A putative enoyl-CoA hydratase contributes to biofilm formation and the antibiotic tolerance of *Achromobacter xylosoxidans*

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**INTRODUCTION**

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein that resides at the apical surface of many epithelial cell types. CFTR defects result in abnormal chloride and bicarbonate transport, increasing mucus viscosity in the pancreas, paranasal sinuses, digestive tract, and most notably, the lower airways. Viscous mucus, due to its impaired clearance and abundant nutrient bioavailability, in turn becomes chronically colonized by pathogenic bacteria that are the leading cause of mortality among the CF population. For decades, *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been extensively characterized and recognized as the primary airway pathogens. More recently, however, many other multidrug-resistant opportunistic bacterial species have received attention for their association with CF disease progression.

Among them, *Achromobacter xylosoxidans* is notable given its association with poor pulmonary function scores and apparent patient-to-patient transmissibility. This aerobic, Gram-negative opportunistic pathogen has been found to colonize anywhere from 2 to 20 percent of CF subjects, though its prevalence has risen in recent years, sparking a renewed interest in its pathophysiology. Of particular concern is the intrinsic and acquired resistance of *A. xylosoxidans* to multiple classes of antimicrobial agents, including aminoglycosides, beta-lactams, carbapenems, chloramphenicol and fluoroquinolones, which presents a significant burden for infection control.

Also complicating treatment of *A. xylosoxidans* is its ability to form robust biofilms, thought to be the predominant in vivo lifestyle among CF pathogens. biofilms confer a highly protective growth environment that shields pathogens against environmental stress, antimicrobials and the host immune response. In addition, nutrient and oxygen gradients throughout microcolonies (or aggregates) result in slowed metabolism among biofilm cells which can also potentiate drug tolerance. The recalcitrance of biofilm cells to antibiotic exposure likely enhances the persistence of *A. xylosoxidans* during chronic infection of the CF lung.

Relative to canonical CF pathogens, comparatively little is known about the molecular mechanisms of biofilm formation and maintenance in *A. xylosoxidans*. This is due, in part, to the paucity of genetic tools available for manipulation of its genome. Therefore, the objectives of this study were two-fold: First, we sought to develop a tractable system to genetically manipulate *A. xylosoxidans*. Using this system, our second objective was to take a transposon mutagenesis approach to study the molecular basis of *A. xylosoxidans* biofilm formation. In doing so, we identified several gene products essential for biofilm development. We chose to further characterize a gene identified most frequently in our screen (*echA*) encoding a putative enoyl-CoA hydratase that, when disrupted, leads to a decrease in biofilm accumulation and increased susceptibility to multiple classes of antibiotics.

**RESULTS**

Identification of biofilm-defective mutants via transposon mutagenesis

To identify genetic determinants of biofilm formation in *A. xylosoxidans*, we generated a mutant library of strain MN001 using random transposon mutagenesis employing the mini-mariner transposable element. From this library, 15,000 mutants were screened for altered biofilm formation. In doing so, we identified a gene, *echA*, encoding a putative enoyl-CoA hydratase, which when disrupted, leads to a decrease in biofilm accumulation and increased susceptibility to multiple classes of antibiotics.

*echA* encodes an enoyl-CoA hydratase, which is a key enzyme in the biosynthesis of fatty acids and other lipids. Mutants in *echA* were found to have reduced biofilm formation, suggesting that the enzyme plays a role in biofilm development. Further studies are needed to determine the precise mechanism by which *echA* affects biofilm formation and to explore the potential for targeting this enzyme as a therapeutic strategy for treating CF infections.
crystal violet (CV)/microtiter dish assay. After an initial round of screening, 134 putative biofilm-defective mutants were identified that were then re-tested (n = 4) to verify mutant phenotypes. After secondary screening and eliminating mutants with general growth defects, 31 transposon mutants were confirmed to be defective in biofilm accumulation (Fig. 1, Table 1).

