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Laboratoire de Recherche sur les Biomatériaux et Biotechnologies LR2B
Equipe « BIOFILM »
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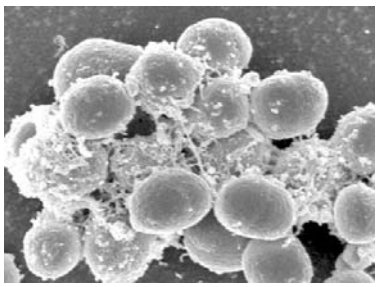
Contribution to study of Staphylococcal biofilms: a chemical approach



Habilitation à Diriger des Recherches

Irina Sadovskaya, PhD

Examination commission:

M. J-C.Michalski	**Professor, Université des Sciences et Technologies de Lille**	**Président**
Mme M. Caroff	**Directrice de Recherche au CNRS, Université de Paris XI**	**Rapporteur**
M. A. Filloux	**Professor, l'Université de la Méditerranée**	**Rapporteur**
M. A. Peschel	**Professor, University of Tübingen, Germany**	**Rapporteur**
M. J. P. O'Gara	**Senior Lecturer, University College Dublin, Ireland**	**Examinateur**
M. S. Jabbouri	**Professor, Université du Littoral-Côte d'Opale**	**Examinateur**
M. P.Hardouin	**Professor, Université du Littoral-Côte d'Opale**	**Examinateur**

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Curriculum vitae

Sadovskaya (Dobkina) Irina
Née le 12.04.61
46 rue Maryse Bastié
62930 Wimereux France
Tel. 03.21.99.77.09 (residence)
03.21.99.45.20 work)
06.87.94.13.94

Nationality: Canadian

Married, 2 children

Diplomas

1999 PhD Thesis in Biochemistry, defended in Université du Litoral-Côte d'Opale (ULCO) : "Contribution to a study of bacterial polysaccharides: cell surface carbohydrate structures of *Vibrio anguillarum*, *Vibrio ordalii* serotype O:2 and *Pseudomonas aeruginosa* serotype O5."

1983 Equivalent of M.Sc. in chemistry, Moscow State University, specialization: Chemistry of Natural Compounds

Employment

1998-2007 Ingénieur d' Etudes, LBCM-LR2B, ULCO.
1998 Assistant Ingénieur, LBCM-LR2B, ULCO
1996-1998 Technical officer TO-3 ; Institut for Biological Sciences, National Research Council (IBS/NRC), Ottawa, Canada
1994-1996 Research Associate, University of Ottawa and IBS/NRC, Ottawa
1991-1994 Research Technician; University of Guelph, and IBS/NRC, Ottawa
1983-1990 Junior Researcher, Zelinski Institute of Organic Chemistry Academy of Sciences of USSR, Laboratory of Plant Polysaccharides

Languages: English, French, Russian (mother tongue)

List of publications

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List of communications

Posters presented at international conferences

Stevens N, Sadovskaya I., Jabbouri S, O'Gara JP, Greene CM, Humphreys The *Staphylococcus epidermidis* biofilm adhesin, Polysaccharide Intercellular Adhesin, potentially activates pro-inflammatory mediators in human astrocytes, 107-th ASM General Meeting, Toronto, Canada, 21-25 May 2007

Sadovskaya I, Chokr A, Vinogradov E, Kogan G, Li J, Jabbouri S Chemical composition of the extracellular matrix of biofilm-forming staphylococci, *12th International Symposium on Staphylococci & Staphylococcal Infections*, Maastricht, The Netherlands, 3-6 September 2006

Sadovskaya I, Kogan G, Chokr A., Chagnon P, Vinogradov E, Jabbouri S. Carbohydrate-containing components produced *in vitro* by some staphylococcal strains, associated with orthopaedic prostheses. *13-th European Carbohydrate Symposium,* Bratislava, Slovakia, Aug. 21-26, 2005

Sadovskaya I, Vinogradov E, Li J, Jabbouri S Structural elucidation of the extracellular and cell-wall teichoic acids of a reference biofilm-positive strain *Staphylococcus epidermidis* RP62A. *The Carbohydrate Workshop,* Research Center Borstel, Borstel, Allemagne. 17-20 mars 2004

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Flahaut S, Igguy R, Sadovskaya I, Jeanfils J, Jabbouri S Growth of *Staphylococcus epidermidis* on staphylococcal extracellular polymeric substances. Xth international congress of bacteriology and applied microbiology of IUMS, Paris. 27 juil-1er août 2002

Petersen BO, Vinogradov E, Sadovskaya, I., Jabbouri S., Duus, JO. Structure of a 23 residue oligosaccharide from *Pectinatus* 43rd Experimental Nuclear Magnetic Resonance Conference, April 2002 Asilomar, California

Sadovskaya I, Flahaut S, Vinogradov E, Jeanfils J, Jabbouri S Polysaccharide composition of *Staphylococcus epidermidis* biofilms, *In* 10th Bratislava Symposium on Saccharides, September 1-6, 2002, Smolenice, Slovakia

Invited seminars

Sadovskaya I. Staphylococcal polysaccharides and biofilm : where do we stand ? Seminar of SFB 267, Research Center Borstel, Borstel, Allemagne. 5 December 2003 (on the invitation of Prof. U. Zaëhringer)

Posters presented on national and local conferences

Stevens N, Sadovskaya I., Jabbouri S, O'Gara JP, Greene CM, Humphreys The *Staphylococcus epidermidis* biofilm adhesin, Polysaccharide Intercellular Adhesin, potentially activates pro-inflammatory mediators in human astrocytes,, Shepards Prise Meeting 20 February

2007 and Royal College of Surgeons Research Day, 11 April 2007 ; Royal College of Surgeons, Dublin, Ireland

Wagstaph JL, Sadovskaya I, Vinogradov E, Jabbouri S, Howard MJ Identification of key *S. epidermidis* biofilm components by NMR spectroscopy, Departemental Graduate Symposium, 14 June 2006, Department of Biosciences, University of Kent, Canterbury, UK

Chaignon Ph, Sadovskaya I, Watier D, Jabbouri S. Traitement enzymatique des biofilms des Staphylocoques, Réseau National Biofilm, 1ières Journées Thématiques, 27-28 octobre 2005, Lorient, France

Supervision of students and young scientists

- S. Faure, *Master 2*, University of Rennes 1, on a subject« Potential use of an antigenic component of staphylococcal biofilm for an early diagnostics of orthopedic implant-related infections» (2004-2005)
- A. Fontaine, BTS ("Brevet de technicien Supérieur"), lycée Valentine Labbé. Projects of 6 weeks in 2006 and 2007
- R. Herro (Master 2, in 2002-2003, joined supervision)
- R. Igguy (Master 2, in 2001-2002 ; joined supervision)
- Dr. Ph. Chaignon (Post-doctoral contract, ANVAR) Enzymatic degradation of staphylococcal biofilms (2003-2005)

Teaching : Laboratory in Masters 1 « Natural Environment », ULCO. Study of carbohydrate molecules participating in the symbiosis of *Rhizobium* using DOC-PAGE (4 hrs, March 2006 and March 2007, with Prof. S. Jabbouri)

Reception of invited scientists

- Dr. G. Kogan (Bratislava, Slovakia), Nord-Pas-de Calais Region Fellowship for 11 months, in 2003-2004. Study of the chemical composition of clinical staphylococcal strains.
- Dr. E. Vinogradov, Invited Scientist in ULCO, May 2006 .
- Dr. X. Châtellier, Géosciences Rennes, UMR 6118 CNRS, University of Rennes 1 (2006, one week on his request). Preliminary study of cell-surface carbohydrates of a cyanobacterium.

Initiated collaborations

- Dr. E. Vinogradov (National Research Council, Ottawa, Canada). This successful collaboration, started in 2000, allowed us to apply NMR techniques for the structural studies of carbohydrate-containing components of staphylococcal biofilm. We also studied the structure of the carbohydrate backbone of LPS from non-type strains of *Pectinatus frisingensis* (beer contaminating bacterium)

- Dr. I. Helander, VTT Biotechnology and University of Helsinki. Structural elucidation of the carbohydrate backbone of LPS from non-type strains of *Pectinatus frisingensis* (2000-2003)

- Dr. M. Howard, University of Kent in Canterbury, Department of Biosciences. Use of selective excitation TOCSY techniques for the analysis of components of staphylococcal biofilm in crude extracts (started en 2004)

- Dr. J. B. Kaplan, (New Jersey Dental School, Newark, NJ). Structure and functions of poly-N-acetyl-glucosamines (PGA) from biofilms of Gram negative bacteria, a swine pathogen *Actinobacillus pleuropneumoniae,* and a human dental pathogen *Aggregatibacter actinomycetemcomitans* (started in 2003)

- Dr. C. Greene, Prof. G. O'Gara, N. Stevens (Royal College of Surgeons in Ireland and University College Dublin. Effect of purified components of staphylococcal biofilms as pro-inflammatory mediators of human astrocytes (started in 2006)

Other scientific contacts (see also chapter III.4):

- Prof. G. Pier, Dr. T. Maira-Litràn, Harvard Medical School, Boston, MA, USA
- Dr. W. Ziebuhr, Institut für Molekulare Infektionbiologie, Würzburg, Germany
- Dr. H. Rohde, Institut für Infektionsmedizin Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany
- Dr. U. Zaehringer, Research Center Borstel, Germany

SUMMARY OF POST-DOCTORAL RESEARCH

Foreword

The results presented here summarize my research work in the Microbiology (later "Biofilm") team of LR2B. In the first part (volume 1), I describe the work related to the main research topic of the team in 2001-2007, the study of staphylococcal biofilm.. Having a solid background in carbohydrate chemistry and taking advantage of a strong collaboration with my former colleague, Dr. Evgueny Vinogradov in IBS, NRC Canada, one of the world leading specialists in chemical analysis of bacterial carbohydrates, I emphasized my efforts on the elucidation of the carbohydrate-containing components of staphylococcal biofilms.

After a short review of literature data (chapter I), the results of our work are exposed (chapter II). First, the problem of the chemical identity of poly-*N*-acetylglucosamine containing polymers (PNAG), known as major components of staphylococcal biofilms, is addressed. PNAG was studied in details for model biofilm-forming strains (chapter II.1) and for a number of clinical strains from the strain collection of our team (chapter II.2). A chemically related polysaccharide, isolated for the first time from biofilms of some gram-negative bacteria, was also characterized (chapter II.3). The use of this polysaccharide as an antigen for the sero-diagnosis of biofilm-related staphylococcal infections of orthopaedic prosthesis, was investigated (chapter II.8)

We have found that another carbohydrate-containing polymer, the extracellular teichoic acid (EC TA), was a part of biofilm of model strains (chapter II.4) and clinical strains of our strain collection (chapter II.5). A detailed chemical structure of TAs of two model strains have been established (chapter II.6). A sensitivity of staphylococcal biofilms and their components to different enzymatic and chemical treatments, in view of practical applications, was investigated (chapters II.5.2 and II.7). Volume 1 is concluded with the discussion of the results and future prospects of the work (chapter III).

Full texts of five published articles (articles 1-5) which I consider as a "core" of my research work on this subject are presented in the first part (Volume 1) of this manuscript. In Volume 2 of the manuscript, full texts of other publications on this subject (articles 6-9) are presented (Annex 1). In the Annex 2, I present the results of the research on another subject, related to the research topic of the team Microbiology in 1998-2001 ("Structural elucidation of the LPS of non-type strains of *Pectinatus frisingensis*", as a part of the team project on *Pectinatus* spp., beer contaminating bacteria), and full texts of corresponding articles 10-12.

Acknowledgements

The summary presented in this part of the manuscript concerns the research performed in the team "Biofilm" of the LR2B in 2000-2007. I am thankful to Prof. Saïd Jabbouri, our group leader, for proposing me this interesting subject, and for his support. I also thank Prof. Pierre Hardouin, director of LR2B, for his encouragements and help for the preparation of the manuscript. I am grateful to Dr. Philippe Chaignon for the editorial help for the French version of the manuscript, and my colleagues in LR2B for their support.

I am very thankful to Dr. E. Vinogradov (National Research Council, Ottawa, Canada). This work could be accomplished due to his extraordinary expertise in the field of the structural analysis of carbohydrates.

I would like to express my gratitude to all who contributed to my professional training : my teachers in Moscow State University, my older colleagues in Zelinski Institute of Organic Chemistry, Moscow (1983-1990) and National Research Council—Canadian Bacterial Diseases Network (1991-1997). Apart from technical knowledge, they taught me respect and sensitivity to the demands of specialists in other disciplines. Thanks to that I was able to develop some projects in collaboration, presented below.

I am obliged to Dmitii for his advice and for his help.

