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Surface migration of *Staphylococcus xylosus* on low-agar media

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Abstract

Staphylococcus xylosus is a commensal species commonly found on the skin of mammals, but also currently used as starter culture for meat fermentation. Most strains of this species colonize by forming a biofilm on abiotic surfaces. We show here that the majority of *S. xylosus* strains also exhibit extensive colony spreading on the surface of soft agar media. This phenomenon seemed to be independent of biofilm-forming ability. It occurred in different culture media and was dependent on temperature. Formation of a giant *S. xylosus* colony did not involve a biosurfactant. Microscopic observation showed that the front of the giant colony comprised a single layer of spacing cells with more packed cells in the median area. Supplementation of the soft media with DNase I increased *S. xylosus* colony spreading, indicating that extracellular DNA may be involved in limiting the phenomenon. The ability of *S. xylosus* to spread on semi-solid surfaces may constitute an advantage for surface colonization.
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Keywords: *Staphylococcus xylosus*; Motility; Giant colony

1. Introduction

It is increasingly believed that communities of bacteria act together rather than as a group of individual cells. It would be interesting to understand how these communities are formed and colonize a variety of natural habitats. Differing invasive behaviors are used by bacteria for the acquisition of nutrients, and motility constitutes a benefit. Many bacteria translocate by the propeller function of flagella [24]. Some bacteria, such as mycobacteria and streptococci, can spread on semi-solid surfaces without locomotor organelles [2,18]. A mechanism called sliding is generally used by these bacteria [9]. It is provided by the expansive forces of a growing culture in combination with special surface properties of the cells, resulting in reduced friction between the cell and its substrate [8]. Sliding motility results in a uniform sheet of closely packed cells in a single layer [9]. A phenomenon called darting has been observed in some staphylococcal species [9] and involves slow surface translocation produced by expansive forces developed in an aggregate of cells. Micromorphologically, cells or aggregates of cells are distributed randomly with empty areas in between [9]. In other Firmicutes, spreading motility independent of flagella was observed. In *Bacillus subtilis*, a sliding type of surface motility was detected in a non-flagellated mutant [13]. Secreted surfactin and extracellular potassium ions are required for spreading of *B. subtilis* [14]. Recently, rapid *Staphylococcus aureus* colony spreading on soft agar medium was reported [12]. Surface translocation of *S. aureus* differed from the darting of *Staphylococcus epidermidis*, involving slow surface translocation on solid agar medium, as described by Henrichsen [9].

Staphylococcus xylosus is a non-motile Gram-positive coccus belonging to the *Staphylococcus saprophyticus* group. *S. xylosus* is a common inhabitant of the skin of a variety of mammals and occasionally of humans [16]. This species is usually isolated from dairy and meat products [10] and [3,17,21] is commonly used as starter culture for meat products [27]. It is also isolated from environmental sources such as surfaces in food processing plants or those of medical equipment [1,4,5]. It probably colonizes the skin and surfaces by forming a biofilm [23]. Surface translocation could also

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contribute to surface colonization. The capacity of *S. xylosus* to translocate from the site of inoculation on soft agar medium was first reported by Kloss et al. [15]. In the present study, we characterized the ability of *S. xylosus* to migrate widely on semi-solid surfaces, and we suggest an important role for extracellular DNA in cell–cell aggregation.

2. Materials and methods

2.1. Strains and media

The *S. xylosus* strains used in this study are listed in Table 1. Bacteria were routinely grown in BHI (brain-heart infusion) broth (BD Becton, Dickinson and Company, Sparks, MD, USA) at 30 °C and with orbital shaking of 200 rpm.

