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Resistance of Biofilms Containing Alginate-producing Bacteria to Disintegration by an Alginate Degrading Enzyme (AlgL)

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Pure culture biofilms of *Pseudomonas aeruginosa* (strains 8830 and ATCC 700829) and mixed population biofilms composed of *Pseudomonas aeruginosa* (ATCC 700829), *Pseudomonas fluorescens* (ATCC 700830), and *Klebsiella pneumoniae* (ATCC 700831) were treated with an alginate-degrading enzyme (AlgL). The enzyme effectively depolymerized the mannuronic acid rich (92%), partially *O*-acetylated bacterial alginate produced by *P. aeruginosa* (8830), both in dilute solution and in a gel-like, concentrated state. However, both biofilms were unaffected by the presence of the enzyme. These findings suggest either that bacterial alginates do not contribute significantly to the cohesiveness of biofilms or that the alginate is protected from enzymatic degradation in biofilms.

Keywords: biofilms; alginate lyase; AlgL; bacterial alginate; biofilm structure; EPS

Symbols:

$[\eta]$	Intrinsic viscosity
η_{sp}	Specific viscosity
a	The exponent used to calculate intrinsic viscosity from the molecular weight
K	The pre-exponential constant used to calculate intrinsic viscosity from the molecular weight
c	Polymer concentration (w/v)
k	Pseudo-first-order rate constant for the cleavage of glycosidic linkages
k'	Constant in Huggins equation
M_0	The monomer equivalent weight
M_W	Molecular weight (weight average)

INTRODUCTION

The cohesiveness, gel-like appearance and viscoelastic properties of biofilms have led to the widely recognized (although sometimes questioned) view that such properties are caused by extracellular, high molecular weight polymers, termed extracellular polymeric substances (EPS). In contrast to the extensive knowledge of the chemistry of extracellular (capsular or soluble) polysaccharides produced by industrially or medically important bacteria when cultured by conventional techniques, little is

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known about the identity, concentration, and distribution of EPS involved in biofilm formation. A general problem is the lack of experimental methods that permit identification and quantification (including spatial distribution) of specific EPS within biofilms, as opposed to the large number of methods that may be used for dissolved biopolymers. In addition, the amount of EPS in biofilms may be very low. The EPS that is responsible for the formation of the cohesive intercellular, hydrated gel may not be the major EPS. Non-gelling proteins and other macromolecules may dominate, even if they do not contribute to biofilm cohesiveness. There is a clear need for novel approaches in order to identify the role of specific EPS in biofilms. In this work, the potential of highly specific enzymes that will depolymerize only certain EPS, in this case bacterial alginates, is explored.

Bacteria such as *Pseudomonas aeruginosa* and *Azotobacter vinelandii* produce alginates (Rehm & Valla, 1997). Alginates are linear polysaccharides composed of two different sugar residues, namely 1,4-linked β-D-mannuronic acid (M) and its 5-epimer, 1,4-linked α-L-guluronic acid (G). Bacterial alginates, especially those produced by *Pseudomonas* sp., differ considerably from the well-known algal alginates (obtained from brown algae such as *Laminaria hyperborea*, *Macrocystis pyrifera* and others) that are used industrially as gelling agents (Skjåk-Bræk *et al.*, 1986; Christensen, 1999). The polymer chains of algal alginates contain numerous blocks (>5 consecutive residues) of L-guluronic acid (G-blocks), thus enabling intermolecular cross-linking *via* selective binding of Ca^{2+} ions to form gels. In contrast, *Pseudomonas* alginates do not contain G-blocks. In this case, the G residues (0–40%) occur as single residues (..MGM.. type), and such alginates will not form rigid gels in the presence of Ca^{2+}. In addition, bacterial alginates are commonly *O*-acetylated (M residues), which further counteracts gelation with Ca^{2+}. The only type of gels that normally may be expected in *Pseudomonas* alginates are entanglement gels. This is a general mechanism of gelation requiring polymers of high molecular weight at relatively high concentrations, but no cross-linking agent (Christensen, 1999). Such gels are unstable and dissolve (presumably slowly) upon dilution. However, other types of interactions with proteins or other polymers may result in some type of gelation, either by an associative mechanism or by phase separation (segrative mechanism).

