Clonality, virulence and the occurrence of genes encoding antibiotic resistance among *Staphylococcus warneri* isolates from bloodstream infections

Ewa Szczuka, Sylwia Krzymińska and Adam Kaznowski

Coagulase-negative *Staphylococcus warneri* is an opportunistic pathogen capable of causing several infections, especially in patients with indwelling medical devices. We evaluated the virulence-associated properties of 23 clinical isolates recovered from blood specimens. In addition, the carriage of biofilm-associated genes, as well as antibiotic-resistant genes, was identified. *S. warneri* isolates appeared to be clonally unrelated and revealed a high degree of genetic diversity. All isolates revealed adhesion to epithelial cells, and 43.5% of strains invaded the cells. Moreover, 52% of isolates formed biofilm in vitro. PCR analysis demonstrated the presence of the ica operon, in two of the 12 biofilm-positive isolates. This indicated that biofilm formation, in this species, is not restricted to strains harbouring icaADBC genes, encoding polysaccharide intercellular adhesion. Analysis by confocal laser scanning microscopy revealed that biofilm-forming strains formed a three-dimensional structure, composed of mainly living cells. All strains revealed cell-contact cytotoxicity that was strongly associated with biofilm formation. Moreover, cell-free supernatants, of 95% of the isolates, expressed a cytotoxic activity which caused the destruction of HeLa cells. *S. warneri* capable of forming biofilm carried significantly more genes encoding resistance to beta-lactams, aminoglicosides and macrolide-lincosamide streptogramin B antibiotics than biofilm-negative isolates. We have shown that tigecycline/rifampicin is effective against bacteria growing as a biofilm. The biofilm inhibitory concentration range of tigecycline/rifampicin was ≤1 µg ml⁻¹. Results indicated that *S. warneri* have the ability to adhere, form biofilm, invade and destroy epithelial cells, which could be important mechanisms contributing to the development of diseases.

INTRODUCTION

Coagulase-negative *Staphylococcus warneri* is an opportunistic pathogen causing severe infections such as bacteraemia, endocarditis, vertebral osteomyelitis, ventriculoperitoneal shunt-associated meningitis, discitis, subdural empyema, urinary tract infections and sepsis in neonates (Kloos & Schleifer, 1975; Kamath et al., 1992; Torre et al., 1992; Wood et al., 1992; Buttery et al., 1997; Mehr et al., 2002; Announ et al., 2004; Stöllberger et al., 2006; Arslan et al., 2011; Legius et al., 2012). These infections occur predominantly in immunocompromised patients and those with indwelling medical devices (Arciola et al., 2005; von Eiff et al., 2006). *S. warneri* does not raise as much concern as other coagulase-negative staphylococci (CoNS). However, some reports noted that *S. warneri* is the second most commonly isolated species among CoNS (Mehr et al., 2002; Cimiotti et al., 2007). The adherence of staphylococci to epithelial cells, or an artificial surface, is considered to be the first step in colonization and subsequent biofilm formation. Biofilm can be defined as sessile communities of surface-attached cells encased in an extracellular matrix. The main component of staphylococci biofilm is a polysaccharide intercellular adhesion (PIA) which is synthesized by an enzyme encoded by the *icaADBC* operon. In addition to PIA, other polysaccharides, proteins, extracellular teichoic acids, lipids and extracellular nucleic acids are recognized as biofilm components (Otto, 2004). The treatment of bacteria in the biofilm mode of growth is challenging due to the resistance of bacteria to both antimicrobials and host defenses. Therefore, biofilm-grown bacteria cause acute and...
chronic infections (Cerca et al., 2014). It was demonstrated that rifampicin has the ability to efficiently penetrate staphylococcal biofilm and achieve a high concentration, as well as show an excellent bacteriocidal effect. However, rifampicin monotherapy quickly results in rifampicin resistance and, for this reason, at least one further active antimicrobial agent was required to be used with rifampicin (Yin et al., 2005). Tigecycline, a novel, glycy cycline antibiotic, has demonstrated a good activity in various animal models against foreign-body infections due to meticillin-resistant Staphylococcus aureus (MRSA) (Murphy et al., 2000; Vaudaux et al., 2009). It is important to point out that no antagonism was observed between tigecycline and rifampicin (Petersen et al., 2006).

