A Brazilian lineage of *Staphylococcus lugdunensis* presenting rough colony morphology may adhere to and invade lung epithelial cells

Eliezer M. Pereira,1,2† Cesar Augusto A. Teixeira,3† Ana Luiza M. Alvarenga,1,2 Ricardo P. Schuenck,1 Márcia Giambiagi-deMarval,1 Carla Holandino,3 Ana Luiza Mattos-Guaraldi4 and Kátia R. N. dos Santos1

1Department of Medical Microbiology, Institute of Microbiology, Rio de Janeiro Federal University, Rio de Janeiro, Brazil
2Federal Institute of Education, Science and Technology of Rio de Janeiro, Rio de Janeiro, Brazil
3Faculty of Pharmacy, Rio de Janeiro Federal University, Rio de Janeiro, Brazil
4Faculty of Medical Sciences, Rio de Janeiro State University, Rio de Janeiro, Brazil

*Staphylococcus lugdunensis* is an unusually virulent coagulase-negative species, which causes serious infection similar to *S. aureus*. We evaluated the expression of virulence factors such as *S. lugdunensis* synergistic haemolysin (SLUSH), fibrinogen-binding protein (Fbl), biofilm production and biofilm-production-related genes in 23 *S. lugdunensis* clinical isolates and one type strain that had been previously characterized for their genotypes. In addition, the biofilm composition and the ability of isolates to adhere to and invade human epithelial lung cells were also investigated. The PCR method used detected the presence of slush and intercellular adhesin (ica) virulence genes in all isolates. All isolates produced the Fbl protein and, with the exception of the type strain, all isolates produced the SLUSH haemolysin. Fourteen (60.9 %) isolates produced biofilms. The detachment assay, using sodium metaperiodate or proteolytic enzymes to analyse the biofilm composition, showed protein-mediated biofilms in two representative isolates, one for each colony type (rough and smooth). All strongly biofilm-producing isolates, including three with rough colony morphology, had the same prevalent PFGE pattern. However, among the representative strains tested, only the *S. lugdunensis* isolate that formed rough colonies was able to adhere to and invade A549 cell monolayers in the same quantities as those observed with *S. aureus* isolates (P<1.000). No significant adhesion or invasion was observed for the other isolates in comparison with the *S. aureus* isolate, independent of biofilm production or clonality. Our results could explain the incredible ability of this pathogen to cause infections that are as aggressive as *S. aureus*. In addition, the ability of *S. lugdunensis* to adhere to and invade eukaryotic cells was also noticed for isolates with rough colony morphology, reinforcing the increased virulence in this species.

INTRODUCTION

*Staphylococcus lugdunensis* is a coagulase-negative *Staphylococcus* (CNS) that has been recognized as an emerging and important human pathogen, occasionally causing serious infections, such as a highly destructive form of native valve endocarditis (Frank et al., 2008). *S. lugdunensis* colonizes the inguinal area and is an unusually virulent CNS that has been identified as responsible for both nosocomial and community-acquired infections. These infections can range from superficial skin lesions to life-threatening endocarditis that evolves aggressively and with similar severity to *Staphylococcus aureus* infections (Frank et al., 2008).

Like *S. aureus*, this micro-organism may express several virulence factors, such as fibrinogen-binding protein, haemolysins and biofilm production (Donvito et al., 1997; Mitchell et al., 2004; Frank & Patel, 2007; Pereira et al., 2010). According to a report by Otto (2008), biofilm...
production is an important factor in staphylococcal pathogenesis. The major component of biofilm structure is the polysaccharide intercellular adhesin, a protein encoded by the *icaADBC* locus. In fact, the biofilm produced by *S. lugdunensis* also seems to be composed of protein (Frank & Patel, 2007).

The ability to adhere to and invade eukaryotic cells has previously been described for *S. aureus* (de Bentzmann et al., 2004; Karauzum et al., 2008). Additionally, Amaral et al. (2005) showed the enhanced ability of a predominant lineage of methicillin-resistant *S. aureus* (MRSA) isolated from Brazilian hospitals to invade epithelial cells. One report describes the analysis of *S. lugdunensis* interaction with eukaryotic cells, but the authors only evaluated the loss of viability of fibroblasts in a monolayer after exposure to clinical isolates (You et al., 1999).

