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New Method Showing the Influence of Matrix Components in *Leuconostoc mesenteroides* Biofilm Formation

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Abstract Studying biofilm formation and influence of the matrix composition was heavy because only old and long methods were employed up to now: confocal microscopy, fluorescent chemical markers, and/or dying techniques. In this context, an innovative tool, the BioFilm Ring Test®, was here employed to explore the role of exopolysaccharides, proteins, and nucleic acids in the formation of biofilm by *Leuconostoc mesenteroides*. The principle is to add magnetic particles in the culture medium. When a biofilm is formed, particles are unable to migrate in the media to form a ring when a magnet is brought nearer to the well. Therefore, culture media supplemented with proteases, glycanases, and/or nucleases allowed us to identify the involvement of these substances in *L. mesenteroides* biofilm formation. The results permitted to demonstrate that dextran, proteins, and nucleic acids are implied in biofilm formation.

Keywords Biofilm · Magnetization · Matrix components · Exopolysaccharides · *Leuconostoc mesenteroides*

Introduction

In the natural environment, bacteria spend a lot of energy to elaborate a biofilm structure and secrete macromolecules as an extracellular matrix. This structure not only confers a protection to microorganisms but also increases their virulence too. Even if this matrix is described as mainly polysaccharidic, it contains also a large part of water [1]. Effectively,

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polysaccharides and more especially exopolysaccharides are known to form a slime layer, which increases the medium viscosity. However, their role in biofilm formation is ambiguous as Wozniak et al. [2] exhibited that alginate was not the significant component of *Pseudomonas aeruginosa* biofilm matrix. That is the way the role of polysaccharide in the biofilm can be envisaged in the interaction with other macromolecules as proteins and nucleic acids. In this context, studies revealed narrow interactions between polysaccharides and proteins in some matrices where proteins represented the largest fraction of macromolecules (~75%) [3]. Moreover, a group of surface proteins, the biofilm-associated protein are active constituents of biofilm formation, involved with polysaccharides in cell-to-cell association during the biofilm maturation [4]. This confusion relative to the real role of polysaccharide and proteins in the biofilm is increased by the presence of other macromolecules and notably by nucleic acids. At this time, the respective function of each biofilm component seems to be dependent on bacterial species as extracellular matrices might be as diverse as the biofilms [5].

Leuconostoc mesenteroides is unique in producing both water-soluble and water-insoluble dextrans, when grown on sucrose [6]. This (1,6)-α-D-glucan of high industrial value is often suspected to play a significant role in biofilm formation by this bacterial strain.

To elucidate the role of dextran in biofilm formation by *L. mesenteroides*, we applied an innovative tool, the BioFilm Ring Test®. It evaluates the bacterial biofilm formation without any washing and staining step thanks to magnetic particles added in the culture medium [7]. When a magnet is approached, they aggregate or not. If the biofilm is formed, particles are trapped in the exocellular matrix, and they cannot form a visible spot opposite to the magnet. With the magnets applied to the culture medium of *L. mesenteroides*, supplemented or not with degrading enzymes, this test allowed us to validate the biofilm formation by this strain and to evaluate the role of each macromolecule family potentially present in the matrix.

Materials and Methods

Bacterial Strain and Growth Conditions

Leuconostoc mesenteroides subsp. mesenteroides CIP 106146 was from the Pasteur Institute. Bacteria were regenerated at 26 °C in Man Rogosa Sharpe (MRS from Fluka) medium, pH 6.2. The brain heart infusion (BHI; from Becton Dickinson), pH 7.4, was employed for *L. mesenteroides* growth at 26 °C and biofilm investigations using the BioFilm Ring Test®. This medium is matched with most strains tested with the kit. Absorbance (A_{600}) of all cultures in BHI was adjusted to 0.1 with sterile BHI before the beginning of the test to obtain the initial bacterium suspension.

Trichoderma harzanium IHEM 5176 was cultivated on 1 L of *Trichoderma* minimum medium supplemented with dextran (20 g/L) as the single carbon source [8]. The strain grew at 25 °C on a rotary shaker (120 rpm) for 72 h. At the end of incubation, the culture broth was filtered successively through 160- and 0.45-μm filters to take off mycelia. Afterward, the extracellular medium was concentrated down to 90 mL using an Amicon stirred cell holding a 10-kDa polyethersulfone ultrafiltration disc (Millipore, Bedford, MA, USA). Considering that 1 U of activity corresponds to the release of 1 μmol of glucose per minute, 204.5 U of dextran hydrolase activity was collected.