2.2. Spreading experiments

Colony spreading was performed in BHI medium solidified with 0.4% w/v agar (Difco, Detroit, MI, USA). In some experiments, agar content varied between 0.3 and 1.5% w/v. Colony spreading was also tested in two other media, Luria–Bertani (LB) medium containing 10 g/L tryptone peptone (Difco), 5 g/L yeast extract (Difco), 5 g/L NaCl (pH 7.0), and a minimal medium, MX medium developed specifically for the *S. xylosus* C2a strain by Fiegler and Brückner [6] and containing (per L): 1 g Na_3-citrate · $2H_2O$, 7 g $Na_2HPO_4 \cdot 2H_2O$, 3 g KH_2PO_4, 1 g NaCl, 1 g KCl, 4 g $(NH_4)2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.0147 g $CaCl_2 \cdot 2H_2O$, three vitamins, 0.012 mg biotin, 4.6 mg nicotinic acid, 2 mg thiamine hydrochloride and trace elements, 1.5 mg $FeCl_2 \cdot 4H_2O$, 0.07 mg $ZnCl_2$, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.006 mg boric acid, 0.19 mg $CoCl_2$, 0.002 mg $CuCl_2 \cdot H_2O$, 0.024 mg $NiCl_2 \cdot 6H_2O$ and 0.036 mg $Na_2MoO_4 \cdot 2H_2O$. Different carbon sources were added to MX medium: 5 g/L of glucose, fructose or lactose. Media were autoclaved and 20 mL or 65 mL were poured into plates of 8.5 or 14 cm diameter, respectively. Plates containing agar medium were freshly prepared before inoculation and dried, open for 15 min, in a laminar flow chamber. For standard tests, the centers of agar plates were inoculated in triplicate with 2 μl of overnight culture containing approximately 10^9 CFU/mL. After inoculation, open plates were dried in a laminar flow chamber for 10 min. The initial inoculum formed a spot with a diameter of 6 mm. Unless indicated otherwise, plates were incubated at 25 °C for up to 96 h before a negative reaction was recorded. Plates were photographed using a digital camera (Kodak EasyShare DX6490). In some cases, plates were inoculated from dilutions of overnight culture containing approximately 10^7, 10^5 or 10^3 CFU/mL, or from 10× or 100× concentrated bacterial suspension obtained after centrifugation of overnight culture. In other experiments, the BHI and MX media were supplemented with different substances: 0.025% Tween 80, 5 μg/mL surfactin from *B. subtilis* (Sigma-Aldrich, Saint Louis, MO, USA), 50 or 250 μg/mL trypsin (Sigma-Aldrich), 90 units/mL DNase I (Roche, Mannheim, Germany) or 1 mg/mL DNase-free RNase A (Sigma-Aldrich). To measure spreading rates, the diameter of the growth zone radiating from the point of inoculation on triplicate plates was measured each hour after surface migration commenced.

Table 1
S. xylosus strain ability to spread at 25 °C on 0.4% agar BHI medium and to form biofilm on microplate test

Strain	Origin	Spreading	Biofilm
DSM20266	Human skin	−	+
DSM20267	Human skin	−	+
C2a	DSM20267 cured	−	+
839	Meat starter	++	−
840	Meat starter	−	+
S01002	Meat starter	++	−
S01003	Meat starter	++	+
S01004	Meat starter	−	−
S01006	Meat starter	+++	+
S01007	Meat starter	+++	+
S01008	Meat starter	−	+
S04002	Meat starter	+++	+++
S01001	Dairy starter	+	+
S04003	Dairy starter	++	++
S03187	Sausage	+++	++
S03191	Sausage	+	−
S06173	Raw milk	++	+
S06171	Cheese	++	+
S06175	Cheese	+++	++
S06176	Cheese	+	+++
S06178	Cheese	−	+
S00290	Environment of food plant	++	+
S06167	Environment of food plant	+++	++
S06179	Environment of food plant	++	+
S06186	Environment of food plant	−	++
S04009	Cow mastitis	+++	+++
S04010	Cow mastitis	+++	+
S04012	Cow mastitis	++	+
S04013	Cow mastitis	+++	−
S04016	Cow mastitis	−	+
S04017	Cow mastitis	−	−
S04018	Cow mastitis	+	++
S04011	Goat mastitis	++	++
S04014	Goat mastitis	−	+
S04019	Goat mastitis	+++	+
S04020	Goat mastitis	+++	++
S04021	Goat mastitis	+	+
00-1747	Mouse dermatitis	−	++
S07002	Mouse	+*	++
S07003	Mouse	−	+
S07001	Poultry feces	++	++
S07010	Dog feces	−	−
S07011	Rabbit feces	+	+
S06222	Clinical, prosthetic heart valve	+++	+++
S07005	Clinical, endocarditis	+*	++
S06223	Clinical, liquid	++	++
S06224	Clinical, urine	++	++
S07006	Clinical, cerebrospinal fluid	++	++
S07007	Clinical, blood culture	++	++
S07008	Clinical, blood culture	+	+++
S06225	Clinical, blood culture	++	++

