Autolytic activity and molecular characteristics of *Staphylococcus haemolyticus* strains with induced vancomycin resistance

Jung Wook Kim, Gyung Tae Chung, Jung Sik Yoo, Yeong Seon Lee and Jae Il Yoo

The aim of this study was to investigate the molecular characteristics of induced vancomycin resistance in *Staphylococcus haemolyticus*. Autolytic properties and phenotypic characteristics of passage-selected vancomycin-resistant *S. haemolyticus* strains were examined. In addition, expression of autolysis-related genes (*atl*, *lrgAB*, *sarA* and *lytS*) was investigated using the RNase protection assay (RPA). The RPA results indicated that only the expression of the *atl* gene was significantly upregulated (2.5- to 6-fold increase) in vancomycin-intermediate and vancomycin-resistant strains. The vancomycin-resistant strains exhibited lower expression of murein hydrolase proteins and reduced autolytic activity compared with the parent strain. In addition, a reduced growth rate, cell wall thickening and higher survival rate in the presence of lysostaphin were observed in vancomycin-intermediate and vancomycin-resistant induced strains compared with the parent strain. In conclusion, altered autolytic properties, in particular upregulation of the *atl* gene, may contribute to vancomycin resistance in *S. haemolyticus*.

**INTRODUCTION**

Coagulase-negative staphylococci (CoNS) have been recognized as important causative agents of nosocomial bacteremia. In particular, the frequency of *Staphylococcus haemolyticus* infection is second only to that of *Staphylococcus epidermidis* among clinical isolates of CoNS (Vignaroli et al., 2006). Some recent reports have recognized *S. haemolyticus* as the cause of severe infections, including meningitis, skin and skin structure infections, prosthetic joint infections, bacteremia and even endocarditis (Yu et al., 2010). Because of the rising incidence of meticillin resistance, glycopeptides have been recommended as therapeutic agents for serious staphylococcal infections (Nunes et al., 2006). However, the extensive use of glycopeptides has decreased the susceptibility of staphylococcal species to these agents. In addition to the tendency of *S. haemolyticus* to develop resistance to multiple antibiotics, it was the first Gram-positive pathogen to acquire glycopeptide resistance; this occurred earlier than in other staphylococcal species and enterococci (Biavasco et al., 2000). However, the molecular characteristics of glycopeptide resistance in *S. haemolyticus* are not yet completely understood.

Various mechanisms of vancomycin resistance have been reported in vancomycin-intermediate *Staphylococcus aureus* (VISA) (Cui et al., 2003; Finan et al., 2001; Pfeltz et al., 2000; Sieradzki & Tomasz, 1997, 1999). Furthermore, a few reports have indicated that the mechanism underlying glycopeptide resistance in *S. epidermidis*, *S. haemolyticus*, *Staphylococcus hominis* and *Staphylococcus sciuri* is similar to that described in VISA and hetero-VISA strains (Sieradzki et al., 1998, 1999). An *S. haemolyticus* strain with reduced vancomycin susceptibility has been reported to exhibit a thicker cell wall than that of the parent strain and variable rates of autolysis (Nunes et al., 2006). However, the molecular characteristics of vancomycin resistance in *S. haemolyticus* are poorly documented. In this study, we induced vancomycin resistance in susceptible *S. haemolyticus* and investigated the phenotypic characteristics and expression of autolytic activity-related genes in the induced strains.

**METHODS**

**Clinical strains.** Staphylococcal isolates were collected from tertiary hospitals in Korea. The staphylococcal strains were identified as CoNS by catalase, coagulase and DNase testing. The CoNS were identified to the species level by using the VITEK system (bioMérieux). In this study, 10 staphylococcal strains were identified as...
**S. haemolyticus**. Among the *S. haemolyticus* strains, the *S. haemolyticus* GS52 strain showed a vancomycin-susceptible MIC (MIC 4), which was the lowest MIC for vancomycin among the collected *S. haemolyticus* strains. To induce vancomycin resistance in a susceptible strain, the *S. haemolyticus* GS52 strain was selected for further study. This strain has been described in a previous study (Yoo et al., 2010).

**Generation of mutants.** Mutants were selected from the clinical *S. haemolyticus* GS52 strain by streaking 10 μl of an overnight culture onto brain heart infusion (BHI) plates containing 4 μg vancomycin ml⁻¹ at 35 °C. Cultures were grown in BHI broth (Difco) containing 4 μg vancomycin ml⁻¹. This procedure was repeated with increasing gradient concentrations of vancomycin until stable mutants with vancomycin MIC values of 16 (GS52V₁₆) and 48 (GS52V₄₈) μg ml⁻¹ were obtained.

