Appl Microbiol Biotechnol (2002) 59:718–720
DOI 10.1007/s00253-002-1044-2

SHORT CONTRIBUTION

X. Chen · P. S. Stewart

Role of electrostatic interactions in cohesion of bacterial biofilms

Received: 19 November 2001 / Revised: 24 April 2002 / Accepted: 3 May 2002 / Published online: 30 July 2002

Abstract Significant decreases in the apparent viscosity of a bacterial biofilm suspension were measured following addition of sodium, potassium, magnesium, or calcium salts, whereas iron salts increased the viscosity. Electrostatic interactions contribute to biofilm cohesion and iron cations are potent crosslinkers of the biofilm matrix.

Introduction

Little is known about the cohesive forces that hold the matrix of a bacterial biofilm together, although there is a growing literature on the material properties of biofilms (Ohashi and Harada 1994, 1996, 1999; Mayer et al. 1999; Stoodley et al. 1999, 2001; Korstgens et al. 2001). The building blocks of the biofilm matrix appear to be polysaccharides and proteins excreted by the bacteria (Cooksey 1992). But how do the extracellular polymeric substances (EPS) made by one bacterium associate with the EPS produced by a second cell to form a larger structure? Some of the possible physical and chemical cohesive forces that could connect polymer strands include electrostatic interactions between oppositely charged polymer strands, electrostatic interactions between like-charged polymer strands bridged by multivalent counterions, physical entanglement, covalent bonding, hydrophobic interactions, and hydrogen bonding (Mayer et al. 1999). These forces are likely to be important in maintaining the multicellular structures that allow bacteria to cooperate metabolically and to withstand antimicrobial challenges. If the cohesive interactions in the biofilm matrix are better understood, it might become possible to design new strategies for controlling detrimental biofilms based on disrupting the matrix rather than killing the microorganisms.

One popular model of EPS cohesion is the *Pseudomonas aeruginosa* alginate paradigm (Davies et al. 1993). Alginate is an anionic polysaccharide, synthesized by *P. aeruginosa*, that can be gelled by the addition of divalent or trivalent cations. Calcium is the most often mentioned of these counterions. There is some experimental evidence showing a positive correlation between calcium concentration and amount of biofilm accumulation (Turakhia et al. 1983; Turakhia and Characklis 1989; Huang and Pinder 1995). Turakhia et al. (1983) showed that immediate and substantial detachment of a *P. aeru-ginosa* biofilm could be effected by addition of the calcium-specific chelant ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA). It is interesting that some electrolytes, such as sodium chloride, have been found to reduce bacterial EPS gel strength to the same degree as EGTA (Gordon et al. 1991). Stoodley et al. (1997) observed reversible contraction and swelling of biofilms subjected to pH changes and they hypothesized that this behavior resulted from a change in the fixed charge on the matrix polymers. Both electrostatic and hydrophobic interactions in the biofilm matrix of *P. fluor-escens* were implicated in a study by Marshall et al. (1989). The capsular polysaccharide of *Klebsiella pneumoniae* is known to contain uronic acid residues, so this polymer has the potential to participate in crosslinking interactions with multivalent cations.

We conjectured that if electrostatic interactions were involved in cohesion of the biofilm matrix, then the apparent viscosity of a biofilm suspension would be affected by changes in ionic strength and composition. This communication reports preliminary measurements of this type.