*Strains spreading after 48 h.

2.3. Biofilm evaluation with crystal violet staining

This assay is based on the colorimetric measure of crystal violet incorporated by sessile cells [22]. For each strain,

200 µl of an overnight culture adjusted to OD_{600} 0.01 was loaded into a 96-well polystyrene microtiter plate. As a control, 200 µl of sterile BHI medium was used. The ''non-biofilm-forming'' strain *Staphylococcus carnosus* UT TM300 was employed as a negative control [7]. After 24 h of incubation at 30 °C, the medium was removed and wells were washed once with 200 µl sterile demineralized water to remove non-adherent bacteria. Two hundred µl per well of a 0.1% (v/v) crystal violet solution in water (Merck, Fontenay-sous-bois, France) were added for 10 min. After the staining step of adhered cells, the wells were washed two times with 200 µl of sterile demineralized water to remove excess stain. The dye incorporated by the adherent cells was solubilized with 200 µl of 33% (v/v) glacial acetic acid (Sigma-Aldricht, St Quentin Fallavier, France). One hundred and fifty µl of the solubilized solution or adapted dilution were transferred to a new microtiter plate. The OD of each well was measured at 595 nm using a microtiter plate reader (iEMS, Thermo Electron Corporation, Courtaboeuf, France). Absorbance was measured from the assay OD_{595} minus control OD_{595}. The assay was performed at least in triplicate with four repeats for each strain.

2.4. Microscopy observation

One strain used as a model for spreading ability was grown for 16 h on 0.4% agar BHI medium. A giant colony on a plate was transferred directly to a glass coverslip. Optical microscopic analyses were performed using a phase-contrast Zeiss Axioplan 2E. For electron microscopic analyses, the cells on glass coverslips were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for 1 h, dehydrated using a graded ethanol and acetone series, placed on stubs and coated with gold in an EM-SCOPE SC500, and observed with a SEM 505 Philips scanning microscope.

3. Results

3.1. Screening of spreading phenomenon in S. xylosus *strains*

Fifty-one *S. xylosus* strains described in Table 1 were inoculated on BHI low-agar medium plates at 30 °C. Thirty-seven *S. xylosus* colonized the surface of the soft medium, after 16 h of incubation (Table 1 and Fig. 1). Fourteen strains did not spread from the point of inoculation and showed a 6 mm colony diameter, which was the size of the spotted area before incubation (Fig. 1B). Efficacy of spreading and shape of the resultant giant colonies were distinct for the different *S. xylosus* strains (Table 1 and Fig. 1). The majority of efficiently spreading strains formed a uniform surface film growth (Fig. 1A). Other morphologies were observed showing a fractal edge of migration (Fig. 1C) or protrusions (Fig. 1D–F). The less efficient strains usually spread with the formation of protrusions (Fig. 1E,F). The giant colonies varied in thickness. No correspondence between ecological niches of *S. xylosus* strains and their spreading capacity was noticed.