CoNS strains are known for their ability to spread rapidly in the hospital environment. It was documented that particular clones of CoNS are responsible for nosocomial outbreaks (Klingenberg et al., 2007). The ability of endemic clones to spread within the hospital environment, seems to depend on their virulence factors and antibiotic resistance (Ziebuhr et al., 2006). Many antibiotic resistance genes are located on mobile genetic elements and thus are supposed to be horizontally transferable between bacterial pathogens. The mecA gene that encodes an alternative binding protein with decreased binding affinity to meticillin, and others for beta-lactam antibiotics, are part of a mobile genetic element called the staphylococcal chromosome mec (SCCmec). The genes code for methylases, erm(A) and erm(B), that modify the target site in 23S rRNA and thereby inhibit the binding of macrolide-lincosamide streptogramin B (MLSb) antibiotics, and are associated with transposons Tn554 and Tn917/Tn551, while the erm(C) gene was identified on small plasmids (Le Bouter et al., 2011). The Imu(A) gene encodes a lincosamide nucleotidyltransferase, which confers resistance to lincosamides and is located on small plasmids (Le Bouter et al., 2011). The aac(6′)/aph(2′) genes that code for bifunctional enzymes, which inactivate a broad spectrum of aminoglycoside antibiotics, are located on transposon Tn4001. The aph(3′)-IIIa gene codes for a phosphotransferase, which mediates resistance to kanamycin, neomycin and amikacin, naturally occurring on plasmids and in transposons (Ardic et al., 2006; Woegerbauer et al., 2014). The ant(4′)-la gene codes for adenylation transferase, which covers resistance to kanamycin, neomycin and tobramycin and is located on plasmid pUB110 (Wendlandt et al., 2013).

The pathogenetic mechanisms of S. warneri infections have not yet been clearly elucidated. In this study, we examined the putative virulence factors of the isolates from bloodstream infections. We assessed biofilm production ability, adhesion, invasion and cytotoxic activity to human epithelial cells. Moreover, the molecular relatedness of S. warneri and the presence of biofilm-associated genes, as well as antibiotic resistance genes, was determined. We also investigated the efficacy of the tigecycline/rifampicin combination on biofilm-growing bacteria.

METHODS

Bacterial strains. Twenty three strains of S. warneri were isolated from patients with bloodstream infections. The strains were identified using a VITEK 2 system (bioMérieux). We also used Escherichia coli K12C600 and Yersinia enterocolitica O3:4 (pYV). All strains were frozen at −70°C, in a 50% (v/v) glycerol broth until the commencement of this study.

Cell culture. Human epithelial cells from the cervix (HeLa) were obtained from the Department of Molecular Virology, Adam Mickiewicz University in Poznan and were maintained in RPMI, supplemented with a heat-inactivated 5% FCS (Gibco), streptomycin (100 µg ml−1), penicillin (100 U ml−1) and 2 mM l-glutamine (Nawrot et al., 2010). The cells were seeded in a suspension of 1 × 10⁶ cells ml⁻¹ per well and incubated at 37°C, in an atmosphere of 5% CO₂.

BOX-PCR analysis. PCR was performed using a primer with the sequence (5′-CTACGGCAAGGCCAGCTGAAG-3′) complementary to BOX elements of bacterial genomic DNA (Versalovic et al., 1991). Amplification products were electro-phoresed in a 1.5 % agarose gel. The DNA in gels was stained with ethidium bromide, viewed under a UV-light transilluminator and documented with a V.99 Bio-Print system (Vilber Lourmat). The results of the BOX-PCR were analysed using Gel-Compar II (version 3.0; Applied Maths) software. The percentages of fingerprint similarities were analysed using the Dice coefficient. The unweighted pair group method with arithmetic mean (UPGMA) clustering method was used to create a dendrogram.

