Serum and tissue protein binding and cell surface properties of *Staphylococcus lugdunensis*

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Summary. Eleven strains of *Staphylococcus lugdunensis* from different clinical sources were investigated for their ability to bind ^{125}I -labelled collagen (Cn) type I and IV, fibronectin (Fn), vitronectin (Vn), laminin (Lm), fibrinogen (Fg), thrombospondin, plasminogen (glu- and lys-form) and human IgG. All the strains bound these proteins, although a higher degree of binding was obtained for Cn types I and IV and IgG with mean values of 36 %, 32 % and 26 % binding, respectively. In tests with proteins immobilised on latex beads in a particle agglutination assay, eight of the 11 strains bound Cn type I and seven bound Fg, whereas no strain bound immobilised IgG. Binding to immobilised Cn-I, Fg, Lm and Vn was abolished when the bacterial cells were treated with proteases or heat, indicating cell-surface receptors with protein characteristics. Cell-surface extracts of *S. lugdunensis* 2342 were able to totally inhibit binding of the homologous strain and *S. aureus* Cowan 1 to latex-immobilised proteins Cn-I, Lm, Vn, Fn and Fg. The binding of ^{125}I -labelled Cn IV by *S. lugdunensis* 2342, was heat sensitive, whereas the binding to *S. aureus* Cowan 1 was heat resistant. The strains gave negative results in tests for the presence of protein A with a *S. aureus* protein A gene probe and with sensitised red blood cells. No production of heat-stable nuclease (TNase) could be detected by monoclonal antibodies against TNase or by the polymerase chain reaction with an oligonucleotide sequence from *S. aureus* TNase as primer. When the cell surface characters of the *S. lugdunensis* strains were studied, five were found to be hydrophobic and negatively charged, four hydrophilic and positively charged and two hydrophobic with positive net charge.

Introduction

Coagulase-negative staphylococci (CNS) are well recognised as opportunist pathogens causing infections in neonates and neutropenic patients.¹ They are the most important pathogens in infections associated with intravascular catheters and grafts, peritoneal catheters and prostheses in various organs.² With the recent description of two new species of CNS, *Staphylococcus lugdunensis* and *S. schleiferi*,³ there are at present 24 species of CNS. *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus* are the species isolated most frequently from human infections.¹

Coagulase-positive staphylococci, i.e., *S. aureus*, are more virulent than CNS.¹ Numerous studies have focused on presumptive virulence factors of *S. aureus*, and latterly on those of CNS.⁴ Of these factors, only protein A and coagulase are consistently absent in CNS. The presence of heat-stable nuclease is characteristic of coagulase-positive staphylococci but has not been ascribed any role in pathogenicity.¹ All other toxins, enzymes and outer-membrane proteins of *S. aureus* have been detected in CNS strains with varying

frequency.^{5,6} Production of extracellular polysaccharide material, “slime”, is more prominent in CNS than in *S. aureus*, and slime has been discussed as an important virulence factor of CNS in biomaterial-associated infections.⁷ This has been an area of controversy.⁸

S. lugdunensis was originally described as a cause of endocarditis.^{9,10} Subsequent studies have shown that *S. lugdunensis* forms part of the normal skin flora but is frequently isolated from endocarditis, arthritis or soft tissue infections, i.e., *S. lugdunensis* appears to be more virulent than other CNS species.^{11,12} This study was undertaken to investigate presumptive virulence factors of *S. lugdunensis* strains isolated from various kinds of infections and from normal skin, particularly for factors which may influence the initial adhesion to host tissue—binding to connective tissue and serum proteins, cell-surface hydrophobicity, and net cell-surface charge.

