Rapid evaluation of the antibiotic susceptibility of fuel ethanol contaminant biofilms


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A B S T R A C T
Bacterial contaminants from commercial fuel ethanol production facilities were previously shown to form biofilms as mixed cultures under laboratory conditions. In this study, a rapid assay was developed to simultaneously compare isolates for their ability to form biofilms as pure cultures. A total of 10 strains were isolated from a dry-grind fuel ethanol plant that routinely doses with virginiamycin. These were identified by sequence analysis as six strains of Lactobacillus fermentum, two strains of L. johnsonii, and one strain each of L. mucosae and L. amylovorus. Isolates exhibited a range of susceptibility to virginiamycin in a planktonic assay, with MIC’s (minimum inhibitory concentration) of ≤0.5–16 µg/ml. Even though all strains were isolated from a mixed culture biofilm, they varied greatly in their ability to form biofilms as pure cultures. Surprisingly, growth as biofilms did not appear to provide resistance to virginiamycin, even if biofilms were grown for 144 h prior to antibiotic challenge.

1. Introduction

Commercial fuel ethanol production is not conducted under pure culture conditions and chronic bacterial contamination is expected, even though it is believed to reduce ethanol yields (Connolly, 1999; Makanjuola et al., 1992; Narendranath et al., 1997). Contaminants compete with yeast for carbon and growth factors. Contaminants also produce byproducts that are inhibitory to yeast, particularly lactic and acetic acids (Narendranath et al., 2001; Thomas et al., 2002). At times contamination can lead to “stuck” fermentations, requiring that facilities be shut down for cleaning. In a survey of corn-based fuel ethanol plants, most contaminants were identified as species of lactic acid bacteria, with the genus Lactobacillus predominating (Skinner and Leathers, 2004). Penicillin and virginiamycin are commercially sold to treat bacterial infections of fuel ethanol fermentations, and some facilities use these antibiotics prophylactically (Hynes et al., 1997; Bayrock et al., 2003). Although the overall efficacy of antibiotic use is unclear, it does appear that the use of antibiotics may select for resistance among contaminants (Bischoff et al., 2007). Fuel ethanol contaminants were recently shown to produce biofilms in mixed cultures under laboratory conditions (Skinner-Nemec et al., 2007). Biofilms are generally considered to be more resistant to antimicrobials than free-living planktonic cells (Gilbert and Brown, 1995; Stewart et al., 2004). Biofilms are also more resistant to cleaning efforts, and this may explain the persistence of contaminants observed in fuel ethanol plants (Skinner and Leathers, 2004).

In our previous study, mixed culture biofilms were produced in the laboratory from fuel ethanol fermentor samples (Skinner-Nemec et al., 2007). In the current study, we compare pure cultures for their ability to form biofilms and for their resistance to virginiamycin. Our previous studies were performed using the CDC biofilm reactor, which provides accurate measurements of biofilm growth with the ability to regulate a number of parameters. However, only one organism can be tested at a time in this reactor, and each assay requires approximately 23 L of medium under our conditions. More rapid methods have been developed for simultaneous assays of numerous organisms under various conditions. The Calgary Biofilm Device utilizes 96-well microtiter plates (MTP) fitted with a lid with 96 pegs (Ceri et al., 1999; Ali et al., 2006). O’Toole and Kolter (1998) developed a colorimetric assay based on crystal violet staining of biofilm growth in MTP wells. Gross et al. (2007) modified this method to rapidly measure biofilm growth on MTP replicator lid pins. In the present study, we further modified this method for use with lactic acid bacteria found as contaminants of fuel ethanol production. This technique will allow rapid assessment of biofilm-forming capacity and potential biofilm-inhibiting agents.
2. Methods

2.1. Isolation of bacterial strains

Fermentor samples from a commercial corn-based, dry-grind ethanol facility were used to inoculate a CDC biofilm reactor as previously described (Skinner-Nemec et al., 2007). A jacketed CDC biofilm reactor (Biosurface Technologies Corp., Bozeman, MT) equipped with stainless steel sample coupons and with a working volume of 350 ml was filled with deMan-Rogosa-Sharpe broth (Difco MRS, Becton Dickinson, Sparks, MD) and equilibrated at 37 °C with nitrogen sparging. The method was modified slightly to eliminate the need for the antifoam, by decreasing the flow of the N₂. The reactor was inoculated with 1.0 ml of fresh fermentor sample and incubated for 12 h with stirring at 100 rpm. The reactor was then shifted to continuous flow operation for biofilm growth, with fresh medium pumped through the reactor at 29 ml/min with stirring at 180 rpm. Biofilm reactor coupons were sampled by the method of Heersink (2003). Bacterial isolates were obtained from sample coupons as previously described (Skinner and Leathers, 2004). Isolates were single-colony purified three times on MRS plates, and incubated anaerobically at 37 °C using the BBL GasPak Anaerobic System (Becton Dickinson).

