Insights into Clostridium phytofermentans biofilm formation: aggregation, microcolony development and the role of extracellular DNA

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Biofilm formation is a critical component to the lifestyle of many naturally occurring cellulose-degrading microbes. In this work, cellular aggregation and biofilm formation of Clostridium phytofermentans, a cellulytic anaerobic bacterium, was investigated using a combination of microscopy and analytical techniques. Aggregates included thread-like linkages and a DNA/protein-rich extracellular matrix when grown on soluble cellobiose. Similar dense biofilms formed on the surface of the model cellulosic substrate Whatman no. 1 filter paper. Following initially dispersed attachment, microcolonies of ~500 μm diameter formed on the filter paper after 6 days. Enzymic treatment of both the biofilm and cellular aggregates with DNase and proteinase resulted in significant loss of rigidity, pointing to the key role of extracellular DNA and proteins in the biofilm structure. A high-throughput biofilm assay was adapted for studying potential regulators of biofilm formation. Various media manipulations were shown to greatly impact biofilm formation, including repression in the presence of glucose but not the β(1→4)-linked disaccharide cellobiose, implicating a balance of hydrolytic activity and assimilation to maintain biofilm integrity. Using the microtitre plate biofilm assay, DNase and proteinase dispersed ~60 and 30% of mature biofilms, respectively, whilst RNase had no impact. This work suggests that Clostridium phytofermentans has evolved a DNA/protein-rich biofilm matrix complementing its cellulytic nature. These insights add to our current understanding of natural ecosystems as well as strategies for efficient bioprocess design.

INTRODUCTION

Surface-attached microbial communities embedded in an extracellular polymeric matrix, known as biofilms, are a critical component of global biogeochemical cycles and thus proper ecosystem function (Madsen, 2011; Paerl & Pinckney, 1996). Biofilms can protect microbial cells by altering diffusion rates, which in turn provides for niche metabolic processes (Fux et al., 2005), increased proximity of cells, enzymes and substrate to surfaces to enhance substrate utilization (Lu et al., 2006), and improved robustness to environmental and antibiotic stresses (Moons et al., 2009; Zuroff et al., 2010). The formation of biofilms by cellulytic micro-organisms is of particular interest, and is thought to promote efficient cellulose hydrolysis and thus carbon recycling (Zhang & Lynd, 2005). Biofilm formation by cellulytic Clostridium spp. is, however, a relatively unstudied field with only a small number of previous reports (Desvaux, 2005; Dimitrache et al., 2013a, b; Wang et al., 2011). To date, no studies have described the details of biofilm formation by Clostridium phytofermentans. This natural isolate represents a strong candidate for the conversion of lignocellulose to biofuel based on its ability to degrade many lignocellulose components, produce ethanol at yields of ~0.32 g ethanol (g carbohydrate)−1 and cooperate with other fuel-producing microbes (Lee et al., 2012; Tolonen et al., 2011; Warnick et al., 2002; Zuroff et al., 2013). Clostridium phytofermentans has been observed to attach to cellulosic surfaces (Tolonen et al., 2011), which...
is presumably a mechanism to facilitate degradation of lignocellulosic substrates. Such behaviour is most energetically favourable when cells, enzyme and substrate are in close proximity, with an estimated optimum distance of ~500 μm (Lynd et al., 2002; Wang & Chen, 2009). In fact, a proposed mechanism by which the inactivation of the single cellulase (Cphy_3367) resulted in an inability of Clostridium phytofermentans to degrade cellulose is that this knockout interfered with the cells’ ability bind to the cellulose surface (Tolonen et al., 2009). The manner by which Clostridium phytofermentans attaches to and initiates cellulose hydrolysis, however, is still unknown and may contrast with other cellulolytic clostridia as Clostridium phytofermentans lacks cellulosomes (Warnick et al., 2002). Understanding this process will provide insight into naturally occurring carbon cycles and could allow for optimization of lignocellulosic biofuel conversion technologies.