It is thus unclear whether bacterial alginates are involved in the formation and turnover of biofilms, even though the bacteria are capable of producing alginates. One strategy to study the role of a specific polymer in a biochemical process is to use specific polymer degrading enzymes (Christensen, 1999). Both *P. aeruginosa* and *A. vinelandii* contain a gene (*algL*) encoding for an alginate lyase (AlgL), which effectively depolymerizes bacterial alginates *in vitro* (Boyd *et al.*, 1993; Ertesvåg *et al.*, 1998). It has been reported (Boyd & Chakrabarty, 1994) that the expression of AlgL in *P. aeruginosa* can be associated with biofilm detachment. It should be noted that this effect was only seen after overproducing the protein 100 times, and cell detachment was most pronounced when lyase production was induced simultaneously with biofilm development. It has further been speculated (Boyd & Chakrabarty, 1994) that the depolymerization of alginate resulting from the presence of AlgL was the mechanism whereby the weakening of the biofilm matrix and subsequent detachment took place. Thus, it is reasonable to expect that the addition of alginate lyase to a biofilm reactor will erode the biofilm by the same mechanism. The goal of this study was to test this hypothesis.

In the authors' previous studies, AlgL was produced, purified, and successfully used to degrade pure alginates, both of algal and bacterial origin (Ertesvåg *et al.*, 1998). In this work, AlgL activity was tested against pure

culture biofilms of *P. aeruginosa* (strain 8830), whose alginate production is well understood. The activity was further tested against pure culture biofilms of *P. aeruginosa* (ATCC 700829), a strain which has been frequently used in biofilm studies, but which has not been studied with respect to alginate production. Finally, a mixed biofilm containing *P. aeruginosa* (ATCC 700829), *P. fluorescens* (ATCC 700830), and *Klebsiella pneumoniae* (ATCC 700831) was investigated.

MATERIALS AND METHODS

AlgL

AlgL was obtained by culturing *Escherichia coli* strain JM109 (pHE113) (Ertesvåg *et al.*, 1998) encoding the *A. vinelandii* alginate lyase gene, *algL*, in L broth. The cells were harvested by centrifugation, washed, re-suspended and sonicated. AlgL was isolated from the resulting supernatant by filtering, followed by anion exchange chromatography (HiTrap Q, Pharmacia, Sweden) and hydrophobic interaction chromatography (HiTrap Phenyl Sepharose6 Fast Flow, Pharmacia). The detailed procedures have been described by Ertesvåg *et al.* (1998). Enzyme activity was determined by monitoring the absorbance at 230 nm caused by the formation of unsaturated urinates which are formed as a consequence of the lyase action on alginates (Ertesvåg *et al.*, 1998). One unit was defined as the amount of enzyme that increased the absorbance by 1.0 absorbance unit per minute. The rate of chain cleavage was calculated from the rate of decrease in the specific viscosity (η_{sp}) of a bacterial alginate solution containing AlgL as described by Hjerde *et al.* (1994) (see Results section).

Bacterial Alginate

Pure bacterial alginate was obtained from *P. aeruginosa* strain 8830 roughly as described by Skjåk-Bræk *et al.* (1986). In brief, the cells were subcultured at 32°C on medium A (Skjåk-Bræk *et al.*, 1986) for 48 h on a gyratory shaker, and then grown on agar plates [medium B (Skjåk-Bræk *et al.*, 1986)] at *ca* 24°C for 48 h. The cells and the viscous exopolymer were scraped off from the surfaces of the gels, and stirred for 1 h with 0.9% NaCl. The suspension was centrifuged and the alginate-containing supernatant was decanted off. This extraction procedure was repeated once. The combined supernatants were filtered (1.2 μm) and the bacterial alginate was precipitated by adding 1 volume of 2-propanol in the presence of 0.2% NaCl. The alginate was re-dissolved in pure water and lyophilized.