Bacterial adhesion and the invasion of epithelial cells. Adhesion and invasion of S. warneri to epithelial cells were determined in a gentamicin/lysozyme protection assay according to the methods of Bur et al. (2013) and Kryzmirska et al. (2015). HeLa cells were infected with bacteria at an m.o.i. of 100 (1 × 10⁶ cells were infected with approx. 1.5 × 10⁶ bacteria) and cultivated for 90 min at 37°C. To assess the total number of bacteria that adhered and invaded epithelial cells, the infected monolayer was washed three times with PBS, lysed with 1% Triton X-100 and c.f.u. were determined by plating serial dilutions on brain heart infusion agar (BHI, Oxoid). To enumerate the number of intracellular bacteria, infected epithelial cells were incubated with 100 µg gentamicin ml⁻¹ and 20 µg lysozyme ml⁻¹ for 2 h, washed three times with PBS and lysed with 1% Triton X-100. The invasive bacteria were determined by plating serial dilutions of the lysates on BHI agar. The number of attached bacteria was determined by subtracting the number of intracellular bacteria from total counts. The adhesion index (AI) was expressed as the mean total number of c.f.u. of associated bacteria per well (1 × 10⁶ cells). The invasion index (InI) was determined as the number of internalized bacteria per well after gentamicin/lysozyme treatment.

Quantitative determination of biofilm formation. The ability of the S. warneri strains to form biofilm on an abiotic surface was quantified essentially as described elsewhere (Kim et al., 2008; Fredheim et al., 2009). In brief, an overnight culture grown in tryptic soy broth (TSB; Difco, Beckton Dickinson) with 0.25 % glucose at 37°C was diluted 1:100 in the medium and 100 µl was transferred into 96-well polystyrene microtiter plates. After overnight incubation at 37°C, the medium was removed from each of the wells and the plates were gently washed three times with PBS. The wells were then stained with a 0.4 % crystal violet solution, washed, and the crystal violet from the biofilm was dissolved using an ethanol/acetone mixture (70:30, v/v) and absorbance was determined. All isolates were tested in six wells in two parallel runs. For each parallel run, the highest and the lowest OD values were removed to exclude outliers, and the remaining values were mean.

The strains were considered biofilm-positive if OD₉₀₀>0.250. A biofilm-producing strain, Staphylococcus epidermidis ATCC 35984, was used as
positive control; a biofilm-negative strain, *S. epidermidis* ATCC 12228, was used as negative control.

**Biofilm detachment assay.** Detachment assay was performed as described by Fredheim *et al.* (2009). The major components of *S. warneri* biofilms were analysed using sodium metaperiodate (NaIO₄) to degrade polysaccharide, protease K to degrade proteins and DNaseI to degrade DNA. Percentage detachment was calculated on the basis of the average difference between wells subjected to NaIO₄ and subsequent bacterial growth was measured by OD₆₅₀ nm.

**Detection of the ica operon.** Chromosomal DNA was extracted using a Genomic DNA prep Plus kit (A&A Biotechnology). PCR was applied to detect the presence of icaADBC (Chokr *et al.*, 2006).

**Confocal laser scanning microscopy (CLSM).** The three-dimensional biofilm structure was investigated using CLSM. Twenty-four-hour cultures of isolates were added to nine Lab-TekII cell-culture chamber wells (Nalge Nunc) and incubated for 24 h. The peg lids were rinsed three times and placed into a new microtiter plate containing twofold dilutions of antibiotics. After 18–20 h, biofilm cells were disrupted into a recovery medium using sonication and an OD at 650 nm was measured on a plate reader. The plate was incubated for 6 h and subsequent bacterial growth was measured by OD₆₅₀. Biofilm growth was defined as a mean OD₆₅₀ difference (OD₆₅₀ at 6 h minus OD₆₅₀ at 0 h) ≥0.05 for biofilm control. Biofilm inhibitory concentration (BIC) values were defined as the lowest concentration without growth.