The goal of this work was to perform a detailed microscopic analysis of Clostridium phytofermentans biofilm formation on model substrates as a means of understanding the organism’s surface attachment mechanisms. A wealth of information is reported here describing the formation of Clostridium phytofermentans biofilms on soluble, insoluble and non-nutritive substrates. We also report the discovery that Clostridium phytofermentans appears to utilize an extracellular matrix rich in both extracellular DNA (eDNA) and protein, and this mature biofilm matrix is highly susceptible to enzymic dispersal.

METHODS

Media and chemicals. GS2 medium (Cavedon et al., 1990) was used in the majority of experimental cultures with the following composition (g l⁻¹): 6.0 yeast extract, 2.9 K₂HPO₄, 2.1 urea, 1.5 KH₂PO₄, 3.0 tri-Na citrate·H₂O, 2.33 cyanisteine-HCl·H₂O, 1.0 MOPS, 0.1 MgCl₂, 0.6H₂O, 0.0113 CaCl₂ anhydrous, 0.000125 FeSO₄·7H₂O and 0.01 % resazurin. The salts solution (MgCl₂·6H₂O, CaCl₂ anhydrous and FeSO₄·7H₂O) and resazurin solution were made and sterilized separately, and added after autoclaving the base components. The pH of the basal components was adjusted to pH 7.0 prior to sterilization. Inoculum cultures contained 3 g l⁻¹ of a 100 ml aliquot of XT stock solution. The XT solution (200 ml of 1.5 mM PI stock solution in deionized water, 1 ml of 5 mM SYTO 9 in DMSO) were thawed and diluted with 0.5 ml distilled water to reach 0.5 mM. Then, 2 ml aliquots of the 0.5 mM solution were made into microcentrifuge tubes which were diluted to 100 μl at a final concentration of 0.8 μg ml⁻¹. SYTO 9 (Invitrogen) stocks (2 μl of 5 mM SYTO 9 in DMSO) were thawed and diluted with 18 μl distilled water to reach 0.5 mM. Then, 2 μl aliquots of the 0.5 mM solution were made into microcentrifuge tubes which were diluted to 100 μl at a final concentration of 10 μM. The diluted Calcofluor White solution was pipetted onto the rinsed filter paper strips and allowed to incubate protected from light at room temperature for 7 min. The diluted SYTO 9 solution was then added and the strip was incubated for another 5 min. The strips were then removed from the staining solutions and rinsed twice with 200 μl sterile distilled water. Strips were placed onto glass microscope slides and gently covered with glass coverslips. When propidium iodide (PI) staining was included, 8 μl of 1.5 mM PI stock solution in deionized water (stored at 4 °C protected from light) was diluted to 100 μl with water at a final concentration of 0.12 mM. This solution was used to stain the filter paper strips for 5 min prior to Calcofluor and SYTO 9 staining.

Bright-field and epi-fluorescence. Bright-field and epi-fluorescence microscopy was conducted using a Zeiss AxioScope-A1 with a Zeiss Strains and culturing conditions. Inocula originated from cultures of Clostridium phytofermentans prepared from cryogenically (~80 °C) frozen stocks in 7.5 ml screw-cap test tubes with 5 ml GS2 medium. Oxygen was removed from Clostridium phytofermentans culture medium by degassing (until de-pinked) in a Coy anaerobic chamber with a 1.5 % H₂/98.5 % N₂ atmosphere or by purging with O₃-free N₂. Initial cultures were allowed to grow at 32 °C to OD₆₀₀ ~0.3. Cultures were then restarted by placing 100 μl initial culture into 4.9 ml fresh GS2 medium. Experiments were initiated from these inoculum cultures by adding −100–300 μl inoculum to give an initial OD₆₀₀ ~0.0125–0.025, which corresponds to ~10⁶ c.f.u. ml⁻¹ (Myers et al., 2013). The amount of soluble carbohydrate carried into filter paper biofilm experiments was considered negligible as it did not permit growth of Clostridium phytofermentans without filter paper (data not shown).