Studies analysing the characteristics associated with virulence in *S. lugdunensis* clinical isolates are rare in literature. Moreover, the interaction of *S. lugdunensis* with host cells has, to our knowledge, not been described. This study aims to assess the phenotypic and molecular characteristics of 23 *S. lugdunensis* clinical isolates that were described by our group in a previous study (Pereira et al., 2010), evaluating their ability to express virulence factors and virulence-factor-related genes. Biofilm composition and ability to adhere to and invade human respiratory epithelial cells were also evaluated.

**METHODS**

**Bacterial isolates.** We evaluated 23 *S. lugdunensis* clinical isolates that were previously identified and genotyped by PFGE (Pereira et al., 2010) from the collection of the Hospital Infections Laboratory, Microbiology Institute, Rio de Janeiro Federal University, Brazil, obtained between 1997 and 2008 from seven hospitals in Rio de Janeiro, Brazil.

**Detection of virulence-factor-encoding genes**

(a) **DNA extraction.** Five to six bacterial colonies, previously grown on agar base (Oxoid) supplemented with 5% sheep’s blood, were suspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA; pH 7.8) and heated to 100°C for 10 min. After centrifugation at 20 000 g for 30 sec, the supernatant was collected for the PCR (Pereira et al., 2010).

(b) **PCR detection.** For the *ica* gene (287 bp fragment), PCR was performed according to published protocols (Potter et al., 2009), and for the *slush* gene fragment, amplification was described as described by Pereira et al. (2010) using the primers slush-F (5’-GAGGACACAATATAAGGAG-3’) and slush-R (5’-CCTGCACATGTTAAATCCC-3’), designed in this study. The *slush* gene sequence was obtained from the GenBank sequence database (accession number U73444). Primers were designed using OligoExplorer (Integrated DNA Technologies) and BioEdit software (Ibis Biosciences). Primer specificity was tested against database sequences using BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A 203 bp amplicon was expected. *S. lugdunensis* DSM 4804T, *S. epidermidis* strains ATCC 35984 (*ica-positive*) and ATCC 12228 (*ica-negative*), and *S. haemolyticus* ATCC 29970T (*slush-negative*) were used as controls.

Amplified products were analysed by electrophoresis on a 1% agarose gel, followed by staining with ethidium bromide and visualization on UV transilluminator.

**Analysis of virulence factors**

(a) **Fibrinogen-binding protein (Fbl).** Western-blotting was performed as described previously (Mitchell et al., 2004), with modifications, in order to detect *fbl* gene expression. Membranes were incubated for 2 h with *S. lugdunensis* anti-Fbl polyclonal antibodies (kindly provided by Professor Timothy Foster, Trinity College, Dublin, Ireland), which were diluted 1:1000. Immune reactivity was detected by incubation for 40 min with peroxidase-conjugated goat anti-rabbit immunoglobulin G (KPL), diluted 1:2000. Bound antibodies were detected using a solution of DAB (3,3'-diaminobenzidine tetrahydrochloride) (0.5 mg ml⁻¹, Sigma) and 0.01% hydrogen peroxide in PBS (pH 7.4). *S. lugdunensis* DSM 4804T and *Escherichia coli* ATCC 25920T were used as positive and negative controls for Fbl production, respectively.

(b) **SLUSH production.** Detection of SLUSH production was performed as described previously (Hébert, 1990). Briefly, *S. intermedius* ATCC 49052 (a β-haemolysin producer) was streaked vertically on the centre of a base agar plate containing 5% sheep’s blood and test strains of *S. lugdunensis* were streaked perpendicularly, without touching the *Staphylococcus intermedius* inoculum. The plates were incubated aerobically at 35°C for 18–20 h and then kept at room temperature for 4–6 h before the reactions were read. A zone of complete haemolysis (where the test strain was growing) within the zone of incomplete haemolysis caused by the β-haemolysin from *S. intermedius* growth was considered a positive test.

(c) **Biofilm formation.** The ability of *S. lugdunensis* to attach to, and form a biofilm on, sterile 96-well polystyrene microtitre plates (TPP 92096) was tested according to Stepanović et al. (2007). Each assay was performed in triplicate and repeated three times. *S. epidermidis* strain ATCC 35984 (formerly RP62A), a biofilm producer, and *S. epidermidis* ATCC 12228, which is unable to produce a biofilm, were used as controls. The comparative analyses were performed according to Stepanović et al. (2007), using the wells inoculated with *S. epidermidis* ATCC 12228 as negative controls.