**Assessment of cytotoxic activity of extracellular and cell-associated bacterial toxins.** To examine the effect of extracellular toxins, the bacteria were grown in TSB at 37°C, for 24 h. Next, the cultures were centrifuged at 2000 g for 30 min and sterilized through a membrane filter with a pore size of 0.22 µm (Roth). A monolayer of HeLa cells was incubated with 100 µl of supernatants, for 24 and 5 h. To determine whether cell contact is required for *S. warneri* cytotoxicity, we used trans-well system plates with tissue culture inserts (Nunc) with a membrane of 0.2 µm pore diameter. Epithelial cells were cultured in the lower chamber. Bacterial cells at m.o.i. of 100 were added into the upper chamber and incubated for 5 h. Extracellular and cell-contact cytotoxicity were measured by means of the mitochondrial-dependent reduction of colourless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to a blue formazan, which was solubilized in isopropanol as described previously by Krzymińska *et al.* (2011). Absorbance at A₅₇₀ nm was measured with a spectrophotometer.

**Detection of antibiotic resistance genes.** The detection of meca, erm(À), erm(B), erm(C), msr(À), lnu(À), aac(6′)-aph(2′”), aph(3′)-IIa and anti(4′)-Ia genes, PCR assays were performed as described by Geha *et al.* (1994), Ardic *et al.* (2006) and Le Bouter *et al.* (2011).

**Statistical analysis.** Results of biofilm formation, AI, InI and the percentage of cytotoxicity are presented as means. They represent two independent experiments performed in triplicate. A one-way ANOVA with Tukey’s post-hoc test was performed. Association between biofilm formation and AI, InI and cytotoxicity were determined with a Mann–Whitney U test. The cytotoxic index was the percentage of damaged cells. Statistical analyses were done with Statistica 9.1 (StatSoft). P values of <0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Population structure**

The clonal relationship of *S. warneri* isolates was analysed on the basis of a BOX analysis (Fig. 1). *S. warneri* strains showed a high degree of genetic diversity and all strains had unique BOX-PCR patterns. This result indicates that there is no endemic clone in the hospital setting.

Previously, Center *et al.* (2003) reported the presence of an endemic clone of *S. warneri* with enhanceable resistance to vancomycin in a neonatal intensive care unit in a hospital in Philadelphia.
**S. warneri adhesion and the invasion of human epithelial cells**

Bacterial adhesion and the invasion of human epithelial cells were quantified by a gentamicin/lysostaphin survival assay. All *S. warneri* strains have the ability to adhere to HeLa cells (Table 1). AI ranged from $0.1 \times 10^5$ to $3.7 \times 10^4$ c.f.u. per $1 \times 10^5$ cells. The highest index, ranging from $1.2 \times 10^5$ to $3.7 \times 10^4$, was revealed for three (13 %) strains. The lowest one, from $0.1 \times 10^5$ to $0.6 \times 10^5$, observed for six strains (26.1 %), was higher in comparison with the index of non-pathogenic *E. coli* K12C600 ($0.015 \times 10^5$ c.f.u.). The index of enteroinvasive *Y. enterocolitica* O:3/4 positive control reached $4.6 \times 10^5$ c.f.u. Ten *S. warneri* strains (43.5 %) revealed invasion of epithelial cells. Inl ranged from $4.7 \times 10^4$ to $0.1 \times 10^5$ c.f.u. per $1 \times 10^5$ cells. The highest index ranged from $1.1 \times 10^4$ to $4.7 \times 10^4$ and was observed for two (8.7 %) strains. The lowest invasion, between $0.1 \times 10^5$ to $0.5 \times 10^5$ c.f.u., was revealed by 21.7 % of isolates. The index of *Y. enterocolitica* O3/4 was $1.1 \times 10^4$ c.f.u. Control *E. coli* K12C600 did not invade any HeLa cells.

