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Formation of biofilm by *Staphylococcus xylosus*

S. Planchon [a], B. Gaillard-Martinie [a], E. Dordet-Frisoni [a], M.N. Bellon-Fontaine [b], S. Leroy [a], J. Labadie [a], M. Hébraud [a], R. Talon [a,*]

[a] *INRA Clermont-Ferrand/Theix, UR 454, Microbiologie, 63122 Saint-Genès Champanelle, France*
[b] *INRA, UR Bioadhésion et Hygiène des Matériaux, 91300 Massy, France*

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Abstract

The ability of 12 *Staphylococcus xylosus* strains to form biofilm was determined through the study of different criteria. Eleven out of the 12 strains were able to form biofilm, 10 preferentially on hydrophilic support (glass) and one, *S. xylosus* C2a, on both hydrophilic and hydrophobic (polystyrene) supports. The determination of bacterial surface properties showed that all strains were negatively charged with five strains moderately hydrophobic and seven hydrophilic. The *bap* and *icaA* genes, important for biofilm formation of some staphylococci, were searched. All strains were *bap* positive but *icaA* negative. Furthermore, *S. xylosus* strain C2a was studied on two supports widely used in the food industry, polytetrafluoroethylene (PTFE, hydrophobic) and stainless steel (hydrophilic) and appeared to adhere preferentially on stainless steel. Addition of 20 g/l of NaCl to Tryptic Soy Broth medium (TSB) did not improve significantly its adhesion but enhanced both bacterial growth and cell survival, which were optimum in this medium. Environmental scanning electron microscopy showed that *S. xylosus* C2a colonized the surface of stainless steel chips with intercellular spaces. The strain formed cell aggregates embedded in an amorphous polysaccharidic matrix. Indeed, synthesis of polysaccharides increased during growth on stainless steel chips in TSB.

Keywords: *Staphylococcus xylosus*; Biofilm; Hydrophobic supports; Hydrophilic supports; Bacterial surface properties; Polysaccharides

1. Introduction

Staphylococcus xylosus, a common bacterial species from the skin microflora of mammals, is frequently isolated from milk, meat, and other food products such as cheeses and sausages (Kloos and Schleifer, 1986; Talon et al., 2002). This species is also used as starter in combination with lactic acid bacteria for sausage and cheese manufacturing, in which it contributes to their flavour (Montel et al., 1996; Talon et al., 2002). In sausages, *S. xylosus* participates through its nitrate reductase activity to the development of the colour and it avoids their rancidity thanks to its anti-oxidant enzymes (Barrière et al., 2001). Furthermore, some strains of *S. xylosus* produced inhibitory substances in laboratory media that prevent development of *Listeria monocytogenes* (Villani et al., 1997; Norwood and Gilmour, 2000).

S. xylosus is frequently isolated from soils and surfaces of food-processing plants (Kloos and Schleifer, 1986). Its presence could be linked to its capacity to form biofilm often involved in the colonization of biotic and abiotic surfaces. Several staphylococcal species form biofilms, among them *Staphylococcus epidermidis* and *Staphylococcus aureus* are responsible of infections related to medical devices and therefore are the most threatening (Peters et al., 1982; Christensen et al., 1985; Götz, 2002;). For these two species, the mechanisms involved in biofilm formation are well characterised unlike other staphylococci such as *S. xylosus* (Heilmann et al., 1997; Cramton et al., 1999).

Biofilm formation is a two-step process; it involves first attachment of cells to a solid surface and second the accumulation and aggregation of cells sticking together by intercellular adhesion (Christensen et al., 1985). Biofilm formation is a complex process regulated by diverse

* Corresponding author. Unité de Microbiologie, INRA, Centre de Clermont-Theix, 63122 Saint-Genès Champanelle, France. Tel.: +33 4 73 62 41 70; fax: +33 4 73 62 42 68.
E-mail address: talon@clermont.inra.fr (R. Talon).

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characteristics of support, bacterial cell surface, growth medium and their interactions (Donlan, 2002). Attachment is governed by physicochemical interactions between the support and the bacterial surface including hydrophobicity, Van der Waals forces and Lewis acid–base properties (Bellon-Fontaine et al., 1996; Briandet et al., 1999; Krepsky et al., 2003). The physicochemical characteristics of the medium such as ionic strength (Barnes et al., 1999) may play a role in the rate of biofilm formation. In particular, sodium chloride induced biofilm formation in *S. aureus* and *S. epidermidis* (Götz, 2002). Also divalent cations such as calcium or magnesium enhanced the adhesion of *S. epidermidis* (Dunne and Burd, 1992). In addition, these divalent cations significantly enhanced slime production by *S. epidermidis* and seemed important in maintaining the biofilm structure (Ozerdem Akpolat et al., 2003). The slime or Polysaccharide Intercellular Adhesin (PIA) excreted by *S. aureus* as well as *S. epidermidis* strains participates to biofilm formation (Götz, 2002) and PIA is encoded by the *icaADBC* operon and its presence is associated with strong biofilm formers (Heilmann et al., 1996; Gerke et al., 1998). This polysaccharide is mainly involved in intercellular adhesion (Heilmann et al., 1996). For some staphylococci, another surface component seemed important in biofilm formation: the biofilm associated protein (Bap). The *bap* gene was identified in bovine mastitis isolates of different species, it is involved in primary attachment and intercellular adhesion (Cucarella et al., 2004).