**Biofilm detachment assay.** The Biofilm detachment assay was performed according Frank & Patel (2007) for the two strongly biofilm-producing *S. lugdunensis* clinical isolates 546 and 541, which presented rough and smooth colony morphologies, respectively, (Table 1) to verify if attachment and biofilm production were carbohydrate- or protein-mediated. Briefly, after biofilm formation by the isolates in 96-well polystyrene microtitre plates (TPP 92096), three different detachment methodologies were performed: 40 mM sodium metaperiodate (NaIO₄, Vetec) in water was used for detachment of a carbohydrate-mediated biofilm; and 100 µg ml⁻¹ proteinase K in 10 mM Tris/HCl (pH 7.5) and 10 U ml⁻¹ trypsin in 10 mM Tris/HCl (pH 7.5) were used for detachment of protein-composed biofilms. Heat-inactivated (by boiling for 40 min) proteinase K and trypsin were also used as controls. *S. epidermidis* ATCC 35984, a well characterized carbohydrate biofilm-producer (*ica*-positive and biofilm-associated protein (*bap*)-negative) and *S. aureus* V389, a known protein-mediated biofilm producer (*ica*-negative, *bap*-positive) (Cucarella et al., 2001) were used as controls. All tests were performed in triplicate.

**Adherence and invasion assays.** The ability of *S. lugdunensis* to adhere to and invade cells was evaluated according to Menzies & Kourteva (2000) with modifications, using the human lung carcinoma cell line A549 (ATCC CCL-185). This cell line presents many of the characteristics of type II alveolar epithelial cells. In order...
**Table 1.** Details and characteristics of the 24 *S. lugdunensis* isolates used in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Isolation date (month/year)</th>
<th>Clinical specimen</th>
<th>PFGE pattern</th>
<th>Colony morphology</th>
<th>Biofilm formation</th>
</tr>
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<tbody>
<tr>
<td>97</td>
<td>HNMD</td>
<td>03/1999</td>
<td>Blood</td>
<td>A1</td>
<td>Rough</td>
<td>+</td>
</tr>
<tr>
<td>98</td>
<td>HNMD</td>
<td>03/1999</td>
<td>Surgical site</td>
<td>A2</td>
<td>Rough</td>
<td>+</td>
</tr>
<tr>
<td>546</td>
<td>HNMD</td>
<td>06/2007</td>
<td>Blood</td>
<td>A3</td>
<td>Rough</td>
<td>++</td>
</tr>
<tr>
<td>451</td>
<td>HNMD</td>
<td>06/2004</td>
<td>Urine</td>
<td>A4</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>644</td>
<td>HNMD</td>
<td>09/2008</td>
<td>Prosthesis secretion</td>
<td>A5</td>
<td>Smooth</td>
<td>++</td>
</tr>
<tr>
<td>541</td>
<td>HNMD</td>
<td>07/2006</td>
<td>Blood</td>
<td>A6</td>
<td>Smooth</td>
<td>++</td>
</tr>
<tr>
<td>540</td>
<td>HNMD</td>
<td>03/2006</td>
<td>Skin wound</td>
<td>A7</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>632</td>
<td>HNMD</td>
<td>02/2005</td>
<td>Blood</td>
<td>A7</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>157</td>
<td>HNMD</td>
<td>10/2002</td>
<td>Blood</td>
<td>A8</td>
<td>Smooth</td>
<td>++</td>
</tr>
<tr>
<td>611</td>
<td>HNMD</td>
<td>10/2007</td>
<td>Blood</td>
<td>A9</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>619</td>
<td>HNMD</td>
<td>06/2008</td>
<td>Blood</td>
<td>A10</td>
<td>Smooth</td>
<td>+</td>
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<tr>
<td>468</td>
<td>HUCFF</td>
<td>04/2005</td>
<td>Blood</td>
<td>A11</td>
<td>Smooth</td>
<td>++</td>
</tr>
<tr>
<td>545</td>
<td>HNMD</td>
<td>06/2007</td>
<td>Skin wound</td>
<td>B1</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>612</td>
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<td>05/2008</td>
<td>Blood</td>
<td>B2</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>515</td>
<td>INTO</td>
<td>01/2006</td>
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<td>+</td>
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<td>B4</td>
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<td>+</td>
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<tr>
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<td>Smooth</td>
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</tr>
<tr>
<td>33</td>
<td>HUCFF</td>
<td>01/1997</td>
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</tr>
<tr>
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<td>LSF</td>
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<td>C1</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>4804</td>
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<td>ND</td>
<td>ND</td>
<td>C2</td>
<td>Smooth</td>
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</tr>
<tr>
<td>142</td>
<td>HNMD</td>
<td>08/2002</td>
<td>Blood</td>
<td>C3</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>HGB</td>
<td>08/1996</td>
<td>Blood</td>
<td>C4</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>HUAP</td>
<td>01/1997</td>
<td>Catheter tip</td>
<td>D1</td>
<td>Smooth</td>
<td>+</td>
</tr>
<tr>
<td>645</td>
<td>IFF</td>
<td>10/2008</td>
<td>Nare</td>
<td>D1</td>
<td>Smooth</td>
<td>++</td>
</tr>
</tbody>
</table>