In order to colonize a host, *S. warneri* displays the ability to adhere to epithelial cells. Interaction of the strains with the cells is the first stage towards the successful establishment of biofilm-associated infections and the bacterial invasion of the host. We have shown, for the first time to our knowledge, that clinical isolates of *S. warneri* have the ability to invade nonphagocytic cells. The mechanism allows *S. warneri* cells to evade the host immune response and facilitates their persistence and, therefore, could be an important virulence factor. Previously, Valour *et al.* (2013) suggested that invasion of osteoblasts by *S. epidermidis* contributes to bone and joint infections caused by these bacteria. Pereira *et al.* (2012) indicated that *Staphylococcus lugdunensis* was able to invade epithelial cells.

**Biofilm formation and in vitro activity of rifampicin combined with tigecycline on bacteria growing as a biofilm**

The results showed that 12 (52 %) *S. warneri* strains had the ability to adhere to polystyrene surface and to form biofilm *in vitro* (Table 1). Eleven isolates were poor biofilm formers under our experimental conditions and were classified as biofilm-negative strains (OD$_{190}<$0.250). Among biofilm-positive strains, only two carried ica genes. In order to estimate the chemical composition of the biofilm matrix of *S. warneri*, the 24 h biofilm was treated with NaIO$_4$, proteinase K and DNase (Fig. 2). Treatment with proteinase K resulted in a biomass reduction (from 45 to 80%), suggesting that proteinase K degrades matrix material and causes biofilm detachment from the polystyrene surface of the wells. The biofilm was also sensitive to the action of NaIO$_4$, a polysaccharide-degrading agent. When DNase was added to the biofilm growing in polystyrene microtiter plates, the biofilm of six *S. warneri* strains was reduced by about 15 %. DNase had no effect on the remaining biofilm-forming strains.

Thus, we can conclude that DNA plays a minor role in the biofilm structural integrity of *S. warneri* strains.

To obtain more detailed information about staphylococcal biofilm, we analysed their structures in CLSM (Fig. 3). The images showed 24 h *S. warneri* biofilms to be three-dimensional structures. The common characteristic of the biofilms was a low number of dead cells observed in mature biofilms. We observed differences in biofilm density and the area of the growth chamber covered by these structures. The thickness of the biofilm formed by these isolates ranged from 13 µm to 19 µm.

All the biofilm-positive strains were included for further study that aimed at determining the efficacy of the tigecycline/rifampicin combination on biofilm-growing bacteria. All planktonic forms of *S. warneri* strains were sensitive to rifampicin and tigecycline. The BIC value for tigecycline/rifampicin against cell growth in the biofilm ranged from 0.062 to 1 µg ml$^{-1}$. Thus, none of the isolates formed biofilm exhibiting resistance to tigecycline (defined according to EUCAST planktonic susceptibility breakpoints).

