Reduced expression of the *atl* autolysin gene and susceptibility to autolysis in clinical heterogeneous glycopeptide-intermediate *Staphylococcus aureus* (hGISA) and GISA strains

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Objectives: To assess a link between resistance to Triton X-100 induced autolysis (TIA) and lowered *atl* expression in a collection of clinical glycopeptide-intermediate *Staphylococcus aureus* (GISA) and heterogeneous GISA (hGISA).

Methods: Nine clinical GISA, 11 hGISA and 11 glycopeptide-susceptible *S. aureus* (GSSA), including three pairs of related isolates, were analysed using TIA assays. Lysostaphin MICs were determined by a broth microdilution technique and reverse transcriptase PCR was used to compare *atl* expression levels in all isolates.

Results: Eight of nine clinical GISA and six of 11 hGISA exhibited lower susceptibility to TIA and higher MICs of lysostaphin than GSSA. Eight of nine GISA and all hGISA strains had lowered *atl* expression levels compared with GSSA.

Conclusions: The majority of GISA and hGISA isolates exhibited lowered susceptibility to TIA and lysostaphin and reduced *atl* expression when compared with GSSA isolates. These factors could contribute to, or predispose to the development of, a thickened cell wall and glycopeptide-intermediate resistance.

Keywords: antibiotic resistance, cell wall, mechanism of resistance

Introduction

Glycopeptide-intermediate-resistant *Staphylococcus aureus* (GISA) and heterogeneous GISA (hGISA) strains share the common characteristic of a thickened cell wall. The role of the thickened cell wall in the mechanism of glycopeptide resistance is uncertain, as is the precise mechanism of cell wall thickening. However, it is possible that reduced expression of murein hydrolases, including *Atl*, may play a role. In two recent studies, laboratory-derived GISA and four clinical GISA strains exhibited lowered susceptibility to Triton X-100 induced autolysis (TIA).¹² The laboratory-generated GISA also showed reduced hydrolase activity, but no such change was seen in clinical GISA.¹² To investigate this further, we examined expression levels of the major autolysin gene, *atl*, autolytic activity and susceptibility to lysostaphin in a number of clinical GISA, hGISA and glycopeptide-susceptible *S. aureus* (GSSA) isolates.

Materials and methods

Bacterial strains

Glycopeptide susceptibility was determined by population analysis profile–area under curve (PAP-AUC) using the criteria: <0.9, GSSA; 0.9–1.29, hGISA; and ≥1.3, GISA.³ Expression of *atl* and susceptibility to TIA and lysostaphin were studied in nine clinical GISA (isolates MI, NJ, GL3700, GL2759, SW307, SL, PC3, LIM3 and Mu50), 11 clinical hGISA (isolates Fduf, AGN, SH23, NW1018, SW309, SL6096, SMH2, LIM1, PC1, LLE and Mu3) and 11 clinical GSSA (randomly selected on the basis of glycopeptide susceptibility; from the UK, including EMRSA-15 and EMRSA-16 clonal types). This collection included three pairs of related isolates (LLA-GSSA/LLE-hGISA, PC1-hGISA/PC3-GISA and LIM1-hGISA/LIM3-GISA). LLA and LLE were clonal strains isolated from an 82-year-old male with chronic renal failure; LLA prior to vancomycin therapy and LLE after 22 days of vancomycin.

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\textbf{Autolytic assays}

Cultures were grown in brain–heart infusion broth (BBL, Cockeysville, MD, USA) and harvested at mid-log phase (OD\textsubscript{600} 0.7). Cells were pelleted, washed in ice-cold water, then resuspended in 0.05% Triton X-100. Optical density at 600 nm was read at time 0 and at 30 min intervals. Percentage lysis was calculated by dividing OD by initial OD × 100 and an AUC was calculated. A Kruskal–Wallis statistical test was performed on AUC data.

\textbf{Lysostaphin MIC determination}

Exponentially growing cells were transferred to tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) containing serial two-fold dilutions of lysostaphin (range 0.06–64 mg/L; Sigma, Poole, UK) in round-bottomed microtitre wells. Plates were incubated at 37°C for 16 h and the MIC was taken as the lowest concentration to inhibit all cell growth.

\textit{atl} gene expression: reverse transcriptase (RT)–PCR and \textit{atl} sequencing

RNA was recovered from exponentially-growing cells in TSB (OD\textsubscript{600} 0.7) using a dedicated kit (Qiagen Rneasy Mini 74104) and stored at −20°C. DNA was removed from RNA extractions using DNase (according to the manufacturer’s instructions) and RNA concentration was quantified by spectrophotometry (Promega, USA). One microgram of RNA was used per RT–PCR (Qiagen One-step RT-PCR Kit, 210210, USA) together with gene-specific primers (atl-F: 5’-CAGTTAGCAAGATTGCTCAAG-3’, atl-R: 5’-CCGTTACCTGT-TCTTAAATGG-3’, atl-promoter F: 5’-GGAAAGGATCGAGCAT-3’, atl-promoter R: 5’-GGGTATATGCAACCAT-3’). Expression levels of \textit{atl} in GISA were evaluated in triplicate on agarose gels, using expression levels in GSSA as equivalent to normal expression. Control amplifications, using 16S rRNA primers, were performed on every isolate to eliminate artefactual expression differences resulting from different template concentrations. Sequence analysis of the \textit{atl} promoter region was determined by the Advanced Biotechnology Centre (Imperial College School of Medicine, London, UK) and compared using DNASTAR-SeqMan 5.0 software (DNASTAR Inc., USA).