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Abbreviations

Aap	Accumulation associated protein
ANVAR	Agence Nationale de Valorisation de la Recherche
BHI	Brain Heart Infusion
CE-MS	Capillary electrophoresis – Mass Spectrometry
CoNS	Coagulase Negative Staphylococci
D-Ala	D-alanine
DOC	Deoxycolate
DspB	Dispersine B
EC TA	Extracellular Teichoic Acid
EFS	Etablissement Français du Sang
ELISA	Enzyme-linked Immunosorbent Assay
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GLC	Gas-Liquid chromatography
Gro	Glycerol
HMBC	Heteronuclear Multiple Bond Correlation
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
MSCRAMM	Microbial Surface Component Recognizing Adhesive Matrix Molecules
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectrometry
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
PAGE	Polyacrylamide Gel Electrophoresis
PIA	Polysaccharide Intercellular Adhesin
PGA	Poly-γ-DL-glutamic acid
	Poly-β-(1,6)-N-acetylaglucosamine
PNAG	Poly-*N*-acetyl-glucosamine
PS/A	Polysaccharide/Adhesin
SAA	Slime-associated Antigen
SAE	*S. aureus* exopolysaccharide
SDS	Sodium Dodecyl Sulfate
TCA	Trichloroacetic acid
TA	Teichoic acid
TLC	Thin Layer Chromatography

Contribution to study of staphylococcal biofilms: a chemical approach

I. Introduction

1. Medical implant-related infections

Use of different kinds of medical implants had significantly grown for the last decades. If a frequent use of such implants gives good functional results, it is not devoid of complications, nosocomial infections being the most important ones. These infections are mainly caused by Staphylococci, members of family Micrococcaceae. One can distinguish coagulase- positive staphylococci with *S. aureus* as a prevalent species, and Coagulase-negative staphylococci (CoNS) represented mostly by *S. epidermidis*. While *S. aureus* is known as a pathogen with a number of virulence factors (ex. exotoxins and enzymes), *Staphylococcus epidermidis* is mainly a normal inhabitant of the healthy human skin and mucosal microflora, and as a commensal bacterium it has a low pathogenic potential. In recent decades, however, *S. epidermidis* and other CoNS have emerged as a common cause of numerous nosocomial infections, mostly occurring in association with the use of medical devices such as pace maker electrodes, synthetic vascular drafts, urinary tract catheters and orthopedic implants (Ziebuhr *et al.*, 2006). The pathogeneicity of *S. epidermidis* is mostly due to its ability to colonize indwelling polymeric devices and form a thick adherent biofilm. Biofilms are often the cause for the difficulty to eradicate *S. epidermidis* infections on an indwelling device, due to an impaired penetration of antibiotics and a decreased immune response (Vadyvaloo & Otto, 2005).

The ability to form biofilm is considered as a main virulence factor of *S. epidermidis* (Mack *et al.*, 2001).

2. Biofilm formation

The formation of biofilms during a foreign body-related infection proceeds in two main steps. The first step consists in the adherence of bacteria to an inert surface. During the second step, the accumulation of biofilm, bacteria produce the extracellular matrix in which they are embedded (Fig. 1).

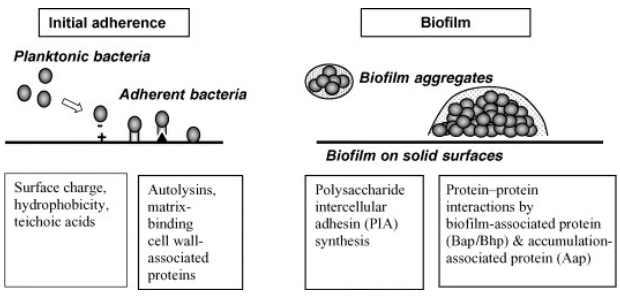

Figure 1. Two steps of biofilm formation (Ziebuhr *et al.*, 2006)

The first step, a primary adherence of bacteria, depends on the characteristics of bacterial surface and the nature of the support. It involves non-specific physicochemical interactions such as hydrophobicity and surface charge. Following these reversible interactions, specific interactions between the bacteria and the biomaterial take place, and the adhesion becomes irreversible (Donlan & Costerton, 2002).

In vivo, when a foreign body is implanted, it is quickly recovered by eukaryotic cell matrix proteins, such as fibrinogen, fibrin, fibronectin, laminine and certain collagens. These host factors could serve as specific receptors for colonizing bacteria (Herrmann *et al.*, 1988) and therefore favor their irreversible interaction with the extracellular matrix of the host. Specific adhesins interacting with host proteins are present on the surface of *S. aureus*. They belong to the family of MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules). In most cases, MSCRAMMs are covalently linked to the cell-wall peptidoclycan (Foster & Hook, 1998).

A number of adhesins specific for host extracellular matrix proteins were also characterized in *S. epidermidis* and other CoNS. Among them, fibrinogen and fibronectine binding proteins, encoded by *fbe* and *embp* gene loci (Nilsson *et al.*, 1998), (Pei & Flock, 2001)); autolysins, such as AltE in *S. epidermidis* or AltC in *S. caprae* (Heilmann *et al.*, 1997), (Allignet *et al.*, 2002). An autolysin Aae, also capable to bind vitronectin, was recently described (Heilmann *et al.*, 2003).

Second phase of biofilm formation, called accumulation or colonization, is characterized by the production of extracellular polymeric substances and the formation of multi-layered cell clusters. Microscopic studies show that biofilm bacteria grow in matrix-enclosed microcolonies interspersed with less dense regions of the matrix which include permeable water channels. The channels allow convective flow of liquids and therefore rapid molecular and ionic equilibration with the fluid, providing necessary nutrients (Costerton *et al.*, 1994).

The extracellular matrix provides optimal conditions for survival of embedded bacteria, which are present in an altered physiological state (von Eiff *et al.*, 2002). Within the biofilm, bacteria can exchange specific signals which permit them to adapt their behavior to the changing environment in synchronic fashion (Webb *et al.*, 2003). Furthermore, biofilms impair the penetration of antibiotics, prevent normal immune responses, and increase the difficulty of eradicating biofilm infections. Biofilm cells are capable of persisting in the presence of antimicrobials at concentrations that are 1000-fold higher than those necessary to eradicate a planktonic population (Cerca *et al.*, 2005);(Hamilton, 2002). Biofilm-related infections become a major threat to public health (Reid, 1998).

The occurrence of the medical device-related infections is not surprising since the implanted artificial surfaces of the indwelling devices predispose to bacterial and myotic infection (Printzen, 1996). *S. epidermidis*, an important member of human skin and mucous membranes microflora,

can contaminate the surface of the material during the surgical implantation of the device (von Eiff *et al.*, 2002). Bacterial infections represent one of the most serious and devastating complications following orthopedic implant surgery. They have an important impact in terms of morbidity, mortality, and medical cost (Campoccia *et al.*, 2006). Treatment for chronic infection usually requires removing of the prosthesis, cleaning the bone interface, and new authoplacy (Lortat-Jacob *et al.*, 2002). It often results in long periods of hospitalization, severe functional impairment and sometimes increased mortality.

The knowledge of the composition and chemical structure of the extracellular matrix could help finding therapeutic tools to fight nosocomial biofilm-related infections.

In the present study, we focused on elucidation of the chemical composition of the extracellular polymeric substances of staphylococcal biofilm.

3. Composition of staphylococcal biofilm

Due to a certain existing inconsistency in terminology, we use one adopted by Hussain *et al*. and use the term "biofilm" to refer to an accumulated biomass of bacteria and extracellular material on a solid surface. The term "slime" is defined as a mucoïde extracellular substance holding a community of bacterial cells as cell aggregates (Hussain *et al.,* 1993).

Extracellular markers of staphylococci related to biofilm production have been intensively investigated in the past decade. Depending on the authors, different major biofilm components have been identified. Two biofilm-forming staphylococcal strains were mainly studied by different research groups : *S. epidermidis* RP62A (ATCC 35984) and *S. aureus* MN8m.

- "Discrete macromolecules" (Karamanos *et al.*)

According to Karamanos *et al.*, biofilm of several strains of *S. epidermidis*, including the strain RP62A, is composed of several "discrete macromolecules ": a "20-kDa sulfated polysaccharide", an "80-kDa peptidoclycan" and a "teichoic acid-like substance" (Karamanos *et al.*, 1995; Karamanos *et al.*, 1997). The chemical structure of these molecules has not been elucidated. Nevertheless, the "20-kDa polysaccharide" has been proposed as an antigen to use in diagnosis of *S. epidermidis* infections by detecting its specific antibodies in blood sera (Karamanos *et al.*, 1997), (Georgakopoulos *et al.*, 2002), (Lamari *et al.*, 2004). These results, however, have not been used for medical applications.

- The role of the teichoic acid (TA)

According to Hussain *et al*., "slime" of *S. epidermidis* RP62A, cultivated in a chemically defined medium, is composed of teichoic acid (80%) and proteins (20%) (Hussain *et al.*, 1993).

Chemical composition of the extracellular substance of this strain was studied and compared to the composition of its cell-wall teichoic acid (CW TA). Both polymers were composed of Glc, GlcNAc, phosphate, glycerol and D-alanine (D-Ala). The exact chemical structures of these polymers have not been established.

According to recent studies, CW TA significantly enhances adhesion of *S. epidermidis* to fibronectine-coated surfaces (Hussain *et al.*, 2001), suggesting a potential role of CW TA as a bridging molecule between microorganisms and immobilized fibronectine in early steps of *S. epidermidis* pathogenesis, the primary attachment of bacteria to artificial surfaces. D-Alanine esterification which decreases the total negative charge of TA, seems to affect the susceptibility of *S. aureus* to glycopeptide antibiotics and its initial binding to plastic surfaces (Peschel *et al.*, 1999), (Peschel *et al.*, 2000), (Gross *et al.*, 2001).

Knowledge of the chemical structure of the CW of staphylococci is essential for clear understanding of bacterial pathogenesis and the designing of new drugs against staphylococcal infections. Surprisingly, the exact chemical structure of TAs of *S. epidermidis* was not well elucidated (see chapter II.6).

- Poly-*N*-acétylglucosamines

To date, the poly-*N*-acetyl-β–(1,6)-glucosamine remains the most studied and well-characterized component of staphylococcal biofilm. Since its discovery in 1988 by the researchers of G. Pier's group (Harvard Medical School, Boston, MA) (Tojo *et al.*, 1988), this polymer attracts attention of different research teams. Use of several strains, as well as differences in culture conditions and methods of purification and characterization caused a disagreement among the research teams (Tab. 1). The GlcNAc-rich polysaccharide, isolated by Tojo *et al*. from culture supernatant of *S. epidermidis* RP62A and called PS/A (Polysaccharide/Adhesin), has been reported to be the component of the bacterial cell surface and biofilm layer, mediates cell adherence to biomaterials and protects the cells from host defenses (Tojo *et al.*, 1988). A Slime-Associated Antigen (SAA), isolated two years later, had similar functions but was claimed to be different form PS/A due to a relatively high glucose content (Christensen *et al.*, 1990), (Baldassarri *et al.*, 1996). Later, Mack et al. (Universitätsklinikum Hamburg-Eppendorf) described a GlcNAc-containing polysaccharide (Polysaccharide Intercellular Adhesin, PIA) playing an important role in biofilm accumulation (Mack *et al.*, 1994). Using a combination of analytical methods and NMR spectroscopy, PIA was identified as a linear poly-β-(1,6)-glucosaminoglucane containing ca. 130 GlcNAc residues, partially *O*-succinylated, partially de-*N*-acetylated and apparently phosphorylated (Mack *et al.*, 1996), Fig. 2.

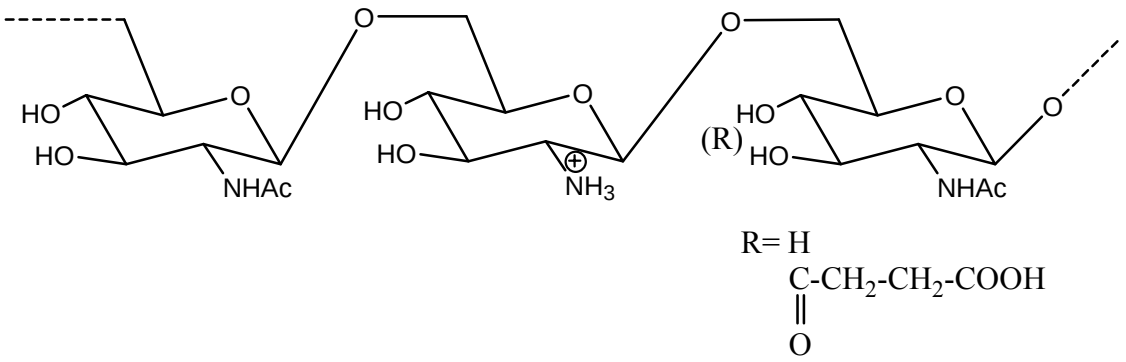

Figure 2. Chemical structure of PIA (Mack *et al.*, 1996)

The genes encoding PIA biosynthesis are organized in *ica* (intercellular adhesion) operon *ica*ADBC, which consists in four open reading frames (Gerke *et al.*, 1998), (Heilmann *et al.*, 1996). The product of *ica*A is a glycosyltransferase, responsible for the biosynthesis of the polymeric GlcNAc chain. Co-expression of *ica*A and *ica*D is necessary for the optimal catalytic activity (Gerke *et al.*, 1998). Ica C might be involved in the export of PIA. IcaB is a membrane-bound protein, involved in the partial de-*N*-acetylation of the poly-GlcNAc chain. The free NH_2 groups thus introduced (Fig. 3) seem to be essential for the attachment of PIA to the bacterial surface, bacterial adhesion to the epithelial cells, biofilm formation and other biological properties in the mouse model of device-related infection (Vuong *et al.*, 2004).

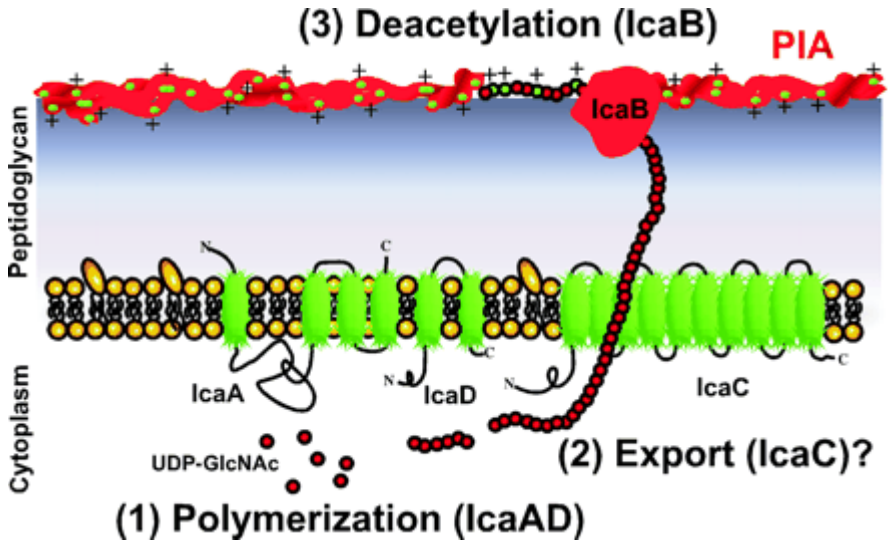

Figure 3. Model of PIA biosynthesis (Vuong *et al.*, 2004)

Three steps of PIA biosynthesis. (1) IcaA and IcaD synthesize a poly-β-(1,6)-*N*-acetyl-glucosamine chain using the UDP-GlcNAc. (2) IcaC might be involved in the export of PIA. (3) After export PIA polymer is deacetylated by IcaB to introduce positive charges, which are crucial for its biological function.