Assays

Protein concentrations were determined by the Bradford method [9] using the Bio-Rad reagent and bovine serum albumin as standard.

The reducing sugars released were quantified using the 2,2′-bichinchoninate method [10] and glucose as standard.

Biofilm Formation by *Leuconostoc mesenteroides*

The BioFilm Ring Test® (BioFilm Control, Saint Beauzire, France) includes microplates (12 polystyrene strips of eight wells, SBS format), Toner (magnetic beads solution), Contrast Liquid (a nontoxic and inert opaque oil used for reading step), dedicated Bloc Test (magnet support), and Plate Reader (scanner).

In each well, 200 μL of medium (BHI)+Toner were added. The final concentration of the Toner was 18 μL/mL. In these experiments, *L. mesenteroides* was filled in six wells of a strip (six repeats were performed at time); the two others represented the control (sterile BHI+Toner). One microplate per time of lecture (0, 2, 4, 6, 8, 24, and 48 h) was incubated at 26 °C in a moist box.

Before the reading, the Contrast Liquid was added in each well. The plate was scanned with the Plate Reader, and we obtained an image (I0) with no spot visible. During 1 min, the strip was placed on the Bloc Test to magnetize the beads. In the bloc, 96 magnets were placed opposite to the wells. A second reading gave the image (I1). If there was no biofilm, a central dot was formed because beads were attracted in the center of the bottom wells. No spot was detected when a biofilm was present, since they remained in place, trapped in the biofilm matrix. Chavant et al. published scanning electron microscopy pictures of microorganisms with magnetic beads in wells on different bacterial strains. Images of each well before (I0) and after (I1) magnetization were compared with the Biofilm Control® Software as described previously giving a value named the Biofilm Index (BFI) ranging from 0 to 20. A high BFI value corresponds to a high mobility of beads under magnet action (i.e., control wells), while a low value corresponds to a full immobilization of beads (no difference between I0 and I1). A full immobilization is never obtained; that is why BFI never takes a value lower than 2. When BFI reached a value of 2, the biofilm was formed [7]. To verify the viability of microorganisms and/or the presence of contamination, a plate of the medium was inoculated from wells.

Enzymes and Polysaccharide

Dextran (from *Leuconostoc* ssp., Fluka) was dissolved at different concentrations from 4 to 50 g/L in a sterile BHI medium. One strip per concentration was filled in a microplate of the BioFilm Ring Test®, and the BFI value was determined.

Sterile BHI was supplemented with dextranases isolated from *T. harzanium* at the concentration of 3.3×10^{-3} mg/mL, with pronase (from *Streptomyces griseus*, Roche) at 2 mg/mL, proteinase K (from *Tritirachium album*, Sigma) at 0.3 mg/mL, and DNase I and RNAse A (from pancreas bovine, Sigma) at 1 mg/mL.

All the enzyme solutions were sterilized by filtration (0.22 μm).

Bacteria, which A_{600} was adjusted to 1, were added directly in the medium supplemented with enzymes, to be diluted 250 folds. We tested the actions of pronase, proteinase K, dextranases, or nucleases but also a mix of them. One strip per enzyme or mix of enzymes was filled.

A control was tested; enzymes were just added in sterile BHI, and the evolution of the aspect of beads was followed.

Results and Discussion

In a first experience, the biofilm formation by *L. mesenteroides* was validated with the BioFilm Ring Test® (Fig. 1). A significant decrease in the BFI was detected after 6 h of incubation, and it reached a value of 2 after 24 h. The BFI increase observed on each curves during the first 4 h of experiences is due to beads sedimentation in the depths of wells [7].

To identify the role of all macromolecular compounds implicated in the extracellular matrix, investigations were first performed on dextran. With yields of extracted dextran comprising between 8 and 20 g/L [11, 12], immobilization of beads was first studied with different concentrations of dextran. A concentration of 4 g/L corresponds to a BFI of 11.5 and to a BFI of 8.5 at a concentration of 50 g/L. BFI decreased when the medium is concentrated with more than 20 g/L of dextran. This result associated to the presence of insoluble dextrans in *L. mesenteroides* culture media led us to conclude a role of dextran in the biofilm formation [6]. In fact, this insoluble dextran was not taken into account in dextran yields as it was removed during bacteria extraction. According to that, *Leuconostoc* sp. is able to produce more than 20 g/L of polysaccharide. Nonetheless, BFI did not reach a value corresponding to that in the biofilm formation as it is in Fig. 1. That is the reason why we assumed that other constituents could take part in this formation.

