Use of an Enzyme-Linked Lectinsorbent Assay To Monitor the Shift in Polysaccharide Composition in Bacterial Biofilms

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An enzyme-linked lectinsorbent assay (ELLA) was developed for quantification and characterization of extracellular polysaccharides produced by 1- and 4-day biofilms of 10 bacterial strains isolated from food industry premises. Peroxidase-labeled concanavalin A (ConA) and wheat germ agglutinin (WGA) were used, as they specifically bind to saccharide residues most frequently encountered in biofilms matrices: β-glucose or β-mannose for ConA and N-acetyl-β-glucosamine or N-acetylmuramic acid for WGA. The ELLA applied to 1- and 4-day biofilms colonizing wells of microtiter plates was able to detect that for Stenotrophomonas maltophilia and to a lesser extent Staphylococcus sciuri, the increase in production of exopolysaccharides over time was not the same for sugars binding with ConA and those binding with WGA. Differences in extracellular polysaccharides produced were observed among strains belonging to the same species. These results demonstrate that ELLA is a useful tool not only for rapid characterization of biofilm extracellular polysaccharides but also, in studies of individual strains, for detection of changes over time in the proportion of the exopolysaccharide component within the polymeric matrix.

Biofilm is defined as a community of “cells immobilized on a substratum and frequently embedded in an organic polymer matrix of microbial origin” (7). The main polymers of this matrix are polysaccharides and proteins (10, 14) which could play a role in survival of biofilm bacteria to stresses (1, 6, 35). The most common methods developed to quantify exopolysaccharides are designed for those produced by planktonic bacteria. When adapted to biofilms, these techniques present some limits: successive steps (5, 33) may lead to loss of part of the material (3); solubilization of the exopolymers is dependent on the choice of the extraction fluid (30); and because the quantity of extracellular substances present in biofilm is small (microgram range), it is often necessary to increase the total area colonized by the cells in order to detect these products (5).

Easier approaches involve the use of specific dyes directly applied to biofilms (13, 32, 34). However, these cationic dyes, whose specificity to polyanions was empirically established, are not always reliable as detectors of exopolysaccharides (12, 15). As underlined by Sutherland (29), there is a need for development of methods for the in situ analysis of small amounts of exopolysaccharides capable of detecting relatively minor differences.

The binding specificity of lectins toward simple sugars appears to be a specific way to characterize and quantify exopolysaccharides. The specificity of lectins has been widely used in microbiology for the determination of components of microbial cells (17, 22). The emergence of fluorochrome-conjugated lectins allowed for the direct visualization of the extracellular substances of biofilm by epifluorescence microscopy (12, 23, 24, 25). More recently, Thomas and coworkers (31) successfully developed an enzyme-linked lectinsorbent assay (ELLA) to quantify in situ the N-acetyl-β-glucosamine components of biofilm exocellular matrix material produced by Staphylococcus epidermidis.

In this paper we describe a similar ELLA using two lectins that recognize saccharide residues most frequently encountered in biofilm matrices (8): concanavalin A (ConA), which binds to β-glucose and β-mannose residues (17, 22); and wheat germ agglutinin (WGA), which recognizes specifically N-acetyl-β-glucosamine and N-acetylmuramic acid, a sialic acid (26). This assay was applied to 1- and 4-day biofilms belonging to various bacterial genera in order to determine (i) if the use of one lectin is sufficient to monitor biofilm exopolysaccharide production over time and (ii) which strains could be characterized by the lectin binding methods.

(Materials and methods)

Strains and culture conditions. Micrococcus sp. strain C714.1, Brevibacterium linens B337.1, coryneform E629.2, Pseudomonas fluorescens E9.1, P. fluorescens D32.2, Stenotrophomonas maltophilia B110.1, and coagulase-negative staphylococci (Staphylococcus sp. strains C778.1, E601.1, and E512.2) were obtained from the laboratory culture collection of SOREDAB (La Boisserie Ecole, France) and were isolated from surfaces of cheese factories in France. Staphylococcus sciuri CCL101 was isolated from the floor of a catering business and belongs to the stock culture collection of AFSSA Lerpac (Maisons-Alfort, France). Long-shelf-life stock cultures stored at ~80°C and monthly stock cultures placed at 3°C were made according to the procedure of Leriche and Carpentier (21). Bacterial suspensions adjusted to 5 × 10^8 CFU ml^-1 in physiological saline were prepared as described by Leriche and Carpentier (21).