To identify DNA sequences (~200–400 bp) flanking transposon insertion junctions, mutants were screened by arbitrarily primed PCR. Insertions were mapped to loci predicted to encode various classes of gene products, including membrane proteins, glycosyltransferases, flagella, transcriptional regulators, in addition to several proteins with no assigned function (Table 1). Arbitrary PCR of three transposon insertion loci generated sequences with no homology to published gene sequences, and we were unable to obtain transposon-flanking sequences for five additional mutants. Of note, a putative enoyl-CoA hydratase, an exonuclease, and a propionate hydroxylase were each hit multiple times. We took interest in the gene encoding a putative enoyl-CoA hydratase (Axylo_0405) given the importance of homologous proteins in fatty acid signal biosynthesis and biofilm development among diverse bacterial species.

We named this gene echA (for enoyl-CoA hydratase A) and sought to characterize its effects on A. xylosoxidans biofilm physiology in greater detail.

### Table 1. List of independent biofilm-defective transposon mutants identified in this study

| Mutant | Axyl Locus Tag | Annotation | Insertion coordinate |
|--------|----------------|------------|---------------------|
| 18-C10 | 2959           | Extracellular serine protease precursor | 3300783 |
| 21-A5  | 0405           | Enoyl-CoA hydratase | 458026 |
| 21-B10 | 2974           | DoxX family protein | 3318225 |
| 21-F3  | 0405           | Enoyl-CoA hydratase | 458968 |
| 37-D9  | No sequence    |            |                     |
| 29-D11 | 1017           | Branched chain amino acid transport protein | 1140377 |
| 29-F4  | 3436           | Flagellar basal body rod protein FlgB | 3820439 |
| 34-H5  | 1568           | Carboxymethylenebutenolidase | 1767696 |
| 44-D4  | No sequence    |            |                     |
| 45-B11 | 1417           | Outer membrane protein assembly factor BamD | 1595377 |
| 52-D3  | 2959           | Extracellular serine protease precursor | 3296785 |
| 56-D9  | No sequence    |            |                     |
| 61-A2  | 4754           | Hypothetical protein | 5265710 |
| 61-G3  | 0328           | Hypothetical protein | 374132 |
| 68-D1  | 2959           | Extracellular serine protease precursor | 3303694 |
| 75-D6  | 0867           | Propionate hydroxylase | 961203 |
| 77-F11 | 2959           | Extracellular serine protease precursor | 3302134 |
| 79-B9  | 0165           | Glycosyltransferase CsbB | 200048 |
| 83-E10 | 0126           | Hypothetical protein | 152964 |
| 84-D4  | 0867           | Propionate hydroxylase | 961404 |
| 86-E10 | 0405           | Enoyl-CoA hydratase | 458180 |
| 87-B2  | 3872           | Glycosyltransferase MshA | 4315184 |
| 90-D7  | No sequence    |            |                     |
| 95-D11 | 1003           | Hypothetical protein | 1124527 |
| 103-G8 | 5298           | L-asparaginase | 5841569 |
| 114-B2 | 0867           | Propionate hydroxylase | 961364 |
| 114-E11| No sequence    |            |                     |
| 115-C6 | 0951           | LysR family transcriptional regulator DmiR | 1065410 |

Fig. 1  Biofilm formation by transposon mutants of A. xylosoxidans MN001. Mean crystal violet absorbance for each mutant is expressed relative to the parental wildtype strain. Error bars represent standard deviation (n = 4).
that have been described in diverse bacterial species for their role in mediating virulence, motility and biofilm development.\textsuperscript{24-29,33,34} In \textit{P. aeruginosa}, for example, the DSF \textit{cis}-2-decenolic acid (cis-DA) is known to play a critical role in biofilm dispersion.\textsuperscript{24,35} Therefore, to test whether the observed biofilm impairment phenotype in \textit{A. xylosoxidans} was also mediated by a DSF-like signaling mechanism, we exogenously added synthetic cis-DA (310 nM)\textsuperscript{35} to the \(\Delta echA\) mutant during biofilm development. Despite having no effect on the WT strain, addition of cis-DA to \(\Delta echA\) resulted in a significant restoration of biofilm biomass relative to the untreated control (\(p = 0.024\)) (Fig. 2). These data suggest that biofilm formation in MN001 is directly mediated by a DSF-like metabolite generated by an enoyl-CoA hydratase.