Materials and methods

Biofilm growth and sample preparation

Two-species biofilms of *P. aeruginosa* (ATCC 700888) and *K. pneumoniae* (ATCC 700831) were co-cultured on a minimal-salts

X. Chen · P.S. Stewart (✉)
Center for Biofilm Engineering and
Department of Chemical Engineering,
Montana State University – Bozeman,
Bozeman, Montana 59717-3980, USA
e-mail: phil_s@erc.montana.edu
Tel.: +1-406-9942890, Fax: +1-406-9946098

Current address:
X. Chen, SuperSolutions, Eden Prairie, Minn., USA

medium with 40 mg glucose l^{-1} as the sole carbon source. The medium contained (per liter): 14.4 mg NH_4Cl, 4.0 mg $MgSO_4 \cdot 7H_2O$, 382 mg Na_2HPO_4, 408 mg of KH_2PO_4, and trace elements as detailed by Chen et al. (1993). Experiments were conducted at room temperature (25±1 °C). Biofilms were grown on 316L stainless steel slides in a continuous-flow rotating annular reactor. Salient features of the reactor are summarized by Chen and Stewart (2000) and a detailed description of the reactor and operating conditions is given by Chen et al. (1993). The reactor was inoculated with 1.0 ml of thawed stock culture (10^8 cells ml^{-1}) of each microorganism and grown in batch mode for 24 h before starting influent flow. The biofilm was allowed to grow for 7–9 days before harvesting. The mean protein density at this time was 2.1±0.6 mg cm^{-2} and the common logarithm of the mean cell density (measured as colony-forming units, cfu) was 10.5±0.2 cfu cm^{-2}. A biofilm suspension was prepared by scraping the biomass from all accessible wetted surfaces of an annular reactor, yielding 20–30 ml of material, into 100 ml of phosphate buffer. This suspension was stored at 4 °C until used.

Viscosity measurement and analysis

The apparent viscosities of biofilm suspensions were measured in a cone and plate viscometer (model LVDV-11++CP, Brookfield Engineering Laboratories). Data collection and instrument operation were automated with a computer interface, using WinGather software supplied by the instrument manufacturer. The measurement temperature was controlled at 25.0±0.1 °C, using a water bath (Neslab model RTE-221) connected to a jacket on the viscometer plate. The rotation rate of the cone was fixed at 0.6 rpm to avoid shear thinning and to approximate the wall shear stress experienced in the annular reactor of approximately 4 dyne cm^{-2}.

A 0.5-ml volume of biofilm suspension collected as described above was loaded into the viscometer and data were recorded over a period of 5 min to establish the pretreatment viscosity. The cone and plate were then disassembled and 0.1 ml of the desired treatment solution was added. The viscometer was reassembled and the post-treatment apparent viscosity was recorded. Viscosity reduction percentages were calculated as the post-treatment viscosity divided by pre-treatment viscosity subtracted from one and multiplied by 100.

Results

Addition of salts or chelating agents to a biofilm suspension caused immediate changes in the apparent viscosity of the suspension ranging from an 87% reduction to a 56% increase (Table 1). When water was used as the treatment, there was a negligible change in the apparent viscosity (2% increase). Control experiments in which the viscometer was loaded with 0.5 ml of water rather than biofilm suspension and then amended with a treatment chemical showed that most treatment chemicals caused a slight increase in apparent viscosity. This shows that viscosity reductions measured with biofilm suspensions reflect a change in the physical properties of the biofilm material rather than a property of the treatment chemical itself.

Ionic treatments had little effect on the apparent viscosity of biofilm suspensions at ionic strengths below about 0.3 (Fig. 1). Isoosmotic treatments (equivalent to 0.3 M NaCl) with NaCl, $MgCl_2$, and $CaCl_2$ reduced the apparent viscosity by 82%, 87%, and 64%, respectively. Treatment with $FeCl_2$ and $Fe(NO_3)_3$ increased the apparent biofilm viscosity by 56% and 44%, respectively.

Table 1 Summary of viscosity reduction of biofilm suspension caused by various chemical treatments.