Fluorescent marker construction and conjugation. Plasmid pQexpEG7 containing an anaerobic fluorescent protein (EC7) was constructed by digesting both pQexp (Tolonen et al., 2009) and the synthetic oligomer EC7 (Table S1, available in the online Supplementary Material) with BamHI and KpnI. The purified products were ligated overnight and transformed into Escherichia coli DH5α, which was used to isolate the plasmid for sequence verification and transformation into E. coli S17-1 (Simon et al., 1983) for conjugal transfer to Clostridium phytofermentans. The conjugation was performed in accordance with Tolonen et al. (2009) with a few minor changes; E. coli S17-1 was used as a conjugal donor on polyethersulfone membranes without the use of trimethoprim as dual selection against E. coli following mating.

Microscopy

Image manipulation. Only brightness, contrast and false colouring were applied to the images presented; no non-linear adjustments were performed. All images were converted from their original format to tagged image file format (TIFF). Images captured using the Olympus Fluoview 1000 microscope were manipulated using Olympus FV10-ASW Viewer software. All other images were manipulated using open-source ImageJ software (http://rsbweb.nih.gov/ij/).
EC Plan-NEOFLUAR × 100/1.3 oil immersion objective. No sample preparation was necessary for simple visualization of cells and cellular aggregates. The same final concentrations described above were used for SYTO 9 and PI staining with green (excitation at 470 ± 40 nm and emission at 525 ± 50 nm) and red (excitation at 545 ± 25 nm and emission at 605 ± 70 nm) fluorescent protein filters to visualize the two stains.

Confocal. Confocal laser scanning images were obtained using an Olympus Fluoview 1000 inverted microscope with UPlanAPO × 10/0.40, UPlanFL × 20/0.50 and PlanApo × 40/1.4 oil objectives, and laser excitation at 405 nm (10 mW) and 488 nm (10 mW) (blue argon). Additional images (Figs 5c and 6b) were captured with a Zeiss Cell Observer spinning disk microscope with a Zeiss EC Plan-NEOFLUAR × 10/0.3, EC Plan-NEOFLUAR × 20/0.5 or Plan-Apochromat × 63/1.4 oil objective, and 405 nm (50 mW), optically pumped semiconductor laser 488 nm (100 mW) diode laser and 561 nm (40 mW) diode laser for excitation.

Scanning electron. For scanning electron microscopy (SEM) images, samples were fixed for 24 h in a solution containing 2 % (w/v) glutaraldehyde and 0.025 M sodium phosphate, pH 7.2, followed by three washes in 0.1 M sodium phosphate buffer, pH 7.2. Samples were then washed in sterile water and dehydrated in ethanol for 24 h. Samples were coated with gold/palladium (60/40) for 2 min and visualized using a scanning electron microscope model JSM-5410 (JEOL).

Biofilm assay. Polystyrene microtitre plates were used for the biofilm quantification assay based on an adapted procedure (Djordjevic et al., 2002). Briefly, Clostridium phytofermentans was grown in DM medium anaerobically at 30 °C until reaching OD600 ~0.3, then 100 μl cell culture was added to fresh medium to achieve an initial OD600 ~0.0125. Aliquots of 200 μl were transferred into eight polystyrene microtitre wells per trial. Each plate included eight wells of non-inoculated DM medium and eight wells of inoculated DM + 3 g cellobiose l⁻¹ medium as controls. The cell turbidity in the microtitre plate wells was monitored using a microtitre plate reader (Bio-Rad or TECAN) at OD595. The mean OD595 from the non-inoculated control wells was subtracted from the OD595 of all test wells to normalize to background absorbance. To assay biofilm formation the medium was removed and the wells were gently rinsed three times to remove loosely associated bacteria. Plates were dried for at least 1 h at 35–60 °C and then each well was stained with 200 μl 0.1 % (w/v) crystal violet solution in water for 30 min. After staining, the plates were washed three times with sterile distilled water to remove the non-biofilm-associated crystal violet. At this point biofilms were visible as purple rings formed on the walls of the well. To destain the wells, 200 μl 95 % ethanol was added and allowed to incubate for 30 min. The ethanol extract in each well was then transferred to a new microtitre plate and OD595 values were measured. Unless otherwise specified, the assay was independently performed three times for all samples as the basis for calculating the SD. The mean OD595 for the biofilm from the non-inoculated control wells was subtracted from the test wells to account for possible background crystal violet staining.