We observed that another important factor in the pathogenesis of *S. warneri* infections is biofilm formation. We found that 52 % of isolates recovered from patients with bloodstream infections produce biofilm. More recently, Paluch-Oléš *et al.* (2011) reported that a *S. warneri* strain isolated from a child with otitis media did not produce biofilm. According to Bradford *et al.* (2011), even harmless, skin commensal *S. epidermidis* strains may be induced to produce biofilm using specific chemicals or growth conditions. However, biofilm density was significantly greater in CoNS strains responsible for bacteraemia, than CoNS strains isolated from the skin of healthy neonates (de Silvia *et al.*, 2002). It should be emphasised that most *S. warneri* strains form biofilm via ica-independent mechanisms. Previously, Chokr *et al.* (2006) reported that four *S. warneri* strains carried icaADBC genes in their genomes. However, only one of those strains had the ability to produce biofilm *in vitro*. In contrast, de Silvia *et al.* (2002) did not find ica genes in eight *S. warneri* strains. Our findings indicate, that proteins play an important role in maintaining the structure and integrity of biofilms. Also, polysaccharides and extracellular DNA are present in the biofilm matrix of *S. warneri*. Because of the protective nature of the biofilm matrix, antibiotics usually fail to eradicate staphylococcal infections. There is evidence that the *S. epidermidis* matrix remarkably decreases the activity of vancomycin and teicoplanin (Singh *et al.*, 2010). The efficacy of erythromycin, clindamycin, gentamicin, tobramycin, amikacin and daptomycin is also moderately affected by the biofilm matrix (Singh *et al.*, 2010). Alternatively, rifampicin demonstrated an ability to penetrate biofilms formed by *S. epidermidis* (Zheng & Stewart, 2002). In the present study, we determined the efficacy of the rifampicin combination with tigecycline on biofilm-growing bacteria. The BIC range of tigecycline/rifampicin was $\leq 1$ µg ml$^{-1}$. Therefore, all isolates forming a biofilm exhibited susceptibility (defined according to EUCAST planktonic susceptibility breakpoints).
Table 1. Biofilm formation, AI, InI, cytotoxic activity and presence of genes encoding antibiotic resistance in *S. warneri* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Biofilm formation* (mean OD value±sd)</th>
<th>Presence of <em>ica</em> genes</th>
<th>AI † (× 10^3)</th>
<th>InI ‡ (× 10^3)</th>
<th>Cytotoxic index§ Extracellular (%)</th>
<th>Cell-contact (%)</th>
<th>Presence of genes encoding antibiotic resistance¶</th>
<th>BIC (g ml⁻¹) of tigecycline/ rifampicin§</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPU Sw9</td>
<td>0.42±0.016</td>
<td>+</td>
<td>36.9</td>
<td>3.6</td>
<td>89.2</td>
<td>76.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MPU Sw1</td>
<td>0.38±0.021</td>
<td>+</td>
<td>21.4</td>
<td>10.8</td>
<td>81.4</td>
<td>76.9</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MPU Sw10</td>
<td>0.31±0.018</td>
<td>−</td>
<td>12.3</td>
<td>0</td>
<td>64.1</td>
<td>70.7</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>MPU Sw13</td>
<td>0.20±0.015</td>
<td>−</td>
<td>9.1</td>
<td>46.7</td>
<td>11.6</td>
<td>21.1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MPU Sw17</td>
<td>0.19±0.016</td>
<td>−</td>
<td>6.9</td>
<td>0</td>
<td>18.9</td>
<td>16.1</td>
<td>−</td>
<td>−</td>
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<tr>
<td>MPU Sw4</td>
<td>0.15±0.012</td>
<td>−</td>
<td>3.8</td>
<td>1.4</td>
<td>6.4</td>
<td>11.7</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MPU Sw19</td>
<td>0.18±0.021</td>
<td>−</td>
<td>1.9</td>
<td>0</td>
<td>4.8</td>
<td>9.9</td>
<td>−</td>
<td>−</td>
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<tr>
<td>MPU Sw21</td>
<td>0.19±0.021</td>
<td>−</td>
<td>11.6</td>
<td>0</td>
<td>28.4</td>
<td>31.3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MPU Sw25</td>
<td>0.39±0.014</td>
<td>−</td>
<td>10.5</td>
<td>0.1</td>
<td>24.7</td>
<td>71.6</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MPU Sw6</td>
<td>0.32±0.025</td>
<td>−</td>
<td>1.2</td>
<td>0.3</td>
<td>8.7</td>
<td>63.5</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MPU Sw16</td>
<td>0.36±0.022</td>
<td>−</td>
<td>1.1</td>
<td>0.5</td>
<td>4.1</td>
<td>74.2</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MPU Sw8</td>
<td>0.30±0.017</td>
<td>−</td>
<td>1.1</td>
<td>0</td>
<td>2.6</td>
<td>70.3</td>
<td>−</td>
<td>+</td>
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<tr>
<td>MPU Sw15</td>
<td>0.14±0.013</td>
<td>−</td>
<td>1.07</td>
<td>0</td>
<td>1.9</td>
<td>63.5</td>
<td>−</td>
<td>−</td>
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<tr>
<td>MPU Sw22</td>
<td>0.10±0.008</td>
<td>−</td>
<td>1.02</td>
<td>0</td>
<td>43.1</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>MPU Sw24</td>
<td>0.29±0.019</td>
<td>−</td>
<td>0.9</td>
<td>0.6</td>
<td>2.4</td>
<td>76.3</td>
<td>−</td>
<td>+</td>
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<tr>
<td>MPU Sw14</td>
<td>0.33±0.018</td>
<td>−</td>
<td>0.8</td>
<td>0</td>
<td>4.7</td>
<td>68.7</td>
<td>−</td>
<td>+</td>
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<tr>
<td>MPU Sw12</td>
<td>0.27±0.024</td>
<td>−</td>
<td>0.7</td>
<td>0</td>
<td>2.1</td>
<td>65.3</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MPU Sw11</td>
<td>0.32±0.022</td>
<td>−</td>
<td>0.6</td>
<td>0.2</td>
<td>4.3</td>
<td>71.9</td>
<td>−</td>
<td>+</td>
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<tr>
<td>MPU Sw18</td>
<td>0.08±0.007</td>
<td>−</td>
<td>0.6</td>
<td>0</td>
<td>1.4</td>
<td>39.4</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*Biofilm formation measured using the crystal violet staining method.
†AI calculated as described in Methods.
‡InI calculated as described in Methods.
§Cytotoxic index: Extracellular (%) + Cell-contact (%).
¶Presence of genes encoding antibiotic resistance: mecA (6¢) or aac(3')-IIIa (2¢), aph(2') (3¢), ant (4')-Ia, emr (A), erm (C), msr (A), lnu (A), BIC (g ml⁻¹) of tigecycline/ rifampicin.
||BIC (g ml⁻¹) of tigecycline/ rifampicin.
Table 1. cont.