- **ica gene**: No data.
- **Phenotypic expression**: +++, poor biofilm production; +++, moderate biofilm production; ++++, good biofilm production.

**RESULTS**

**Phenotypic and molecular analysis of virulence factors**

Fbl protein production was detected by Western blot analysis using *S. lugdunensis* lysates and anti-*S. lugdunensis*-Fbl polyclonal antibodies. The test revealed two bands.
present in all 23 clinical isolates and the reference strain DSMZ 4804T. The bands, at ~120 and ~160 kDa, were characteristic of the Fbl protein and its precursor, respectively, as reported previously (Mitchell et al., 2004).

All 23 S. lugdunensis clinical isolates produced SLUSH although, curiously, the type strain did not; however the slush gene fragment (203 bp) was detected in all isolates.

All isolates were PCR-positive for the ica gene. Fourteen (60.9 %) clinical isolates produced biofilms, five (21.7 %) being good biofilm-producers, four (17.4 %) being moderate biofilm-producers and five (21.7 %) being poor biofilm-producers. Biofilm production was observed mainly among pulsotype A isolates (83.3 %), a PFGE type described previously by Pereira et al. (2010). Moreover, all the isolates presenting good biofilm production or rough colony morphology were included in this pulsotype. These data are shown in Fig. 1.

Biofilm detachment assay

Biofilm detachment results are shown in Fig. 2. The strongly biofilm-producing S. lugdunensis clinical isolates 546 (rough morphology) and 541 (smooth morphology) produced protein-mediated biofilms. The results were similar to those found for S. aureus strain V389 (protein-mediated biofilm-producer) and different to those found for S. epidermidis ATCC 35984 (carbohydrate-mediated biofilm-producer). Treatment with sodium metaperiodate was not able to detach biofilms produced by these isolates, while proteolytic enzymes were significantly effective in detaching this bacterial component (P<0.05). Incubation with pre-heated proteolytic enzymes confirmed this finding.

Adherence and invasion assays

Five S. lugdunensis isolates were evaluated by the adherence and invasion assays: three non-biofilm producers and two good biofilm producers. One biofilm-producing isolate (isolate 546), which also presented a rough colony morphology, was the only one able to adhere to and invade A549 cells in the same proportion as the S. aureus isolate HU25 (P=1.000), as shown in Fig. 3a. All other S. lugdunensis isolates evaluated (541, 611, 612 and 142) showed low levels of adherence and invasion compared to S. lugdunensis 546 and S. aureus HU25 (P<0.005). The morphology of rough and smooth colonies is shown in Fig. 3(b, c), respectively. Fig. 3(d) shows the adhesion levels of these isolates as determined by optical microscopy by using Giemsa staining. S. aureus adhered homogeneously all over the cell surface, whereas S. lugdunensis adhered in localized aggregates on the eukaryotic cell membrane.