Enzyme treatment. Stocks of DNase I (New England BioLabs), RNase A (Alfa Aesar) and Proteinase K (New England BioLabs) at 2000 U ml⁻¹, 20 mg ml⁻¹ (~80–100 U ml⁻¹) and 20 mg ml⁻¹ (30 U ml⁻¹) were obtained/prepared and stored at −20, 4 and −20 °C, respectively. All treatments were performed at room temperature without agitation for ~40–45 min at a final concentration of 45 KU (Kunitz units) ml⁻¹, 166 U ml⁻¹ and 60 U ml⁻¹, respectively. For aggregate treatments, 30 μl aggregate from an exponentially growing liquid culture grown on 10 g cellobiose l⁻¹ was placed with the appropriate volume of enzyme stock to reach the desired final concentration. For filter paper biofilm treatment, aliquots of the stock solutions were added to sterile distilled water to reach 200 μl at the desired final concentration and this solution was gently pipetted onto the filter paper samples. Enzyme treatments for the biofilm assay were performed by adding the appropriate volume of the stock solution directly to stationary-phase cultures grown in 96-well microtitre plates. Values for enzyme treatments of biofilms grown on polystyrene are given as a percentage of control biofilms, where 100 % is ~7.4 ± 2.5 OD595 of ethanol/OD595 of growth (i.e. biofilm/OD).

RESULTS AND DISCUSSION

Planktonic aggregate formation

Clostridium phytofermentans planktonic cultures grown on soluble cellobiose displayed slime-like cell aggregates (Fig. 1a, b) that were composed of tightly clustered cells linked together by fine thread-like structures (Fig. 1c–f). The ‘threads’ appeared to have a distinct structure as compared with the flagella noted on Clostridium phytofermentans when grown on xylan (Tolonen et al., 2011). Numerous studies have shown that thread or fibre-like appendages mediate aggregation and biofilm formation in a wide array of micro-organisms. Such non-flagellar filamentous appendages are referred to by various terms, such as fimbiae, curli, pili and filaments (Ottow, 1975). When combined with gyrotary shaking the aggregates periodically formed elegant string-like structures spanning a 5 ml liquid volume (Fig. 1b). In addition to the thread-like appendages visible by SEM, the aggregated cells were clearly embedded in additional extracellular polymeric substances (EPSs) as evidenced by their immobilization compared with their free-floating counterparts. A striking number of cells and endospores existed within the well-defined aggregate with only a small number present in the bulk medium (Fig. 1c, d). The native structure of the matrix material is difficult to visualize due to the destructive nature of electron microscopy preparation, which likely removes some of the EPSs. Nonetheless, it is clear that Clostridium phytofermentans produces a significant amount of extracellular material leading to compact cellular aggregation.

Fluorescent markers for Clostridium phytofermentans

To study Clostridium phytofermentans biofilm formation and the role of EPSs, a robust fluorescent marker was sought. A new class of anaerobic fluorescent proteins based on flavin mononucleotides (Drepper et al., 2010) was explored as a suitable marker. A synthetic Clostridium-optimized gene based on the protein sequence of Pp1 from Evocatal (called EC7) was inserted immediately downstream of the constitutive ferrodoxin oxidoreductase promoter in Qexp and then transferred to Clostridium phytofermentans via conjugation. Fluorescence was confirmed, although at long exposure times and with less photo-stability relative to a WT control stained with SYTO 9 (Fig. S1). These factors rendered the EC7 protein
impractical for confocal biofilm studies which require extended excitation times and intense emission; therefore, SYTO 9 was used for the remaining work to label living cells. A similar conclusion was made in a recent attempt to express the Pp1 protein in *Clostridium cellulolyticum* (Wang et al., 2013).