Since the CV staining approach used in the initial mutant screen relies on a dye that stains not only cells, but all biomass adhering to the microtiter plate, we elected to use additional biofilm assays to further characterize the biofilm phenotype of \(\Delta echA\), and whether disruption of the putative enoyl-CoA hydratase negatively impacts a specific stage of biofilm development (e.g. attachment, matrix production, maturation). We first used a modified adhesion assay\textsuperscript{36} to test for defects in attachment to the surface of a chamber slide. Under static conditions, \(\Delta echA\) exhibited no difference in surface attachment relative to the wildtype, demonstrating that initial stages of biofilm formation are not affected (Fig. 3a). We then used a common colony morphology assay that relies on Congo red staining as a macroscopic means of identifying the capacity for extracellular matrix production.\textsuperscript{37,38} Over the course of 6 days of growth, biofilm colonies showed no apparent differences in morphology or color between strains, suggesting that the \textit{echA} gene product has negligible effect on matrix production in \textit{A. xylosoxidans}. Finally, we used scanning electron microscopy to visualize microcolony ultrastructure in mature biofilms grown for 72 hours under static conditions (Fig. 3c). Relative to WT, the \(\Delta echA\) mutant exhibited notable phenotypic differences in biofilm architecture, corroborating observations made using the microtiter plate assay. First, the mutant strain demonstrated less robust biofilm growth and reduced surface coverage relative to WT. In addition, higher magnification images revealed a less-dense packing order to mutant biofilm cells, suggesting that cell-cell signaling mediated by enoyl-CoA hydratase-derived metabolite is central to biofilm ultrasstructural development in \textit{A. xylosoxidans}.

\(\Delta echA\) exhibits increased susceptibility to antibiotic treatment

Biofilm-associated bacteria can be orders of magnitude more tolerant to antibacterial compounds than their planktonic counterparts.\textsuperscript{20} Given its defect in biofilm formation and more diffuse nature of mature microcolonies shown by SEM, we hypothesized that the \(\Delta echA\) mutant would exhibit increased susceptibility to antimicrobial treatment. To test this, mature biofilms were grown statically in 8-chamber coverslip slides for 72 h and treated with various concentrations of two commonly used therapeutics to which \textit{A. xylosoxidans} is tolerant, levofloxacin and tobramycin (Fig. 4). Despite not showing any difference in minimum inhibitory concentrations for planktonic cells of the WT and mutant strains (100 \(\mu\)g/mL for tobramycin; 4 \(\mu\)g/mL for levofloxacin), Live/Dead staining of treated biofilms, visualization by microscopy (Fig. 4a), and quantification of dead biomass (Fig. 4b) revealed a statistically significant increase (\(p < 0.001\)) in susceptibility of the \(\Delta echA\) biofilm to both antibiotics relative to WT. These data demonstrate a central role of \textit{echA} in biofilm antibiotic tolerance.
**DISCUSSION**

*A. xylosoxidans* is recognized as an emerging nosocomial pathogen and is associated with a range of infections, including bacteremia, endocarditis, meningitis, and pneumonia in immunocompromised individuals. In addition, the prevalence of *A. xylosoxidans* is now estimated to be as high as 20% among individuals with CF, which is of increasing concern given its reported correlation to lung function decline, patient-to-patient transmissibility, and multi-drug resistance phenotypes. Unfortunately, little is known about the physiology and molecular biology of this pathogen, and a greater understanding is critically needed to inform new treatment strategies. This study is an important step in that direction as it identifies several molecular determinants of biofilm growth, thought to play a critical role in the persistence and pathogenesis of *A. xylosoxidans* in the CF airways.