The *ica* locus was later found in a number of *S. aureus* strains, and its presence was related to the ability of the bacteria to form biofilm *in vitro* (Cramton *et al.*, 1999). A recombinant strain

of *S. carnosus* TM300 containing a plasmid pCN27 with *ica*ABC locus, acquired a capability to adhere and form biofilms on glass surface. *N*-succinylated polysaccharide (poly-*N*-succinyl-glucosamine, PNSG) was isolated from this mutant strain. Later identified as a major surface antigen of *S. aureus* clinical strains, isolated from cystic fibrosis patients (McKenney *et al.*, 1999) and as a target for protective antibodies, it was proposed as a promising vaccine candidate against staphylococcal infections (McKenney *et al.*, 2000). However, subsequent studies carried out by the same group showed that the presence of the *N*-succinyl substitution was an analytical artifact (Joyce *et al.*, 2003).

For the studies of immunological properties of PS/A, a "PS/A overproducing strain" *S. aureus* MN8m, was used by the researchers from the group of G. Pier (Boston, MA). Chemical properties of this polysaccharide, identified as PNAG (poly-*N*-acetylglucosamine) were studied (Maira-Litran *et al.*, 2002). Detailed NMR studies in combination with the chemical modifications allowed a complete assignment of NMR spectra of SAE (*S. aureus* exopolysaccharide), isolated from the same strain *S. aureus* MN8m (Joyce *et al.*, 2003). The molecular weight of SAE was estimated as > 300 kDa. According to these authors, the main differences between SAE and PIA were : phosphorylation (absence of phosphate substitution in SAE) and molecular mass (>300 kDa for SAE and ~30 kDa for PIA (Mack *et al.*, 1996))

Polysaccharide	Strain	Analytical artifacts	Reference
PS/A Polysaccharide/Adhesin	*S. epidermidis* RP62A		(Tojo *et al.*, 1988)
SAA Slime Associated Antigen	*S. epidermidis* RP62A *S. epidermidis* clinical strains	Presence of glucose	(Christensen *et al.*, 1990) (Baldassarri *et al.*, 1996)
PIA Polysaccharide Intercellular Adhesin	*S. epidermidis* RP62A *S. epidermidis* 1457	Phosphate ?	(Mack *et al.*, 1996)
PS/A, PNSG Poly-*N*-succinyl-glucosamine	*S. carnosus* (pCN27) *S. aureus* MN8m	*N*-succinyl substitution	(McKenney *et al.*, 1998)
SAE *S. aureus* exopolysaccharide	*S. aureus* MN8m		(Joyce *et al.*, 2003)
PS/A, PNAG Poly-*N*-acetylglucosamine	*S. aureus* MN8m		(Maira-Litran *et al.*, 2002)

Table 1. Poly-*N*-acetylglucosamine-containing polysaccharides of staphylococci (literature data)

Apart from structural differences, different biological properties were attributed to PS/A and PIA : PS/A would be involved in the primary adhesion of staphylococci, while PIA would be an intercellular adhesin, involved in the biofilm accumulation phase (von Eiff *et al.*, 2002). However, it was later found that these conclusions were based on results of studies of adherence of

the recombinant *S. carnosus* strain to plastic tissue culture plates, and the distinction between PS/A and PIA was due to trivial differences in manufacture of tissue culture plates in Europe and USA (Maira-Litran *et al.*, 2004).

To date, despite the interest of the scientific community to the relationship between PIA and PS/A (Götz, 2002), no direct comparative structural study of PS/A (PNAG, SAE) and PIA has been carried out.

There were also still some ambiguities regarding the composition of staphylococcal slime.

For several years, PIA has been considered as a major and most important component of staphylococcal biofilm. According to a recent review, " ..in most cases "slime" was very likely PIA" and "in particular, in the well-studied strains *S. epidermidis* RP62A and O-47 "slime" and PIA are the same" (Götz, 2002). This postulate contradicted previous data of Hussain *et al*., where *S. epidermidis* "slime" was considered to consist mainly of the teichoic acid; or the data of Karamanos *et al*. (see above). Other recent studies indicate the presence of an alternative, PIA-independent mechanisms of biofilm formation: involving the product of a limited proteolysis of the accumulation-associated protein, Aap (Rohde *et al.*, 2005), or an *ica*-independent mechanism in clinical strains of *S. aureus* (Fitzpatrick *et al.*, 2005). Preliminary screening of the biofilm-positive CoNS strains from our collection indicated that some *ica*$^-$ and /or PNAG$^-$ (determined on the basis of the immuno dot-blot) strains were nevertheless biofilm producers (Chokr *et al.*, 2006).

4. Objectives of the study

In the present study, we attempted:

a) To clarify the ambiguities concerning the differences between PIA et le PS/A ;

b) To elucidate the composition of biofilms, produced *in vitro* by a model biofilm-forming strain, *S. epidermidis* RP62A ;

c) To elucidate the composition of biofilms, produced *in vitro* by clinical orthopedic-prosthesis related strains of *S. aureus* et CoNS from our collection (Chokr *et al.*, 2006) ;

d) To clarify the role of the teichoic acids (TAs) in staphylococcal biofilms; elucidate the chemical structure of the TAs of model biofilm-forming strains.

II. Summary of the results

1. Characterization of the *N*-acetylglucosamine-containing polysaccharides of model strains

Presented in Article 1 (Sadovskaya *et al.*, 2005) :

Sadovskaya, I., Vinogradov, E., Flahaut, S., Kogan, G. & Jabbouri, S. (2005). Extracellular carbohydrate-containing polymers of a model biofilm-producing strain *Staphylococcus epidermidis* RP62A. *Infect Immun* **73**, 3007-3017.

1.1.Optimization of PIA production and purification

We established a protocol of a large-scale bacterial biofilm culture. This allowed us to be able to purified the constituents of biofilm in quantities sufficient for analysis by chemical methods, NMR spectroscopy and mass-spectrometry. We have also elaborated, in collaboration with Dr. S. Flahaut, a mild extraction method which efficiently liberated the elements of the extracellular matrix with the minimal cell lysis, thus avoiding the contamination of the extracellular matrix polymers with the inter-cellular contaminants. The purification conditions used here were less drastic that ones proposed in the literature, and therefore the label substituents such as *O*-succinates were preserved. On the other hand, we avoided the use of buffers which could contaminate the final products. As a result, the developed protocol allowed us to purify the GlcNAc-containing polysaccharides directly from the biofilm matrix, avoiding the contaminations or chemical modifications. Potential carbohydrate contaminants, such as polysaccharides from the culture media, were analyzed in parallel in order to take it into account during the analysis.

1.2. « PIA » and « PS/A »: two different polysaccharides?

Two polysaccharides to which different functions were attributed, the "PS/A" and the "PIA" (chapter I.3, Table 1) have been initially isolated from the same strain, *S. epidermidis* RP62A, by two research teams: an American (Tojo *et al.*, 1988) and a German one (Mack *et al.*, 1994), (Mack *et al.*, 1996). Consequently, american researchers worked with another model "PS/A overproducing" strain, *S. aureus* MN8m. Over the years, PS/A was also referred to as PNSG, PNAG and SAE. According to the recent analysis ((Joyce *et al.*, 2003), chapter I.3.), the main differences between the PIA and the exopolysaccharide of *S. aureus* MN8m were : the phosphorylation (absence of the *O*-phosphorylation in the exopolysaccharide from *S. aureus*) and the molecular weigh (>300 kDa for the SAE and ~ 30 kDa for the PIA (Mack *et al.*, 1996). In order to resolve this ambiguity, we carried out a comparative study of polysaccharides from the two model strains, *S. epidermidis* RP62A and *S. aureus* MN8m, using the same growth conditions and the same method of extraction and purification of the GlcNAc-containing polysaccharides. We compared the elution profiles of the crude biofilm extracts of the two strains and the ^{1}H-NMR spectra of the PNAG fractions. As a result, we have demonstrated that:

1) The poly-*N*-acetylglucosamine from *S. epidermidis* RP62A did not contain any phosphate substitution; the presence of phosphate demonstrated by Mack *et al*. (Mack *et al.*, 1996) was probably due to the contamination by phosphate buffer used during purification;

2) Molecular weights of the two polymers were close; the lower MW of PIA, estimated by Mack *et al* was probably due to an artifact (partial alkaline degradation of the polysaccharide during methylation, which was used to access its MW);

3) The chemical structure of the two polysaccharides was identical except for the degree of partial *N*-deacetylation and *O*-succinylation.

These results, being in agreement with the literature data regarding the differences in adhesion of a recombinant strain of *S. carnosus* to the 96-wells tissue-culture plates manufactured in Europe and the USA (Maira-Litran *et al.*, 2004), (chapter I.3), confirm that PIA and PS/A (SAE) represent a molecule with the same basic chemical structure – a poly-β-(1,6)-*N*-acetylglucosamine (PNAG). Our results contributed to clarify the ambiguities « PS/A vs PIA » (Prof. G. Pier, personal communication: « …as it was once said, **Best biology is chemistry** »). The PNAG could have two basic functions: primary adhesion of bacteria during the first stage of biofilm formation, and the intercellular adhesion during the second phase, the biofilm accumulation.

ARTICLE 1

2. Structural elucidation of PNAG of clinical strains

Presented in Article 2 (Sadovskaya *et al.*, 2006) :

Sadovskaya, I., Chaignon, P., Kogan, G., Chokr, A., Vinogradov, E. & Jabbouri, S. (2006). Carbohydrate-containing components of biofilms produced in vitro by some staphylococcal strains related to orthopedic prosthesis infections. *FEMS Immunol Med Microbiol* **47**, 75-82.

In chapter II.1 we described our contribution to a comparative study of PNAG from two model strains, *S. epidermidis* RP62A et *S. aureus* MN8m. Up till now, only the PNAG of these two strains and a PGA from a recombinant strain of *E. coli* (see chapter II.3.1) have been studied in details. To date, no structural study of polysaccharides from biofilms of clinical Staphylococcal strains have been carried out.

Sixty six strains of CoNS, isolated from the infected orthopedic prosthesis explanted in l'Hôpital Mignot, le Chesnay, France, have been recently characterized in our laboratory (Chokr *et al.*, 2006).

Screening of these strains was performed following three criteria: capacity to form biofilm *in vitro*, presence of the *ica*ADBC locus and a capacity to produce PIA. The latter property has been determined by immuno dot-blot using an anti-PIA polyclonal antiserum (generous gift of Prof. D. Mack, Universitätsklinikum Hamburg-Eppendorf).

We further studied the composition of the extracellular matrix of these strains, and in particular the chemical structure of PNAG as a component of their biofilm. We have chosen for this study several *S. epidermidis* strains, as well as other Staphylococci : *S. aureus, S. lugdunensis, S. warneri. S. lugdunensis* is a recently described human pathogen causing more acute infection as usually associated with CoNS. It may be confused with *S. aureus* due to its clinical manifestations and certain microbiological characteristics (Ebright *et al.*, 2004).

The clinical strains were grown statically in TSB in conditions optimal for biofilm formation and production of PNAG. Extracellular polymers were extracted and fractionated using the method established for the model strain *S. epidermidis* RP62A. This purification method allowed us to prepare the PNAG of clinical strains avoiding any contamination and chemical modification, in their native form, due to the mild purification conditions and the absence of ion-exchange chromatography steps (Sadovskaya *et al.*, 2005) (see II.1.1).

Fractions corresponding to PNAG were collected, lyophilized and analyzed by ^{1}H-NMR spectroscopy and chemical methods. The degree of de-*N*-acetylation was measured using two colorimetric methods: Elson-Morgan (Enghofer & Kress, 1979) for the estimation of total

glucosamine and Smith and Gilkerson method (Smith & Gilkerson, 1979) without preliminary hydrolysis for the estimation of the free aminogroups of glucosamine. PNAG of six clinical strains was thus analyzed: *S. epidermidis* 5, 341, 444, 521a ; *S. aureus* 343 and *S. lugdunensis* 18a. ^{1}H-NMR spectra of all PNAG analyzed were similar to those of the model strains *S. epidermidis* RP62A and *S. aureus* MN8m, with the only differences in the degree of de-*N*-acetylation of GlcNAc and of the *O*-succinyl substitution. PNAG of *S. epidermidis* 444 and *S. aureus* 343 had a lower degree of de-*N*-acetylation (5±1 et 2±1% of $GlcNH_2$, compared to ~20% in the PNAG of the model strain *S. epidermidis* RP62A). The NMR spectrum of *S. epidermidis* 5 was more heterogeneous and resembled one of the model strain *S. epidermidis* RP62A. This was confirmed by the results of chemical analysis (17±1% of $GlcNH_2$). ^{1}H-NMR spectrum of *S. lugdunensis* 18a was similar to one of the model strain *S. aureus* MN8m, which showed ~40% of de-*N*-acetylated glucosamine. *O*-succinyl substituents were found in PNAG of *S. epidermidis* 5, 44 and *S. lugdunensis* 18a.

To summarize, we have shown that PNAG of six clinical staphylococcal strains of our collection had a structure similar to PNAG of the model strains. The difference between the PNAG from different strains consisted in different degree of de-*N*-acetylation and *O*-succinylation.

ARTICLE 2

3. Characterization of the PGA of Gram negative bacteria, *Actinobacillus pleuropneumoniae* and *Actinobacillus actinomycetemcomitans*

3.1. Introduction

We have shown above that PNAG is a polysaccharide playing an important role in biofilm formation of staphylococci. And yet, recent studies demonstrated that the functions of this polysaccharide are even more universal.