**Biofilm formation on filter paper**

*Clostridium phytofermentans* was cultured on Whatman no. 1 filter paper submerged in GS2 medium (without cellobiose) for up to 12.5 days. The substrate surface was monitored using CLSM throughout growth by removing, and thus sacrificing, sequential filter paper samples. The samples were stained with Calcofluor White and SYTO 9 to label cellulose and cells, respectively. Initially, *Clostridium phytofermentans* cells were found attached relatively homogeneously throughout the filter matrix with many cells in the bulk medium (Fig. 2a). Unlike *Clostridium thermocellum* (Dumitrache et al., 2013a), no apparent preference in orientation was observed; cells were found attached both parallel and perpendicular to the fibre. A shift from individual cell attachment to thick, multi-cellular biofilms encased in EPSs was apparent at ~3.5–6.5 days along with a significant decrease in the physical rigidity of the filter paper fibres. Eventually distinct areas outpaced others, leading to large microcolonies that had relatively uniform sizes of 200–500 μm (Fig. 3). As the microcolonies grew, they led to localized cellulose hydrolysis which was readily observable by the naked eye. The gases produced at these sites (e.g. CO₂ and H₂) caused bubble formation on and within the filter paper, which provided sufficient buoyancy for the filter paper to float to the surface of the medium (Fig. 2c, upper panel). A similar phenomenon was observed in *Caldicellulosiruptor obsidiansis* and *Clostridium thermocellum* where crating of cellulose substrates was accomplished by localized cell populations (Wang et al., 2011). Such local degradation has been thought to be a critical aspect of cellulose degradation (Costerton et al., 1987) and this evidence in *Clostridium phytofermentans* supports the notion that localized cellulose hydrolysis by surface-bound microbes is common among numerous species with different cellulose hydrolysis mechanisms. The fragility of the filter paper was corroborated by a hydrolysed, visually frayed structure at 12.5 days (Fig. S2). The fine fibrous material which is observed to cover the control (non-inoculated) filter paper fibres was completely degraded and the fibres themselves were almost unrecognizable. At 12.5 days these filter paper biofilms were composed predominantly of endospores with some vegetative cells and cells with terminal endospores.

**Adaptation of the 96-well biofilm formation assay**

In an effort to better understand biofilm regulation and composition, a high-throughput method for quantifying biofilm formation was developed. Microtitre plate biofilm assays are routinely used for assessing biofilm formation (e.g. Djordjevic et al., 2002) and therefore a similar method was sought here. Exponentially growing *Clostridium phytofermentans* cultures were inoculated into fresh medium, placed as 200 μl aliquots in wells of a polystyrene 96-well microtitre plate and allowed to incubate for up to 5 days. Crystal violet staining revealed biofilm as purple stains on the wells of microtitre plates (Fig. 4a), and the amount of biofilm could be quantified using ethanol extraction and subsequent OD₅₉₅ measurement, which is near the absorption maximum for this dye (Myers et al., 2013). Biofilm formation on polystyrene was tested when grown on cellobiose using two different media types: GS2, a complex medium, and DM, a relatively nutrient-poor, defined medium. Biofilm formation did not occur on polystyrene...
when grown in GS2 medium, but significant biofilm formed in DM medium (Fig. 4b). This was surprising as growth was more rapid and achieved a higher density in GS2 medium as compared with DM (Fig. 4c). SEM confirmed the presence of biofilms on the polystyrene surface, and showed thread-like fibres interconnecting the cells and possibly the surface (Fig. 4d, e), suggesting similar attachment mechanisms within the biofilm as observed on nutritive, cellulosic surfaces and in aggregates.