Biofilm development in microtiter plates. Biofilms were developed according to the procedure of Leriche and Carpentier (21), adapted to microtiter plates as substratum: 100-μl volumes of each bacterial suspension containing 5 × 10^8 CFU ml^-1 in physiological saline were placed into eight wells of a sterile 96-well microtiter plate (Luxlon, CML, Angers, France). Cells in the microtiter plate were incubated at 25°C and 95% relative humidity for 3 h to allow adhesion. The nonadherent bacteria were then removed by washing each well with 200 μl of sterilized ultrapure water (MillipQ; Millipore, Saint-Quentin en Yvelines, France) delivered by a handheld multichannel pipettor. The wash volume was pipetted steadily, and 100 μl of a 1:20 dilution of tryptic soy broth (TSB; BioMérieux, Marcy-l’Etoile, France) supplemented with yeast extract (YE; 6 g liter^-1; BioMérieux) was deposited on the adherent bacteria prior to incubation at 25°C and 95% relative humidity for 20 h. Again the nonbiofilm bacteria were removed via washing with sterilized ultrapure water. This preparation constituted a 1-day
biofilm. Four-day biofilms were developed with the same incubation conditions as above but with new culture medium added daily following washing for 3 consecutive days. The last wash of 1- and 4-day biofilms was performed with 300 μl of sterilized ultrapure water that was subsequently removed by inverting the plate.

Estimation of the amount of exopolysaccharides produced by biofilms using ELLA. (i) Preparation of the peroxidase-linked lectin stock solutions. One milligram of peroxidase-labeled ConA from Canavalia ensiformis (Jack bean; Sigma, St. Louis, Mo.) and 1 mg of peroxidase-labeled WGA from Tritium vulgaris (Sigma) were diluted in 1 ml of sterilized ultrapure water and in 1 ml of phosphate-buffered saline (PBS; NaCl, 8 g liter⁻¹; KCl, 0.2 g liter⁻¹; Na₂HPO₄, 1.15 g liter⁻¹; KH₂PO₄, 0.2 g liter⁻¹; pH 7.3), respectively. These 1 mg ml⁻¹ solutions were divided in 100-μl aliquots and stored at –20°C until use.

(ii) ELLA applied to biofilms. Peroxidase-labeled lectin solutions stored at –20°C were diluted in PBS containing 0.05% (vol/vol) Tween 20 diluting buffer to obtain final concentrations of 10 μg ml⁻¹ (ConA) and 1.25 μg ml⁻¹ (WGA). Two hundred microliters of the peroxidase-labeled lectin solution was added to the first of eight wells colonized by the biofilm, and 100 μl was transferred stepwise from the first well to mix with 100 μl of diluting buffer previously added to the second well. Serial half dilutions were therefore performed into the remaining wells. Clean wells or wells covered with growth medium for the same contact time as used for biofilms before being rinsed were submitted to the same procedure and used to estimate the nonspecific binding in the ELLA response.

Microtiter plates were placed at room temperature for 1 h to allow the lectin to bind to the saccharide moieties of the biofilm exopolysaccharides. Peroxidase-labeled lectin solutions were removed from the wells by inverting the plates and tapping on absorbent paper. Following three successive washes with 200 μl of diluting buffer to eliminate unbound enzyme conjugate, the linked peroxidase conjugate was visualized following addition of 100 μl of freshly mixed commercial 2',2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solutions recommended by the manufacturer (Kierkegaard & Perry Laboratories, Gaithersburg, Md.). The reaction was allowed to develop for 15 min in darkness, and the absorbance was measured at 405 nm with a microplate reader (Multiskan RC; Labsystems, Cergy-Pontoise, France). Like in enzyme-linked immunosorbent assay (ELISA) techniques, the quantity of polysaccharide adsorbed to the surface of the wells was estimated by plotting the optical density at 405 nm (OD₄₀⁵) versus the logarithm of the concentration of the peroxidase-labeled lectin added: the greater the saccharide component present, the less peroxidase-labeled lectin needed to reach the plateau of the sigmoidal curve.

(iii) ELLA applied to polysaccharides adsorbed onto surfaces. ELLA was performed using dextran and xanthan (Sigma). One-hundred-microliter aliquots of serial dilutions of 0.5 mg ml⁻¹ aqueous sugars solutions were placed in wells (eight wells per solution) of a sterile 96-well microtiter plate and incubated at 25°C, 95% relative humidity for 3 h. Wells were then rinsed by adding 300 μl of sterilized ultrapure water. The quantity of saccharidic components adsorbed onto the surface was evaluated by the peroxidase-linked ConA assay described above.

Endogenous peroxidase activity. (i) Detection of bacterial peroxidase. Cultures stored at 3°C were transferred to tryptic soy agar (Difco) slopes and incubated for 24 h at 25°C, followed by transfer to Lowenstein-Jensen medium slopes (BBL Microbiology Systems, Baltimore, Md.) and incubated for 24 h at 25°C. The presence of peroxidase was detected according to the procedure used for Mycobacterium species (19).