A genetic locus *pgaABCD* promoting surface binding, intercellular adhesion, and biofilm formation in *E. coli* have been recently identified. Genetic and biochemical studies demonstrated that a *pga*-dependent polysaccharide was a poly-β-(1,6)-GlcNAc (PGA), a polymer with the structure close to staphylococcal PNAG. Despite the fact that the final products are structurally very close, genes and enzymes implicated in the biosynthesis of PNAG and PGA share a limited homology. PgaC and IcaA share 35% amino acid identity and 57% similarity. Both enzymes are supposed to be polysaccharide polymerases using UDP-GlcNAc as a substrate. A small protein PgaD could have functions similar to one of the IcaD. Certain homology between IcaB and PgaB and the 20 amino acid signal sequence indicate that PgaB is a putative deacetylase (Wang *et al.*, 2004).

Homologues of the *pgaABCD* locus are present in the genomes of several pathogenic Gram negative bacteria, such as *Actinobacillus actinomycetemcomitans*, *Actinobacillus pleuropneumoniæ*, *Bordetella pertussis*, *Burkholderia cepacia*, *Pseudomonas fluorescens*, *Yersinia pestis* etc. These pathogens could synthesize hexosamine-containing exopolysaccharides that stabilize biofilms of these species (Wang *et al.*, 2004), (Kaplan *et al.*, 2004b).

A glycosyl hydrolase, capable to degrade *S. epidermidis* biofilms, have been recently identified by Prof. J. Kaplan and his co-workers (New Jersey Dental School, Newark, NJ) (Kaplan *et al.*, 2004a). This enzyme, called dispersin B (DspB), is produced by an oral bacterium *Actinobacillus actinomycetemcomitans* (Kaplan *et al.*, 2003; Kaplan *et al.*, 2004b), and a related swine pathogen *Actinobacillus pleuropneumoniae*. It have been shown that dispersin B endolytically hydrolyses the β-(1,6) –linkages of PNAG (Itoh *et al.*, 2005). This enzyme was capable to rapidly disperse biofilms of four clinical strains *S. epidermidis* isolated from infected catheters (Kaplan *et al.*, 2004a).

Dr. Kaplan and his collaborators have demonstrated that biofilms produced by *Actinobacillus actinomycetemcomitans* et *Actinobacillus pleuropneumoniae* produced a hexosamine-rich polysaccharide encoded by the *pga* locus homologous to one of *E. coli* (Kaplan *et al.*, 2003),(Kaplan *et al.*, 2004b). These polysaccharides have not yet been characterized.

3.2. Results

A structural study of PGA of *A. pleuropneumoniae* and *A. actinomycetemcomitans* are subjects of our collaboration with de Dr. Kaplan and his team.

The results of this study are presented in the following Article 6:

IzanoEA, Saodvskaya I, Vinogradov E, Mulks MH, Velliyagounder K, Ragunath Ch, Kher WB, Ramasubbu N, Jabbouri S, Perry MB & Kaplan JB (2007)

Poly-*N*-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae* Microb Pathogen **43**, 1-9

The polysaccharides have been prepared directly from biofilms of two clinical *A. pleuropneumoniae* strain (IA1 -- serotype1 and IA5—serotype 5), and analyzed by chemical methods and 1- and 2D NMR spectroscopy (Dr. E. Vinogradov). We have shown that polysaccharides of the two strains were β-(1,6)-linked poly- GlcNAc. Depending on the strain and the preparation, a part of GlcNAc residues (1—15%) were *N*-deacetylated. This structure was identical to that of staphylococcal PNAG, except that *A. pleuropneumoniae* PGA did not contain *O*-succinate substitution. In this study, for the first time the PGA was isolated directly from a biofilm of clinical Gram-negative strains. Also, we present first chemical and NMR evidence for the de-*N*-acetylation of PGA from Gram-negative bacterium.

Findings presented in the article suggest that PGA functions as a major biofilm adhesin of *A. pleuropneumoniae*.

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Poly-*N*-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*

Era A. Izano[a], Irina Sadovskaya[b], Evgeny Vinogradov[c], Martha H. Mulks[d], Kabilan Velliyagounder[a], Chandran Ragunath[a], William B. Kher[a], Narayanan Ramasubbu[a], Saïd Jabbouri[b], Malcolm B. Perry[c], Jeffrey B. Kaplan[a,*]

[a]*Department of Oral Biology, New Jersey Dental School, Newark, NJ 07103, USA*
[b]*Laboratoire de Recherche sur les Biomatériaux et les Biotechnologies INSERM ERI 002, Université du Littoral-Côte d'Opale, Boulogne-sur-mer 62327, France*
[c]*Institute for Biological Sciences, National Research Council, Ottawa, Ont., Canada K1A 0R6*
[d]*Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA*

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Abstract

Most field isolates of the swine pathogen *Actinobacillus pleuropneumoniae* form tenacious biofilms on abiotic surfaces in vitro. We purified matrix polysaccharides from biofilms produced by *A. pleuropneumoniae* field isolates IA1 and IA5 (serotypes 1 and 5, respectively), and determined their chemical structures by using NMR spectroscopy. Both strains produced matrix polysaccharides consisting of linear chains of *N*-acetyl-D-glucosamine (GlcNAc) residues in $\beta(1,6)$ linkage (poly-β-1,6-GlcNAc or PGA). A small percentage of the GlcNAc residues in each polysaccharide were *N*-deacetylated. These structures were nearly identical to those of biofilm matrix polysaccharides produced by *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. PCR analyses indicated that a gene encoding the PGA-specific glycoside transferase enzyme PgaC was present on the chromosome of 15 out of 15 *A. pleuropneumoniae* reference strains (serotypes 1–12) and 76 out of 77 *A. pleuropneumoniae* field isolates (serotypes 1, 5 and 7). A *pgaC* mutant of strain IA5 failed to form biofilms in vitro, as did wild-type strains IA1 and IA5 when grown in broth supplemented with the PGA-hydrolyzing enzyme dispersin B. Treatment of IA5 biofilms with dispersin B rendered them more sensitive to killing by ampicillin. Our findings suggest that PGA functions as a major biofilm adhesin in *A. pleuropneumoniae*. Biofilm formation may have relevance to the colonization and pathogenesis of *A. pleuropneumoniae* in pigs.

Keywords: Crystal violet; Dispersin B; *dspB*; *pgaABCD*

1. Introduction

Surface-associated colonies of bacteria known as biofilms play a role in the pathogenesis of many chronic infections [1]. Bacterial cells in a biofilm are encased in a self-synthesized, extracellular hydrogel matrix that holds the cells together in a mass and firmly attaches the bacterial mass to the underlying surface [2]. This matrix, also referred to as the slime layer, glycocalyx, or extracellular polymeric substance (EPS) matrix, can comprise up to 90% of the biofilm biomass [3]. In addition to its structural role, the EPS matrix provides biofilm cells with a protected microenvironment containing dissolved nutrients, secreted enzymes, DNA, and bacteriophages. The EPS matrix also contributes to the increased resistance to antibiotics and host defenses exhibited by biofilm cells [4]. Polysaccharide is a major component of the EPS matrix in most bacterial biofilms [2].

Abbreviations: BHI, brain heart infusion; EPS, extracellular polymeric substance; GlcNAc, *N*-acetyl-D-glucosamine; $GlcNH_2$, glucosamine; MHB, Mueller–Hinton broth; PGA, poly-β-1, 6-*N*-acetyl-D-glucosamine; PBS, phosphate buffered saline; TSA, Tryptic Soy agar

*Corresponding author. Medical Science Building, Room C-636, 185 S. Orange Ave., Newark, NJ 07103, USA. Tel.: +1 973 972 9508; fax: +1 973 972 0045.
E-mail address: kaplanjb@umdnj.edu (J.B. Kaplan).

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The PGA of the oral bacterium *A. actinomycetemcomitans* (strain JK1044, serotype e), was also investigated. Unlike *A. pleuropneumoniae*, the PGA of *A. actinomycetemcomitans* does not seem to be part of its extracellular matrix, but is instead closely bound to its external membrane and might form a complex with its LPS. Other studies indicated that Flp fimbriae (Kachlany *et al.*, 2001), rather than PGA, were major biofilm adhesins of *A. actinomycetemcomitans in vitro.*

This observation is interesting for a consideration of the colonization mechanisms which may be employed by the oral pathogen *A. actinomycetemcomitans*. Excreting dispersine B enzyme, it could degrade biofilms of other competing bacteria in its habitat, and employ itself an alternative, PGA-independent mechanisms of biofilm formation. Complementary studies of these mechanisms could be proposed.

The results of this work are a part on article which have been submitted to "Microbial Pathogenesis" (Article 7).

The discovery of the dispersin B by J. K. Kaplan *et al* opened new opportunities in the search of therapeutic tools against staphylococcal infections. This enzyme has an important potential for the treatment and prevention of orthopedic implants-related infections. For this reason, our collaboration with Dr. J. Kaplan, initiated in 2003, is very important and should be continued.

Article 7

Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in *Aggregatibacter actinomycetemcomitans*

Era A. Izano[a], Irina Sadovskaya[b], Hailin Wang[a], Evgeny Vinogradov[c], Chandran Ragunath[a], Narayanan Ramasubbu[a], Saïd Jabbouri[b], Malcolm B. Perry[c], Jeffrey B. Kaplan[a,*]

[a] *Department of Oral Biology, New Jersey Dental School, Newark, NJ 07103, USA*

[b] *Laboratoire de Recherche sur les Biomatériaux et les Biotechnologies INSERM ERI 002, Université du Littoral-Côte d'Opale, Boulogne-sur-mer 62327, France*

[c] *Institute for Biological Sciences, National Research Council, Ottawa, K1A 0R6 Ontario, Canada*

[*] Corresponding author. Address: Medical Science Building, Room C-636, 185 S. Orange Ave., Newark, NJ 07103, USA. Tel.: +1 973 972 9508; fax: +1 973 972 0045.

E-mail address: kaplanjb@umdnj.edu (J.B. Kaplan).

Abbreviations: CPC, cetylpyridinium chloride; eDNA, extracellular DNA; GlcNAc, N-acetyl-D-glucosamine; PGA, poly-β-1,6-N-acetyl-D-glucosamine; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TSA, Tryptic Soy agar; TSB, Tryptic Soy broth.

Keywords: Congo red, Crystal violet; DspB

Abstract

Clinical isolates of the periodontopathogen *Aggregatibacter actinomycetemcomitans* form matrix-encased biofilms on abiotic surfaces *in vitro*. A major component of the *A. actinomycetemcomitans* biofilm matrix is PGA, a hexosamine-containing polysaccharide that mediates intercellular adhesion. In this report we describe studies on the purification, structure, genetics and function of *A. actinomycetemcomitans* PGA. We found that PGA was very tightly attached to *A. actinomycetemcomitans* biofilm cells and could be efficiently separated from the cells only by phenol extraction. *A. actinomycetemcomitans* PGA copurified with LPS on a gel filtration column. ^{1}H-NMR spectra of purified *A. actinomycetemcomitans* PGA were consistent with a structure containing a linear chain of N-acetyl-D-glucosamine residues in β(1,6) linkage. Genetic analyses indicated that all four genes of the *pgaABCD* locus were required for PGA production in *A. actinomycetemcomitans*. PGA mutant strains still formed biofilms *in vitro*. Unlike wild-type biofilms, however, PGA mutant biofilms were sensitive to detachment by DNase I and proteinase K. Treatment of *A. actinomycetemcomitans* biofilms with the PGA-hydrolyzing enzyme dispersin B made them 3 log units more sensitive to killing by the cationic detergent cetylpyridinium chloride. Our findings suggest that PGA, extracellular DNA and proteinaceous adhesins all contribute to the structural integrity of the *A. actinomycetemcomitans* biofilm matrix.

4. Biofilm composition of the model strain *S. epidermidis* RP62A: role of EC TA

Presented in Article 1 (Sadovskaya *et al.*, 2005), chapter II.1 :

Sadovskaya, I., Vinogradov, E., Flahaut, S., Kogan, G. & Jabbouri, S. (2005). Extracellular carbohydrate-containing polymers of a model biofilm-producing strain *Staphylococcus epidermidis* RP62A. *Infect Immun* **73**, 3007-3017.

We have established optimal culture conditions for large-scale biofilm production and the procedure for the extraction of extracellular matrix polymers with the minimal cells lysis (see II.1.1). It allowed us to obtain the total extract of extracellular biofilm polymers, minimizing the contaminations with macromolecules from culture media and cellular polymers. Fractionation of the crude biofilm extract of a model strain *S. epidermidis* RP62A by gel-permeation chromatography on Sephacryl S-300 column allowed us to isolate, along with PNAG, an important amount of proteins and another carbohydrate-containing polymer with a lower MW. This polymer contained both neutral and aminosugars. After the elimination of the majority of proteins by phenol, phenol-chloroform extractions and the re-purification of the carbohydrate-containing polymer on a Sephadex G-50 column, we have identified it as an extracellular teichoic acid (EC

TA). Its structure was elucidated by chemical methods, mass-spectrometry and NMR spectroscopy.

It was shown that the EC TA was a poly(glycerol phosphate) substituted with D-Ala, α-Glc, α-GlcNAc et α-Glc6OAla. The structure of the EC TA was identical to the cell-wall teichoic acid (CW TA) of this strain ((Sadovskaya *et al.*, 2004) ; chapter II.6.1). While the TAs were identified among the extracellular polymers of some CoNS grown on chemically defined medium (Hussain *et al.*, 1991), it is for the first time that we show the EC TA is a constituent of staphylococcal biofilm.

We have also studied the composition of the biofilm (or of the extracellular matrix) of the model strain *S. epidermidis* RP62A depending on the growth conditions. It seemed interesting to understand if, for a given staphylococcal strain, the composition of biofilm varied depending on the growth conditions. This study could shed light to a further understanding of the behavior of staphylococci in their natural habitat.