The inherent and acquired drug resistance of *Achromobacter* spp. not only limits therapeutic approaches but has also led to a paucity of tools (i.e. selection markers) available for molecular biology studies of *A. xylosoxidans* and manipulation of its genome. Here, we systematically screened numerous vectors and determined that among antibiotics tested, only tetracycline and gentamicin resistance markers were compatible with both *E. coli* and *A. xylosoxidans*. Tet cassette was then used to generate (to our knowledge) the first transposon mutant library and a null deletion strain in *A. xylosoxidans*, facilitating a detailed study at the molecular level. These tools paved the way for addressing critical questions about the physiology of this bacterium and its role in pathogenesis.

Screening of our transposon library revealed several genetic determinants of biofilm formation. Statistically, genome coverage in our library was unsaturated (~92%), though 31 hits in genes in diverse functional categories suggests that the regulation of biofilm development in *A. xylosoxidans* integrates a complex network of cellular processes and environmental stimuli. Several homologs of genes identified in this screen have been linked to biofilm formation in other bacteria. For example, *IglB*, encoding a flagellar basal body rod protein, has been implicated in biofilm development in both *Bordetella bronchiseptica* and *P. aeruginosa*. It is noteworthy that other flagellar-associated genes are downregulated in *A. xylosoxidans* biofilms relative to planktonically grown cells. In addition, *LysR* family regulators, such as *Axylo_0951* that was identified in our screen, have been implicated in biofilm morphology and mucosal colonization in other respiratory pathogens such as *P. aeruginosa, Burkholderia cenocepacia, and Klebsiella pneumoniae*. Further characterization of these and other genes identified in our screen (see Table 1) will undoubtedly generate new insights into the many aspects of *Achromobacter* biofilm physiology that remain uncharacterized.

We took most interest in *Axylo_0405* (*echA*), encoding a putative enoyl-CoA hydratase (one of eight in strain MN001) with homology to *DspI* in *P. aeruginosa*. *DspI* and other bacterial homologs (e.g. *RpfF* in *X. campestris*) are key enzymes used in the synthesis of diffusible signaling factors (DSFs) that are monounsaturated fatty acids of medium chain length containing a cis-2 double bond thought to be central in their mechanism of action. These metabolites broadly regulate bacterial behaviors such as motility, iron uptake and virulence, and data presented here adds to the growing appreciation of their widespread role in biofilm development across diverse Gram-negative bacteria. While synthetic cis-DA was able to partially rescue the biofilm defect in *ΔechA*, the specific identity of the fatty acid signal produced by *A. xylosoxidans* remains to be determined. It is also possible that *A. xylosoxidans* may respond to multiple DSF-like signals produced endogenously or exogenously, as interspecies signaling mediated by cis-2 fatty acids has also been reported. Ongoing work is now aimed at characterizing the signal structure, its biosynthetic pathway, sensing mechanism(s) and downstream phenotypic effects in addition to biofilm development.

Respiratory pathogens that adopt a biofilm lifestyle in vivo exist in a protective environment against antimicrobials and host defense mechanisms. Furthermore, biofilm cells generally reduce their metabolic activity relative to planktonic cells, further enhancing their intrinsic resistance to therapy. *A. xylosoxidans* has been shown to form robust biofilms both in vivo and in vitro, which is corroborated by microscopy data presented here. However, our data demonstrating a heightened susceptibility of *ΔechA* to antibiotic treatment suggest that interfering with fatty acid-mediated cell-cell signaling may represent a viable approach to managing *A. xylosoxidans* biofilms in the lower airways, particularly when used in combination with conventional CF antibiotics. Here we elected to test two compounds to which *A. xylosoxidans* has high resistance, levofloxacin and tobramycin, though it will be interesting to test whether this effect holds true for other antibiotics with different mechanisms of action.