3.2. Culture conditions for S. xylosus *spreading*

Four efficiently spreading *S. xylosus* strains, S04002, S04009, S06175 and S06222, were chosen to characterize colony spreading on semi-solid media. Four non-spreading *S. xylosus* strains on agar 0.4% at 30 °C in BHI medium, C2a, S04016, S07003 and S07010, were also tested in different conditions of growth. These selected *S. xylosus* strains were from various ecological niches (Table 1). Different agar concentrations were tested ranging from 0.3 to 1.5% of agar (w/v) in BHI and LB media. *S. xylosus* strains S04002, S04009, S06175 and S06222 were spread on either LB or BHI medium. For these four strains, colony spreading was observed from 0.3 to 0.9% of agar, but above 0.5% of agar, a significant decrease in the size of surface migration was noticed. Optimal spreading was observed on 0.3–0.4% agar (wt/vol). Colony spreading was observed for *S. xylosus* S04009 and S04020 strains in MX medium containing glucose, lactose and fructose as carbon sources with 0.4% agar. The S06222 strain was not able to growth in MX medium containing fructose as carbon source, but spread on glucose and lactose MX medium. *S. xylosus* S06175 did not grow in MX medium. *S. xylosus* strains which are able to spread on the MX medium spread less efficiently on this medium than on BHI or LB media due to a low rate of growth (data not shown). It is important to note that whatever the media, colony spreading decreased when the period of plate drying before inoculation was prolonged (data not shown). The surface migration of the four spreading *S. xylosus* strains was observed at different temperatures ranging from 10 °C to 42 °C. The strains showed good growth at all tested temperatures (data not shown). For all four strains, surface migration was observed at temperatures ranging from 20 °C to 35 °C, except for strain S04009 which spread up to 37 °C, with an optimum between 25 °C to 30 °C. At 10 °C, cellular translocation was slow and was observed after almost 48 h of incubation.

Non-spreading *S. xylosus* strains C2a, S04016, S07003 and S07010 were not able to spread whatever the agar concentration, media or temperature of growth used.

Kinetic measurements under optimal conditions for *S. xylosus* spreading (BHI medium solidified with 0.4% of agar, 25 °C) indicated that the diameters of the growth zone did not increase between 0 and 6 h for the four spreading strains, after which the growth zone diameter increased continuously and rapidly, reaching speeds of 126, 97, 144 and 63 µm/min for S04002, S04009, S06175 and S06222 strains, respectively.

To investigate whether initial cell density influenced surface migration, we tested different bacterial inocula. The overnight culture of S04002, S04009, S06175 and S06222 was 100- or 10,000-fold diluted in BHI broth, or 10- or 100-fold concentrated. Two µl of these suspensions or pellets were spotted. No differences in colony spreading were observed after inoculation of the dense cell suspensions. The start of spreading was delayed with the diluted suspensions.