The chemical composition (content and distribution of G- and M-residues) and intrinsic viscosity were characterized using procedures described by Abrahamson *et al.* (1996).

Degradation of Alginate Gels

The ability of AlgL to erode a viscoelastic, concentrated solution of bacterial alginate was tested by transferring bacterial alginate (0.7 ml, 20 mg ml^{-1}) to a test tube. The solution was so viscous that no flow occurred during several minutes after turning the tube upside-down. Then, a drop of AlgL solution (1 unit ml^{-1}, 0.7 ml in 50 mM TRIS buffer, 0.35 M NaCl, pH 8.1) was placed on top of the gel. Mixing of the two phases was not observed during the time of exposure. The test tube was incubated at room temperature. The shifting position of the gel–liquid interface was measured after turning the tube upside-down for a few seconds, which also allowed mixing of the upper liquid phase.

Biofilms

In the present study, three biofilms were used, *viz.* 1) a pure culture of *P. aeruginosa* (strain 8830), 2) a pure culture of *P. aeruginosa* (ATCC 799829) and 3) a mixed population biofilm of *P. aeruginosa*

(ATCC 700829), *P. fluorescens* (ATCC 700830), and *K. pneumoniae* (ATCC 700831). Strain 8830 is a stable producer of bacterial alginate (Darzins & Chakrabarty, 1984) with well-characterized properties (Abrahamson *et al.*, 1996). ATCC 700830 has been used as a model organism in a series of biofilm investigations (Yang & Lewandowski, 1995; Beyenal & Lewandowski, 2000).

The biofilms were grown at room temperature on microscope slides (2.5×2.5 cm) and placed on the bottom of an open channel flow cell made of polycarbonate (3.5 cm deep, 2.5 cm wide, and 34 cm long with a total working volume of 120 ml) (see Xia *et al.*, 1998, for details). First, the microscope slides were placed at the bottom of the reactor, then the lid of the reactor was sealed with a silicon rubber to prevent contamination, and the entire system was sterilized with 70%. The reactor was then rinsed with sterile water (autoclaved) until all the alcohol was removed. Tubings, connectors, air filters and the growth medium were autoclaved at 121°C.

The broth solution was made of KH_2PO_4 (0.35 g l^{-1}), Na_2HPO_4 (1.825 g l^{-1}), $(NH_4)_2SO_4$ (0.1 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.01 g l^{-1}), yeast extract (0.01 g l^{-1}) and glucose (0.05 g l^{-1}). The glucose and yeast extract were autoclaved separately and then added to the growth medium. This medium is essentially the same as that used for producing bacterial alginate (medium B).

The following procedure was used to inoculate the reactors. One millilitre portions of frozen stock of *P. aeruginosa* (ATCC 700829), *P. fluorescens* (ATCC 700830), and *K. pneumoniae* (ATCC 700831) were inoculated into separate sterile flasks (50 ml working volume) containing growth medium (0.2 g l^{-1} yeast extract, 0.5 g l^{-1} glucose and the nutrients given above). The flasks were shaken for 24 h (150 rpm), and 15 ml of each culture from the flasks was used to inoculate the open channel flow cell. For pure culture *P. aeruginosa* biofilms, 30 ml of culture from the flasks were inoculated. The reactor was first operated in batch mode (recycling) for 12 h and then in the continuous feed mode 5–7 d.

Peristaltic pumps (Cole-Parmer, Chicago, IL) were used to maintain flow and recycle rates. The reactor was continuously fed with sterile growth medium. The recycle ratio (the ratio of volumetric flow rates of recycle and feed) was >100 and the hydraulic retention time was <30 min to prevent growth of microorganisms in suspension, and to keep the substrate concentrations constant along the reactor. Recycling was used to maintain a flow velocity of 2 cm s^{-1}, which provided the shear force necessary for the biofilms to adhere to the substratum. The recycled solution was continuously aerated in a mixing chamber before entering the reactor and the dissolved oxygen concentration was kept near the saturation value.