In summary, we developed a robust genetic system for use in *A. xylosoxidans* that we leveraged to generate a transposon mutant library. Further study of this library revealed a suite of genes essential for biofilm development in vitro and invoked an essential role for a putative enoyl-CoA hydratase in biofilm ultrastructure and tolerance to antimicrobials, likely mediated by a
cis-2 fatty acid signaling compound. While a clear picture of the clinical impact of *A. xylosoxidans* in CF airway disease is only beginning to emerge, the continued study of the molecular basis of its biofilm formation and physiology will undoubtedly lead to improved treatment strategies for this important respiratory pathogen.

**METHODS**

**Bacterial strains, plasmids, and growth conditions**

Bacterial strains used are listed in Supplementary Table 1. *A. xylosoxidans* MN001 and *Escherichia coli* B were grown in lysogeny broth (LB) at 37°C unless otherwise specified. When necessary, growth media were supplemented with ampicillin at 100 μg/ml (*A. xylosoxidans*), kanamycin at 20 μg/ml (*E. coli*), tetracycline at 30 μg/ml (*A. xylosoxidans*), carbonic anhydrase (300 μg/ml), or chloramphenicol (30 μg/ml). *E. coli* strain B2155 was supplemented with 360 μM diaminopimelic acid (DAP).

Transposon mutagenesis

The transposon delivery vector pTnTet containing the hyperactive mariner transposon\(^*\) was introduced by transfection into the *E. coli* donor bacterial strain B2155.\(^{55}\) *A. xylosoxidans* MN001 and *E. coli* B2155 (carrying pTnTet) were grown overnight in LB and LB containing chloramphenicol (30 mg/ml) and 360 μM DAP, respectively, at 37°C. Cells were combined in a donor-to-recipient ratio of 5:1, centrifuged at 4000 × g for 5 min, resuspended in 200 μL fresh LB-DAP and spot-plated (10 μL) onto LB agar. Mating proceeded for 8 h at 37°C, at which point cells were harvested and resuspended in 1 mL of LB. Cell suspensions were diluted 1:5, and 100 μL aliquots were plated on tetracycline agar and allowed to grow for 48 h at 37°C. Colonies were randomly selected for downstream biofilm assays.

Biofilm microtiter plate assay

To identify determinants of biofilm development, transposon mutants were screened using a modified crystal violet (CV) assay approach.\(^{25}\) Briefly, individual mutants were transferred to single wells of a 96-well microtiter plate containing 200 μL LB per well, and incubated while shaking at 37°C. Following 24 h of growth, 2 μL was transferred to 198 μL ABT medium [15 mM (NH\(_4\))\(_2\)SO\(_4\), 40 mM NaH\(_2\)PO\(_4\), 20 mM KH\(_2\)PO\(_4\), 50 mM NaCl, 1 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), and 0.01 mM FeCl\(_3\) supplemented with 0.5% casamino acids and 0.5% glucose\(^{25}\) in a new microtiter plate, and grown for an additional 24 h at 37°C in a humidified chamber. Plates were first measured spectrophotometrically (OD\(_{600}\)) to determine culture cell density. Supernatants were discarded, and plates were washed 3X with ultrapure water. Plates were dried in a bio-safety hood for 2.5 h and stained with 200 μL of 0.1% CV for 20 minutes. Plates were then washed 5X to remove excess stain, air-dried for 4 h, and 200 μL 30% acetic acid was added to each well. Following a 15 min incubation, CV absorbance was measured spectrophotometrically (OD\(_{595}\)) and normalized to culture density. Wells exhibiting less than 50% absorbance than the wildtype (*A. xylosoxidans* MN001) were considered putative hits (134 total) and were subjected to a secondary screen using the same protocol (n = 4 for each mutant). Transposon mutants showing a significant reduction in biofilm growth (31 total, as determined by a Mann-Whitney U test) were stored for further characterization.