(ii) Influence of the endogenous peroxidase activity on the ELLA response. To check whether the bacterial peroxidase interfered with the action of the peroxidase conjugates, each biofilm colonized with a 3-day biofilm was overlaid, after the final wash, with a 100-μl volume of 0.02% (wt/vol) sodium azide (Sigma) or sterilized ultrapure water (control) for 10 min. The solutions were removed by inverting the plates, and wells were rinsed two times. Plates were subjected to the ELLA as described above.

Estimation of bacterial attachment to the substratum. Bacterial attachment was estimated indirectly by the crystal violet microplate bacterial adhesion assay described by Shea and Williamson (27), with the following modifications. The dye was removed by aspiration with a pump using a fine-tip Pasteur pipette, and the wells were then rinsed three times by the addition of 300 μl of sterilized ultrapure water followed by inversion of the plate and taps on absorbent papers. A significant linear relation was found between the OD measured at 560 nm with this method and the number of CFU per square centimeter (data not presented).

Statistical analysis. Analysis of variance was performed with Statgraphics software (version 3.3; Manugistics, Rockville, Md.).

RESULTS AND DISCUSSION

Dose-response curves with pure dextran and xanthan adsorbed to surfaces. For each pure bacterial extracellular polysaccharide, (dextran and xanthan) adsorbed onto the surface, the intensity of the ELLA signal was positively correlated with the initial sugar concentration (Fig. 1). OD₄₀⁵ values measured for dextrans (Fig. 1a) were higher than those obtained with xanthan (Fig. 1b). These lower values associated with xanthan are likely due to the fact that xanthan contains several residues which are not all recognized by ConA, whereas dextrans are exclusively composed of α-D-glucopyranosyl units (8).

This finding shows that the signal generated with the ELLA was proportional to the number of simple sugar residues adsorbed for 24 h to the biofilm surface.

Use of ELLA to quantify exopolysaccharides produced by biofilms. (i) Influence of nonspecific bindings in the ELLA response. ConA did not bind to the clean surface, as average values of OD₄₀⁵ remained low (0.05) for all concentrations of lectin conjugate tested (Fig. 2a). When molecules of the growth medium were allowed to adsorb for 20 h onto the surface, the OD₄₀⁵ increased as the concentration of peroxidase-labeled ConA increased, but it did not exceed 0.3 for the highest concentration of the lectin conjugate tested. With WGA (Fig. 2b), OD₄₀⁵ Values obtained following adsorption of growth medium remained low and constant (range, 0.05 to 0.1). In common with ELISA techniques, surfaces of the microtiter plates are saturated with protein solutions in order to quench and minimize nonspecific binding. In our case, low OD values obtained on clean surfaces or on surfaces covered with growth medium suggest that there were no or very few lectin-specific sites present and that pretreatment of the microtiter plates was not necessary.

(ii) Bacterial peroxidase activity. Many bacteria possess peroxidase enzymes (11) which could induce an oxidation of the chromogen (ABTS) and thereby a false-positive response. Endogenous peroxidase production was detected in all bacteria tested except in Staphylococcus strain; P. fluorescens D32.2 gave the strongest response (data not shown). To determine whether this peroxidase activity interfered with the peroxidase-
linked lectin sorbent assay, 1-day biofilms of *P. fluorescens* D32.2 and *Micrococcus* strain C714.1 were treated for 10 min with a solution of sodium azide, a cytochrome oxidase inhibitor, and extents of ABTS oxidation of treated and untreated biofilms were compared by the peroxidase-labeled ConA assay (Fig. 3). Results show that for both peroxidase-producing strains, sodium azide-treated and control biofilms displayed the same dose-response curve, suggesting that endogenous peroxidase activity which may exist in biofilms was not sufficient enough to interfere with the peroxidase-linked lectin sorbent assay.

(iii) Repeatability and reproducibility. Repeatability of the peroxidase-linked lectin sorbent assay was evaluated on 1-day biofilms by comparing the OD values obtained in the same biofilm following several repetitions of the assay. When the assay was applied to the growth medium adsorbed to the surface or 1-day biofilms, the coefficients of variation of the measurements did not exceed 15% (data not shown).