To go further in the comprehension of the macromolecules sharing in the decrease in BFI, degrading enzymes were employed (Figs. 2, 3, 4, and 5). As noted in "Materials and Methods," the viability of microorganisms after growth in BHI supplemented with degrading enzymes was verified by inoculation on BHI plates with wells analyzed at the

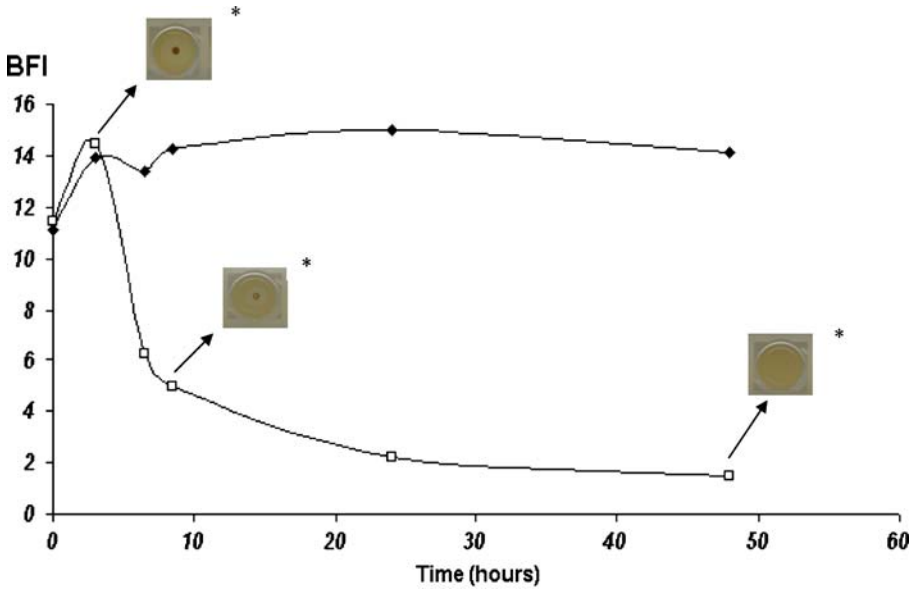

Fig. 1 Evolution of BFI during incubation of *Leuconostoc mesenteroides* in BHI medium: (—◆—) Control without microorganism, (—□—) *Leuconostoc mesenteroides.* *Images of the bottom wells

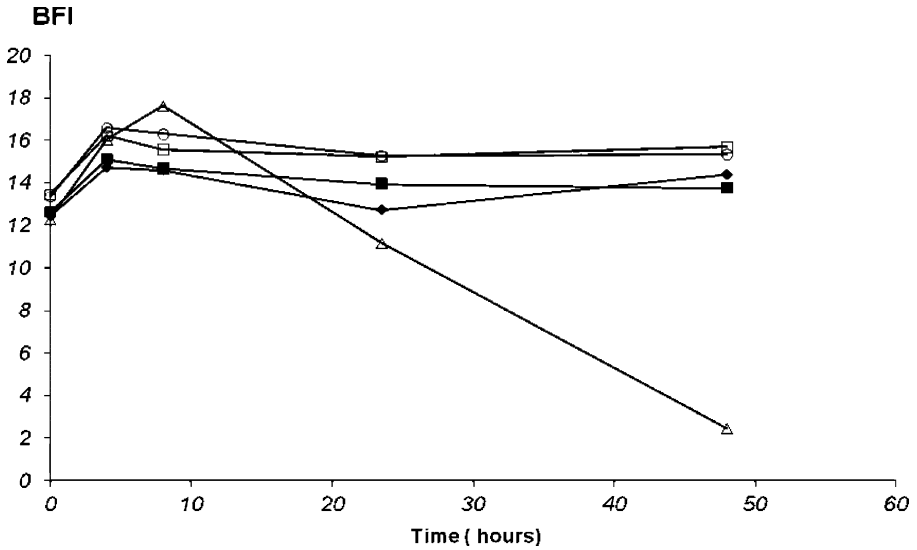

Fig. 2 Test of controls: sterile medium supplemented with enzymes: (—◆—) sterile BHI, (—□—) BHI+ dextranases, (—○—) BHI+RNAse, (—■—) BHI+proteinase K, (—△—) BHI+pronase

final time. First, the influence of enzymes addition in the medium was checked on. Figure 2 indicates that only the strip in which pronase is present induced a wrong behavior of magnetic beads even if the wells remain sterile. Therefore, pronase was not fitted with the experiment.