Biofilm Treatment

To test biofilm degradability by the AlgL enzyme, the microscope slides with the deposited biofilms were transferred to Petri dishes, 25 ml working volume. Before adding the enzyme, the nutrient solution was replaced with 0.025 M phosphate buffer containing 0.35 M NaCl, pH 8.1. In different tests, AlgL activities of 0.084 U ml^{-1} and 0.840 U ml^{-1} were used. The microscope slides with the biofilm were placed at the bottom of uncovered Petri dishes. Then the buffer solution was added to submerge the slides, and the petri dishes were placed on the stage of a Nikon Diaphot 200 inverted microscope. Mixing of the solution was accomplished by recycling the medium using a peristaltic pump set at a flow rate of 100 ml min^{-1}.

Observation and Quantification of Biofilm Structural Changes

The changes in biofilm structure were monitored through the bottom of the Petri dishes with a 4× objective (total magnification, 40×). Images of biofilms were captured by a computer software

using a COHU® camera (Closed Circuit, CA, model no 2222-1040/0000) and Flashpoint® frame grabber (Integral Technologies Incorporated, Indianapolis, IN). The images were stored in TIFF format, 640×480 pixels, using a commercial image analysis software, Image Pro® (Media Cybernetics, MD). After adding the enzyme, images of the biofilm were taken at 5 min intervals during the first 2 h and at 1 h intervals thereafter. From the biofilm images, the temporal changes of areal porosity, fractal dimension, and total length of cell cluster perimeters were determined. The measurements were done using an Image Structure Analyzer (ISA), a software package developed for the purpose of quantifying biofilm structure (Yang *et al.*, 2000). Images were converted from gray scale to binary using the gray-scale threshold value of 150 on 256 gray scale.

RESULTS AND DISCUSSION

Bacterial Alginate

A batch of bacterial alginate was isolated from *P. aeruginosa* strain 8830 following cultivation on agar. The major characteristics of this alginate have been reported previously (Abrahamson *et al.*, 1996). The present batch had an intrinsic viscosity of 1600 mg ml^{-1}. ^{1}H-NMR analyses showed that it contained 8% G-residues (F_G=0.08), but no GG diads or G-blocks, as expected for *Pseudomonas* alginates. The degree of *O*-acetylation was 0.71 (acetyl groups per sugar residue).

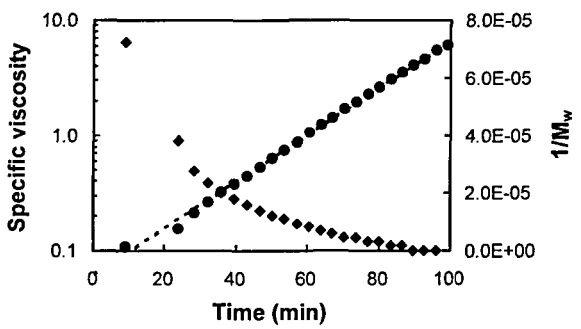

FIGURE 1 Depolymerization of *O*-acetylated alginate from *P. aeruginosa* by the alginate lyase AlgL. Alginate concentration: 0.9 mg ml^{-1}, AlgL: 0.091 U ml^{-1}, 50 mM tris buffer in 0.35 M NaCl, and pH 8.1. The measurements were carried out at 20°C. The specific viscosity (η_{sp}) (◆) was continuously monitored in a capillary viscometer. The corresponding weight average molecular weight (M_w) (●) was calculated from the specific viscosity.