Using the extraction and fractionation method developed previously, we have studied the chromatographic profiles of crude extracellular extracts of *S. epidermidis* RP62A grown in different culture media (TSB and BHI) and in different conditions of aeration. We have also developed an analytical method of quantification of the EC TA using gas-liquid chromatography. We have found that the relative amounts of PNAG and EC TA considerably varied depending on the growth conditions : in TSB without shaking, optimal conditions for PNAG production, relatively small amounts of the EC TA were produced (equiv to <50 µg glycerol per liter of culture). In the same aeration conditions in BHI medium, the biofilm formation was similar to one in TSB. However, the extracellular matrix formed in these conditions contained much less PNAG, and more EC TA (equivalent to ~300 µg glycerol per liter) and proteins. In both media, vigorous shaking favorized the production of EC TA and proteins, but decreased the production of PNAG. These results show that that for the same staphylococcal strain, the biofilm composition is different depending on the culture media and conditions of aeration. A similar phenomenon would most probably take place in the bacterial natural habitat.

5. Biofilm composition of clinical staphylococcal strains

Presented in the articles 2 (Kogan *et al.*, 2006) and 3 (Sadovskaya *et al.*, 2006) :

Kogan, G., Sadovskaya, I., Chaignon, P., Chokr, A. & Jabbouri, S. (2006). Biofilms of clinical strains of Staphylococcus that do not contain polysaccharide intercellular adhesin. *FEMS Microbiol Lett* **255**, 11-16 (chapter II.2);

Sadovskaya, I., Chaignon, P., Kogan, G., Chokr, A., Vinogradov, E. & Jabbouri, S. (2006). Carbohydrate-containing components of biofilms produced in vitro by some staphylococcal strains related to orthopedic prosthesis infections. *FEMS Immunol Med Microbiol* **47**, 75-82.

5.1. Chemical analysis of biofilms

Having clarified the problem of a biofilm composition for the model strain *S. epidermidis* RP62A (chapters II.1 and II.4), we studied the composition of biofilm of clinical strains from our collection, produced *in vitro* in the conditions optimal for biofilm and PNAG production, which were elaborated earlier. We have chosen fifteen biofilm-positive strains: fourteen CoNS such as *S. epidermidis*, *S. lugdunensis*, *S. warneri* (Chokr *et al.*, 2006) and one strain of *S. aureus* (Eleaume & Jabbouri, 2004). Among them, certain strains were found not to produce PIA (detection using an immuno dot-blot with a polyclonal rabbit anti-PIA antiserum, generous gift of Prof. D. Mack). In this study, we attempted to understand the nature of other biofilm components of these strains.

All the strains were grown in the same conditions, statically in TBS without agitation. Their crude biofilm extracts were prepared, partially deproteinated and fractionated on a Sephacryl S-300 column. Their chromatographic profiles were compared with the one of the model strain *S. epidermidis* RP62A. High MW fractions were collected, lyophilized and analyzed by ^{1}H-NMR spectroscopy. Thus, we studied the structural features of PNAGs from clinical strains (chapter II.2). In certain cases, PNAG was not present in the crude extracellular biofilm extracts.

We observed three major types of chromatographic profiles:

a) Profiles similar to ones of the model strain *S. epidermidis* RP62A with important quantities of PNAG (*S. epidermidis* 5, 444 and a CoN *S. aureus* 343). *S. epidermidis* 5 was producing more PNAG as a model strain *S. epidermidis* RP62A, and could therefore be considered as a PNAG-overproducing strain;

b) Profiles corresponding to quantities of PNAG inferior to ones corresponding to the model strain *S. epidermidis* RP62A (*S. epidermidis* 341 and *S. lugdunensis* 18a) ;

c) Profiles corresponding to the absence of PNAG, characteristic for most of the strains studied.

In all three cases, the peak corresponding to the EC TA was present in the crude biofilm extract. In order to confirm that this peak indeed corresponded to the EC TA and therefore the EC TA was an integral part of the biofilm, we analyzed the composition of the EC TAs and compared them to one of the cell-wall (CW) TAs of corresponding strains. The TAs were prepared using the methodology previously elaborated for the model strain *S. epidermidis* RP62A (Sadovskaya *et al.*, 2004). For each of the eight strains studied, the composition of the EC TA closely corresponded to one of the CW TA. The TA of *S. epidermidis* 5 and *S. lugdunensis* 47 had a composition similar to one of the TA from *S. epidermidis* RP62A and contained glycerol, Glc and GlcNAc (Sadovskaya *et al.*, 2004). Analysis of TAs of other strains showed, in addition, the presence of ribitol and the 1,5-anhydro-ribitol. Thus, the composition of the TAs from *S. epidermidis* 392, 495, *S. warneri* 446, *S. aureus* 383 was similar to one of the model strain *S. aureus* MN8m (Vinogradov *et al.*, 2006), chapter II.6.2. To summarize, we have shown that biofilm, produced *in vitro* by different staphylococcal strains contained proteins and EC TAs. It could also contain the PNAG in variable quantities.

5.2. Susceptibility of staphylococcal biofilms to enzymatic and chemical treatments

It have been shown that *E. coli* biofilms, which integrity is maintained due to PNAG, are degraded by methaperiodate ($NaIO_4$ or HIO_4), a PNAG-modifying agent. These biofilms are not susceptible to the treatment with proteases, such as the proteinase K (Wang *et al.*, 2004). On the contrary, biofilm of an *ica*$^-$ strain *S. epidermidis* 1572-R, which uses a mechanism of formation independent from PNAG production but involving the Accumulation Associated Protein Aap, is completely dispersed by proteinase K (Rohde *et al.*, 2005). Taking into account these published phenomena, we tried to understand whether there was a correlation between the chemical composition of biofilm of staphylococcal strains from our collection and their sensitivity to the agents which specifically degrade different biofilm components. We studied the susceptibility of a number of staphylococcal strains to dispersine B, a PNAG-degrading enzyme (Kaplan *et al.*, 2003), (Kaplan *et al.*, 2004b), (Itoh *et al.*, 2005), to metaperiodate and to the proteinase K. We used two groups of strains: PNAG-producers PNAG (*S. epidermidis* 5, 444 et *S. aureus* 343) and strains forming biofilm without PNAG (*S. aureus* 383, *S. epidermidis* 455a, 28 , *S. lugdunensis* 18a ; II.4.1). Biofilm of the model strain *S. epidermidis* RP62A was used as a reference. The experiments on enzymatic degradation of biofilms were carried out by Dr. Ph. Chaignon.

We have found that the susceptibility of staphylococcal biofilms to enzymatic treatments were in good agreement with their chemical composition. Biofilms containing important quantities of PNAG were readily degraded by dispersine B, while biofilms not containing PNAG were not sensible to this treatment. In contrast, proteinase K did not disperse the biofilm of PNAG-

producing strains. It was relatively efficient (elimination of 30-80% of biofilms in 2 hrs) for the degradation of biofilms of strains which did not produce PNAG. This indicated that biofilms of this group of strains were stabilized by proteins rater than carbohydrate molecules.

ARTICLE 3

6. Structural characterization of the TAs of the model strains *S. epidermidis* RP62A and *S. aureus* MN8m

We have shown (chapters II.4 and II.5) that the EC TA was a part of the biofilm of all staphylococcal strains studied. In addition, the CW TA seems to play an important role during the first step of biofilm formation. Surprisingly, the chemical structure of the staphylococcal TAs, especially the pattern of D-Alanine (D-Ala) substitution – an important feature for the pathogenecity of this microorganism— have not been elucidated in details.
The chemical structure of CW TAs of *S. aureus* et *S. epidermidis* is known thanks to the pioneer studies of Baddiley *et al*. in the 60-th and 70-th. These studies have shown that the TA of *S. aureus* was a (1,5)-linked poly(ribitol phosphate), substituted in the position 4 of the ribitol residue with a β-GlcNAc (Fig. 4, a) (Baddiley et al., 1961), (Baddiley et al., 1962b), (Baddiley et al., 1962a). The lipoteichoic acid (LTA) of *S. aureus* was a (1,3)-linked poly(glycerol phosphate), attached to the diacylglycerol lipid anchor *via* a diglucosyl (gentobiosyl) unit (Fig. 4, b) (Duckworth *et al.*, 1975). The CW TA of *S. epidermidis* I2 was also a (1,3)-linked poly(glycerol phosphate), containing β-Glc and D-Ala residues (Fig. 4, c) (Archibald *et al.*, 1968).

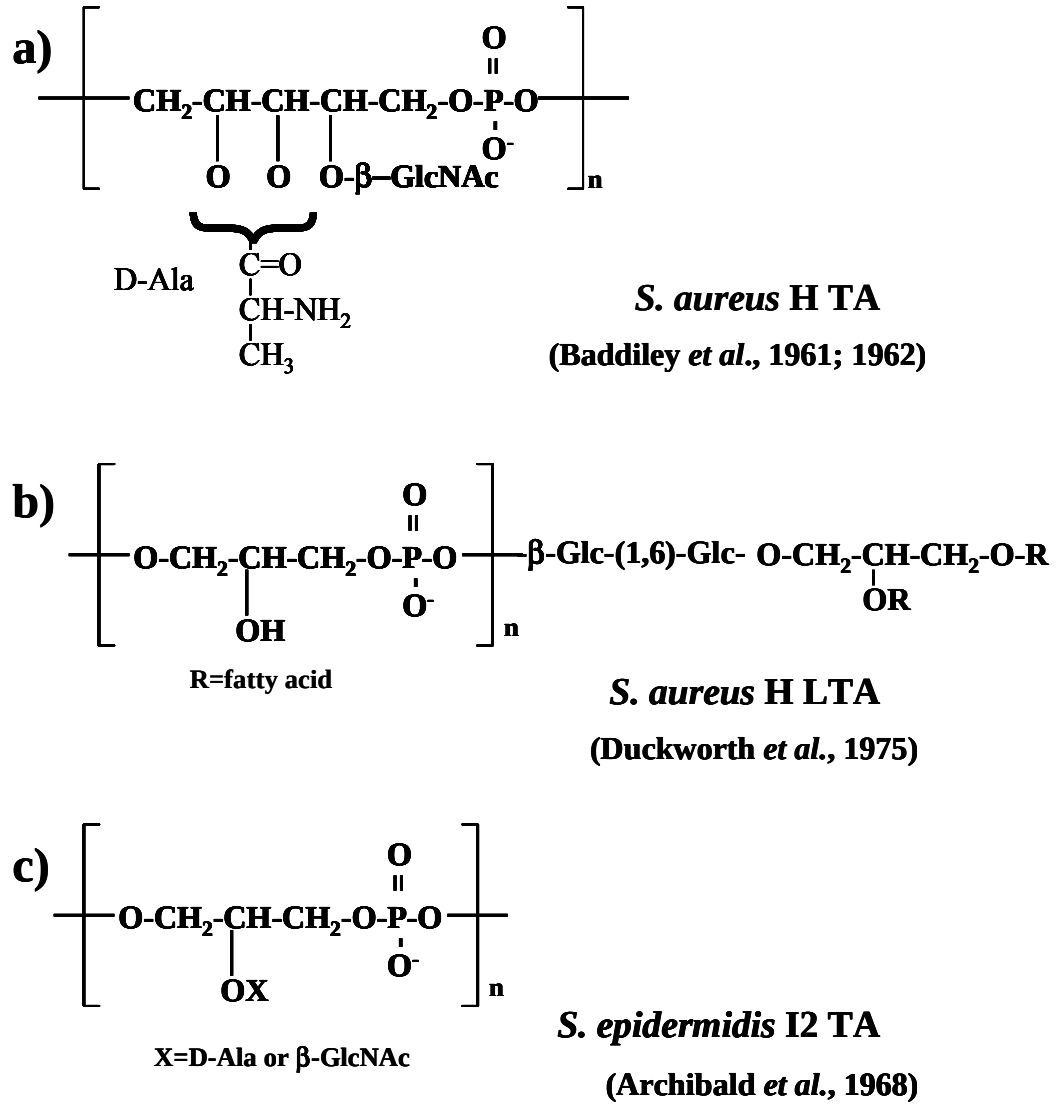

Figure 4. Structural representation of the TA and LTA of *S. aureus* and the TA of *S. epidermidis* (literature data)

Later, End *et al*. (Endl *et al*., 1983) studied the composition of the CW TAs of several strains of *S. aureus* and CoNS ; however, the pattern of D-Ala substitution has not been studied.

6.1. Structure of the TAs of a model strain *S. epidermidis* RP62A

Presented in article 4 (Sadovskaya *et al.*, 2004) :

Sadovskaya, I., Vinogradov, E., Li, J. & Jabbouri, S. (2004). Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus epidermidis* RP62A, a reference biofilm-positive strain. *Carbohydr Res* **339**, 1467-1473.

We studied the chemical structure of the CW and EC TAs of the model strain *S. epidermidis* RP62A. Earlier, Hussain *et al*. studied the composition of the CW TA and of the "extracellular

substance" of this strain. Both preparations contained glycerol, phosphate, Glc, GlcNAc and D-Ala (Hussain *et al.*, 1992). The chemical structure of these TAs was not established.

To prepare the CW TA, we used the method described by Signoretto *et al* for the extraction of the CW TA of *Enterococcus faecalis* (Signoretto *et al.*, 2000) with some modifications. Before the extraction, all the lipid-containing substances, including the LTA, were removed by boiling with 4% SDS. The CW TA was consequently extracted with the 5% trichloroacetic acid (TCA). Chemical analyses of the CW TA showed the presence of glycerol (Gro), phosphate, D-Ala; as well as D-Glc et D-GlcNAc in the ratio of 5:1. The detailed chemical structure of the TA was established by NMR spectroscopy.

^{1}H and ^{13}C-NMR spectra were assigned using 2D homo- and heteronuclear correlation techniques. Residues of α-Glc, α-GlcNAc, D-Ala, glycerol and acetylated glycerol were identified.