Curves representing OD$_{405}$ versus the logarithm of the concentration of the peroxidase-labeled lectin obtained from three (Fig. 2a) or two (Fig. 2b) separate experiments were superimposed (for clarity, only the average OD$_{405}$ values obtained for each strain are represented). The coefficients of variation of the measurement calculated for each strain were lowest when the OD$_{405}$ ranged from 0.6 and 1. For these OD intervals, coefficients of variations were less than 14.7% (ConA) and less than 19.7% (WGA). Since the sigmoidal curves (Fig. 2) were clearly well separated from each other at a zone of OD$_{405}$ ranging from 0.6 to 1, a cutoff value of 0.8 was chosen to estimate the amount of exopolysaccharide produced by 1-day biofilms. This estimation was realized in each experiment by determining the logarithmic concentration of the lectin conjugate necessary to add to the wells to obtain an OD of 0.8. Analysis of variance of the results (Fig. 2) indicates that the reproducibility of the method using peroxidase-labeled ConA is sufficient enough to distinguish three significantly different groups ($P < 0.0001$) among the six biofilms studied: coryneform (group 1), whose lectin was the most productive of glucose and mannose residues; *S. sciuri* and *Micrococcus* sp. (group 2); and *S. maltophilia*, *B. linens*, and *P. fluorescens* D32.2 (group 3), whose exopolysaccharides contain few mannose and glucose residues. When WGA was used as the lectin conjugate, four significantly different groups of biofilms ($P < 0.0001$) were detected: *P. fluorescens* D32.2 (group 1), whose exopolysaccharides were rich in WGA-specific binding residues; *S. sciuri* (group 2); *Micrococcus* sp. (group 3); and coryneform species, *B. linens*, and *S. maltophilia* (group 4).

**Exopolysaccharide diversity.** As mentioned above, the amount of exopolysaccharide produced by 1- and 4-day biofilms was estimated from the sigmoidal curves obtained for each bacterial strain by determining the logarithmic concentration of lectin conjugate needed to obtain an OD of 0.8. Two of the ten strains studied did not produce detectable ConA-specific binding sugars: *P. fluorescens* E9.1 and *Staphylococcus* sp. strain E512.2 (Fig. 4a). All *Staphylococcus* species appeared to produce great amounts of N-acetylglucosamine and/or N-acetylmuraminic acid in their extracellular matrix (Fig. 4b). Hussain et al. (18) previously observed that the slime of coagulase-negative staphylococci consists of teichoic acids containing N-acetyl-β-glucosamine and/or N-acetyl-μ-glucosamine mixed with a small quantity of several proteins. But among the four strains of *Staphylococcus* spp. studied herein, one (*S. sciuri*) exhibited a high content of ConA-specific binding residues, whereas the amount of such residues was below the detectable level of the method for another strain (*Staphylococcus* sp. strain E512.2). A great difference also appeared between the two strains of *P. fluorescens*: although both strains had nearly the same population (Fig. 5), no signal or only a very slight signal was detected for *P. fluorescens* E9.1 with peroxidase-labeled ConA and WGA, whereas vast amounts of WGA binding exopolysaccharides...
were detected in *P. fluorescens* D32.2 biofilms. Such differences in exopolysaccharide production may help to explain difference in behavior of the two strains observed toward colonization by *L. monocytogenes* (20).

Change of extracellular polysaccharide content over time. The amounts of extracellular polysaccharides, when detected, were higher in 4-day biofilms than in 1-day biofilms for the majority of strains (*P*, 0.007 for both lectins tested). This is in agreement with many works reporting that the synthesis of extracellular substances increases with the age of biofilms (2, 28, 37). It is interesting, however, the production of exopolysaccharides was not correlated with the biofilm population, evaluated with the crystal violet staining method (Fig. 5). This is in agreement with previous results obtained by Bayston and Rodgers for *Staphylococcus epidermidis* (cited in reference 31). For some strains, increased exopolysaccharide production was observed although the population of 1- and 4-day biofilms was stabilized (*S. maltophilia*, *B. linens*, and *Staphylococcus* strain C778.1). For others, an increase of exopolysaccharides occurred when the biofilm population either increased (*Micrococcus* sp.) or decreased (*Staphylococcus* strains E601.1 and CCL101; coryneform). It has already been reported that exopolysaccharides can consolidate the adhesion of the bacterial cells on the surfaces (4) or, conversely, can promote their detachment (36).

The average ratios between the concentration of conjugated ConA and the concentration of conjugated WGA necessary to reach an OD of 0.8 were calculated for 1-day (*R*<sub>1D</sub>) and...
mer components could help us to better understand some peculiar behaviors detected within biofilms. However, for most of the strains studied herein, the use of only one lectin was insufficient to monitor biofilm exopolysaccharide production over time. The use of several, complementary lectins specific to different sugars may constitute a further tool to study biofilm extracellular polymers and could help us to better understand the role of saccharidic components within the biofilm community. Unfortunately, the currently available enzyme-labeled lectins do not allow for the detection of all simple sugars. There is, to our knowledge, no lectin specific for the uronic acids of bacterial alginates [linear polymer of β-(1→4)-d-mannuronic acid and α-(1→4)-l-guluronic acid], polysaccharides secreted by several species like Pseudomonas aeruginosa and Azotobacter vinelandii (35). The use of cationic dyes (Alcian blue (34)) and/or enzyme-linked antibodies (ELISA) (for example, specific to alginate (16)) in association with ELLA could help in situ characterization of biofilm exopolysaccharides.

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REFERENCES