\textbf{Results and discussion}

All GISA, except Mu50, showed reduced susceptibility to TIA in comparison with hGISA and GSSA isolates. Figure 1(a) shows mean autolysis profile AUCs from GISA, hGISA and GSSA. Susceptibility to TIA decreased with increasing glycopeptide-intermediate resistance, with GISA strains exhibiting the lowest susceptibility, followed by hGISA and GSSA isolates (means were significantly different; \( P = 0.02 \)). The reduced susceptibility to TIA in clinical GISA and hGISA, but not in Mu50 or Mu3, agrees with data presented in other studies,\(^1\)\(^2\) where it was suggested that lowered susceptibility enhanced the capability of \textit{S. aureus} to evade the lysis-inducing effect of vancomycin. In clinically related pairs of isolates, resistance to TIA, indicated by AUC, was also consistently higher in the more glycopeptide intermediately resistant isolates (Figure 1b).

MICs of lysostaphin revealed a trend towards higher resistance among GISA isolates compared with hGISA and GSSA isolates (Figure 1c). Lysostaphin resistance in \textit{S. aureus} is associated with the genes \textit{epr} and \textit{femA}, the former responsible for increasing serine content and decreasing glycine content of the peptidoglycan interpeptide bridge and the latter for shortened glycine bridges.\(^3\) However, evidence suggests that the peptidoglycan of clinical and laboratory-generated GISA isolates has glycine bridges of normal length and composition.\(^4\)\(^5\)

Eight of nine GISA and all 11 hGISA exhibited lower \textit{atl} expression, as indicated by less intense bands on agarose gels, when compared with \textit{atl} expression in all GSSA isolates, which suggests a correlation with glycopeptide resistance. Expression of \textit{atl} in all isolates of the related pairs (including the GSSA, LLA; Figure 2)
appeared lower than in control GSSA isolates. For pair LLA and LLE, **atl** expression appeared to be greater in the hGISA isolate, the reasons for which are unknown. The lower **atl** expression exhibited by LLA may indicate that reduction in **atl** expression takes place prior to development of glycopeptide-intermediate resistance and therefore that it could be a predisposing factor of the GISA/hGISA phenotype. Previous investigations into **atl** have reported similar sequences in glycopeptide-resistant and susceptible strains, although expression rates could be altered by a mutated promoter region. A 250 bp region upstream of **atl** was sequenced and compared in all isolates and found to be identical, confirming that it is not a causative factor in altered expression levels (data not shown).

This study is in accordance with the findings of two previous reports. In the first case, a single laboratory-generated GISA mutant (COL-VR1) showed reduced methicillin-induced autolysis compared with a parental strain, and lower intensity of a >83 kDa band in zymographic profiles, which was thought by the authors to be related to the **atl** gene product. In the second case a clinically related hGISA and GISA pair (IL-A, IL-F) exhibited reduced susceptibility to TIA and similar hydrolase profiles, except for a 116 kDa product, which was less abundant in both isolates compared with a vancomycin-susceptible parent.

Although it is assumed that the characteristic thickened cell walls in GISA and hGISA may account for lowered susceptibility to TIA, this does not seem to be the case for Mu50 and Mu3, which appear to be exceptions of the phenotype in this respect. This suggests that the thickened cell wall is not primarily responsible for the decreased autolysis susceptibility and it is possible that cell wall composition is different in GISA, or that protease production is increased giving rise to reduced autolysin levels and hence varied autolysis profiles.

In this study all GISA (except Mu50) and six of 11 hGISA showed reduced susceptibility to lysostaphin and TIA, with eight of nine GISA and all hGISA exhibiting lowered expression of **atl**. The **atl** sequences of all GISA/hGISA strains studied here were identical to those of two GSSA strains, N315 and MW2, which indicates that the altered expression did not result from faulty primer annealing. Exceptions included Mu50, Mu3, NJ and LLA, the former two exhibiting lowered **atl** expression but similar autolytic susceptibility to GSSA, NJ exhibiting resistance to TIA but not lowered **atl** expression and the latter, LLA, showing reduced susceptibility to lysostaphin and TIA and expression of **atl**. As **atl** plays a fundamental role in cell division and separation, lowered expression may produce build-up of peptidoglycan layers contributing to a thickened cell wall. In NJ, the gene appears to be functioning normally; however, post-translational modification may occur to reduce the activity of Atl, or an increase in protease production may be responsible for diminished lysostaphin and Triton X-100 susceptibility. Previously, a two-step hypothesis model was proposed for GISA where lowered susceptibility to TIA precedes the development of vancomycin-intermediate resistance. This theory could be expanded to include reduced expression of the **atl** gene prior to reduced susceptibility to TIA. In the related clinical strains LLA (GSSA) and LLE (hGISA), both **atl** expression and susceptibility to TIA was reduced in LLA compared with other GSSA, suggesting that autolysis resistance may occur prior to the onset of the hGISA phenotype. These characteristics would contribute to a thicker cell wall, and in turn, decrease the susceptibility of the cell to induced autolysis. This hypothesis has exceptions, such as Mu50, the archetypal GISA in terms of vancomycin susceptibility, but not in terms of either autolytic resistance or sequence comparisons in certain genes. This suggests that the GISA/hGISA phenotype, including thickened cell wall, is not mediated through a single set of events, but can be achieved by alternate means.

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**References**


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