% Bactotryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) at 37°C with vigorous shaking. Ampicillin (50 μg/ml) was used for the selection of plasmid-containing transformants. The genomic DNA was prepared from *S. aureus* ATCC 6538p according to the method of Sambrook & Russell [13]. The primers 5'-AAACCACATATCGATAATTATC-3' and 5'-TTATTTGACTTCTGTAGCTACAA-3' were used for PCR amplification of the *srtA* gene (with the exception of N-terminal membrane anchor sequence) from the genomic DNA [17]. Amplification of the *srtA* gene was performed in a volume of 100 μl containing 10 μg of template DNA, 0.5 U of *Taq* polymerase, 10 μl of 10× *Taq* DNA polymerase buffer, 16 μl of dNTP mixture, and 7 μl of forward and reverse primers (100 pmol/μl, each). PCR amplification was carried out in the following conditions on a thermal cycler (OmniGene, Hybaid): hot start at 94°C for 5 min, 30 cycles of denaturation (94°C, 1 min), annealing

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(45°C, 1 min), extension (72°C, 2 min), and a final extension at 72°C for 10 min. The amplified PCR fragment was purified from 0.7% Seakem GTG agarose (FMC, U.S.A.) gel by suprec-01 (Takara Shuzo Co., Japan) and ligated into the pBAD/Thio-TOPO vector for TA cloning. The ligation mixture was transformed into *E. coli* TOP10 and the transformant was selected on LB agar plates containing ampicillin. The construct was confirmed by restriction enzyme digestion. The recombinant plasmid was isolated from *E. coli* transformants and subjected to nucleotide sequencing with an ABI PRISM 377 DNA sequencer using two synthetic primers: 5'-TCACAACCCGGGCAC-TGCGCCG-3', forward primer; 5'-AGACCCCACACTA-CCATCGGCG-3', reverse primer.

The positive transformant was grown in an LB medium supplemented with 50 μg/ml of ampicillin at 37°C for 13 h. The pre-incubated cells were inoculated into 10 ml of LB broth containing ampicillin and incubated with shaking at 37°C until OD_{600} reached approximately 0.5. At this point, arabinose was added to the medium to a final concentration of over 0.002% and incubation was continued for an additional 6 h to induce the fused srtA expression. Cells were harvested by centrifugation at 3,000 ×g for 30 min and lysed by lysozyme treatment and sonication [13]. Clear extracts containing fused protein were applied on a Xpress™ protein purification system (Invitrogen) equilibrated with 20 mM sodium phosphate and 500 mM NaCl (pH 7.8). The column was washed twice with 4 ml of the wash buffer (20 mM sodium phosphate and 500 mM NaCl, pH 6.0). The bound proteins were eluted three times with 1 ml of elution buffer (20 mM sodium phosphate, 500 mM NaCl, and 50 mM imidazole, pH 6.0). Fractions containing N-terminal fused protein were collected and treated with enterokinase [50 mM Tris-HCl, 1 mM $CaCl_2$, and 0.1% Tween-20 (pH 8.0); incubation for 16 h at 37°C] for eliminating amino-terminal residues. After cleavage, the reaction mixture was dialyzed by using UFV4BGC25 membrane (Millipore, Bedford, MA, U.S.A.) against an enzyme reaction buffer (50 mM Tris-HCl buffer and 150 mM NaCl, pH 7.5) and filtered through a 0.22 μm pore-size membrane filter. The cleared reaction mixture was subjected to an AKTA purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) and the srtA was purified. The expressed recombinant protein was analyzed by SDS-PAGE [8].

To determine the enzyme activity quantitatively, reactions were carried out in 2 ml containing 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 2.5 μg synthetic LPXTG peptide substrate, 4-(4-dimethylaminophenylazo)benzoic acid (Dabcyl)-QALPETGEE-5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (Edans), and 5 mM $CaCl_2$ with increasing amount of srtA. After incubation for 1 h at 37°C, the increase in fluorescence intensity was recorded by fluorescence spectrophotometery (F-2000, Hitachi, Tokyo, Japan), using 350 nm for excitation and 495 nm for recordings. In addition, the effect of pHMB (*p*-hydroxymecuribenzoic acid), a known srtA inhibitor [16], on the srtA enzyme activity was investigated. Five millimoles of pHMB and 320 μg of srtA were added to a 2-ml reaction mixture and treated as stated above.