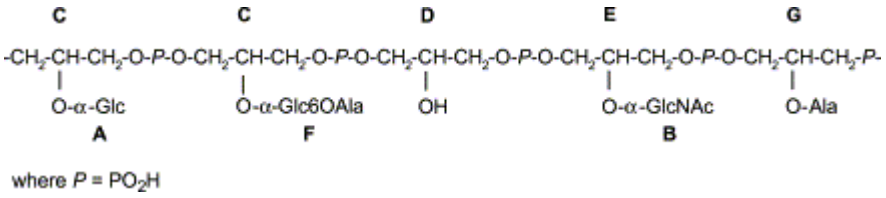

Figure 5. Structure of the CW and EC TAs from *S. epidermidis* RP62A

Structure of the TAs is shown in Fig. 5. It is a (1, 3)-linked poly(glycerol phosphate), substituted in the position 2 of glycerol with the residues of α-Glc, α-GlcNAc, α-Glc6Ala and D-Ala. The unusual feature of this structure is a partial esterification of the α-Glc with D-Ala in position 6. The substitution site was determined due to the HMBC correlation between the C1 of D-Ala and the H6 of Glc, and the characteristic chemical shifts of C5 and C6 of the acylated Glc residue. The substitution of the sugar and not the polyol residue with D-Ala is a unique structural feature for a staphylococcal TA. A substitution of a sugar residue with D-Ala was described for two strains of group D *Streptococci* (Wicken & Baddiley, 1963), but this assignment was uncertain and the site of substitution have not been established. Very recently, Glc6Ala was identified in the capsular polysaccharide of *Enterococcus faecalis* strain 12030 (Fabretti *et al.*, 2006).

We used the capillary electrophoresis – mass spectrometry (CE MS) to confirm the site of D-Ala substitution and to see if α-Glc, α-GlcNAc and α-Glc6Ala were the part of the same macromolecule or were part of two different polymers. CE - MS and the MS - MS analysis of characteristic fragments confirmed that glycerol residues, substituted with Glc, Glc6Ala and GlcNAc were part of the same polymeric chain.

The EC TA was purified from the extracellular matrix of *S. epidermidis* RP62A after gel-filtration on a Sephacryl S-300 column, deproteinisation by phenol extraction, followed by gel-filtration on a Sephadex G-50 column and ion-exchange chromatography. This protocol has been established during the present work. The extracellular matrix was prepared by sonication in conditions with a minimal cell lysis. The integrity of bacterial cells was verified by plating before and after sonication. DNA was absent in the extracellular extract. Therefore, the EC TA was not contaminated with the CW TA, which is important for a comparative study.
Proteins were eliminated by extraction with phenol and phenol-chloroform. This mild deproteinisation method, largely used for the purification of nucleic acids, was shown to be efficient for the EC TA of *S. epidermidis* RP62A and other staphylococcal strains (chapter II.6.1). During the extraction, we avoided the use of buffers with pH>7, in order to minimize the hydrolysis of ester-linked substituents. Presence of the D-Ala was verified with thin layer chromatography (TLC) after *O*-deacetylation (10% NH_4OH).
The structure of the EC TA was studied by 1D and 2D NMR, and was found to be identical to one of the CW TA, only with the slight difference in the degree of D-Ala substitution.

ARTICLE 4

6.2. Structure of the TAs of a model strain *S. aureus* MN8m

Presented in the Article 5 (Vinogradov *et al.*, 2006) :

Vinogradov, E., Sadovskaya, I., Li, J. & Jabbouri, S. (2006). Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus aureus* MN8m, a biofilm forming strain. *Carbohydr Res* **341**, 738-743.

Biofilm of the model strain *S. aureus* MN8m (PNAG overproducer) contained some EC TA. The EC TA was purified, and the CW TA of this strain was prepared using the method developed for *S. epidermidis* RP62A (chapter II.6.1). These TAs were composed of ribitol, glycerol, phosphate and GlcNAc. The TA of *S. aureus* was described as a poly(ribitol phosphate) (Navarre & Schneewind, 1999), while the poly(glycerol phosphate) chain was characteristic for the TA of *S. epidermidis* (Archibald *et al.*, 1968), (Endl *et al.*, 1983) and the lipoteichoic acid (LTA) of *S. aureus* (Duckworth *et al.*, 1975). Interestingly, this unusual composition, containing both glycerol and ribitol, was found in TA of several clinical staphylococcal strains of our collection (chapter II.5.1). The structure of the TA of the model strain *S. aureus* MN8m was therefore studied in details.

6.2.1. Structural elucidation of the TA by NMR spectroscopy and mass-spectrometry

^{1}H and ^{13}C-NMR spectra of the CW TA were assigned by 2D mono- and heteronuclear correlation techniques. Spectra contained signals of spin systems of ribitol, β-GlcNAc, D-Ala, glycerol and acylated glycerol. A NOE interaction between H1 of the GlcNAc and the H4 of ribitol, as well as the HMBC correlation between H1 of the GlcNAc and the C4 of ribitol showed that GlcNAc was linked to ribitol at position 4. It was clear that ribitol was substituted with β-GlcNAc at nearly 100%. This poly(ribitol phosphate) structure corresponded to one described in literature for *S. aureus* H (Baddiley *et al.*, 1961).

Glycerol residues were (1, 3)-linked. Most of these residues were unsubstituted, with ~20% of residues acylated with D-Ala at position 2.

A question of whether glycerol and ribitol units were present on one or separate polymeric chains was solved using CE-MS, employing in-source fragmentation strategy (Li *et al.*, 2005). No fragments containing simultaneously glycerol and ribitol were observed, pointing to the presence of separate poly(glycerol phosphate) and poly(ribitol phosphate) chains. Additional proof of this comes from the fact that lower orifice voltage (decluster potential) was needed to observe ions originating from poly(glycerol phosphate) chains. Consequently, the two polymeric chains, the poly(glycerol phosphate) and the poly (ribitol phosphate) are not connected (Fig. 6).

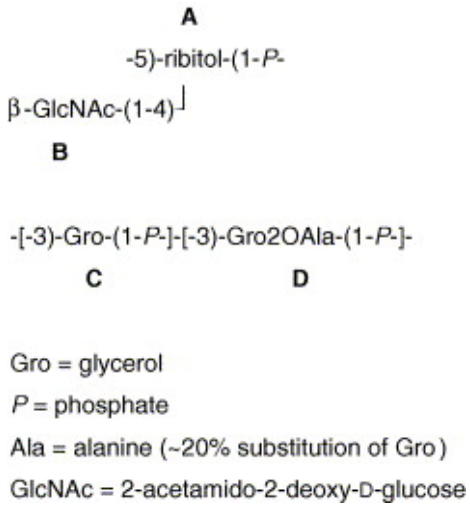

Figure 6. Structure of the CW and EC TAs of *S. aureus* MN8m

6.2.2. Comparative analysis of the TA and the LTA preparations

We have shown that the TA of *S. aureus* MN8m contained a mixture of two polymers:

a) a (1,5)-linked poly(ribitol phosphate), substituted with β-GlcNAc – a usual structure for a *S. aureus* TA;
b) a poly(glycerol phosphate), quite unusual for a TA of *S. aureus* and rather characteristic for the TA of *S. epidermidis* (Archibald *et al.*, 1968), (Endl *et al.*, 1983).

On the other hand, the published structure of the LTA of *S. aureus* comprises a poly(glycerol phosphate) chain of 28-30 units linked *via* diglucosyl (gentobiosyl) unit to diacylglycerol (Duckworth *et al.*, 1975). In order to confirm that our preparations really contained the TA and not a mixture of the TA and LTA, we subjected the crude TA preparation to a hydrophobic interaction chromatography on Octyl-Sepharose. The LTA is retained on this type of column because of the presence of a lipid moiety. This method is largely used for the purification of LTA (Iwasaki *et al.*, 1986). No phosphate-containing material was retained on the column. The chemical composition of the TA preparations before and after the chromatography was identical. In order to verify the efficiency of the hydrophobic interaction chromatography for the separation of TA and LTA of *S. aureus* MN8m, the LTA was extracted from defatted cells with aqueous phenol by a conventional method (Iwasaki *et al.*, 1986) , and purified on the column with Octyl-Sepharose in the same conditions as TA sample. The LTA was found in the retained fraction, and its composition (glycerol and Glc in the ratio ~10 :1) were in good agreement with the published structure (Duckworth *et al.*, 1975). We could therefore conclude that the poly(glycerol phosphate) chains indeed belong to the TA of *S. aureus* MN8m.

ARTICLE 5

7. Enzymatic degradation of staphylococcal biofilms

7.1. Introduction

Knowledge of the biofilm composition of clinical strains allows to better target the biofilm-degrading enzymes.

The interest of the scientific community to the enzymatic degradations of bacterial biofilms of different origins remains very high, since biofilm formation is a cause of industrial, environmental and medical problems in many areas. Different enzymes or enzyme mixtures were recommended for elimination of a biofilm in general (ex. (Johansen *et al.*, 1997); (Marion *et al.*, 2005)), often with no reference to chemical composition of the biofilm of specific bacterial species.

In case of implant-associated infections, biofilms impair the penetration of antibiotics, prevent normal immune responses, and increase the difficulty of eradicating biofilm infections (chapter I). Biofilm (sessile) cells are capable of persisting in the presence of antimicrobials at concentrations that are 1000-fold higher than those necessary to eradicate a planktonic population ((Cerca *et al.*, 2005);(Hamilton, 2002); chapter I). Treatment of these infections usually requires appropriate surgical intervention combined with a prolonged course of antimicrobial therapy (Trampuz & Zimmerli, 2005). In certain cases of infection, washing – draining procedures of the infected device with solutions containing antibiotics are employed, in order to maintain the implant if possible. The use of an agent which would disintegrate the bacterial biofilm, release the planktonic cells into environment and therefore allow the appropriate antibiotic to eliminate infection would greatly improve the efficiency of this medical procedure. Complete elimination of biofilm could thus help to avoid the removal of the orthopedic implant.

The purpose of the present study was to search for enzymes capable to specifically degrade the constituents of the extracellular staphylococcal matrix. These enzymes could be further used in clinical procedures for treatment of orthopedic implant-associated infections. We tested different enzymes and enzyme preparations in view of their capacity to disintegrate biofilms formed by staphylococcal strains related to orthopedic prosthesis infections. The chemical composition of the biofilm of these strains from our collection was studied earlier ((Kogan *et al.*, 2006), (Sadovskaya *et al.*, 2006), chapter II.5). Unlike most of the previous studies, we tried to specifically target the biofilm constituents. We compared the efficiency of different biofilm-degrading agents with the chemical composition of the biofilms. We have also examined the effect of some of these agents on the purified carbohydrate components of staphylococcal biofilms, PNAG and teichoic acid, and tested the proteolytic activities on crude biofilm extracts.

7.2. Results

This study was mostly carried out by Dr. Ph. Chaignon (postdoctoral fellowship in 2004-2005, ANVAR, project director: Prof. S. Jabbouri). I have participated in many aspects of this work, namely in the studies of the effect of enzymatic preparations on the purified biofilm components and the assessment of proteolitic activities.

The results are presented in the Article 8 (Chaignon *et al.*, 2007) :

Chagnon, Ph., Sadovskaya, I., Kaplan, J. B., Ragunah, C., Ramasubbu N. & Jabbouri, S. (2007). Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition, *Appl Microbiol Biotechnol,* **75**: 125-132

We have tested the activities of different enzymes : dispersin B, proteases (proteinase K, trypsin), pancreatin and Pectinex Ultra SP preparation (PUS, Novozyme) on the biofilms formed by different staphylococcal strains of our collection (Chokr *et al.*, 2006). The capacity of these preparations to degrade biofilm is variable on different strains. Despite the fact that all biofilms contain proteins, the three proteases efficiently degraded only biofilms of strains which do not produce PNAG. For the PNAG-producing strains, dispersin B was shown to efficiently disperse their biofilms. The hydrolytic activity of the dispersin B and proteinase K on biofilm components was confirmed by their direct action on PNAG and the protein fraction of biofilms, respectively. Our results indicate that a treatment with dispersin B followed by a protease (proteinase K or trypsin) could be capable of eradicating biofilms of a variety of staphylococcal strains on inert surfaces.

Appl Microbiol Biotechnol
DOI 10.1007/s00253-006-0790-y

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition

P. Chaignon · I. Sadovskaya · Ch. Ragunah · N. Ramasubbu · J. B. Kaplan · S. Jabbouri

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Abstract Bacterial infections are serious complications after orthopaedic implant surgery. Staphylococci, with *Staphylococcus epidermidis* as a leading species, are the prevalent and most important species involved in orthopaedic implant-related infections. The biofilm mode of growth of these bacteria on an implant surface protects the organisms from the host's immune system and from antibiotic therapy. Therapeutic agents that disintegrate the biofilm matrix would release planktonic cells into the environment and therefore allow antibiotics to eliminate the bacteria. An addition of a biofilm-degrading agent to a solution used for washing–draining procedures of infected orthopaedic implants would greatly improve the efficiency of the procedure and thus help to avoid the removal of the implant. We have previously shown that the extracellular staphylococcal matrix consists of a poly-*N*-acetylglucosamine (PNAG), extracellular teichoic acids (TAs) and protein components. In this study, we accessed the sensitivity of pre-formed biofilms of five clinical staphylococcal strains associated with orthopaedic prosthesis infections and with known compositions of the biofilm matrix to periodate, Pectinex Ultra SP, proteinase K, trypsin, pancreatin and dispersin B, an enzyme with a PNAG-hydrolysing activity. We also tested the effect of these agents on the purified carbohydrate components of staphylococcal biofilms, PNAG and TA. We found that the enzymatic detachment of staphylococcal biofilms depends on the nature of their constituents and varies between the clinical isolates. We suggest that a treatment with dispersin B followed by a protease (proteinase K or trypsin) could be capable to eradicate biofilms of a variety of staphylococcal strains on inert surfaces.