**Perturbation of biofilms**

The assay was used to investigate the impact of numerous medium components to understand what role the nutritional environment plays in biofilm regulation. DM medium was supplemented with potassium phosphate (KH$_2$PO$_4$) to achieve 0.05, 0.2 and 0.5% (or 0.005, 0.02 and 0.5 g l$^{-1}$). This concentration range may impact medium buffering, but the changes did not significantly impact growth (Fig. S3). The highest levels (0.5%)
inhibited biofilm formation nearly completely, whilst only slightly altering (increasing) the maximum achievable OD. A similar sensitivity was observed for biofilm formation when the calcium chloride concentration was altered. Concentrations of 0.01 and 0.05 mg l\(^{-1}\) (1000 and 5000 times baseline levels, respectively) completely inhibited biofilm formation, whilst only slightly impacting suspension growth. The addition of EDTA, which has been thought to interfere with the ability of cells to attach the polystyrene surface (Banin et al., 2006; Chang et al., 2012), had almost no effect on growth or biofilm formation except at very high concentration (approximately equal to molar levels of iron – the primary chelating function of EDTA). These sensitivities are not unexpected considering biofilm formation by cellulolytic microbes has already been shown to be highly regulated by environmental conditions similar to organisms which attach to non-nutritive surfaces (Stanley & Lazazzera, 2004). For example, biofilm formation and curdlan (EPS) production in *Cellulolomas uda* was shown to be strongly upregulated under nitrogen-limited conditions which promoted cellulose hydrolysis as a possible competitive mechanism for access to nitrogen (Young et al., 2012), although a similar response was not observed in *Clostridium phytofermentans* (unpublished results).

Finally, considering that biofilm formation by cellulolytic microbes is involved in conversion of substrate to soluble carbohydrates, the carbon source was varied in DM medium. Galactose at both 3 and 6 g l\(^{-1}\) strongly inhibited biofilm formation on polystyrene, whilst xylose at these concentrations promoted the formation of biofilms even though planktonic growth was limited (Fig. S3). *Clostridium phytofermentans* did not grow in DM medium on either sucrose or lactose and, correspondingly, no biofilm was observed. Biofilm formation when grown on glucose rather than cellobiose was also limited even though growth rates were quite similar (Fig. 5a, b). Glucose inhibition of biofilm formation was validated when *Clostridium phytofermentans* was grown on filter paper in GS2 medium (Fig. 5c). The addition of 3 g cellobiose l\(^{-1}\) had little impact on biofilm formation compared with the control without added carbohydrates. However, glucose inhibited the long-term attachment of cells to the surface. Both cellobiose and glucose significantly inhibited cellulose hydrolysis as evidenced by the intact paper strips after 9 days of incubation (Fig. 5c, insets). This finding has important implications to the strategy of utilizing *Clostridium phytofermentans* for biofuel production. For instance, in our recent work (Zuroff et al., 2013) we demonstrated that the rate of cellulose hydrolysis limited ethanol productivity in a symbiotic consortium with *Saccharomyces cerevisiae* cdT-1. In monocultures, glucose and other soluble celloextrins accumulated which, based on this work, would inhibit attachment to the surface as well as impede cellulose

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**Fig. 4.** Development of a biofilm assay for *Clostridium phytofermentans*. (a) Demonstration of crystal-violet-stained *Clostridium phytofermentans* biofilm formed on polystyrene microtitre plate wells. (b) Biofilm formation and (c) growth in DM (●) and GS2 (□) medium. (d, e) *Clostridium phytofermentans* cells adhered to the polystyrene substrate as individual cells and clusters (d) and evidence for presence of small thread-like connecting fibres (e). Biofilm and growth results are representative of at least three independent experiments. Bars, 5 μm (d); 1 μm (e).
hydrolysis. The addition of *Saccharomyces cerevisiae* cdt-1 provides a means of maintaining zero soluble carbohydrate concentrations, which would promote attachment and cellulose degradation. Furthermore, any strategies to optimize cellulose hydrolysis capabilities of *Clostridium phytofermentans* must consider the impact of soluble carbohydrate accumulation, again emphasizing the benefit of the consortia-mediated bioprocessing approach.