After the genomic DNA was extracted from *S. aureus* ATCC 6538p, the *srtA* was amplified by PCR with the exception of the N-terminal membrane anchor sequence. Resulting PCR product was cloned into pBAD/Thio-TOPO vector and transformed into *E. coli* TOP10. The sequence of the *S. aureus* ATCC 6538P srtA clone showed 99% identity at the amino acid level with the published *S. aureus* SM317 srtA (assession number AF162687) [10] (data not shown).

The bacterial expression system used in this study provides a one-step cloning strategy for the direct insertion of amplified PCR products into the plasmid vector (pBAD/Thio-TOPO) for soluble, regulated expression and simplified protein purification in *E. coli*. Recombinant proteins were expressed as cleavable fusion protein to His-Patch (HP) thioredoxin for high-level expression and simple purification. Expression in *E. coli* is driven by the *ara*BAD promoter (P_{BAD}). The *ara*BAD promoter is both positively and negatively regulated by the product of the *ara*C gene [12, 14]. AraC is a transcriptional regulator that forms a complex with L-arabinose, therefore expression of the recombinant gene can be induced by adding arabinose. There was no significant difference in the induction of gene expression by the arabinose concentration over

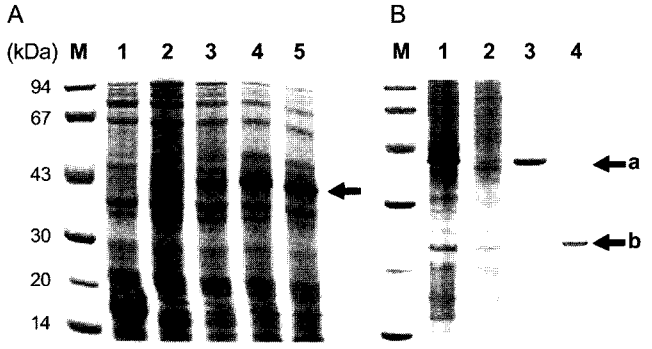

Fig. 1. SDS-PAGE analysis of the sortase expressed in *E. coli*.
(A) The samples were loaded on a 15% polyacrylamide gel. M, protein molecular weight standard; lane 1, total proteins of host with pBAD/Thio-TOPO; lane 2, total proteins of host without pBAD/Thio-TOPO; lane 3, total proteins of host with pBAD/Thio-TOPO-*srtA* in a 4-h induction; lane 4, 6-h induction; lane 5, 8-h induction. The overexpressed recombinant sortase is indicated by an arrow. (B) The sortase was purified by a Ni-NTA affinity column chromatography. The eluted protein was treated with enterokinase for cleavage between amino-terminal residues for affinity and recombinant sortase. Lane 1, total protein of host with pBAD/Thio-TOPO-*srtA* (6-h induction); lane 2, unbound fraction of Ni-NTA affinity column; lane 3, eluted fraction containing the fused sortase; lane 4, enterokinase-treated recombinant sortase separated from amino-terminal residues for affinity. Label "a" indicates the overexpressed recombinant sortase (ca. 36 kDa) and "b" indicates enterokinase-treated (cleaved) recombinant sortase (ca. 20.8 kDa).

0.002%. Therefore, the gene expression was induced by adding arabinose to a final concentration of 0.002% as described above.

The concentration of expressed thio-srtA was analyzed by SDS-PAGE, as shown in Fig. 1. The concentration of fusion protein content was about 20% of total protein produced in *E. coli* and there was no significant difference in the expressed protein depending on the induction time (Fig. 1A). Because the protein was fused with HP-thioredoxin that has a Ni-binding property at pH 7.8, the expressed thio-srtA was purified by a Ni-NTA affinity column. Nonspecific binding could be reduced by washing the resin with wash buffer. Finally, the thio-srtA was eluted by including a high concentration of imidazole (50 mM) in the wash buffer. The molecular mass of the protein was calculated to be 36 kDa. Considering the fusion protein, this molecular weight seemed to be reasonable. After purification, thio-srtA was treated with enterokinase to cleave the fused HP-thioredoxin. The cleaved HP-thioredoxin was separated by AKTA purifier and the mature form of the recombinant srtA was purified (Fig. 1B).