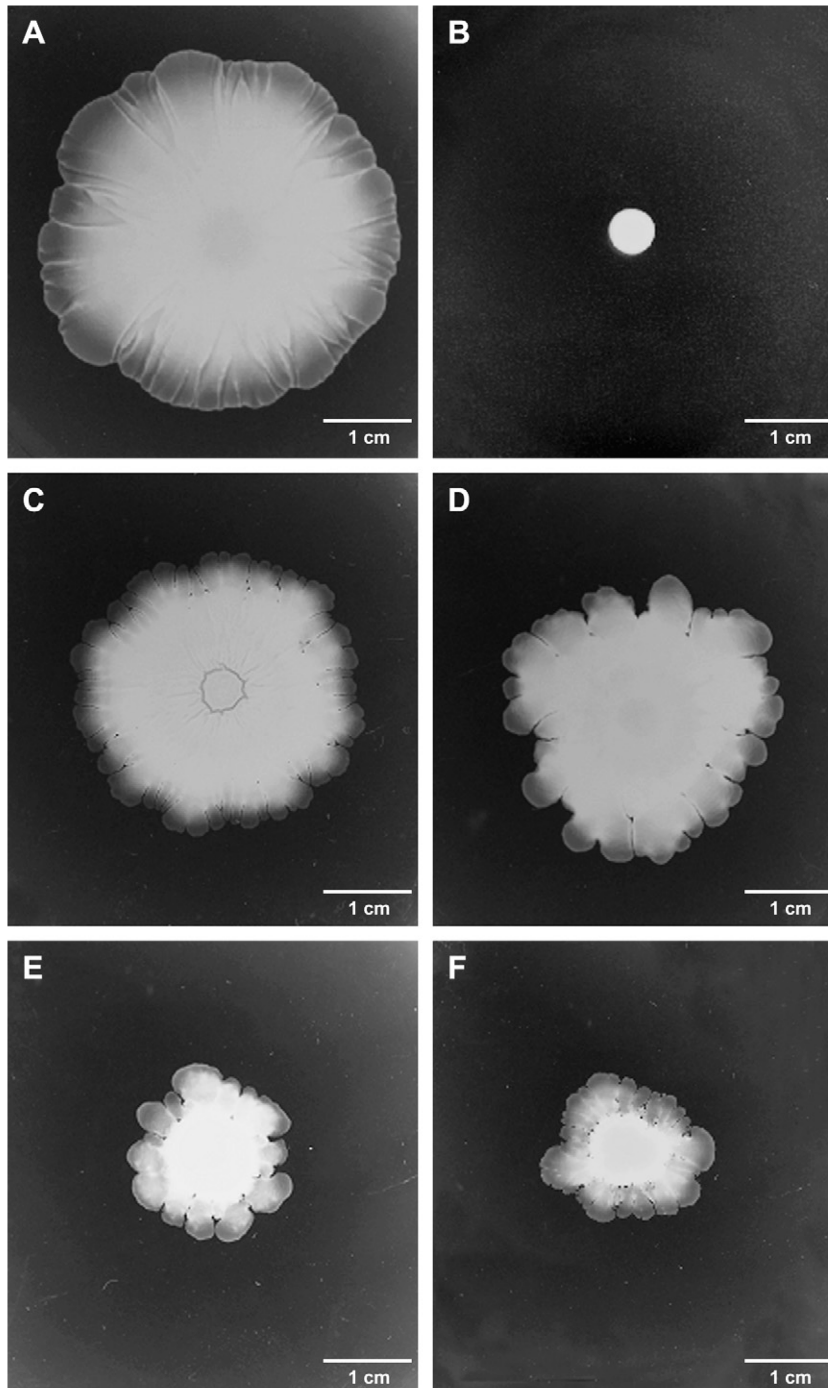

Fig. 1. Surface colonization by *S. xylosus* strains on low-agar medium plates. Two μl of overnight cultures were spotted in the center of 0.4% agar BHI medium and incubated at 25 °C for 16 h. (A) Spreading growth zone of strain S04002; (B) growth on non-spreading C2a strain; (C), (D), (E) and (F) spreading growth zone of strains S04011, 839, S04018, and S01001, respectively.

3.3. Absence of a biosurfactant

To examine the mechanism of surface migration, we tested for production of a surfactant that could increase the wettability of the agar surface. We were unable to detect surfactant in *S. xylosus* cultures of strains S04002, S04009, S06175 and S06222 by the drop collapsing test of Jain et al. [11]. Moreover, 10 μl water drops applied to the surface of the agar medium near the edge of the giant colonies did not cause water spotting, confirming the absence of a surfactant. The addition of Tween 80, an anionic surfactant, resulted in disruption of surface film growth and formation of some protrusions of the spreading strains (data not shown) and did not promote spreading of non-motile strains C2a, S04016, S07003 and S07010. Supplementation of BHI with surfactin did not influence growth but decreased the spreading of S04002, S04009,

S06175 and S06222 strains. It did not induce the formation of giant colonies by C2a, S04016, S07003 and S07010 strains (data not shown).

3.4. Molecules which increase the S. xylosus rate of surface migration

Spreading of *S. xylosus* S04002, S04009, S06175 and S06222 was weakly enhanced by the addition of trypsin to BHI medium (data not shown). An increase in the trypsin concentration increased the rate of colony surface migration. The addition of DNase I but not RNase A to the BHI medium strongly enhanced the surface film growth of the four efficient strains. The surface of the 14 cm diameter plate was completely covered after 22 h of growth on DNase I-supplemented BHI medium for S04002, 25 h for S06175 and 30 h for S04009 and S06222. Speeds of spreading were increased and were 175, 143, 167 and 138 μm/min for S04002, S04009, S06175 and S06222 strains, respectively. The giant colonies were thinner on media supplemented with DNase I (data not shown). The same observation was made when DNase I was added to MX medium containing glucose for the three spreading strains which can grow on this medium. Trypsin and DNase I did not induce spreading in the four non-spreading *S. xylosus* strains.