**Biofilm regulatory signalling**

The regulatory systems behind aggregate and biofilm formation in *Clostridium phytofermentans* are of great interest. Although *Clostridium phytofermentans* does not have identifiable homoserine lactone quorum sensing networks, it does appear to have typical two-component regulatory systems (an illustrative example is shown in Fig. S4). The histidine kinase extracellular sensing components of the circuits are readily annotated; the *Clostridium phytofermentans* genome contains 62 such putative histidine kinases. The characteristic GGDEF motif associated with the catalytic domain of diguanylate cyclase to generate cyclic-di-GMP is annotated in 13 genes, five of which also contain the phosphodiesterase EAL domains which degrade this well-established signal of sessility and biofilm formation (Hengge, 2009). The gene Cphy_1136 contains both the cyclic-di-GMP and histidine kinase genes, which is a recognized tie between these signalling systems associated with biofilm formation (Hickman et al., 2005). Given this extensive network of biofilm-related signals, microarray data (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/) available for media conditions promoting (cellobiose) and inhibiting (glucose) biofilm formation were examined for differential expression. Sets of histidine kinases and adjacent transcriptional regulators revealed minimal differential regulation aside from the modestly downregulated pairs Cphy_0864/0863 and Cphy_3211/3212, which both contain AraC family transcriptional regulators that are known to participate in biofilm formation of *Staphylococcus aureus* (Lim et al., 2004). Further elucidation of regulatory function would require gene knockouts, complementation and a semiquantitative biofilm formation assay such as that described in this work.

**Extracellular polymer composition**

The composition of the extracellular matrix produced by *Clostridium phytofermentans* during growth on soluble and insoluble substrates was probed using staining, enzymic treatment and the microtitre plate biofilm assay. Although not shown here, no significant carbohydrates were detected using a modified version of the Dreywood anthrone method.

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**Fig. 5.** (a) Growth and (b) biofilm formation of *Clostridium phytofermentans* with cellobiose (●) or glucose (○). (c) Validation of glucose biofilm inhibition in filter-paper-grown cultures without any added carbohydrate ('None'), with 3 g cellobiose l⁻¹ added ('+ Cellobiose') and with 3 g glucose l⁻¹ added ('+ Glucose'). Inset in (c) shows the filter paper strips immediately before removal for microscopy and demonstrates inhibition of hydrolysis by added carbohydrates. Cells were stained with SYTO 9 and cellulose was stained with Calcofluor White. Assay results are representative of at least three independent experiments; CLSM results are representative of at least three randomly selected areas on the filter paper and were consistent between two independent experiments. Bars, 20 μm.
Clostridium phytofermentans biofilms and eDNA

Fig. 6. (a) *Clostridium phytofermentans* aggregates stained with SYTO 9 (green) and PI (red) show diffuse background fluorescence indicative of eDNA. A 40 min treatment with DNase I and Proteinase K resulted in loss of background fluorescence, whilst RNase A treatment had little impact. (b) PI staining also revealed eDNA in biofilms formed on Whatman no. 1 filter paper fibres which is again removed with DNase and proteinase treatment. Cells were stained with SYTO 9 (green) and PI (red), and cellulose fibres are highlighted with Calcofluor White (blue). (c) Treatment of pre-formed polystyrene biofilms with DNase, RNase and proteinase recapitulates the critical role of eDNA (and to some extent protein) in biofilm structure and dispersal. The control biofilm levels (100 %) are shown by the dashed line. Bars, 10 μm (a); 20 μm (b).

The presence of eDNA and proteins in the extracellular matrix of *Clostridium phytofermentans* biofilms formed on filter paper was also investigated (Fig. 6b). Consistent with the aggregate studies, both DNase and proteinase were capable of removing the background PI staining of eDNA in pre-formed biofilms, whilst RNase had little impact. Cells were still present on the surface after DNase treatment, whilst proteinase apparently stripped the majority of the biofilm from the cellulose fibres. Enzymic deconstruction was quantified in biofilms formed on polystyrene treated with DNase, RNase and proteinase. More than half of the total biofilm was removed via DNase treatment and ~30 % via proteinase treatment, whilst RNase had no impact (Fig. 6c). Similar results for DNase treatment have been observed in a numerous other microbial species (Jakubovics et al., 2013), validating a critical role for eDNA and protein as structural components of *Clostridium phytofermentans* biofilms.

