Alterations in phage-typing patterns in vancomycin-intermediate \textit{Staphylococcus aureus}

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The ability of phage-typing and \textit{Sma}I chromosomal RFLPs to conclude appropriate strain relatedness between a collection of 12 well-characterized \textit{in vitro}-selected vancomycin-intermediate \textit{Staphylococcus aureus} (VISA) strains and their seven vancomycin-susceptible parent strains is reported. Generally, no \textit{Sma}I RFLP alterations were observed in VISA strains when they were compared with their respective parent strains, and clonal relationships between isogenic strains were clearly evident. Unlike the \textit{Sma}I RFLP results, parent strains and VISA derivatives generally did not share similar phage-typing profiles. Depending on the phage set investigated, some VISA strains even became untypable by this method. Loss of phage infectivity is probably due to cell wall (phage receptor) alterations that are expressed by the VISA strains investigated. Collectively, these findings indicate that inappropriate relationships between VISA and vancomycin-susceptible parents might be drawn if only phage-typing and antibiotic susceptibility are utilized to determine epidemiological relationships.

Introduction
Analysis of \textit{Sma}I chromosomal RFLPs produced using PFGE is very effective in tracking \textit{Staphylococcus aureus} clones (Jorgensen et al., 1996; O’Brien et al., 1999; Simor et al., 2002). Phage typing is also used to track outbreak, epidemic and community strains of \textit{S. aureus} effectively (Blair & Williams, 1961; O’Brien et al., 1999; O’Neill et al., 2001). While phage typing is often valuable in discerning strain relationships quickly and inexpensively, the technique is not always helpful, since non-typable \textit{S. aureus} strains exist (Blair & Williams, 1961; Andrasevic et al., 1999; Tambic et al., 1997).

The glycopeptide antibiotic vancomycin is still the major drug of choice for treatment of severe disease caused by infection with methicillin-resistant \textit{S. aureus} (MRSA). Since 1997, vancomycin-intermediate \textit{S. aureus} (VISA) have become an emerging clinical reality (Hiramatsu et al., 1997a).

Vancomycin-intermediate expression in \textit{S. aureus} is mediated by chromosomal mutation(s) (Avison et al., 2002; Pfeltz et al., 2000; Sakoulas et al., 2002; Walsh & Howe, 2002) and is not due to horizontal gene transfer of vancomycin-resistance determinants (\textit{van}) from organisms such as the vancomycin-resistant enterococci. VISA strains harbour a chromosomal mutation(s) that leads to alterations in cell wall synthesis and structure, which are thought to impart resistance by sequestering glycopeptide molecules away from their target (for review, see Walsh & Howe, 2002). PFGE and \textit{Sma}I chromosomal RFLP analysis have been used to demonstrate that VISA can disseminate in a clonal fashion (Chesneau et al., 2000; Hiramatsu et al., 1997b; Kim et al., 2002). Two reports have now appeared on high-level vancomycin-resistant \textit{S. aureus} (VRSA) that have acquired the enterococcal \textit{vanA} determinant (Miller et al., 2002; Sievert et al., 2002). Outbreaks with VISA or VRSA have not yet become a common occurrence.

We now report on the ability of phage typing and PFGE to conclude appropriate strain relatedness between a collection of 12 \textit{in vitro}-selected VISA and their seven vancomycin-susceptible parents.
Results and Discussion

Small chromosomal RFLPs of the isogenic parents (BB255, BB270, SH108, BB399, 13136p, BB568 and COL) and in vitro-selected VISA strains (BB255V3, BB270V5, BB270V15, SH108V5, BB399V5, BB399V12, 13136pV3, BB568V5, BB568V15, COLV5 and COLV10) and a dendrogram generated from these RFLPs are shown in Fig. 1. It should be noted that strain BB270 is a methicillin-resistant transductant of BB255, and strains BB568 and COL are isogenic (Pfeltz et al., 2000); therefore, as expected, these strain pairs cluster together (Fig. 1). Small restriction profiles also revealed that, with the exception of VISA strain BB399V12, no significant alterations occurred in the Small chromosomal RFLPs of most strains (BB255, BB270, SH108, 13136p–m+, BB568 and COL) as they were step-selected to become vancomycin-intermediate. These data confirm that major genetic alterations are not required for S. aureus to develop the VISA phenotype. In addition, clonal relationships between the collection of in vitro-selected VISA mutants and isogenic parent strains are clearly evident. Other investigators have also demonstrated that in vitro-selected VISA retain parental Small RFLP patterns (Schaaff et al., 2002). Small chromosomal RFLPs are also similar between VISA that arise from vancomycin-susceptible strains during vancomycin therapy in a single patient (Rotun et al., 1999; Sieradzki et al., 1999a; Smith et al., 1999).