In subsequent experiments, pure cultures were grown in the CDC biofilm reactor under the same conditions using a 1 mL inoculum from a 24 h culture grown in MRS broth.

2.2. Identification of bacterial strains

Strains were identified by the sequences of their 16S rRNA genes as previously described (Bischoff et al., 2007). Primers were U1 (5'-CCAGAGGCGGTAATACG-3'), corresponding to nucleotides 518 to 537 of the Escherichia coli 16S rRNA gene) and U2 (5'-ATCAGYGTACCTTGTTACGACTTC-3', corresponding to nucleotides 1513 to 1491 of the same gene), as described by Lu et al. (2000). PCR utilized the HotMaster Taq DNA Polymerase kit (5 Prime, Gaithersburg, MD). Buffer contained 25 mM TAPS, pH 9.3, 2.5 mM MgCl₂, 50 mM KCl, 1.0 mM β-mercaptoethanol, and 0.2 mM dNTPs. Denaturation was at 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 45 °C for 10 s, and 65 °C for 50 s.

The resulting products (about 1000 bp) were sequenced by standard methods using the U1 primer. GenBank accession numbers are reported in Table 1. Sequences were compared with those in GenBank using BLASTn (Altschul et al., 1997) available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Putative species identifications were based on similar strains with the highest BLAST scores, which in all cases shared greater than 98% identity to a known species.

2.3. Determination of MIC for virginiamycin

Minimum inhibitory concentrations (MICs) for virginiamycin were determined by an antimicrobial susceptibility method analogous to those described by the Clinical Laboratory Standards Institute (CLSI). For virginiamycin, the agar dilution method was performed on MRS agar plates containing 2-fold serial dilutions of virginiamycin, starting with 64 μg/ml. Virginiamycin was purchased from Research Products International Corporation, Mt. Prospect, IL. Inocula were diluted to a density of 0.5 McFarland units, then spotted on agar plates and incubated for 24 h at 37 °C in an anaerobic chamber.

2.4. Rapid method for biofilm screening

The assay method used was a modification of that developed by Gross et al. (2007). A preinoculum culture of each isolate was grown anaerobically at 37 °C for 24 h in a 15 ml tube containing 10 ml of MRS broth. A master 96-well plate (Nunc, Denmark, polystyrene, non-treated) was prepared containing 150 μL of MRS in each well. Culture broth (20 μL) from each isolate was transferred into each of 8 wells. A 96 pin replicator lid (Nunc, Denmark, oxidized polystyrene) was placed on the plate to prevent contamination. The plate was incubated for 48 h under anaerobic conditions at 37 °C. After 48 h, the cells were agitated by pipet, and a new lid was dipped into the cells and transferred to a new plate with 150 μL of fresh media in each well. One row of 8 wells was left uninoculated to provide a medium control. Since the assay is destructive, replica plates were prepared (in duplicate) for each time point to be tested. All plates were preincubated for 48 h to correspond to the static adhesion phase of a conventional biofilm reactor. Subsequently all lids were transferred to fresh medium plates (time point zero). Biofilm formation was measured at 48, 96, and 144 h time points. At each 48 h time point, replica plate lids were either transferred into fresh medium or analyzed for biofilm production.

Analysis of the biofilm formation was performed by a protocol modified from Gross et al. (2007). The pins were washed in 200 μl of water using gentle agitation. The lids were then dried at 65 °C for 30 min, stained with 200 μl of a 0.1% crystal violet solution for 30 min at room temperature, and washed again in water. The plates were then dried again for 1 h at 37 °C. For destaining, the pins were immersed for 30 min at room temperature in a fresh MTP containing 200 μL of 5% ethanol per well. The amount of crystal violet staining, representing biofilm growth, was measured at 600 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Non-sterile liquid handling aspects of the assay were carried out by a Biomek FXP Laboratory Automation Workstation (Beckman Coulter, Brea, CA).

Table 1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strain number</th>
<th>Species</th>
<th>GenBank accession number</th>
<th>MIC* (μg/mL)</th>
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<td>HQ154615</td>
<td>4</td>
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<tr>
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<td>L. Johnsonii</td>
<td>HQ154616</td>
<td>4</td>
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<td>L. fermentum</td>
<td>HQ154622</td>
<td>≤0.5</td>
</tr>
</tbody>
</table>

* Minimum inhibitory concentration of virginiamycin for planktonic cultures.
Fig. 1. Rapid assay for biofilm growth. Isolates #1–10 were grown for 144 h in the absence of antibiotic.