The effect of proteinase treatment on structure and associated eDNA may be due to a proteinacious matrix which is intertwined with eDNA similar to that thought to be present in certain wastewater anaerobic granules (Xiong & Liu, 2013), *E. coli* biofilms (Goodman et al., 2011) and *Streptococcus mutans* cellular aggregates (Petersen et al., 2005). Proteinase K also removed cells from the surface of filter paper fibres. This result could be explained if cells are bound to the surface via proteins such as carbohydrate-binding modules or via the protein coat (Tolonen et al., 2011). The use of a primarily eDNA/protein matrix is most clearly described in staphylococcal species and not described previously for *Clostridium* species. Deletion of the staphylococcal *ica* locus which encodes extracellular poly-N-acetylglucosamine did not result in a dramatic

(Zuroff et al., 2013), which quantifies numerous carbohydrates that may be found in extracellular polysaccharides (e.g. glucose, fructose, galactose, rhamnose, ribose, arabinose, etc.; Yemm & Willis, 1954). Based on the assumption that polysaccharides played a relatively minor (though not necessarily negligible) role in the matrix of *Clostridium phytofermentans*, the presence of other components was investigated. Aggregates were stained with SYTO 9 and PI to selectively highlight live and dead cells as well as probe for eDNA. PI is a DNA-specific stain that is not membrane-selective and thus will only stain cells with compromised membranes (i.e. ‘dead’). PI has also been used to examine eDNA, which appears as diffuse background staining (Dominiak et al., 2011). Cellular aggregates cultured for 3 days were composed of both live and dead cells, and had a highly red fluorescent background due to PI staining, suggesting that eDNA is a component of the matrix (Fig. 6a). To further validate the presence of eDNA within the matrix, *Clostridium phytofermentans* aggregates were treated with DNase I, RNase A and Proteinase K. Samples treated with either DNase or proteinase showed significant reduction in aggregate structure and almost complete loss of background eDNA staining, whilst similar results were not observed in RNase-treated samples. DNase and proteinase treatments did not retain large aggregates (as observed with the control and RNase treatments), suggesting that dispersal is primarily caused by the enzyme treatments and not a result of inaccurate sampling. In addition to PI labelling of eDNA, filamentous structures with adhered cells were periodically observed in mid-exponential-phase *Clostridium phytofermentans* cultures and fluoresced with SYTO 9 staining similar to eDNA microfilaments observed in *Reinheimera* sp. F8 (Böckelmann et al., 2006; Tang et al., 2013) (Fig. S5).
change in biofilm phenotypes as eDNA and proteins are thought to substitute for the poly-N-acetylg glucosamine polysaccharide (Boles & Horswill, 2011). Furthermore, *Listeria monocytogenes* is thought to rely heavily on eDNA for biofilm adhesion and development due to its lack of ability to produce extracellular polysaccharides (Harmsen et al., 2010). The reverse of this logic could be used to explain the *Clostridium phytofermentans* matrix. *Clostridium phytofermentans* likely uses an eDNA/protein-based biofilm matrix as a strategically orthogonal complement to its robust array of >160 carbohydrate active enzymes (www.cazy.org).

**CONCLUSIONS**

The ability to form biofilm on nutritive surfaces such as lignocellulose is a critical aspect to successful competition and proliferation in the natural environment for many cellulolytic microbes (Costerton et al., 1987; Leschine, 1995; Lynd et al., 2002). It is clear that there are numerous strategies for effective surface attachment, including extracellular polymer production and carbohydrate-binding modules present as part of both cellulosomes and individual cell surface-associated enzymes (Dumitrache et al., 2013a; Kenyon et al., 2005). Such mechanisms demonstrate how surface attachment provides a competitive strategy to acquire substrate where proximity facilitates efficient cellulose hydrolysis and assimilation. The present work provides a detailed description of numerous aspects of *Clostridium phytofermentans* biofilm formation and a biofilm assay for investigation of the impact of media composition on biofilm formation. Further, this study demonstrates a critical role for eDNA and protein in cellular aggregation and surface attachment. These findings are important for understanding the ecological role of this species, and represent the first demonstration of biofilm dispersal in a *Clostridium* species via DNA and protein hydrolysis. This work also has significant implications for the use of *Clostridium phytofermentans* as a potential lignocellulosic biofuel-producing organism because glucose accumulation due to enhanced cellulose hydrolysis will likely inhibit biofilm formation. By appreciating the organism’s natural tendency to aggregate and attach to cellulosic surfaces, it may be possible to better engineer reactor systems to optimize lignocellulosic conversion processes.

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