**Growth curves.** The *S. haemolyticus* strains were grown in BHI broth at 37 °C with aeration. In each experiment, overnight cultures were diluted 1000-fold in 10 ml fresh BHI broth medium and grown at 37 °C with shaking at 200 r.p.m. Growth was followed by monitoring the OD₆₀₀ at hourly intervals.

**Antimicrobial susceptibility testing.** The MICs of vancomycin, teicoplanin, oxacillin, penicillin, imipenem, linezolid, dapto mycin, tigecycline, erythromycin and rifampicin for all derivatives and parent strains were determined by Etest (AB Biodisk) according to the manufacturer’s instructions.

**Autolysis assay.** Autolysis assays were performed for mutant and parental strains as previously described (Four nier & Hooper, 2000). Cells were grown exponentially to an OD₆₀₀ of about 0.3. The cultures were then chilled rapidly and centrifuged; cells were then washed once with ice-cold distilled water and resuspended in 50 mM Tris/HCl (pH 7.5) and 0.1 % Triton X-100 to obtain an OD₆₀₀ of 1.0. The cells were then incubated at 35 °C with shaking, and the changes in OD₆₀₀ were measured. Results were normalized to OD₆₀₀ at time zero (t=0), i.e. per cent lysis at time t=[(OD₆₀₀−OD₆₀₀)/OD₆₀₀]×100. All the results shown represent the mean values of at least two independent determinations.

**Zymographic analysis.** Zymographic analysis of peptidoglycan hydrolase profiles was performed as previously described (Zheng et al., 2007). Briefly, overnight cultures were centrifuged at 10 000 g at 4 °C for 10 min, and the supernatants were collected, filter-sterilized and concentrated 100-fold with a Centricon concentrator (Millipore). The concentration of total proteins in each sample was determined using the Bradford assay (Bio-Rad) according to the manufacturer’s instructions. A total of 30 μg of proteins from each sample was resolved on an 8 % SDS-PAGE gel containing 0.2 % autoclaved and lyophilized wet *S. haemolyticus* cells or *Micrococcus lysodeikticus* cells (Sigma-Aldrich). Following electrophoresis, the gels were washed with water and incubated overnight in renaturation buffer [25 mM Tris/HCl (pH 7.0) containing 1 % Triton X-100] at 37 °C. The gel was stained with 1 % methylene blue in 0.01 % KOH and destained in deionized water. Zones of hydrolysis appeared as white bands in the gels; black bands indicating regions of murein hydrolase activity were also observed.

**RNase protection assay (RPA).** The strains were cultured in 10 ml BHI and the cells were incubated at 37 °C with shaking until they reached an OD₆₀₀ of 0.4. Cells were harvested by centrifugation, and total RNA was isolated from the pellet by using an RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. RNA antisense probes were prepared using a MAXIscript kit (Ambion). RNA antisense probes were transcribed with a template containing a T7 phage promoter. The antisense probe template was prepared by PCR with genomic DNA as the template, and primer sets with the T7 phage promoter were incorporated into the downstream primer (Table 1). PCR products were eluted, dried and resuspended in 20 μl water. The template was transcribed with 2 U T7 RNA polymerase in a reaction mixture containing Ambion 1× transcription buffer, 1 μg template, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.3 mM biotin-labelled UTP and water in a final reaction volume of 20 μl. The reaction mixture was incubated at 37 °C for 1 h and then run on a 10 % polyacrylamide gel to purify the probe. *S. haemolyticus* RNA (10 μg) was hybridized overnight with the labelled RNA probes (150 pg) at 42 °C in hybridization buffer. The unhybridized probe was digested with RNase A/T1 (1:100 dilutions) at 37 °C for 30 min, the RNases were inactivated, and the remaining RNA was precipitated. The pellet was resuspended in formamide gel loading buffer. Then, the sample was run on a 10 % polyacrylamide/7 M urea gel, and transferred to a positively charged nylon membrane. The membrane was incubated with anti-digoxigenin antibody (1:10 000; Ambion) conjugated to alkaline phosphatase. Subsequently, the protected fragments were detected using CDP-Star (Ambion). Chemiluminescent signals were quantified using Labworks software (UVP).

**RT-PCR.** Expression of target genes was confirmed by RT-PCR. Five micrograms of total RNA was reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase for 60 min at 42 °C. The resulting cDNA fragments (1 μl) were used as templates for PCR amplification of target genes. The PCR cycling reaction was performed under the following conditions: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s and 72 °C for 7 min.