Depolymerization of Alginate in Solution

The depolymerization of alginate in dilute solution (c=0.9 mg ml^{-1}) was investigated by continuous monitoring of the specific viscosity (η_{sp}) following addition of the enzyme (Hjerde *et al.*, 1994). This assay is much more sensitive than monitoring the formation of unsaturated urinates (absorbance at 230 nm). In addition, the solution viscosity and the molecular weight are more fundamental parameters which directly influences the gelation properties. The corresponding intrinsic viscosities ([η]) were calculated from the Huggins' equation ($\eta_{sp}/c = [\eta] + k'[\eta]^2 c$) using a value of 0.4 for the Huggins' constant (k') (obtained in a separate experiment). The weight average molecular weight (M_w) was further calculated by the Mark–Houwink–Sakurada equation, ($[\eta] = KM_W^a$), where the constant (K=0.014) and the exponent (a=0.93) had previously been determined for an alginate with 100% D-mannuronic acid (unpublished results). The enzyme concentration was kept low (0.091 U ml^{-1}). Typical results are shown in Figure 1. As expected for a random depolymerization of a linear polymer (Tanford, 1961), a linear increase in M_W^{-1} with time was observed and the *pseudo*-first-order rate constant for cleavage of glycosidic linkages (k) was directly calculated from the slope (slope=$k/2M_0$, where M_0 is the monomer equivalent weight). Substrate saturation was observed for alginate concentrations above 1 mg ml^{-1} (at 0.091 U ml^{-1}). The data in Figure 1 correspond to k=0.019 h^{-1}. It may be noted that an initially viscous solution ($\eta_{sp} \approx 10$) lost its viscosity in 30–60 min in the presence of

AlgL ($0.091\,U ml^{-1}$). The estimated weight average molecular weight after 60 min was about 25 kDa and after 24 h about 1 kDa, the latter corresponding to oligomers of 5 units on average, which is essentially complete degradation. No difference in the degradation kinetics was observed when using a phosphate buffer instead of tris buffer (at the same pH and ionic strength). In the absence of AlgL, the solution viscosity remained constant and no measurable degradation took place.

Depolymerization/erosion of a Concentrated Alginate Solution (Artificial Biofilm Model)

The ability of AlgL to erode a highly viscous and viscoelastic (gel-like) solution of bacterial alginate (a simple biofilm model) was tested by adding the enzyme ($1\,U ml^{-1}$) on top of the gel and measuring the decrease in the gel height. AlgL effectively eroded the gel from the top towards the bottom while maintaining a well-defined interface between the almost solid gel and the upper solution containing enzyme and, presumably, dissolved alginate (Figure 2). The average erosion rate was approximately $100\,\mu m\,h^{-1}$ (during the first 100 h), and the entire gel was dissolved in about 1 week. Thus, the ability of AlgL to erode bacterial alginate gel or viscoelastic solution was clearly demonstrated. It may be noted that the low molecular weight alginate fragments that accumulated in the liquid did not appear to inhibit the enzyme. No measurable degradation took place before adding the enzyme.

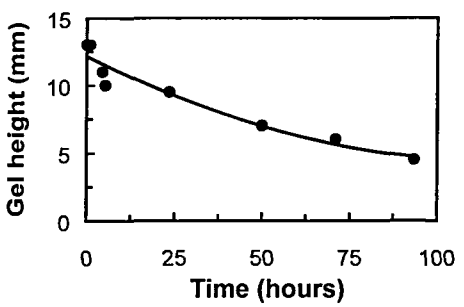

FIGURE 2 Surface erosion of alginate gel cylinder containing *O*-acetylated alginate from *P. aeruginosa* ($20\,mg\,ml^{-1}$) by the alginate lyase AlgL. The alginate was placed in a tube (0.7 ml volume and 13 mm height) and then 0.7 ml ($1\,U ml^{-1}$) of AlgL was added to the top of the gel.

Biofilm Treatment

To avoid changes in biofilm structure due to microbial growth during the test, the nutrient solution was replaced with a phosphate buffer at a desired pH. In separate tests, it was documented that the replacement of nutrient with the buffer did not cause significant changes in biofilm morphology or enzyme activity. Figure 3 shows images of the *P. aeruginosa* (strain 8830) biofilms, average thickness 250 μm, before addition of the enzyme (Figure 3A) and 24 h after adding 1 ml of AlgL (the activity of AlgL was $21\,U ml^{-1}$, which corresponded to the final enzyme concentration of $0.84\,U ml^{-1}$) (Figure 3B). The parameters characterizing biofilm structure measured before and 24 h after the addition of the enzyme were almost identical, *viz.* areal porosity = 0.62, fractal dimension = 1.3320, and total combined perimeter of the cell clusters = 15481 μm. The exposure to the enzyme for 24 h did not change the biofilm morphology. According to the erosion experiments above, the present enzyme concentration of $0.84\,U ml^{-1}$ should correspond to an erosion rate of approximately $84\,\mu m\,h^{-1}$ in a pure alginate gel. An effect of this order of magnitude would be detected easily in the present case. The tests were also extended to 48 h without visible effects on the biofilm structure.