The aim of the present study was to investigate the capacity of *S. xylosus* strains to form biofilm. For 12 strains of *S. xylosus*, development in biofilm was investigated on hydrophobic and hydrophilic supports and their potential of adhesion was characterised by determining their surface properties. Moreover, *bap* and *ica* genes were searched. For *S. xylosus* strain C2a, biofilm development was studied in different growth media and biofilm architecture was visualized by environmental scanning electron microscopy.

2. Materials and methods

2.1. Strains and growth medium

Twelve *S. xylosus* strains were studied, including three reference strains DSM 20266, DSM 20267 and C2a (University of Tübingen) isolated from human skin. The nine other strains were isolated from fermented sausages and identified by PCR (Morot-Bizot et al., 2003). Bacteria were subcultured three times with orbital shaking (150 rpm) at 30 °C for 16 h in Tryptic Soy Broth supplemented with 6 g/l of yeast extract (TSB, Difco, Le pont de Claix, France). The third subculture was used in stationary phase for all the experiments.

2.2. Screening for biofilm formation on glass and polystyrene

The 12 strains were screened for their ability to form biofilm on hydrophilic (glass) and hydrophobic (polystyrene) tubes. The third bacterial subcultures (see Section 2.1) were centrifuged at 4000 × *g* for 10 min (Jouan MR22i, Saint-Herblain, France). Cells were washed three times in saline water (8.5 g/l NaCl) and were adjusted at an optical density (OD) of 0.1 at 600 nm (UV 160A, Shimadzu Corporation, Japan). One milliliter of culture was distributed in each type of tubes. After a 2-h adhesion step at 30 °C, the bacterial suspension was eliminated and replaced by TSB medium. The tubes were incubated for 48 h at 30 °C. Then, the bacterial suspensions were removed and the tubes were washed three times with 1 ml of 0.01 M phosphate buffer saline ($NaH_2PO_4H_2O$ 0.45 g/l, $Na_2HPO_412H_2O$ 1.8 g/l, NaCl 7.4 g/l) and air-dried. The adherent bacterial film was stained during 5 min with 1 ml of 0.25% (w/v) safranine O (Merck, Darmstadt, Germany) and rinsed with distilled water and air-dried. The bacterial film was then solubilized with ethanol 95% (v/v) and the colour was quantified by the absorbance at 540 nm. Tubes inoculated with sterile media were used as negative controls. The strains with an OD ≥ 0.5 were considered as able to form biofilm (Krepsky et al., 2003). Experiments were performed in triplicate with three repetitions by strain for each experiment.

Data were analysed by the Student *t* test (Microsoft Excel software) to compare biofilm development of the strains on polystyrene and glass tubes.

2.3. Surface properties of the 12 strains

2.3.1. Affinity of the strains for the solvents (MATS)

The Microbial Adhesion To Solvents (MATS) method which compares the microbial cell affinities for monopolar and nonpolar solvents was used according to Bellon-Fontaine et al. (1996). Two pairs of solvents were selected as follows: i) chloroform, an acidic solvent (electron-acceptor), and hexadecane, a nonpolar *n*-alkane and ii) ethyl acetate, a basic solvent (electron-donor), and decane, a nonpolar *n*-alkane. The first pair determined the Lewis basic character of the strain and the second the Lewis acid character of the strain.

The third bacterial subculture was centrifuged, washed and resuspended in saline water to give an OD = 0.6–0.7 at 400 nm (*A*0, initial absorbance). A volume of 2.4 ml of this suspension was mixed (vortex) for 60 s with 0.4 ml of a solvent. The mixture was stored 15 min at room temperature to allow the separation of two phases. OD_{400nm} was measured in 1 ml of aqueous phase (A). The percentage of cells in each solvent was calculated by the following equation: % Affinity = $[1-(A/A0)]\times 100$. Experiments were performed in duplicate with three repetitions for each solvent.

2.3.2. Determination of surface charge

The surface charge was determined by the electrophoretic mobility. The third bacterial subculture was centrifuged and suspended in 1.5×10^{-3} M sodium chloride at a final concentration of 10^7 CFU/ml. All strains were studied at pH 6.0 and for 3 strains (20266, 20267, C2a), the pH of the suspension was adjusted within the range of 2 to 8 by adding nitric acid (0.01 N) or potassium bromide (0.01 N). The electrophoretic mobility was measured in a 50 V electric field using a laser Zetameter (CAD Instrumentation, Limours, France). The results were based on an automated video of