Keywords *Staphylococcus* · Biofilm detachment · Enzymatic treatment

P. Chaignon · I. Sadovskaya · S. Jabbouri (✉)
Laboratoire de Recherche sur les Biomatériaux et les Biotechnologies, Université du Littoral-Côte d'Opale, Bassin Napoléon, BP 120,
62327 Boulogne-sur-Mer Cedex, France
e-mail: jabbouri@univ-littoral.fr

P. Chaignon
Institut de Chimie, Université Louis Pasteur/CNRS,
4 rue Blaise Pascal,
67070 Strasbourg, France

C. Ragunah · N. Ramasubbu · J. B. Kaplan
Department of Oral Biology, New Jersey Dental School,
Newark, NJ 07103, USA

Introduction

Staphylococci are responsible for more than 1 million serious hospital-acquired infections per year (Projan and Novick 1997). *Staphylococcus epidermidis*, an important member of human skin and mucous membrane microflora, as well as other coagulase-negative staphylococci (CoNS) are the major cause of infections of various indwelling medical devices, including prosthetic cardiac valves, intraocular lenses, catheters and orthopaedic prostheses (Götz 2002). Bacterial infections represent one of the most serious and devastating complications after orthopaedic implant surgery. Treatment for chronic infection usually requires removal of the prosthesis, cleaning the bone interface and new arthroplasty (Lortat-Jacob et al. 2002). It often results in long periods of hospitalisation, morbidity, severe functional impairment and increased mortality.

The pathogenesis of foreign-body-associated infections of CoNS and particularly of *S. epidermidis* is related to their ability to grow as an adherent biofilm (Mack et al.

8. Toward the elaboration of a diagnostic tool for detection of biofilm-related staphylococcal infections

8.1. Introduction

The early detection of the medical device-related staphylococcal infections is difficult by the classical tools of microbiological analyses. During an implant-related biofilm infection, the quantity of bacteria in the bloodstream is very low, and their direct detection is nearly impossible. Diagnosis is made by bacteriological analyses of the infected site. The diagnosis is often made only at advanced stages of infection, when often severe complications occur: formation of abscesses, pain and unsealing of the prosthetic devices. Specific and non-invasive laboratory tests to diagnose these infections are not yet available.

The major problem in the diagnostics of *S. epidermidis* infections is the presence of this microorganism in commensal microflora. Peptidoclycan, teichoic acids or other cell-wall component, common for invasive and commensal strains can not be used.

The pathogeneicity of *S. epidermidis* is mostly due to its ability to colonize indwelling polymeric devices and form a biofilm (chapter I). A diagnostic test could, therefore, be based on the detection of antibodies specific for biofilm components of CoNS, particularly *S. epidermidis*.

A detection of specific "anti-biofilm" antibodies in the blood serum of patients could serve as convenient non-invasive and inexpensive diagnostic tool for the detection of foreign body-associated infections. However, no antigen specific for staphylococcal infection have been identified.

Different extracellular antigenic preparations have been proposed by different authors as candidates for immunological tests : an extracellular extract of a clinical *S. epidermidis* strain (Staphylococcal Slime Polysaccharide Antigen SSPA, (Selan *et al.*, 2002)), a « 20-kDa sulfated polysaccharide », an « 80- kDa peptidoclycan » (Karamanos *et al.*, 1997), (Kolonitsiou *et al.*, 2001), (Georgakopoulos *et al.*, 2002; Lamari *et al.*, 2004). In all these cases, the chemical structure of the antigens has not been determined. An extracellular « lipid S » of *S. epidermidis*, a molecule close to the LTA of *S. aureus*, was also proposed as candidate for a serodiagnostic test (Lambert *et al.*, 2000). However, to date, none of these antigens led to a commercialized diagnostic test.

We have chosen to test, as an antigen for a serodiagnostic, the PNAG, a characteristic and well characterized component of staphylococcal biofilms. In order to evaluate structural epitopes in the PNAG molecule, we have also tested the chemically de-*N*-acetylated PNAG (dPNAG). The de-*N*-acetylation have been previously shown as an important factor affecting biological activity of

PNAG (Vuong *et al.*, 2004). In addition, it was recently shown that the nonacetylated epitopes of PNAG were the dominant targets of the human antibody response to staphylococcal PNAG (Kelly-Quintos *et al.*, 2005). The comparison of two vaccines, containing PNAG and its deacetylated derivative, dPNAG, conjugated to a carrier protein, showed that the dPNAG conjugated vaccine was more effective in generating opsonic and protective antibodies (Maira-Litran *et al.*, 2005).

As shown above, the composition of staphylococcal biofilm is variable, and certain strains produce biofilms without PNAG (chapter II.5). Therefore, as a first step of our study, we investigated cases of chronic infections caused by the strains known as PNAG-producers. This problem could be addressed thanks to an animal model, developed in our team (Chokr *et al.*, 2007). Briefly, in order to mimic a prosthesis related infection, a multi-perforated teflon tube (tissue cage, TC), filled with beads of materials which are classical components of human implanted medical devices, was implanted subcutaneously in the flanks of the animals. A few days after TC implantation, it was inoculated with a minimum infection dose of a *S. epidermidis* strain (10^3 cfu). Two weeks after inoculation, the tissue cages were removed, washed and sonicated in order to detach bacteria from biofilm on the surface of the implants. Quantitive cultures allowed us to establish the presence of a device-related infection. Strains *S. epidermidis* RR62A and *S. epidermidis* 444, both PNAG-producers, were found capable to cause an implant-related infection in this model. Blood sera of infected animals were used for this study, and sera of guinea pigs before the infection or not infected guinea pigs were used as controls.

We also assessed the presence of antibodies against PNAG and dPNAG (IgG and IgM) in the blood sera of patients with staphylococcal infection of orthopedic prosthesis (artificial hip and knee joints, fracture-fixation devices) and compared it with blood serum of healthy individuals.

A part of this work was done by Mlle Stéphanie Faure (Université de Rennes 1; Microbiologie Fondamentale et Appliquée) during her 6-month stage of Master 2.

8.2.Results

We have shown that in the chosen animal model, the levels of anti-PNAG antibodies were significantly higher in guinea pigs infected with *S. epidermidis* RP62A compared to healthy animals ($P>0.01$). When the evolution of antibody response to PNAG in individual guinea pigs was studied, we observed an increase of the level of antibodies following the implant-related infection.

The results were more ambiguous with human sera. Screening of patent's sera and sera of healthy individuals reveals a relatively high level of anti-PNAG IgGs in the sera of healthy

controls. Level of these IgGs in patient's sera was very variable and overall higher, but the difference was insignificant (P>0.05). The levels of anti-PNAG IgMs were low in both groups, except for one case of an infected patient.

The specificity of antibodies was confirmed by competition ELISA.

To summarize, our results show that PNAG producing strains seem to cause an increase of anti-PNAG IgG antibodies in the implant-related infection guinea pig model. However, PNAG does not seem to be a convenient antigen for a diagnostic tool which could discriminate patients with implant-related staphylococcal infection and healthy individuals. Two main reasons for this are variable and sometimes high IgG titers in healthy individuals[1] and, on the other hand, an high proportion of clinical CoNS which are not PNAG producers.

As complementary to other tests and in cases of infections with a PNAG producing staphylococci, the elaborated ELISA method can be helpful for a follow-up of patients after explanting of the infected prosthesis and consecutive antibiotic treatment. In order to develop a reliable diagnostic tool, other antigens should be investigated.

The results of this study , summarized in Article 9, have been recently submitted to "Clinical and Vaccine Immunology".

[1] This fact could be explained by several considerations: nasal carriage of *S. aureus*, previous infections or exposure to other PNAG-producing pathogen. (ex. *Actinobacillus actinomycetemcomitans*, *Bordetella pertussis, Yercinia pestis*- see chapter II.3.1)

Article 9

Poly-*N*-acetyl-β-(1,6)-glucosamine is not an appropriate antigen in the detection of staphylococcal orthopedic prosthesis-related infections

Irina Sadovskaya[a], Stéphanie Faure[a*], Denis Watier[a], Damien Leterme[a], Ali Chokr[a], H. Migaud[b] and Saïd Jabbouri[a**]

[a]Laboratoire de Recherche sur les Biomatériaux et les Biotechnologies, Université du Littoral-Côte d'Opale, Bassin Napoléon, BP 120, 62327 Boulogne-sur-mer, France
[b]Département d'Orthopédie de l'Université de Lille, Hôpital Salengro 59037 Lille

[*] Present address : Laboratory for the Research and Investigation of Veterinary Drugs and Disinfectants, Pharmacokinetic – Pharmacodynamic Unit, AFSSA Fougères, BP 90203, La Haute Marche, F-35133 Javené, France.
[**] Corresponding author. Tel. :+33 3 21 99 45 17, fax : +33 3 21 99 45 24, *E-mail address* :jabbouri@univ-littoral.fr

9. Conclusion

Biofilm is considered as a main virulence factor of CoNS, major cause of orthopedic implants-associated infections. *Detailed characterization of the chemical composition of the extracellular polymeric substances of biofilms is necessary for the development of therapeutic tools against these infections.*

In the present work, we developed an original and multi-disciplinary approach for this subject, which includes methods of microbiology, analytical biochemistry and structural analysis. Thanks to this approach, important results have been obtained, namely:

- A direct analytical approach and a detailed analysis of literature data allowed us to clarify some ambiguities and to conclude that PIA and PS/A (also referred to as SAA, PNSG and SAE) have the same chemical structure – a poly-β-(1,6)-*N*-acetylglucosamine (PNAG), and differ only by the degree of positive and negative charge due to substitution ;
- For the first time, the PNAG of several clinical strains associated with orthopedic prosthesis infections, were purified and analyzed by chemical methods and NMR spectroscopy. Our study concerned the biofilms of *S. epidermidis* and other staphylococcal species present in the prosthesis-associated infections, such as *S. aureus*, *S. warneri*, *S. lugdunensis*. For the first time, we have also isolated and characterized *pga*-dependent poly-β(1,6)-*N*-acetylglucosamines (PGA) directly from biofilm of clinical strains of Gram negative bacteria, *Actinobacillus pleuropneumoniae* and *Actinobacillus actinomycetemcomitans* ;
- A study of the chemical composition of the extracellular matrix of a model strain *S. epidermidis* RP62A, showed that it contained, in addition to PNAG, the extracellular teichoic acid (EC TA) and proteins. The ratio of these polymers considerably varied depending on the growth conditions. We have shown, for the first time, that the EC TA was an integral component of the biofilm of this strain ;
- A study of the chemical composition of the extracellular matrix of a number of orthopedic implant-associated staphylococcal strains indicated that an important number of these strains were able to form biofilms not containing PNAG, with EC TA as a main carbohydrate-containing component. Other clinical strains formed biofilms with variable amounts of PNAG. A susceptibility of staphylococcal biofilms of a known composition to enzymatic and chemical treatments was also studied; and was correlated to the sensitivity of biofilm constituents to these agents;

- We have established that the chemical structure of the cell-wall and EC TAs of two model strains, *S. epidermidis* RP62A and *S. aureus* MN8m. We have shown some original characteristics in these structures. The TA of *S. epidermidis* RP62A contains, in addition to the D-Ala substituting the glycerol residue at position 2, a D-Ala residue attached to the C6 of glucose. The TA of S. *aureus* MN8m is a mixture of two polymers, a poly(ribitol phosphate), structure typical for the TA of *S. aureus*, and a poly(glycerol phosphate). These unusual results seem to be quite important, since a number of clinical strains of our collection had a composition similar to one of the TA from *S. aureus* MN8m.

These results were summarized in seven publications:
(Sadovskaya *et al.*, 2004; Sadovskaya *et al.*, 2005; Sadovskaya *et al.*, 2006), (Kogan *et al.*, 2006), (Vinogradov *et al.*, 2006); (Chaignon *et al.*, 2007), (Izano *et al.*, 2007) and constitute an important contribution in the field. Our articles were cited in the recent reviews on the subject (Ziebuhr *et al.*, 2006) ; (Mack *et al.*, 2006) ; (Harraghy *et al.*, 2006) ; (Vadyvaloo & Otto, 2005) ; (Lasa, 2006) ; (Stewart, 2006:01) (O'Gara, 2007). Two more articles are submitted for publication.

III. Discussion and future prospects

1. Composition of staphylococcal biofilms

Knowledge of the chemical composition of staphylococcal biofilms is important for the development of therapeutic tools against foreign-body related staphylococcal infections. We have shown that generally the composition of staphylococcal biofilm is variable and strain-dependent. There are three major groups of components of staphylococcal biofilms:

- a polysaccharide, PNAG ;
- a carbohydrate-containing poly (polyol phosphate), teichoic acid (TA)
- proteins

PNAG may be present or absent in staphylococcal biofilms, and its quantity can vary depending on the strain and culture conditions. The elucidation of the protein fraction of staphylococcal biofilms is out of scope of this study. The TA was a part of biofilm in all cases studied, and the chemical composition of the corresponding CW and EC TAs were identical. Thus, it can be assumed that molecules of the TA can detach from the bacterial cell-wall surface and become part of the extracellular matrix, and, therefore, of the biofilm. It still remains unclear if other cell-wall components, such as the lipoteichoic acid (LTA) and peptidoclycan (Fig. 4) are part of staphylococcal biofilm.