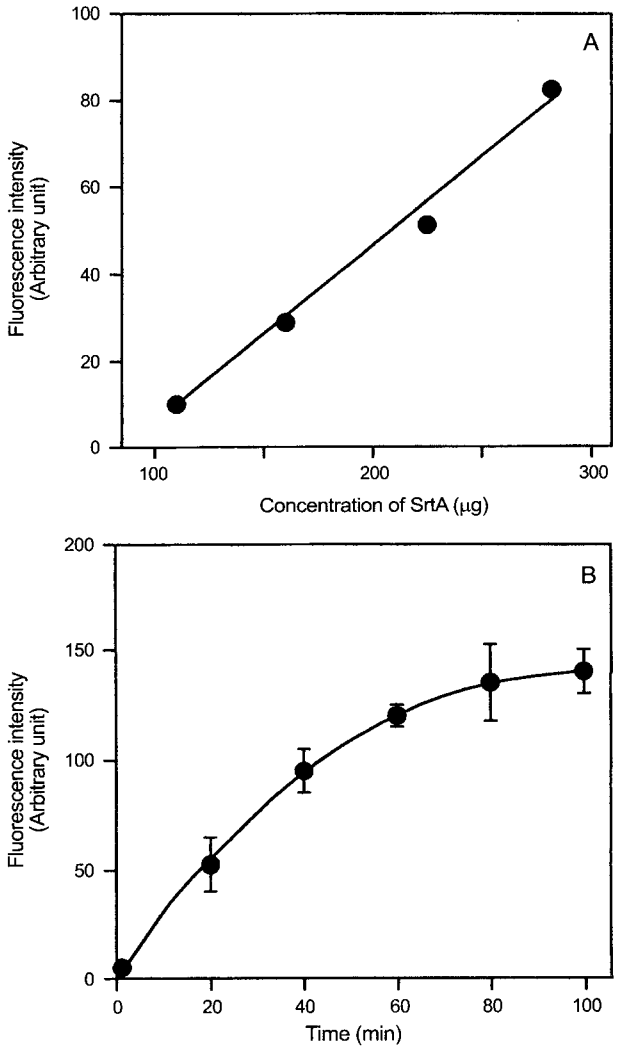

Fig. 2. Dabcyl-QALPETGEE-Edans (LPXTG) cleavage activity of purified sortase in the enzyme reaction.
(A) Effect of enzyme concentration on activity. The enzyme reaction was carried out under the standard assay conditions at 37°C for 1 h. (B) Time course of the enzyme reaction. Reactions were assembled in a volume of 2 ml containing 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 2.5 μg synthetic LPXTG peptide substrate, and 5 mM $CaCl_2$, and 320 μg sortase. The enzyme reaction was carried out at 37°C.

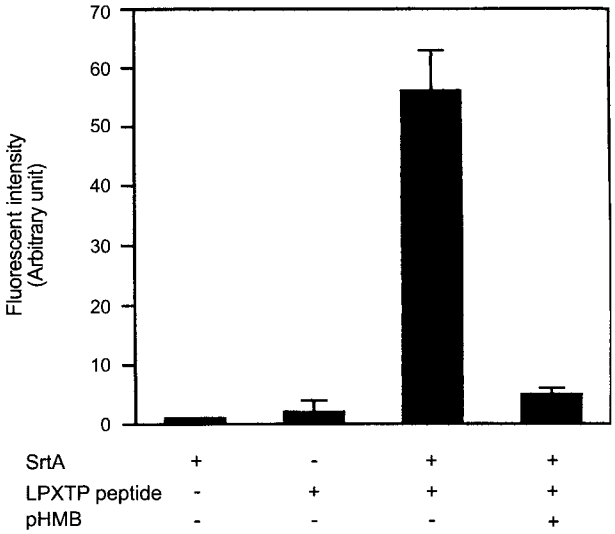

Fig. 3. Cleavage of LPXTG peptide by purified sortase *in vitro*.
Sortase was incubated with the sorting substrate Dabcyl-QALPETGEE-Edans (LPXTG), and peptide cleavage was monitored as an increase in fluorescence. The addition of pHMB (5 mM), a known inhibitor of sortase, inhibited cleavage.

The enzyme activity of srtA could be assayed by monitoring the increment of fluorescence intensity. Fluorescence of the Edans fluorophore within the peptide Dabcyl-QALPETGEE-Edans is quenched by the close proximity of Dabcyl [19, 21]. When the peptide is cleaved and the fluorophore is separated from Dabcyl, an increase of fluorescence is observed [9]. Incubation of the LPXTG peptide with srtA resulted in an increase of fluorescence intensity, as shown in Fig. 2, and the cleavage activity was dependent on the amount of enzyme and incubation time. Next, the effect of pHMB on the srtA-substrate reaction was investigated. When the pHMB was added to the reaction mixture, the increment of fluorescent intensity was diminished, revealing that pHMB prevents the srtA reaction (Fig. 3). These results suggest that the above *in vitro* assay system can be used in the search for sortase inhibitors.

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