Materials and methods

Bacterial strains and culture media

Eleven strains of *S. lugdunensis* were isolated from human infections and typed according to the methods

Fig. 1. Binding of ^{125}I -labelled serum and tissue proteins to strains of *S. lugdunensis* (●), *S. aureus* Cowan 1 (★), *S. haemolyticus* E2498/86 (□) and *S. epidermidis* 3380 (○).

of Freney *et al.*³ with an in-house panel of tests for ornithine decarboxylase, arginine dihydrolase and urease, nitrate reduction, acetoin production, acid production from mannose, sucrose, trehalose, mannitol and xylose, as well as anaerobic growth in semi-solid thioglycollate broth. The strains were tested further for coagulase with human plasma, and for clumping factor with an in-house preparation of human plasma, with Staphyslide® (bioMérieux, Lyon, France) in which fibrinogen is adsorbed to sheep erythrocytes, with Monostaph® (BioNor, Skien, Norway) in which fibrinogen and IgG are adsorbed to monodispersed particles, and with Staphaurex® (Wellcome Diagnostics) in which fibrinogen and IgG are adsorbed to latex particles. Susceptibility to novobiocin 5 µg, polymyxin B 150 µg and furazolidone 50 µg was determined with Neo-Sensitab® (Rosco AS, Taastrup, Denmark). The strains were isolated from the following sites: 2342 and G2-89, blood (endocarditis); G6-87, blood (septicaemia); 49/91, bone marrow (osteomyelitis); SÅ, knee joint (arthritis); G5-87, peritoneal fluid (peritonitis); G58-88 and A251, wound infections; G66-68, suppurating axillary lymph node; and G3A and G16-89, normal skin.

S. aureus Cowan 1, Wood 46, V8 and ATCC 29213, *S. haemolyticus* E2498 and *S. epidermidis* 3380 were used as control strains. Bacterial strains were cultured on blood agar with horse erythrocytes 4% v/v for 24 h at 37°C.

Iodine-labelled protein binding assay

The method has been described previously.¹³ Washed bacterial cells were suspended in 0.07 M phosphate-buffered saline (PBS), pH 7.2, at a concentration of 5×10^8 cells/ml, and 100 µl of bacterial suspension was incubated with 50 µl of ^{125}I -labelled

protein (3×10^4 cpm) in PBS with bovine serum albumin (BSA) 1% for 1 h at 20°C. After washing with PBS-Tween 0.1%, the radioactivity of the pellet was measured, and the percentage binding was calculated. Less than 5% binding was considered to be a negative result.

Inhibition of binding of soluble proteins was studied by pre-incubation of bacterial cells with purified proteins (100 µl/ml, 30 min, 20°C), after which binding of labelled protein was assayed as above.

Particle agglutination assay (PAA)

Proteins were immobilised on latex particles (0.8 µm) as described previously.¹⁴ After adsorption of proteins to 1 ml of latex, the mixtures were centrifuged (9200 g, 5 min, 4°C), and the pellets were resuspended in 2 ml of glycine buffer (0.17 M glycine-NaOH, pH 8.2) with ovalbumin 0.01% and merthiolate 0.01%. The suspensions were kept at 4°C. The PAA was performed on glass slides as described previously.¹⁴ The reactions were scored after observation for 2 min and given a numerical value: strongly positive = 3, moderate = 2, weakly positive = 1, and no visible agglutination = 0.¹⁴ Strains were tested for auto-aggregation by mixing one drop of bacterial cell suspension with one drop of Pp-buffer.¹⁴

Heat treatment

Suspensions (1 ml) of bacterial cells were heated at 80°C for 10 min and chilled rapidly.

Extraction of cell-surface components

Cells of *S. aureus* Cowan 1, and *S. lugdunensis* 2342

Table 1. Reactions of 11 *S. lugdunensis* strains in four methods designed to detect clumping factor

Strain no.	Reaction in tests with			
	Fg	Staphaurex	Staphyslide	Mono-Staph
2342	+	+	+	(+)
SA	+	+	+	+
G3A	—	—	—	—
G5-87	+	+	+	(+)
G2-89	—	—	—	—
G6-87	+	+	+	(+)
G16-89	+	+	+	(+)
G58-88	—	—	—	—
G66-88	+	+	+	(+)
49/91	—	—	—	—
A251	—	—	—	—
Cowan 1	+	+	+	+
E2498	—	—	—	—

(+) , Weak reaction.