3.5. Correlation with biofilm formation

To determine whether colony spreading ability and biofilm formation were related to each other, we evaluated the capacity of the 51 strains to form biofilm (Table 1). Quantification of biofilms was performed by a colorimetric measure of sessile cells stained by crystal violet. A large majority of *S. xylosus* strains formed biofilm in polystyrene microplates (Table 1). Only seven strains were biofilm-negative in our experimental conditions. Among these seven strains, four strains spread at different rates on semi-solid media. Three of the fourteen non-spreading strains did not form biofilm. The highly efficient spreading *S. xylosus* strains showed various biofilm-forming capacities, with one strain unable to form biofilm or strains forming biofilm at different rates.

3.6. Microscopic observations

To examine the giant colony morphology of *S. xylosus*, optical and electron microscopic analyses were performed on spreading strain *S. xylosus* S04002. Optical microscopic observations of the edge of the giant colony on low-agar medium showed a monolayer of cells with spaces between the cells. In the median zone, cells seemed to be more packed (data not shown). This observation was confirmed by electron microscopy (Fig. 2A,B). In the median growth area of the giant colony, cells were aggregated in multiple layers separated by intercellular spaces (Fig. 2B). When the giant colony was formed on medium supplemented with DNase I, the appearance of the median zone was clearly modified and showed few or no aggregated cells (Fig. 2C).