**Transmission electron microscopy (TEM).** All strains were inoculated on BHI agar at 35 °C for 24 h and were then fixed with 2 % paraformaldehyde and 2 % glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 18–24 h. Samples were post-fixed for 2 h at room temperature with 1 % osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2). Cells were then washed, dehydrated with ethanol and embedded. Ultrathin sections (60–70 nm) were stained with uranyl acetate and lead citrate and observed using a JEM 1010 (JEOL) transmission electron microscope operating at 80 kV. Morphometric evaluation of the cell wall was performed using images at a final magnification of × 100 000. At least 30 cells from each strain with nearly equatorial cut surfaces were measured; the results are expressed as mean ± SD values. The statistical significance of increases in the cell wall thickness was evaluated by Student’s t-test (P<0.001).

**Lysostaphin resistance determination.** Strains were cultured overnight in BHI broth and diluted in BHI medium containing 256 μg lysostaphin ml⁻¹ to an OD₆₀₀ of 1.0. After 1 h, the bacterial survival rate was determined by measuring the OD₆₀₀.

**RESULTS**

**Strains and antimicrobial susceptibility testing**

Using the *S. haemolyticus* GS52 strain, derivative strains were selected (*S. haemolyticus* GS52V₁₆, GS52V₄₈) in the presence of vancomycin. After 30–40 serial passages, stable vancomycin-resistant strains (MIC 16, MIC 48) were obtained. The origin of derivative strains was confirmed by PFGE patterns of the parent strain (data not shown). These strains showed decreased susceptibilities.
to vancomycin, teicoplanin, tigecycline and daptomycin compared with the parent strain (Table 2). The MIC of teicoplanin increased in proportion to that of vancomycin. The daptomycin MIC increased sixfold; however, this change was within the susceptible MIC range. The tigecycline MIC changed from susceptible to resistant in the S. haemolyticus GS52V48 strain. The MICs of other antimicrobial agents were unchanged.

**Characterization of the vancomycin-resistant strains**

Cell wall thickening is a common feature of vancomycin-resistant staphylococci. TEM was performed on S. haemolyticus GS52V4, GS52V16 and GS52V48 strains to assess cell wall thickness. TEM revealed that the cell wall thickness of all the derivative strains was significantly higher ($P<0.001$) than that of the parent strain. The parental strain GS52V4 exhibited a cell wall thickness of 21.48 nm, and the derivatives GS52V16 and GS52V48 exhibited an increase in thickness of 28% and 89%, respectively (Fig. 1a). We examined the survival rate for the derivatives GS52V16 and GS52V48 in 256 μg lysostaphin ml$^{-1}$. The survival rate of GS52V4 in lysostaphin was 34.4%, while the survival rates for the GS52V16 and GS52V48 strains were 79.8% and 95.9%, respectively (Fig. 1c).

The growth characteristics of the GS52V4, GS52V16 and GS52V48 strains were examined in BHI broth medium without antibiotics. The S. haemolyticus GS52V16 and GS52V48 strains grew slower than the parent strain in the exponential phase (Fig. 1b).

**Table 1. Primers used in this study**

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**Table 2. Antimicrobial susceptibility profiles of clinical and derivative strains of S. haemolyticus**

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<th>PG</th>
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<td>1.5</td>
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Susceptibility to Triton X-100-induced autolysis

Autolysis rates are shown in Fig. 2. The vancomycin-resistant derivative strains, *S. haemolyticus* GS52V16 and GS52V48, exhibited lower autolysis rates than that of their parent strain (Fig. 2a). In the presence of Triton X-100, 72% of the parent strain cells lysed after 4 h incubation. In contrast, only 12% of the *S. haemolyticus* GS52V48 and 30% of the *S. haemolyticus* GS52V16 cells lysed after 4 h incubation. Zymographic analysis showed that the hydro-lase protein bands at 105 kDa, 57 kDa and 87 kDa were reduced in the derivative strains (arrow in Fig. 2b). SDS-PAGE also showed that proteins corresponding to the lytic bands were decreased in these strains (Fig. 2c).