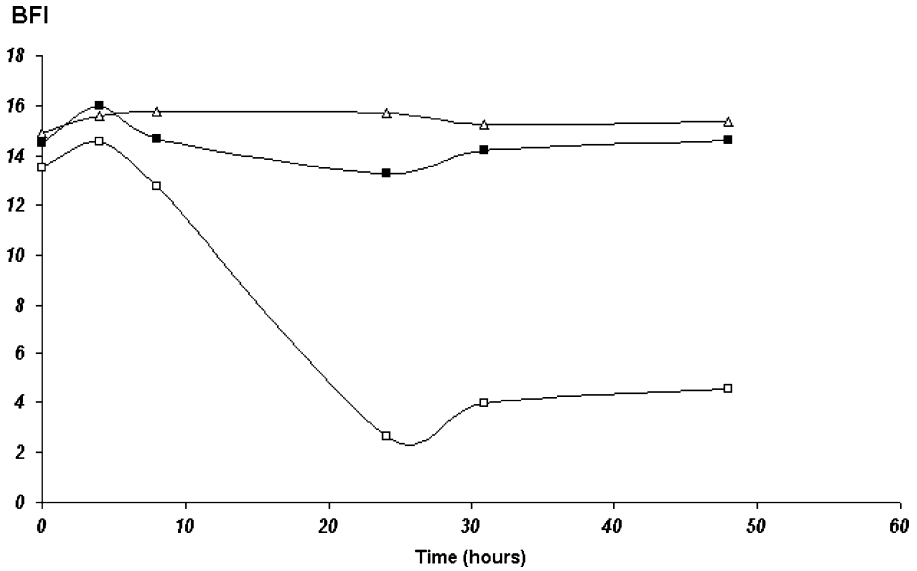

Fig. 3 Evolution of BFI during incubation of *Leuconostoc mesenteroides* in BHI medium supplemented with dextranases and nucleases: (—□—) *Leuconostoc mesenteroides*, (—■—) *Lc. mesenteroides*+ dextranases, (—△—) *Lc. mesenteroides*+nucleases

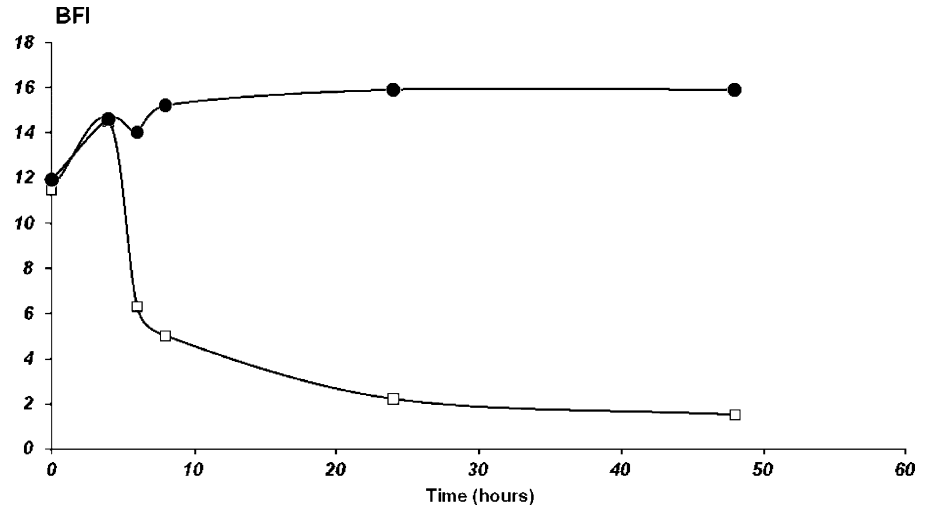

Fig. 4 Evolution of BFI during incubation of *Leuconostoc mesenteroides* in BHI medium supplemented with protease: (—□—) *Lc. mesenteroides* (—●—) *Lc. mesenteroides*+proteinase K

In the presence of dextranases (Fig. 3), no biofilm was detected after 48 h of incubation. In comparison with the effect of BFI measurement of sterile BHI supplemented with commercial dextran, the dextranase activities probably limited the viscosity of the solution. In this context, hydrolyzed dextran did not prevent beads to form a spot. This corroborated that this polysaccharide was implied in the biofilm formation by *L. mesenteroides*.