Table 1. Cont.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Biofilm formation* (mean OD_{560})</th>
<th>Presence of gene encoding antibiotic resistance</th>
<th>Cytotoxic index§</th>
<th>Cell-contact activity (%)</th>
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<tr>
<td>Sw7</td>
<td>0.26±0.030.09</td>
<td>+</td>
<td>6.4</td>
<td>5.7</td>
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<tr>
<td>Sw2</td>
<td>0.12±0.030.18</td>
<td>-</td>
<td>6.4</td>
<td>0</td>
</tr>
<tr>
<td>Sw3</td>
<td>0.09±0.030.013</td>
<td>-</td>
<td>0</td>
<td>1.4</td>
</tr>
<tr>
<td>Sw20</td>
<td>0.05±0.000.008</td>
<td>-</td>
<td>0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*Biofilm formation was examined by crystal violet assay. Strains were considered biofilm-positive if OD_{560}>0.250.
†Mean c.f.u. of adhered bacteria per 1x10^8 cells.
‡Mean c.f.u. of internalized bacteria after gentamicin/lysostaphin treatment per 1x10^8 cells.
§The percentage of damaged cells due to extracellular and cell-contact toxins was determined by reduction of MTT. All results are means of two separate experiments performed in triplicate.
||Presence of genes encoding antibiotic resistance and ica genes in S. warneri strains was detected using PCR assays.
¶BIC of tigecycline/rifampicin was examined by biofilm susceptibility assay.
ND, not determined.

Virulence and antibiotic resistance of Staphylococcus warneri strains

This study clearly shows that tigecycline/rifampicin is a promising treatment option for infections caused by biofilm-producing S. warneri strains. Research on the rabbit model of S. aureus osteomyelitis demonstrated that the association of rifampicin and tigecycline resulted in complete eradication of bacteria from bone (Yin et al., 2005). Similarly, Vergidis et al. (2015) indicated that tigecycline/rifampicin combination treatment decreased bacterial counts in the rat model with implanted stainless steel wire.