Fig. 2. Rapid assay for biofilm growth. Isolates #1–10 were grown for 144 h in the presence of either 2 μg/ml (A) or 32 μg/ml (B) virginiamycin.
2.5. Biofilm antibiotic susceptibility assay

The antibiotic susceptibility assay for biofilms was similar to the rapid method for biofilm screening. Biofilm cultures (plate lids) were allowed to grow from 0 to 144 h in the absence of antibiotic, and then transferred to plates containing MRS medium with either 2 or 32 µg virginiamycin/mL and grown for an additional 48–144 h.

3. Results and discussion

3.1. Isolation of fuel ethanol contaminants

As described in Section 2, 10 organisms were isolated from mixed culture biofilms from a commercial, corn-based dry-grind fuel ethanol plant. Previous studies indicated that species of *Lactobacillus* are most common from such facilities (Skinner and Leathers, 2004). Consistent with this finding, all 10 isolates were identified by sequence analysis as strains of *Lactobacillus* sp. GenBank accession numbers are provided in Table 1. Six strains were identified as *L. fermentum*, two isolates were identified as *L. johnsonii*, and one isolate each was identified as *L. mucosae* and *L. amylovorus* (Table 1).

This fuel ethanol facility doses the fermentation tank with 0.13 ppm (final concentration) of the antibiotic virginiamycin in an effort to control bacterial contamination. Previously, we found that some contaminants were resistant to virginiamycin and other antimicrobials (Bischoff et al., 2007). Consequently, the minimum inhibitory concentration (MIC) was determined for planktonic cells of each isolate (Table 1). As shown, five isolates were susceptible to virginiamycin (MIC < 0.5 µg/mL), one isolate exhibited an MIC of 2 µg/mL, two isolates had an MIC of 4 µg/mL, and two had a relatively high MIC of 16 µg/mL. The maximal recommended application rate for this antibiotic is 6 ppm (6 µg/mL).

Although each strain was originally isolated from a mixed biofilm culture, it does not necessarily follow that a pure culture also will be able to grow as a biofilm. Natural biofilms involve complex interactions between community members (Molin et al., 2004). We wished to compare the ability of all 10 strains to grow as biofilms in pure culture. We also wished to test the effect of biofilm growth on susceptibility to virginiamycin, since biofilms are generally regarded as resistant to antimicrobials (Gilbert and Brown, 1995; Stewart et al., 2004). Testing in a conventional CDC biofilm reactor is time and labor intensive and requires many liters of growth medium. In order to facilitate biofilm studies of numerous combinations of strains and antibiotics, a rapid assay for biofilm growth was developed.

![Fig. 3. Rapid assay for biofilm growth. Isolates #1–10 were grown for 48 h in the absence of antibiotic, then, as shown in the figure, grown for an additional 144 h in the presence of either 2 µg/ml (A) or 32 µg/ml (B) virginiamycin.](image-url)
3.2. Rapid assay for biofilm growth

A rapid assay for biofilm growth was developed as a modification of the method of Gross et al. (2007). As detailed in Section 2, this assay utilizes 96-well microtiter plates equipped with 96-pin replicator lids. Lids are sequentially passed into plates containing fresh medium to promote biofilm growth, measured as crystal violet-staining biomass adhering to the replicator pins over time. This rapid assay allows multiple strains to be directly compared side-by-side. Compared with the conventional CDC biofilm reactor, the 96-well assay also greatly reduces the volume and cost of medium and antibiotics consumed. Furthermore, the rapid assay is considerably less labor intensive in terms of set-up, analysis, and clean-up.

As shown in Fig. 1, all 10 strains showed relatively low levels of crystal violet staining at 48 h of growth. Five strains (isolates #2, #3, #4, #5, and #7) showed no additional growth at 96 h (Fig. 1). However, two of these strains (#2 and #5) showed modest growth at 144 h (>0.1 OD600). The remaining five strains showed much greater biofilm growth at 96 h (isolates #1, #6, #8, #9, and #10). Isolates #6, #8, and #9 produced the most biofilm material at 144 h (Fig. 1). By ANOVA analysis (Student–Newman-Keuls Method, p < 0.05), these three isolates were significantly different from all other isolates, but not from each other at 144 h. L. fermentum strains (#1, #5, #6, #8, #9, and #10) all produced higher levels of biofilm formation (Fig. 1) than non-L. fermentum strains (#2, #3, #4, and #7), suggesting that L. fermentum may predominate in contaminant biofilms.