(c. 1 g wet weight) were washed twice in Pp-buffer, and the cell-surface proteins were extracted with 1.5 ml of 1 M LiCl, pH 5.0, at 45°C for 2 h. After centrifugation at 9750 *g* for 60 min the supernates were dialysed against 0.02 M ammonium bicarbonate with 1 mM EDTA, pH 8.4, for 24 h at 4°C. The extracts were centrifuged for 60 min at 9750 *g* and supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF). The protein content was 100 and 95 µg/ml for *S. aureus* Cowan 1 and *S. lugdunensis* 2342 respectively, as determined by BioRad micro-assay (BioRad, Richmond, CA, USA). The extracts were stored at –20°C until required.

Particle agglutination inhibition assay

Bacterial cell suspensions were incubated with purified proteins (100 µg/ml, 30 min, 20°C) before PAA was performed.

In another series of experiments, bacterial cell-surface extracts were incubated with equal volumes of coated latex beads (30 min, 20°C) and then mixed with bacterial cells. Agglutination was recorded after 2 min and compared to agglutination with untreated coated latex beads.

Protein A determination

The production of protein A in 11 strains of *S. lugdunensis* was assayed with sensitised sheep erythrocytes 3% on glass slides as described previously,¹⁵ and in a tube agglutination test with readings taken after incubation at 37°C for 2 h, and at 4°C for 18 h. *S. aureus* ATCC 29213 and V8 were used as positive controls and *S. aureus* Wood 46 as negative control.

S. lugdunensis strains were hybridised with a DNA gene probe derived from *S. aureus* 8325-4 containing the whole protein A gene.¹⁶ Hybridisation was performed in a dot-blot assay under stringent conditions with *S. aureus* Cowan 1 and 8325-4 as positive controls.

Heat-stable (thermo) nuclease production

The production of thermo-nuclease (TNase) was determined with monoclonal antibodies (MAbs) against TNase,¹⁷ and by the polymerase chain reaction (PCR) with an oligonucleotide sequence from *S. aureus* TNase as primer.¹⁸

Partitioning in aqueous two-phase systems

An aqueous polymer two-phase system containing PEG 6000 7.13% w/v and dextran (Dx, MW 48000) 8.75% w/v in 0.015 M NaCl, pH 6.8 was prepared as described previously.¹⁹ To determine particle negative charge, the negatively charged Dx-sulphate was included at a concentration of 0.40% w/v, replacing an equivalent amount of Dx. Similarly, the positively charged DEAE-Dx at a concentration of 0.40% replaced an equal amount of Dx.

Hydrophobic affinity partitioning was performed by including monosubstituted PEG-palmitate, replacing an equal amount of PEG, and Dx-palmitate, replacing an equal amount of Dx in the two-phase system.¹⁹

Bacterial cell suspensions (100 µl, c. 5×10^8 cells/ml) was added to 0.9 ml of the phase systems, previously homogenised by stirring, and mixed by gentle shaking. The mixtures were allowed to separate into phases for 1 h at 20°C. The concentration of bacterial cells in the PEG-rich top phase and the Dx-rich bottom phase was then determined turbidimetrically at 540 nm, and the recovery of cells in the bottom phase was expressed as a percentage of the original concentration of added cells. Differences in the hydrophobic and charge properties of the different bacterial strains are expressed as delta log G which is defined as:

$$\log G = \log \frac{\text{G value of Dx-sulph}^1 \text{ or PEG-palm}^2 \text{ system}}{\text{G value of the PEG-Dx system}}$$

$$\text{where } G = \frac{\text{percentage of cells in the bottom phase}}{\text{percentage of cells in the rest of the system}}$$

Chemicals

Fibronectin (Fn) was purified from human plasma on gelatin-Sepharose according to Vuento and Vaheri,²⁰ and vitronectin (Vn) from human urea-treated plasma on heparin-Sepharose.²¹ Vitrogen® (containing 95% type I and 5% type III collagen) and collagen type IV were purchased from Collagen Corporation, Palo Alto, CA, USA. Fibrinogen (Fg) and immunoglobulin G (IgG) were supplied by KABI, Stockholm, Sweden. Laminin (Lm), purified from an Engelberth-Holm-Swarm transplantable mouse tumour was kindly supplied by K. Valkonen, Oulu, Finland,²² thrombospondin (Ts) by J. Lawler, Boston, MA, USA,²³ and plasminogen (Pl) by U. Hedner, Novo, Copenhagen, Denmark.²⁴ Ovalbumin, PMSF, and dextran were purchased from Sigma. LiCl was from BDH, and latex beads from Difco. PEG 6000

Fig. 2. The effect of heat treatment of *S. lugdunensis* cells on binding to ^{125}I -labelled Cn-IV (+—+) and laminin (□.....□), and of *S. aureus* Cowan 1 cells on binding to ^{125}I -labelled Cn-IV (●—●) and laminin (⊠.....⊠).