Treatment	Percent reduction ±SD in apparent viscosity
Control	–2±19
NaCl (0.3M)	82±4
$CaCl_2$ (0.21M)	64±12
$MgCl_2$ (0.21M)	87±4
LiCl (0.3M)	76±10
KCl (0.3M)	74±13
$FeCl_2$ (0.1M)	–56±28
$Fe(NO_3)_3$ (0.05M)	–44±33
EDTA (0.01M)	18±3
Dequest 2006 (1000 mg l^{-1})	50±8
Sucrose (0.47M)	5±9
Urea (2M)	46±4

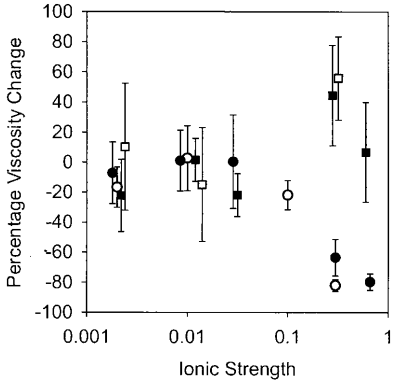

Fig. 1 Effect of ionic strength on the apparent viscosity of a biofilm suspension treated with NaCl (*black circles*), $CaCl_2$ (*white circles*), $Fe(NO_3)_3$ (*black squares*), and $FeCl_2$ (*white squares*). At ionic strengths (0.002, 0.01, 0.03) where data points overlap, the points have been jittered to allow individual error bars to be discriminated

Treatment with 0.47 M sucrose, which is osmotically equivalent to 0.3 M NaCl, induced only a 5% decrease in the apparent biofilm viscosity. Two chelants, ethylenediamine tetraacetic acid (EDTA) and a commercial calcium-chelating agent, Dequest 2006 (Monsanto, St. Louis, Mo.), which is the pentasodium salt of aminotri(methylene-phosphonic acid), resulted in significant viscosity reductions.

Discussion

These results implicate an important role for electrostatic interactions in crosslinking the matrix of this bacterial biofilm. Treatment of a biofilm suspension with chloride salts of lithium, sodium, potassium, magnesium, and calcium sharply reduced the apparent viscosity of a biofilm suspension. This does not appear to be an osmotic effect, since an isoosmotic dose of sucrose did not significantly reduce the apparent biofilm viscosity. As we reported (Chen and Stewart 2000), treatment of intact biofilm with NaCl, $CaCl_2$, or $MgCl_2$, resulted in the rapid de-

tachment of a significant percentage, averaging 43%, of the total biofilm protein. Increasing the ionic strength of the medium presumably screens out crosslinking electrostatic interactions, diminishing biofilm cohesiveness.

Mulivalent cations, calcium in particular, have been hypothesized to crosslink polyanionic matrix polymers in biofilms. It was therefore surprising that treatment with $CaCl_2$ gave rise to a reduction in apparent biofilm viscosity, comparable with that realized by NaCl. Following the hypothesis of calcium as a crosslinking agent would lead one to predict that treatment with $CaCl_2$ should increase the viscosity of a biofilm suspension. A possible explanation for this is that the biofilm was already saturated with respect to calcium. In this case, increasing the calcium concentration would not drive more calcium into crosslinking binding sites. The increased ionic strength of the $CaCl_2$ would, however, function to screen electrostatic binding interactions. The observation that iron salts increase rather than reduce the apparent viscosity of a biofilm suspension suggests that the biofilm was not saturated with respect to iron and also indicates that Fe^{2+} and Fe^{3+} are potent crosslinking cations. Trivalent aluminium may also be a particularly effective crosslinking cation. This is suggested by experiments in which a biofilm became much more rigid after exposure to Al^{3+} (Stoodley et al. 2001). The measurement of reductions in biofilm suspension viscosity following treatment with the chelating agents EDTA and Dequest 2006 is consistent with an important role for crosslinking multivalent cations in biofilm cohesion.

Urea caused a 46% reduction in the apparent viscosity of the biofilm suspension. This suggests a role for hydrogen-bonding interactions in crosslinking the biofilm (Mayer et al. 1999). Concentrated urea is known to disrupt hydrogen bonding.

Acknowledgements This work was supported by NSF award BES-9623233 and was made possible by facilities at the NSF-supported Center for Biofilm Engineering (Cooperative Agreement EEC-8907039).

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