A 300 kb Small fragment present in parent strain BB399 has been replaced by a 275 kb fragment in BB399V12 (Fig. 1), indicating the loss of ~25 kb, which actually allowed BB399V12 to cluster with COL and BB568. Altered Small RFLP patterns have been reported to occur within in vitro-selected VISA strains (Riepert et al., 2003). These alterations have been attributed to loss of the methicillin-resistance determinant (mec) as well as other genes (Riepert et al., 2003; Sieradzki et al., 1999b). BB399V12, however, maintains high-level methicillin resistance, similar to parent BB399 (Pfeltz et al., 2000), indicating that the former has not lost mec. Small RFLP alterations can result from the loss of a prophage (Smeltzer et al., 1994) and, perhaps, during the selection for the VISA phenotype in BB399V12, a prophage excised and was lost.

Unlike the Small RFLP results, parent strains and VISA derivatives generally did not share similar phage-typing profiles using the international basic phage set (IBPS), the International MRSA phage set (IMRSA) or the Australian MRSA phage set (AMRSA) (Table 1). When the IBPS was used, all VISA derivatives demonstrated altered phage types when compared with their respective parent strains. VISA mutant 13136p–mV3 even became non-typable by this phage set. Typing with IMRSA revealed that isogenic strain sets BB255 and BB568 had the same phage types, while strain 13136p–m+ and its VISA mutants were non-typable by this phage set. All other VISA strains exhibited altered IMRSA types compared with their respective parent strains, and SH108V5 became untypable. Typing with AMRSA revealed that the BB255 and BB568 strain sets all had identical phage types, as did COL and its VISA derivative COLV5. All other VISA mutants demonstrated altered AMRSA types compared with their respective parent strains, and BB399V5 became untypable. Other studies have also shown alterations in phage-typing patterns following in vitro acquisition of a VISA phenotype in S. aureus (Daum et al., 1992; Schaaff et al., 2002).

Phage infection can be prevented in Gram-positive bacteria as a result of adsorption inhibition (Chatterjee, 1969; Tran et al., 1999; Wilkinson & Holmes, 1979), restriction modification systems (Kong & Josephsen, 2002), DNA injection-blocking systems (McGrath et al., 2002), lysogenization (Beard-Pegler & Vickery, 1985) and abortive infection systems (Dai et al., 2001). We showed that the in vitro VISA investigated demonstrated minimal to no alteration in Small
RFLPs compared with parent strains; however, alterations in phage types were common. This latter finding is probably due to modifications of phage cell-wall receptors that result from alterations in cell-wall physiology that are expressed by the VISA strains investigated (Pfeltz et al., 2000; Hiramatsu et al., 1997a). Failure of bacteriophage typing to detect an inter-hospital outbreak of methicillin-resistant Staphylococcus aureus (MRSA) in Zagreb subsequently identified by random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE).

Table 1. Phage-typing results of S. aureus strains investigated

<table>
<thead>
<tr>
<th>Strain</th>
<th>IRBP</th>
<th>IMRSA</th>
<th>AMRSA</th>
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<tr>
<td>BB255</td>
<td>88/47/53/(54)/75/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
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<tr>
<td>BB255v15</td>
<td>88/75/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
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<tr>
<td>BB270</td>
<td>88/47/53/54/75/77/84/90</td>
<td>MR8/MR12/38/56B</td>
<td>47T/56B/56C/90A/1648/78M/13M</td>
</tr>
<tr>
<td>BB270v15</td>
<td>88/75/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
</tr>
<tr>
<td>SH108</td>
<td>88/6/53/54/75/83/90</td>
<td>33/38/56B</td>
<td>47T/56B/56C/90A/1648/78R/87M/13M</td>
</tr>
<tr>
<td>SH108v3</td>
<td>29/(52A)/(80)/90</td>
<td>Non-typable</td>
<td>67R/13M</td>
</tr>
<tr>
<td>BB399</td>
<td>80</td>
<td>33</td>
<td>Non-typable</td>
</tr>
<tr>
<td>BB399v15</td>
<td>52A/80/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
</tr>
<tr>
<td>13136p–m+</td>
<td>(88)/53/(54)/75/77/84/90</td>
<td>Non-typable</td>
<td>47T/(56C)/90A</td>
</tr>
<tr>
<td>13136p–v15</td>
<td>Non-typable</td>
<td>Non-typable</td>
<td>47T/(56C)/90A</td>
</tr>
<tr>
<td>13136p–v20</td>
<td>54/85/90</td>
<td>Non-typable</td>
<td>47T/(56C)/90A</td>
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<tr>
<td>BB668</td>
<td>(52A)/(75)/77/84/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
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<tr>
<td>BB668v5</td>
<td>52A/80/77/84/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
</tr>
<tr>
<td>BB668v15</td>
<td>88/52A/(80)/47/90</td>
<td>MR12/33/38</td>
<td>47T/90A/1648/13M</td>
</tr>
<tr>
<td>COL</td>
<td>88/52A/80/77/84/90</td>
<td>MR8/MR12/33/38</td>
<td>47T/90A/1648/13M</td>
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<tr>
<td>COV5</td>
<td>52A/80/90</td>
<td>33</td>
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<td>COV5t10</td>
<td>52A/80/90</td>
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