Fig. 3. Inhibition of laminin binding to *S. lugdunensis* 2342 with increasing amounts of LiCl-extract from *S. lugdunensis* 2342 (+—+) and *S. aureus* Cowan 1 (⊠.....⊠).

and merthiolate were from KEBO, Stockholm, Sweden and Iodobeads® from Pierce Chemical Co., Rockford, IL, USA. Dx-sulphate was from Pharmacia, Uppsala, Sweden, and PEG-palmitate from Aqueous Affinity, Arlöv, Sweden. DEAE-Dx and Dx-palmitate were kind gifts from G. Johansson, Lund. Agar bases were from Oxoid. All salts were of analytical grade.

Results

Binding to soluble proteins

Fig. 1 shows the binding profile of the 11 strains of *S. lugdunensis* and control strains. Most strains expressed medium binding of iodine-labelled collagen types I and IV and IgG (mean value 36, 32 and 26%

binding). Lower grades of binding were obtained with labelled Fg, Lm, Vn, Ts, Fn and Pl, both glu- and lys-forms (mean values 19, 17, 12, 12, 12, 8 and 16% respectively). The positive control strains, *S. aureus* Cowan 1 and *S. haemolyticus* E2498 bound all the proteins tested whereas *S. epidermidis* 3380 either did not bind, or gave low grade binding with all the proteins tested.

Binding to immobilised proteins

When Cn type I and Fg were immobilised on latex beads, eight and seven strains, respectively, agglutinated coated latex beads. Two strains agglutinated immobilised Vn, and one strain Fn, Lm and gelatin. No strain agglutinated immobilised IgG or showed agglutination in the Staphaurex test. However, when *S. lugdunensis* strains were tested in the commercial tests according to the manufacturer's instructions, i.e., without prior washing of the cells, 6 of 11 strains gave positive results, although only weak reactions were noted with the Monostaph tests (table I).

Characterisation of protein binding

Binding of radiolabelled Lm by *S. aureus* Cowan 1 and *S. lugdunensis* 2342 was heat-sensitive whereas binding of Cn type IV by Cowan 1 was heat-resistant and by *S. lugdunensis* was heat-sensitive (fig. 2). Cell-surface extracts from strains Cowan 1 and 2342 inhibited Cn binding and partly inhibited Lm binding to homologous as well as heterologous strains (fig. 3). These cell-surface extracts also inhibited agglutination of all immobilised proteins (table II). Pre-incubation

Table II. PAA-reaction of *S. aureus* Cowan 1 and *S. lugdunensis* 2342 after inhibition by cell-surface extracts of Cowan 1 and 2342

Strain	Extract	PAA-reaction with						
		CnI	Fn	Vn	Lm	Fg	IgG	Staphaurex
Cowan 1	None	3	3	2	2	3	2	3
Cowan 1	Cowan 1	0	0	0	0	0	0	3
Cowan 1	2342	0	0	0	0	0	1	3
2342	None	3	3	3	2	3	1	0
2342	2342	0	0	0	0	0	0	0
2342	Cowan 1	0	0	0	0	0	0	0

of *S. lugdunensis* strain 2342 with Cn inhibited subsequent binding of soluble but not of immobilised Cn (table II).

Thermonuclease production

None of the 11 *S. lugdunensis* strains reacted with MAbs to TNase of *S. aureus*, or with the oligonucleotide sequence of *S. aureus* in PCR.