Biofilm growth of MN001, its ΔechA derivative and complemented strain (see below) were also tested using the same microtiter assay. In these experiments, growth medium was supplemented with 2% ethanol to drive expression of the alcA promoter in pMB84 (below). When indicated, cis-2-decenoyl acid (F13807D, Carbosynth) was also added at a concentration of 310 nM.\(^{55}\)

Arbitrary PCR and mutant sequencing

An arbitrary-PCR-based approach\(^{27}\) was used to identify sequences flanking transposon insertion sites. The PCR method involved two rounds of reactions, with the first using a primer unique for the mariner transposon and one degenerate primer (pair 1, Table S2).\(^{56}\) The second round included nested primers (pair 2) unique to the transposon and 5′ end of the arbitrary primer for amplification of PCR products obtained in the first round. PCR products were sequenced at the University of Minnesota Genomics Center (UMGC) and were mapped to the *A. xylosoxidans* MN001 genome (Accession#:PRJNA288995).

Construction and complementation of an echA deletion mutant

To create a deletion vector compatible with *A. xylosoxidans*, pSV65\(^{55}\) was first digested using Apal. A tetracycline resistance cassette was then amplified from pEX18Tc\(^{55}\) using primer pair 3 (Supplementary Table 2) and digested with Apal. Vector and insert (1:3 ratio) were then ligated using T4 ligase, transformed into *E. coli* UQ950 and selected for on LB agar containing tetracycline (15 μg/mL). Colonies were screened using primers M13F and TetR (pair 4) to confirm insertion orientation. One plasmid, pBMB1, was selected for further analysis.

To generate an in-frame, unmarked deletion of *Axylo1_0405* (echA), ~1 kb sequences flanking echA were PCR amplified using primer pairs 5 and 6 (Supplementary Table 2). These flanking regions were combined and cloned into pMB81 digested with Spel and Xhol using Gibson assembly, resulting in pMB82 (Supplementary Fig. 1). This plasmid was then chemically transformed into UQ950, and positive ligations were screened by PCR. pBMB2 was then transformed into *E. coli* strain WM3064 and mobilized into *A. xylosoxidans* MN001 by conjugation. Recombinants were selected for on LB-tetracycline agar and double recombinants were selected for on LB agar containing 6% sucrose.

Complementation was achieved via exogenous expression of echA (Ax_0405) from pBBR1MCS-5.\(^{55}\) To do so, an alcohol-inducible promoter, alcA\(^{27}\) was first amplified from pGGA008 using primers alcA_F and alcA_R (Supplementary Table 2) before digestion with restriction enzymes HindIII and BamHI. Ligation into similarly digested pBBR1MCS-5 yielded pMB83 (pBBR1MCS-5::alcA). echA was then amplified from *A. xylosoxidans* MN001 genomic DNA using primers echA_F and echA_R, and digested with BamHI and SacI before ligation into pMB83 using T4 ligase. The resulting vector, pMB84 (pBBR1MCS-5::alcAechA; Supplementary Fig. 1), was transformed into *E. coli* UQ950. This vector was then introduced into *A. xylosoxidans* MN001 via conjugation with an *E. coli* donor strain WM3064, and transconjugants were selected on LB agar containing 300 μg/ml gentamicin sulfate. All constructs and positive transformants were verified by Sanger sequencing.

Attachment assay

A modified attachment assay\(^{66}\) was used to assess early attachment of *A. xylosoxidans* to a polystyrene substratum. Briefly, MN001 and its ΔechA derivative were grown for 18 h at 37°C in ABT followed by dilution 1:100 into fresh medium. Cells were then grown to mid-log phase (OD\(_{600}\) = 0.6) before dilution in ABT to an OD\(_{600}\) of 0.1. 200 μL of each culture was added to an 8-chamber coverslip slide (Ibidi, #80824) and incubated at 37°C for 1 h. Following incubation, slides were rinsed twice with 200 μL of PBS to remove unattached biomass, and attached cells were stained using SYTO 9 (Invitrogen) in PBS according to the manufacturer’s protocol. Substrata were imaged using an Olympus IX83 inverted fluorescence microscope with a transmitted light, Koehler illuminator and a 40X objective lens (Olympus). Four images per strain per biological replicate (n = 4) were captured on a Hamamatsu ORCA camera, and post-acquisition analysis was performed using FIJI software\(^{56}\) by calculating the integrated density of SYTO 9.