Cytotoxic activity of S. warneri extracellular and cell-contact toxins

We observed that cell-free supernatants of 20 (87 %) S. warneri strains were cytotoxic to epithelial cells after 24 h of incubation. It has been noted that the differentiated percentage of damaged cells ranged from 1.4 to 89.2 % (Table 1). The highest activity, above 60 %, was observed for three (13 %) strains, the lowest (from 1.4 to 4.7 %) was noted for 9 (39 %) isolates. The supernatants of three (13 %) strains and E. coli K12C600 were not cytotoxic to epithelial cells. A quantitative cell-contact toxic activity assay of S. warneri showed that bacterial cells were able to lyse epithelial cells within 5 h of incubation (Table 1). All strains revealed cell-contact activity, with a cytotoxic index ranging from 1.4 to 76.9 %. The highest activity, from 63.5 % to 76.9 % was observed for 13 (56.5 %) strains. The lowest cytotoxic index, below 5 %, revealed 2 (8.7 %) isolates. The cells of E. coli K12C600 caused the destruction of 0.6 % HeLa cells. After 5 h of incubation, a low cytotoxic activity (below 12.6 % of damaged cells) occurred with bacterial culture supernatant, which suggests that these strains produced extracellular toxins (data not shown). We observed that the presence of biofilm was strongly associated with cell-contact cytotoxicity (P<0.001) but not with the InI (P=0.67) or the cytotoxic activity of cell-free bacterial supernatants (P=0.24). Le et al. (2014) suggested that among multiple molecules, only cell-associated phenol-soluble modulins are the main effectors of staphylococcal biofilm maturation and dispersion. The modulins are a family of cytolytic peptide toxins which play an important role in contact toxicity and S. aureus pathogenicity (Laabei et al., 2014).

Although CoNS are considered a minor pathogen with a low virulence potential, our study revealed that S. warneri strains produce extracellular toxins, causing the destruction of epithelial cells. Verdon et al. (2009) characterized warnericin and delta-toxins I and II, named hemolysins, isolated from S. warneri. The extracellular proteins displayed cytotoxic activity. Otto (2004) reported that bacteria secrete enzymes such as protease, lipase and esterase, which may contribute to virulence via the destruction of host tissues. Interestingly, in the present study we observed activity of cell-contact toxins. It was suggested that the ica class of small staphylococcal peptides, known as phenol-soluble modulins, are cytotoxic to many cell types (Le et al., 2014). The cytotoxic activity of S. warneri strains allows them to evade the host immune system.
The mechanics gene, which codes for resistance to almost all beta-lactam antibiotics, was detected in only three biofilm-positive strains. The most important gene coding for aminoglycoside resistance [aac(6')/aph(2'')] was also detected in four biofilm-positive isolates. Three isolates harboured the gene [aph(3')-IIIa] encoding phosphotransferases and three isolates carried the gene encoding nucleotidyltransferases [ant(4')-Ia]. One strain was positive for both aac(6')/aph(2'') and aph(3')-IIIa genes. MLSb resistance genes erm(A), erm(C), msr(A) and lnu(A) were detected in 2 (10%), 2 (10%), 7 (35%) and 3 (15%) of the S. warneri isolates, respectively. The majority of these strains were biofilm-positive. No isolates harboured any erm(B) genes.

It was observed that antibiotic resistance genes were more frequent in biofilm-positive than biofilm-negative S. warneri isolates. Biofilm structures with cells sitting close to each other may be ideal for horizontal gene transfer and therefore facilitating the spread of antibiotic resistance. As mentioned above, many of the antibiotic resistance genes are located on mobile genetic elements such as SCCmec cassette, plasmids and transposons, which are capable of broad-range gene transfer in bacteria. We found that S. warneri strains carried genes encoding resistance to MLSb antibiotics, aminoglycosides and beta-lactam antibiotics. However, the prevalence of these antibiotic resistance genes was low. Similar findings were reported by Hira et al. (2013), who found low antibiotic resistance in S. warneri strains isolated from neonates treated in hospital. None of the biofilm-forming isolates exhibited resistance to tigecycline.

**Resistance to antibiotics**

Another aspect examined in our study was the estimated prevalence of genes responsible for antibiotic resistance.
CONCLUDING REMARKS

In conclusion, our results provide new insights into the virulence of *S. warneri* strains. The data suggested that the isolates revealed a multifactorial mechanism of pathogenicity. They were able to adhere to, produce biofilm, invade and destroy epithelial cells. The mechanisms allowed evasion of the host’s immune response and facilitated bacterial persistence. Furthermore, we demonstrated that rifampicin/trimetopime has an excellent in vitro activity against commensal bacteria growing as a biofilm.

REFERENCES