It is surprising to note that the same results were obtained using nucleases (Fig. 3). Indeed, when ribonucleic acid and deoxyribonucleic acid are depolymerized, the spot was visible after 48 h. Therefore then function of nucleic acids in biofilm formation by *L.*

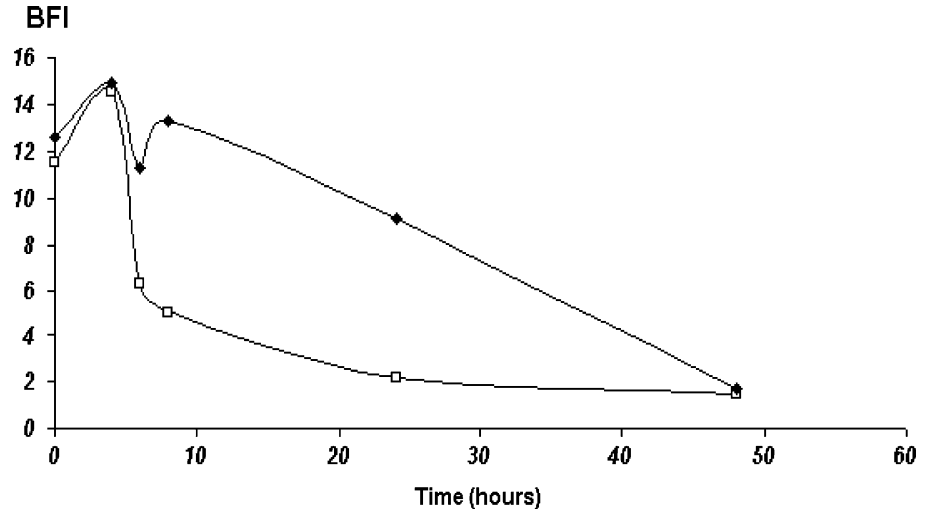

Fig. 5 Evolution of BFI during incubation of *Leuconostoc mesenteroides* in BHI medium supplemented with a mix of enzymes: (—□—) *Lc. mesenteroides*, (—◆—) *Lc. mesenteroides*+dextranases+proteinase K

mesenteroides was revealed by this experiment. The role of these macromolecules was at this time not really depicted as they are present in low quantities (<1–2%) in the matrix [1].

To evaluate the implication of proteins, a protease with low substrate specificity (proteinase K) was added in the culture medium (Fig. 4). Proteinase K prevents the formation of the biofilm. This result reinforces the implication of proteins in the elaboration of the biofilm structure. However, this action of protease could be indirect as dextran is synthesized by extracellular enzymes, the dextran sucrases [13]. So the proteolytic degradation of these glycosyltransferases could stop the biofilm formation by lack of dextran in the extracellular medium. A mixture of proteinase K and dextranases was tested in the following experiment (Fig. 5). In this context, the impact of proteinase K addition in culture medium on biofilm formation was lower than that observed when the proteinase K was added alone. This result underlines the role of dextran. Effectively, if proteinase K digests dextranases, its action against dextran sucrases could decrease. This residual dextran synthesis associated to the presence of nucleic acids could be sufficient to increase the solidity of the matrix. Consequently, the BFI declines longer. To conclude, the implication of the principal macromolecules of the matrix was underlined in the *L. mesenteroides* biofilm with a new rapid method. A clear role of nucleic acid, proteins, and dextran in the elaboration of biofilm has been identified. Another interesting aspect would be to associate proteases with dextranases and/or nucleases to compare their blended action on biofilm formation. The effect of the enzyme/substrate ratio could also be interesting to appreciate notably the role of proteins.

Understanding the impact of the biofilm matrix offers a good way to fight against biofilms. The BioFilm Ring Test® appears as a practical tool to study antibiofilm and probiofilm molecules. Effectively, it is well known that microorganisms associated in a matrix structure are 100- to 1,500-folds more resisting confronted to antibiotics [14]. It would be also a helping technique to identify strains capable of forming a biofilm in the food industry, a line of business that kicks daily against them [15].

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