DISCUSSION

S. lugdunensis is an unusually virulent CNS that is able to produce an arsenal of substances that contribute to its virulence and aggressiveness. It also presents an infection pattern similar to that of S. aureus, mainly in cases of endocarditis (Liu et al., 2010). Although this staphylococcal species was first described more than two decades ago, its virulence genes and their products have rarely been investigated. In this study, we detected slush and ica genes in all 23 S. lugdunensis clinical isolates, which were previously identified by detection of the fbl gene (Pereira et al., 2010). The Fbl protein is an important adhesion factor that contributes to the virulence of S. lugdunensis and its exceptional ability to interact with fibrinogen, as described in a recent study (Geoghegan et al., 2010). Since all the strains in the present study were able to produce this protein, it is possible that the Fbl protein is expressed constitutively by S. lugdunensis, contributing to its adhesion to host tissues and prosthetic materials. All S. lugdunensis strains in our study were positive for SLUSH protein activity, except the type strain DSMZ 4804T, although the slush gene was detected in all strains. In a recent study,

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**Fig. 1.** Dendrogram of the PFGE profiles of Smal-digested genomic DNA from 24 S. lugdunensis isolates. Percentage similarities were derived from analysis using the unweighted pair group method with arithmetic mean (UPGMA) and based on Dice’s coefficient. Isolates showing a similarity coefficient $\geq 70\%$ were considered genetically related.
Rautenberg et al. (2011) showed that SLUSH peptides are phenol-soluble modulins (PSMs), which interact with formyl-peptide receptor 2 (FPR2) of human leukocytes, resulting in their attraction and stimulation. FPR2 is a general receptor for all PSM-like peptide toxins. The release of PSMs correlated closely with the apparent capacity of staphylococcal species to cause invasive infections and with their ability to activate FPR2.

Biofilm formation in *S. lugdunensis* has been characterized previously, either by detecting the presence of the *ica* gene (Chokr et al., 2006; Frank & Patel, 2007) or by biofilm production on microtitre plates (Frank & Patel, 2007). In the present study, we observed that some *S. lugdunensis* isolates were able to produce large amounts of biofilm matrix. Moreover, the biofilm production was observed mainly among predominant pulsotype A isolates. This finding could explain the success of this pathogen in causing native and prosthetic valve endocarditis (Anguera et al., 2005; Liu et al., 2010). An interesting result of our work was the isolation of good biofilm-producing isolates showing a rough colony morphology similar to that of *Bacillus* colonies, which is uncommon for staphylococci (Fig. 3a). These isolates were also included in the prevalent pulsotype A. To our knowledge, this is the first report of this kind of staphylococcal colony morphology.

Since *S. aureus* is recognized as the most pathogenic species in the *Staphylococcus* genera, and its ability to adhere to and invade host cells is widely reported (Sinha et al., 2000;
For the two biofilm-producing *S. lugdunensis* isolates, one with rough colony morphology and the other with smooth colony morphology, the biofilm composition was characterized by the detachment assay with sodium metaperiodate (a carbohydrate-degrading agent) or proteolase K and trypsin proteolytic enzymes. Biofilms produced by these two *S. lugdunensis* isolates were identified as being protein-mediated. When proteolytic enzymes were added after an inactivation step (boiling for 40 min), no detachment was observed, reinforcing our data. Our data are in agreement with those described by Frank & Patel (2007) who detected protein-mediated biofilms in all 15 biofilm-producing *S. lugdunensis* clinical isolates from several sites of infection, including cases of endocarditis and prosthetic infections. Moreover, this protein biofilm matrix has been described for *S. aureus* isolates (Cucarella et al., 2004), demonstrating, once more, a similarity between these two staphylococcal species.

In conclusion, we have shown that all the clinical isolates of *S. lugdunensis* evaluated carried the *fbl*, *slush* and *ica* virulence genes and, with the exceptions of biofilm production and SLUSH protein activity in the type strain DSMZ 4804, all clinical isolates expressed these virulence factors, which may contribute to the atypical infection course and explain the incredible ability of this pathogen to cause infection as aggressive as *S. aureus*. In addition, the ability of *S. lugdunensis* to adhere to and invade eukaryotic cells was also noticed in isolates presenting rough colony morphology, reinforcing the increased virulence in this species. Further studies are necessary to understand how *S. lugdunensis* surface components contribute to cell adherence and invasion.

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