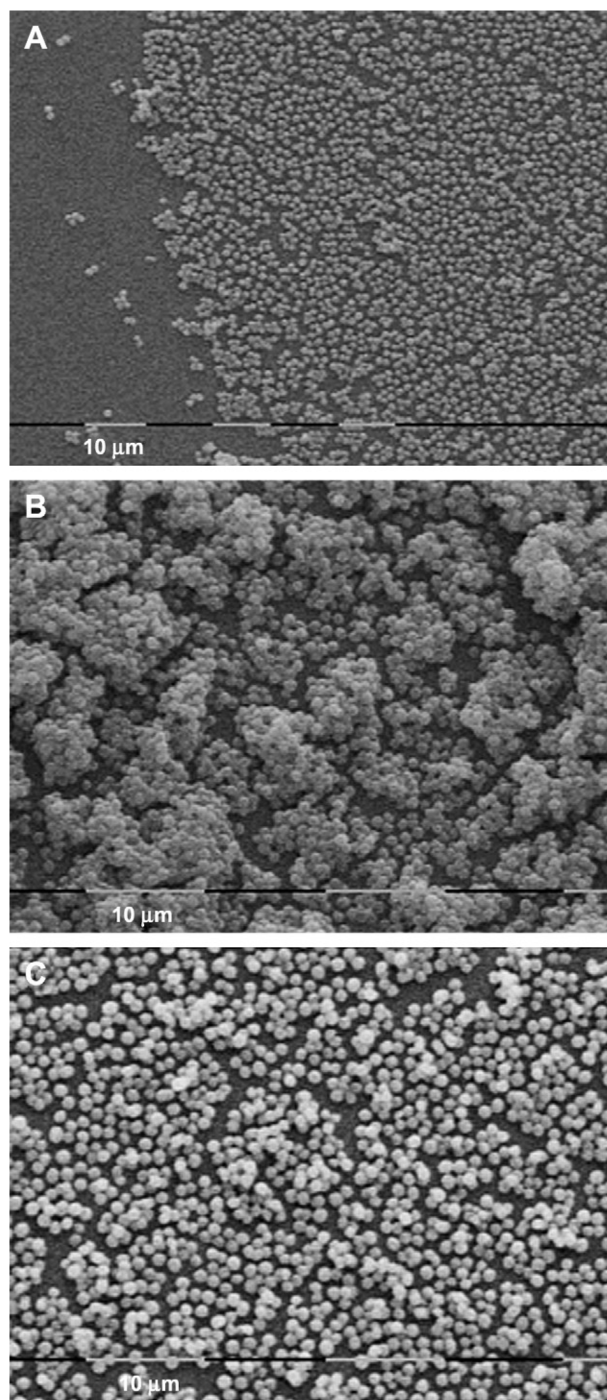

Fig. 2. Electron microscopic observations of the edge (A) and median growth area of a giant colony of *S. xylosus* S04002 strain formed at 25 °C on 0.4% agar BHI supplemented (C) or not (B) with DNase I.

4. Discussion

The majority of *S. xylosus* strains, independently of their origin, spread rapidly on a soft agar medium surface, colonizing the entire surface available. The average size of the giant colonies was dependent on the strain, agar concentration, temperature and time of incubation. Spreading growth of *S. xylosus* was optimally observed on 0.3–0.4% agar media between

25 °C to 30 °C. Surface translocation was limited above 35 °C and inhibited above 37 °C. The two rich BHI and LB media and minimal medium MX with various sources of carbon supported spreading of *S. xylosus* strains. Spreading behavior was thus independent of the growth medium and did not require amino acids, glucose or complex nutrients. Some strains of *S. aureus* can spread rapidly on low-agar medium [12]. Unlike *S. xylosus,* 40 °C was the optimum temperature for surface translocation of *S. aureus*. No spreading of *S. aureus* was observed below 30 °C or when LB medium was used. Moreover, microscopic analyses revealed that the micromorphology of a giant colony of *S. xylosus* was different from that of *S. aureus*. The edge of the *S. xylosus* colony comprised a single layer of spacing cells, while that of *S. aureus* consisted of closely packed cells in several layers [12]. The surface translocation of *S. xylosus* does not appear to correspond to any of the six known bacterial surface translocations described by Henrichsen [9].

The flagellum-independent spread could depend on the production of surfactants, which lower the surface tension of the water and enhance surface migration [13,19]. We were unable to detect a surfactant in *S. xylosus* giant colonies, but colony spreading of this species is probably facilitated by the wettability of the media, as drying of the surface media decreased the phenomenon. But the addition of Tween 80, which is known to modify the surface tension of the medium and improve surface wettability [20], did not have a significant effect on *S. xylosus* colony spreading. Complementation of the medium with *B. subtilis* surfactin did not induce spreading in non-efficient strains and reduced the phenomenon in positive spreading strains.

Addition of trypsin, a serine protease, moderately improved the surface migration of *S. xylosus* strains, probably by limiting cell–cell adhesion due to proteins. But DNase I strongly enhanced *S. xylosus* surface translocation. DNase I acts upon single-chain and double-stranded DNA. It can prevent cell–cell aggregation, which might be induced by extracellular DNA (eDNA) [25]. In *S. xylosus*, the presence of DNase limited the formation of cell aggregates in the median area of the giant colony. Some bacteria, such as *Pseudomonas aeruginosa* and *S. aureus*, produce eDNA, which forms a matrix participating in cell–cell contact in biofilms [26,28]. We presume that eDNA reduces the spreading of *S. xylosus*. We had previously reported the ability of *S. xylosus* to form biofilms [23]. In this study, we have shown that most *S. xylosus* strains isolated from various niches can form biofilm on polystyrene surfaces. No clear correlation between spreading ability and biofilm formation can be established. Biofilm-forming *S. xylosus* strains may or may not spread on semi-solid medium, and conversely, spreading strains may or may not form biofilm. The mechanisms implicated in spreading seem to be independent of those implicated in biofilm formation in *S. xylosus* species.

Colony spreading could be of great benefit for *S. xylosus*, which is ubiquitous and colonizes naturally fermented food. The spreading motility of *S. xylosus* strains may provide some advantages for colonization of surfaces. The biological significance of the ability of *S. xylosus* to spread, and the spreading mechanisms, remain to be elucidated.

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