Protein A production

The positive control strains of *S. aureus* showed strong and rapid haemagglutination in the slide and tube agglutination tests whereas neither the *S. lugdunensis* strains nor the Wood 46 strain showed visible haemagglutination. None of the *S. lugdunensis* strains hybridised with the DNA gene probe for protein A of *S. aureus*; the control *S. aureus* strain did hybridise.

Fig. 4. Physicochemical surface properties of strains of *S. lugdunensis* revealed by two-phase partitioning system. Negative value of delta G hydrophobicity indicates hydrophobicity and negative value of delta G charge indicates negative surface charge.

Physicochemical characteristics of the cell surface

There was no uniform pattern of either cell-surface hydrophobicity or of cell-surface charge in the strains tested. Three strains were neutral and four were positively charged, of which one expressed a hydrophilic cell surface. Four strains expressed a hydrophobic cell surface, of which three were negatively charged (fig. 4). There was no correlation between expression of cell-surface hydrophobicity, surface charge and protein binding.

Discussion

In a series of studies on experimental abscess formation, Lambe *et al.* have confirmed that *S. lugdunensis* is more virulent than other CNS species.^{25,26} However, factors contributing to the higher virulence have not been identified. It is interesting that *S. lugdunensis* strains are isolated from the same types of infections as *S. aureus* strains, i.e., deep or invasive infections, and infrequently from biomaterial-associated infections, where other CNS strains predominate.²

We have compared the expression of the ability to bind extracellular matrix (ECM) proteins and Fg and Vn by *S. lugdunensis* and *S. aureus* strains. Generally, *S. lugdunensis* expresses a higher ability to bind these proteins in a soluble form than other CNS species studied but a lower ability than that of *S. aureus*. Most strains expressed high binding to collagens, intermediate binding to Fg, Vn and Lm, and low binding to Fn. The difference in expression of binding to immobilised proteins is not so pronounced between the various species. The proteins included in this study are known to expose different domains when they are immobilised, compared to when they are in a soluble form.^{27,28} CNS strains generally express higher binding to immobilised proteins²⁹ than to soluble proteins, which is likely to be relevant in biomaterial-associated infections. Our preliminary characterisation of bind-

ing by *S. lugdunensis* to Cn and Lm indicates that the binding is mediated by surface-located proteinaceous structures.

The expression of Fg binding by *S. lugdunensis* strains is interesting from several aspects. First, Fg binding is usually co-expressed with coagulase production. However, *S. lugdunensis* strains do not produce coagulase. Secondly, there are probably a number of Fg-binding proteins in *S. aureus*. Since Fn, Vn and Cn binding proteins differ between *S. aureus* and CNS,²⁹ it will be most interesting to investigate the relationship between Fg-binding proteins in *S. aureus* and *S. lugdunensis* chemically and immunologically, and particularly to study whether the Fg-binding protein of *S. lugdunensis* is related to any of the Fg-binding proteins of *S. aureus*. Expression of Fg binding has been postulated as an important virulence factor of *S. aureus* in catheter-associated infections.³⁰ Thirdly, Fg binding causes false positive reactions in some commercial *S. aureus* tests, which implies under-diagnosis of *S. lugdunensis* clinically.

In the original description of *S. lugdunensis*, the strains were described as unable to produce TNase. Our study confirms this and shows that *S. lugdunensis* strains lack the gene for TNase production. The DNA fragment used for detection of protein A contains the whole gene, i.e., both the IgG-binding and the cell-wall anchoring part. Hence the weak reaction of IgG binding of *S. lugdunensis* is not related to expression of part of protein A.

The findings presented in this study show that strains of *S. lugdunensis* are able to bind to soluble ECM proteins, Vn and Fg in a manner similar to strains of *S. aureus* rather than to other CNS strains. This may shed light on the pathogenesis of *S. lugdunensis* since the clinical spectrum of infections caused by *S. aureus* and *S. lugdunensis* is similar.

This study was supported by grants from the Swedish Board of Technical Development, NUTEK, and Faculty of Medicine, University of Lund. We thank E. Jakab for the purification of fibronectin and vitronectin, B. Guss and G. Brakstad for fruitful collaboration, D. W. Lambe and Dr W. Witte for sharing *S. lugdunensis* strains with us and T. Wadström for constructive criticism.

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