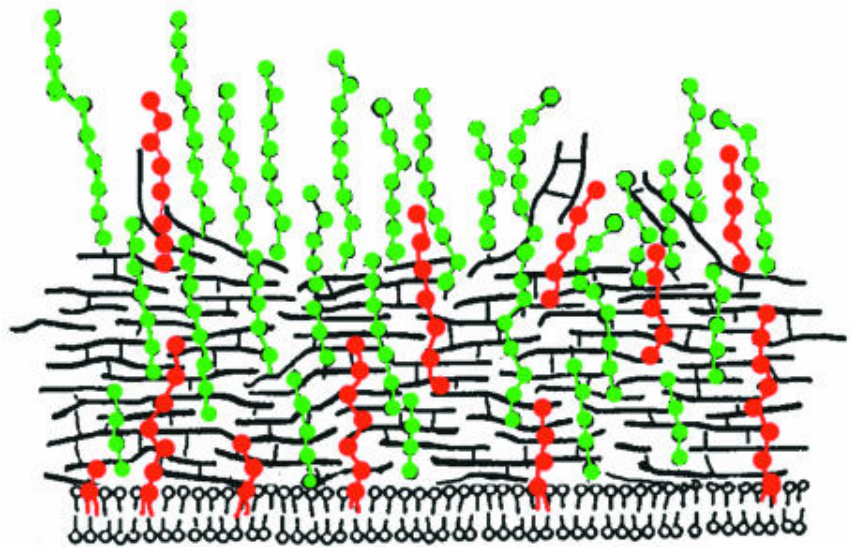

Figure 7. Topography of the CW TA and LTA in the Gram+ cell-wall (Neuhaus & Baddiley, 2003)
Grey symbols represent the CW TA and dark symbols – the LTA. The peptidoglycane and the phospholipids are represented in black.

It was recently shown that proteins involved in biosynthesis of the peptidoglycan were over- expressed in *S. aureus* in sessile state, compared to its planktonic state (Resch *et al.*, 2005; Resch *et al.*, 2006). Lysostaphine, a glycyl-glycine endopeptidase which hydrolyses the staphylococcal peptidoglycan (Fig. 5), was found to be capable to degrade the extracellular biofilm matrix of *S. aureus*, which was shown by scanning electron microscopy (Wu *et al.*, 2003). These studies demonstrate that the presence of the peptidoglycan in biofilm is possible, but, to date, there is no direct evidence of this phenomenon.

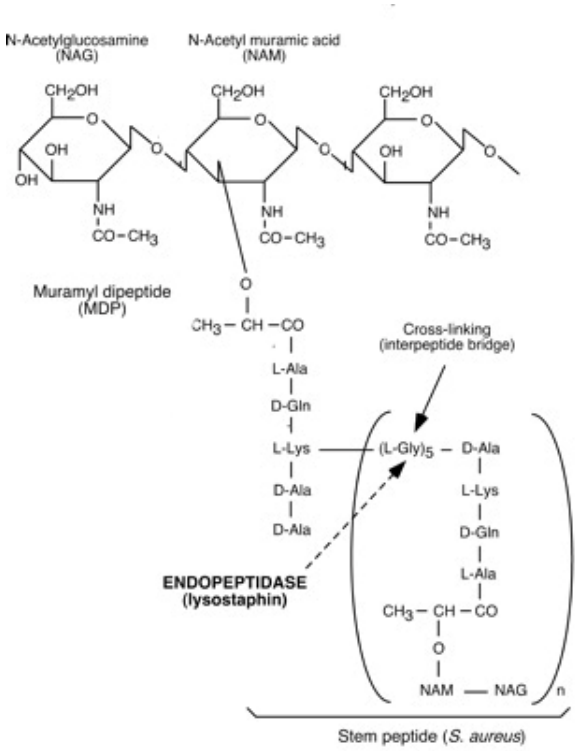

Figure 8. Schematic representation of a peptidoglycane of Gram+ bacteria (adapted from Navarre & Schneewind, 1999).
The cleavage site for lysostaphin is marked with an arrow

Another extracellular component of staphylococci is a poly-γ-DL-glutamic acid (PGA), which was recently identified as a polymer attached to the cell-all of *S. epidermidis* 1457 (Kocianova *et al.*, 2005). This acidic macromolecule constitutes a capsule of *Bacillus anthracis* (Hanby & Rydon, 1946). The PGA plays in important role in the protection of *S. epidermidis* against the host immune system and to high salt concentrations. The *cap* gene locus, coding for the PGA, was present in the genomes of clinical and commensal isolates of *S. epidermidis* and other CoNS, but was absent in *S. aureus* (Kocianova *et al.*, 2005). The role of the PGA in the biofilm formation of CoNS remains unexplored.

Among the proteins involved in staphylococcal biofilm formation, only Aap (Rohde *et al.*, 2005) seems to play a structural role in the extracellular matrix of certain strains.

Proteins of the Bap (biofilm-associated protein) family (Cucarella *et al.*, 2002), (Pernadés, 2006) and a recently described Ipa (intercellular protein adhesin) (Christner *et al.*, 2006), are also involved in biofilm formation. Our analysis of the crude biofilm extracts of several clinical strains show the presence of an important amount of proteins (unpublished data). Identification of these proteins or glycoprteins might be an interesting prospect of our work. Identification of proteins, specific for invasive biofilm-forming strains could give a potential antigen for an immunological diagnostic test of implant-associated infections. Till now, no diagnostic tool for the early detection of this kind of infections has been commercialized (chapter II.8.1).

2. Identification of biofilm constituents in crude extracts by NMR

Extraction and purification of the extracellular components of bacterial biofilms is a long and laborious process, and it would be advantageous to devise a rapid method enabling detection of carbohydrate polymers from biofilm extracts without the need to separate components. As a result of a collaboration with Dr. Mark J. Howard (Department of Biosciences, University of Kent, Canterbury), an original method based on a selective TOCSY NMR technique (Gradwell *et al.*, 1997) have been developed. This method allows having precise information on PNAG, TAs and proteins using crude biofilm extracts. This method allows not only detecting the PNAG in crude extracellular extracts, but also observing some structural details of PNAG, such as the de-*N*-acetylation.

In order to develop the method, we used purified biofilm constituents and crude biofilm extracts of *S. epidermidis* 5, a PNAG-producing strain from our collection.

This technique has an important potential as a tool for rapid characterization of the nature of biofilm constituents. For instance, it could be used for a characterization of the PGA (PNAG) of *Actinobacillus actinomycetemcomitans*, organism that produces dispersin B, wich still remains a challenge (chapter II.3.2). As described above, PGA in this Gram negative bacterium seems to be closely bound to the bacterial cell-wall rather than being part of the extracellular matrix. The purification of the *A. actinomycetemcomitans* PGA is difficult, since it co-purifies with the LPS, perhaps forming a stable complex. The characterization of this PGA directly in a mixture using the selective TOCSY NMR method would significantly facilitate the task.

This promising NMR technique could also be used in the studies of the activity of the PGA deacetylase PgaB, which are currently carried out by Dr. J. Kaplan and his team (J.B. Kaplan, personal communication). This method could be used for a rapid detection of the PgaB activity *in situ.*

This non-destructive NMR method could also serve for the research of other constituents of staphylococcal biofilms, such as fragments of peptidoclycan or poly--γ-DL-glutamic acid (chapter III.1). In further studies, it might be also used for determining the interactions between different components within biofilms.

3. Structure of the teichoic acids of the clinical staphylococcal strains

Structures of staphylococcal TA were largely elucidated in the 60-th and 70-th. The corresponding structural data entered in textbooks of biochemistry and microbiology. The structure adopted for the TA of *S. aureus* is one established by Baddiley *et al* (Baddiley *et al.*, 1961; Baddiley *et al.*, 1962a; Baddiley *et al.*, 1962b) ; and for the TA of *S. epidermidis* – one established by Archibald et al (Archibald *et al.*, 1968) for the strain *S. epidermidis* I2. However, the position of the D-Ala in the TA of *S. epidermidis* I2 was assigned only tentatively (Archibald *et al.*, 1968). Till now, the study of Endl et al (Endl *et al.*, 1983) remains a reference for structures of TAs from the genus *Staphylococcus*. The substitution with D-Ala, an important factor related to virulence of Staphylococci, was not elucidated there. Using NMR spectroscopy and mass-spectrometry, we have shown, for the first time that the TA of S. *epidermidis* RP62A contained two sites of substitution with D-Ala: one in position 2 of glycerol, and the other in position C6 of the glucose residue.
We have found that the TA of *S. aureus* MN8m is a mixture of two polymers, a poly(ribitol phosphate)—usual structure for the TA of *S. aureus*, and a poly(glycerol phosphate). These unusual results seem to be important, since the TAs of several clinical strains of our collection had a composition similar to one of the TA of *S. aureus* MN8m. The presence of the tow types of polymers is therefore not an exception.
PNAG and the TA are two carbohydrate-containing component of staphylococcal biofilm. Both of them are polymers with a modulable charge. For PNAG, the total charge will vary depending on the degree of de-*N*-acetylaion (positive charge) and *O*-succinylation (negative charge). The TAs are polymers with a high negative charge, which can be decreased depending on the degree of the D-Ala substitution. In *S. aureus*, the substitution of the TA with D-Ala is crucial for the initial adhesion to plastic surfaces and biofilm formation (Gross *et al.*, 2001) . It increases the bacterial resistance to certain antibiotics (Peschel *et al.*, 1999; Peschel *et al.*, 2000). Esterification of the TA with D-Ala in Enterococci has a similar effect (Fabretti *et al.*, 2006).
Positively charged D-Ala esters are involved in the ionic interactions with the negatively charged groups in TA, peptidoglycan and proteins (Neuhaus & Baddiley, 2003). Specific interactions between PNAG, TA and proteins in staphylococcal biofilm have not yet been elucidated. In this context, and taking into account the importance of the TA in the virulence of staphylococci, one of the major causes of nosocomial infections, *we propose to carry out a detailed study of the TAs of the clinical strains using modern methods of chemical analysis, such as NMR and mass-spectrometry.*

4. Purified components of cell wall and biofilm: potential applications

In the present work, we developed a relatively simple and efficient protocol of preparation of staphylococcal PNAG, as well as its chemically de-*N*-acetylated derivatives (dPNAG). The expertise in sample preparation of these polysaccharides, poorly soluble in water and convenient buffers at physiological pH, is also helpful for designing experiments for studies of biological properties of these important polymers.

We have shown that for two model staphylococcal strains the EC TAs were structurally identical to the CW TAs. Elaboration and a simple protocol for the purification of staphylococcal TAs allowed us to prepare the TAs from different staphylococcal strains in relatively large quantities. The availability of the purified PNAG and TAs of Staphylococci attracted interest of several researchers working in the area of staphylococcal biofilms.

- The PNAG preparation was used to prepare the rabbit polyclonal anti-PNAG antiserum for microscopic studies of staphylococcal biofilm (***Dr. W. Ziebuhr,*** *Institut für Moleculare Infektionsbiologie, Würzburg University, Germany)*. Dr. W. Ziebuhr and her collaborators also used our preparation of the TA of *S. epidermidis* RP62A.
- We have purified and analyzed the TAs of strains *S. epidermidis* 1572 and *S. epidermidis* 1572-R; which served ***Dr. H. Rohde,*** *Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany* and his team for the studies of the mechanisms of Aap-dependent biofilm formation.
- ***Prof. G. Pier et Dr. T. Maira-Litran,*** *(Channing Laboratory, Harvard Medical School, Boston, MA*), have used our preparations of the TAs of *S. aureus* MN8m and its mutant *S. aureus* MNΔ*ica*, for the studies of cross-reactions of monoclonal anti-PNAG antibody. The CW and EC TA of the mutant strain *S. aureus* MNΔ*ica* was prepared by Mlle A. Fontaine, BTS in lycée Valentine Labbé, la Madeleine, during her stage in May-June 2006.
- Recently, we were contacted by a group of researchers from Ireland, working on a collaborative project investigating *S. epidermidis* biofilm forming capacity in the central nervous system in patients with implanted extraventricular devices (*Prof.* ***James. P. O'Gara****, University College, Dublin ;* ***Dr. Catherine Greene,*** *Prof.* ***Hillary Humphreys, Niall Stevens, Royal College of Surgeons in Ireland***). Our preparation of PNAG and dPNAG will serve to evaluate the capacity of these polysaccharides to stimulate expression of different cytokines by microglyal cells, and to determine the mechanisms od PNAG action (ex *via* Toll-like receptor TLR). In the initial experiments, PNAG in different concentrations was used to stimulate a human glioblastoma astrocytoma cells (cell line

U373 MG). The IL-8 levels in supernatants were measured by ELISA (Niall Stevens, PhD thesis). The results indicate that PNAG is able to stimulate the production of IL-8 as efficiently as other TLR agonsits. Further studies of mechanisms of this stimulation are in progress. dPNAG and TAs from different staphylococcal strains will be also tested.

- We would like, in 2007-2008, initiate a research project in collaboration with ***Dr. F. Roussel,*** Laboratoire de Thermophysique de la Matière Condensée (LTPMC), a research team of ULCO which is a part of UMR CNRS 8024. It will concern a study of mechanisms of biofilm formation using an Atomic Force Microscopy (AFM). The AFM allows to directly assess the reversible and specific interactions between the bacteria and different substrates (Hanna *et al.*, 2003) during the initial adhesion of the bacteria to biomaterial, the first step of biofilm formation (I.2). This method allows to distinguish hydrophobic, ionic and specific interactions between the cells and the biomaterials. It could be used for studies of bacterial adhesion with a very high resolution (Razatos *et al.*, 1998). AFM have been previously used for studies of different components of bacterial cell-wall, such as LPS and colonic acid, in adhesion of *E. coli*, cause of urinary tract infections (Emerson & Camesano, 2004) ; (Hanna *et al.*, 2003). It was also used recently for studies of biofilms of *S. epidermidis* (Chaw *et al.*, 2005).
  Staphylococcal PS/A (PNAG) is known to be involved in initial adhesion of bacteria to biomaterials, as well as the accumulation of biofilm (I and II.1.2). Using the purified PNAG we could study in details its role in the bacterial adhesion by AFM.

5. General remarks

Due to a constant growth of biofilm-related nosocomial infections, an interest to this problem of the scientific community remains high. Several groups of researchers are involved in this area throughout the world. Inter-disciplinary collaborations are greatly beneficial and contribute significantly to the progress of research. Such collaborations developed during this work are an example of quite successful cooperation of researchers with different area of expertise. The important working contacts with different research groups in Europe and the US will make it easier to develop other collaborative projects.

Currently, biofilm mode of growth is considered as predominant for bacteria in different ecosystems (Costerton *et al.*, 1995). Biofilms cause important problems in different fields, such as public health, water treatment, food and oil industries. We hope to be able to contribute to the solution of these problems in the future.

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