Colonies biofilm assay

MN001 and ΔechA were grown overnight in LB medium, diluted 1:100, and 10 μL was spotted on nutrient agar containing 1% tryptone, 1% agar, 20 μg/mL Coomassie Brilliant Blue, and 40 μg/mL Congo red.\(^{56}\) Plates were incubated at 37°C for 6 days and monitored daily for colony morphology.

Scanning electron microscopy

Overnight cultures were diluted 1:10 in fresh LB medium and were added to 48-well microtiter plates containing autocholored Aclar fluoropolymer film (Electron Microscopy Sciences, Hatfield, PA). Biofilms were grown for 48 h at 37°C, shaking at 50 rpm, and prepared for SEM using cationic dye stabilization methods\(^{55,56}\). Briefly, Aclar membranes containing biofilm growth were washed three times in 0.2 M sodium cacodylate buffer, and submerged in primary fixative (0.15 M sodium cacodylate buffer, pH 7.4, 2% parafomaldehyde, 2% glutaraldehyde, 4% sucrose, 0.15% alcian blue 8 G3) for 22 h. Samples were washed three more times prior to a 90 minute treatment with secondary fixative (1% osmium tetroxide, 1.5% potassium ferrocyanide, 0.135M sodium cacodylate, pH 7.4). After three final washes, biofilms were chemically dehydrated in a graded ethanol series (25, 50, 70, 85, 95 [2x] and 100% [2x]) before CO\(_2\)-based critical point drying. Aclar membranes were attached to SEM specimen mounts using carbon conductive adhesive tape and sputter coated with ~5 nm iridium using...
the Leica ACE 600 magnetron-based system. Biofilms were imaged using a Hitachi S-4700 field emission SEM with an operating voltage of 2 kV.

Antibiotic challenge
Biofilm antimicrobial susceptibility testing was performed using a chamber slide assay. Briefly, MN001 and ΔchaA were grown in LB overnight, diluted 1:1000 and grown to an OD_{600} of 0.6 before dilution to an OD_{600} of 0.5. In total 200 μL of each culture was then added to each well of an ibidi 8-chamber coverslip slide and incubated at 37°C in a humidified chamber. After 24 h, medium was replaced with fresh LB and incubated for an additional 24 h. Media was gently aspirated from each well, replaced with LB containing either tobramycin or levofloxacin (0, 10, 100, and 1000 μg/mL), and incubated at 37°C for 6 h. Cells were washed in sterile PBS to remove unattached biomass, stained for 15 minutes using the BacLight Live/Dead viability assay (Life Technologies, #L7012) and visualized by fluorescent imaging as described above. Integrated density for SYTO 9 and propidium iodide (PI) for each image was determined using Fiji, and percentage of dead biomass was determined by (average integrative density of PI)/(average integrative density of PI + integrated density of SYTO 9). Data were generated using four biological replicates (n = 4).

Prior publication
This work was previously published as a preprint.

Reporting Summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
All data generated during and/or analyzed during the current study are included in this article, its Supplementary information files, or are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
L.C. performed experiments, created figures, and helped write and edit the manuscript. B.B performed experiments, created figures, and helped edit the manuscript. C.P. and A.L. performed experiments. L.K. performed SEM experiments and helped edit the figures. R.H. contributed to the conception, experimental design, data interpretation, statistical analysis, and manuscript preparation.

ADDITIONAL INFORMATION
Supplementary information accompanies the paper on the npj Biofilms and Microbiomes website (https://doi.org/10.1038/s41522-019-0093-6).

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