Identification of the expression levels of autolysis-associated genes

To further determine whether reduced autolysis resulted from changes in the transcription of autolysis-related genes, we investigated the expression levels of genes involved in autolysis by RPA. The expression of genes (*atl*, *lrgAB*, *lytSR*, *sarA*) encoding enzymes with previously documented bacteriolytic activities in *S. aureus* was confirmed in our *S. haemolyticus* strain. Among these genes, the transcription of the *atl* gene was much higher (sixfold increase) than that in the parent strain, whereas little or no changes in *lrgAB*, *lytSR* and *sarA* gene transcription were observed in the *S. haemolyticus* GS52V16 and GS52V48 strains (Fig. 3). The change in *atl* transcript level identified by RPA was confirmed by RT-PCR (Fig. 3c).

**DISCUSSION**

Resistance to teicoplanin and vancomycin in *S. haemolyticus* has been previously reported (Schwalbe *et al.*, 1990; Veach *et al.*, 1990), but little is known about its autolytic activity or about the expression of autolytic genes. In this study, we investigated phenotypic changes, including expression of several autolytic activity-related genes, in *S. haemolyticus* strains with laboratory-induced vancomycin resistance.

Our results demonstrate a characteristic change in cell wall thickness similar to that reported in previous studies (Cui *et al.*, 2003; Nunes *et al.*, 2006) on *S. haemolyticus* strains with induced vancomycin resistance. In the present study, the cell wall thickness of the strain with induced vancomycin resistance was almost 1.9-fold higher than that of its parent strain. Cell wall thickness changes led to increased...
survival rates of vancomycin-intermediate and vancomycin-resistance-induced strains when exposed to lysostaphin. The strain with induced vancomycin resistance also showed decreased susceptibility to teicoplanin, tigecycline and daptomycin. The increased MIC of daptomycin, which is known to target the bacterial cell membrane, may be influenced by the cell wall changes.

Autolysis is linked to the process of cell division, and is therefore related to the growth of the cell and the expression of autolysins, which hydrolyse cell wall components. The autolysis rates of *S. haemolyticus* with induced vancomycin resistance were less than those of the parent strain. This suggests that resistance to autolysis indicates reduction of cell wall turnover (Pfeltz *et al.*, 2000; Nunes *et al.*, 2006). In addition, the results of the SDS-PAGE and zymographic experiments showed that expression of autolysin proteins had decreased in *S. haemolyticus* with induced vancomycin resistance. While several autolysin genes have been reported and studied in *S. aureus*, little is known of autolysin genes and their expression in vancomycin-resistant *S. haemolyticus*. Here,
we confirmed the presence of the atl, lrgAB, lytS and sarA genes in vancomycin-resistance-induced S. haemolyticus and compared the expression of these genes in derivative strains and the parent strain. Other reported autolysis genes, such as the cidABC operon, atlRS and mra genes of S. haemolyticus, were not detected. In this study, the expression of the atl gene increased in the vancomycin-resistance-induced S. haemolyticus, but the expression of the other autolysis-related genes – lrgAB, lytS and sarA – did not change. In S. aureus, the atl gene plays a fundamental role in cell division and separation. Decreased atl gene expression in vancomycin-resistant S. aureus may produce a build-up of peptidoglycan layers contributing to a thickened cell wall. However, in the present study, expression of autolysis-related genes such as lrgAB, lytS and sarA showed little change; only atl gene expression was upregulated in the vancomycin-resistance-induced S. haemolyticus strains, while cell wall thickness was much greater than that in the parent strain. This indicates that the atl gene is involved in vancomycin resistance of S. haemolyticus. In this study, decreased autolytic activity and increased expression of atl were observed in vancomycin-resistance-induced S. haemolyticus. In previous studies, the correlation of expression of atl and autolytic activity has been investigated in vancomycin-resistant S. aureus strains, and some showed increased atl gene expression or lower autolytic activity than that of parent strains (Mongodin et al., 2003; Wootton et al., 2005; Nunes et al., 2006). Though the majority of studies have focused on S. aureus, the exact mechanism has not been answered yet.

In conclusion, vancomycin-resistance-induced S. haemolyticus exhibited a typically thick cell wall, decreased cell growth phenotype, decreased autolysis activity and upregulation of the atl gene. Because our study investigated S. haemolyticus strains specifically induced for vancomycin resistance, our findings may have somewhat limited application to other vancomycin-resistant S. haemolyticus strains.

However, to our knowledge, this is the first investigation of the expression of autolysis-related genes in vancomycin-resistant S. haemolyticus. Because S. haemolyticus is prevalent in hospitals and is regarded as an important nosocomial pathogen with a tendency to develop multiple resistance, further understanding of the mechanisms underlying vancomycin resistance should be acquired through future detailed studies.

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REFERENCES