These experiments were repeated using mixed biofilms composed of *P. aeruginosa* (ATCC 700829), *P. fluorescens* (ATCC 700830), and *K. pneumoniae* (ATCC 700831), and pure culture biofilms of *P. aeruginosa* (ATCC 700829). The results were similar to those described above as the enzyme did not appear to degrade the EPS matrix to any detectable extent.

If erosion of a biofilm with purified AlgL had been observed, it would strongly indicate that

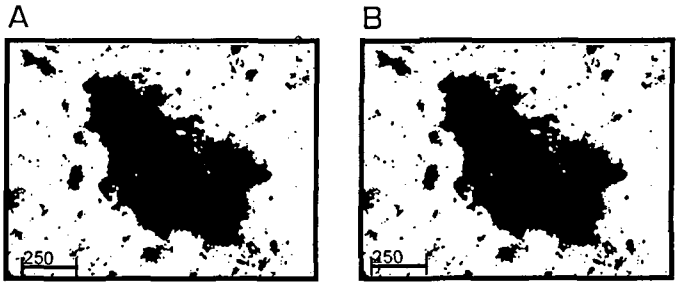

FIGURE 3 Structure of the biofilm made by *P. aeruginosa* (strain 8830). A = before addition of AlgL; B = 24 h after addition of 0.84 U ml^{-1} AlgL. Black areas are cell clusters. Scale bars = 250 μm.

alginate was the major macromolecular component in terms of forming a cohesive, gel-like structure. However, the observed inability of AlgL to degrade biofilms containing alginate-producing bacteria, especially in pure culture, may be interpreted in two different ways. One possibility is that even if alginate is the only exopolysaccharide which is known to be produced by these bacteria, it plays only a minor role in producing a cohesive, gel-like biofilm matrix. Other (unknown) EPS must therefore be involved. The present investigations were performed on a mature biofilm, and the possibility that the susceptibility towards alginate lyase may be different in earlier stages cannot be ruled out. This will be subject to subsequent investigations. Another possibility is that even if alginate is present and accounts for the gel-like properties of the biofilms, the action of alginate lyase may be prevented or inhibited. AlgL is a very robust enzyme, which operates well on dissolved alginates in dilute and concentrated solutions (pseudo-gel state), as shown above. Any action on the cohesive biofilm EPS with a rate constant comparable to that found in pure alginate systems would lead to detectable erosion or disintegration. Prevention or reduction of enzyme activity in biofilms may tentatively be ascribed to poor penetration into the biofilms, for instance due to limited porosity or binding to other biofilm components such as proteins. The need for future work that includes studies on the transport of enzymes and similar substances from the bulk liquid phase across the biofilm–liquid interface and into the biofilm gel-phase is clearly demonstrated.

CONCLUSIONS

Addition of the enzyme AlgL effectively degrades pure bacterial alginates both in dilute solutions and in a viscoelastic gel-like state. However, biofilms containing the alginate producing *P. aeruginosa* strain 8830 as well as strain ATCC 700829 (pure or mixed culture) appear to be totally resistant towards erosion or disintegration by the enzyme under otherwise similar conditions. The inability of AlgL to degrade biofilms may be caused by a) biofilms containing major structure-forming macromolecules other than alginate, or b) enzymatic degradation of bacterial alginate in biofilms may be retarded or prevented due to the presence of other macromolecules. These may interact with the enzyme by preventing its penetration into the cell clusters of biofilms.

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