As a validation test of the rapid 96-well assay, isolates #5, #6, and #9 were tested for biofilm growth in a conventional CDC biofilm reactor as previously described (Skinner-Nemec et al., 2007). Isolates #6 and #9 showed similar cell densities of 6.8 ± 0.2 (standard error) log10 (cfu/cm²) and 7.2 ± 0.1 log10 (cfu/cm²), respectively. By Student’s t-test, these values are not significantly different. Modest biofilm-producing isolate #5 showed a lower cell density of 5.2 ± 0.0 log10 (cfu/cm²). This value is significantly lower than those of the higher biofilm-producing strains at p < 0.07. Poor biofilm-forming isolates, such as #3, #4, and #7, were not tested in the CDC reactor because cell densities would have fallen below the detection limit for this method, approximately 3.0 log10 (cfu/cm²). Thus, quantitative measurements of biofilm growth in a CDC biofilm reactor corresponded well with biofilm growth in the rapid 96-well plate assays.

Fig. 4. Rapid assay for biofilm growth. Isolates #1–10 were grown for 96 h in the absence of antibiotic, then, as shown in the figure, grown for an additional 144 h in the presence of either 2 μg/ml (A) or 32 μg/ml (B) virginiamycin.
3.3. Effect of antibiotics on biofilm formation

All 10 biofilm isolates were grown in the rapid 96-well plate assays in medium containing antibiotic (virginiamycin) from time 0 (following a 48 h pre-incubation for adhesion and establishment of biofilms). Two concentrations of virginiamycin were tested, 2 μg/ml (Fig. 2A), and 32 μg/ml (Fig. 2B). In the lower concentration of antibiotic, only isolates #6 and #8 showed modest growth at 144 h (>0.1 OD$_{600}$). By comparison, in the absence of antibiotic these strains reached an OD$_{600}$ of approximately 0.4 (Fig. 1). Importantly, these two strains also exhibited the highest MIC for virginiamycin (16 μg/ml, Table 1). All other isolates were strongly inhibited by virginiamycin at 2 μg/ml. By ANOVA (Student–Newman-Keuls Method, $p < 0.05$), isolates #6 and #8 were similar at 144 h, but differed significantly only from poorest growing strains #9 and #10. When 32 μg/ml were added at time 0, only strains #6 and #8 showed detectable growth at 144 h. Even isolates that grew well as biofilms, such as #9, failed to grow unless they had a high MIC for virginiamycin. In this case, isolates #6 and #8 were significantly different from all other isolates. Thus, growth as biofilms failed to protect these strains from antibiotic, contrary to the generally accepted notion.

However, it is possible that more well established biofilms gain resistance to antibiotic. In order to test this idea, biofilms were grown for 48 h and then challenged with virginiamycin for an additional 144 h (Fig. 3). At 48 h, isolates grew to an OD$_{600}$ of approximately 0.05–0.1. In the presence of only 2 μg/ml virginiamycin, only isolates #6 and #8 (with high MIC’s for antibiotic) showed further growth (Fig. 3A). All other strains showed a gradual decline in crystal violet staining. At 144 h, only isolates #6 and #8 were not significantly different by ANOVA. At 32 μg/ml virginiamycin, this effect was even more pronounced (Fig. 3B). These results indicate that 48 h of established biofilm growth does not provide resistance to antibiotic.

As a further test, biofilms were grown for 96 h in the absence of antibiotic and then challenged with virginiamycin for an additional 144 h (Fig. 4). In this case, isolates #1, #6, #8, #9, and #10 had grown considerably to an OD$_{600}$ of 0.2–0.3. Again, except for highly resistant strains #6 and #8, even a challenge of 2 μg/ml resulted in a decline in OD$_{600}$. Strains #6 and #8 were inhibited but resumed growth after 96 h. While strains #6 and #8 differed significantly from all other strains at 144 h, they were not significantly different from each other (ANOVA).

Finally, biofilms were grown for 144 h in the absence of antibiotic and then challenged with virginiamycin for an additional 144 h (Fig. 5). Once again, all isolates except for #6 and #8 showed a decline in OD$_{600}$ even in 2 μg/ml virginiamycin (Fig. 5A). Strains
#6 and #8 also were inhibited but resumed growth after approximately 48 h. Again, strains #6 and #8 were significantly different from all of the other strains, but were not significantly different from each other. Thus, even 144 h of biofilm growth did not provide resistance to antibiotic.

4. Conclusions

This study shows for the first time that bacterial contaminants of fuel ethanol production can grow as biofilms in pure culture, and that this ability is strain-specific. Thus, biofilms may play a role in the persistence of contaminants in production facilities. Resistance to virginiamycin was commonly found among these contaminants, and this may limit the efficacy of antibiotic treatments. However, contrary to generally accepted notions, growth as biofilms did not appear to provide resistance to the antibiotic.

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References


