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Differential Protein Expression in *Streptococcus uberis* under Planktonic and Biofilm Growth Conditions▽†

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The bovine pathogen *Streptococcus uberis* was assessed for biofilm growth. The transition from planktonic to biofilm growth in strain 0140J correlated with an upregulation of several gene products that have been shown to be important for pathogenesis, including a glutamine ABC transporter (SUB1152) and a lactoferrin binding protein (gene *lbp*; protein SUB0145).

Approximately 33% of bovine mastitis cases within the United Kingdom are attributable to *Streptococcus uberis*, and infections can be both persistent and resistant to antimicrobial treatment (10, 17). In this study, we assess the ability of *S. uberis* to form biofilms *in vitro*. We also analyze the *S. uberis* clinical isolate 0140J under planktonic and biofilm growth conditions *in vitro* by using gel electrophoresis liquid chromatography (GeLC)-tandem mass spectrometry (MS/MS) and demonstrate that the changes that 0140J undergoes in the transition from planktonic to biofilm growth include factors strongly implicated in pathogenesis.

Biofilm formation by *S. uberis* isolates. To establish biofilm formation among different strains of *S. uberis*, we analyzed *S. uberis* isolates 0140J (a strain of high virulence for the lactating bovine mammary gland), EF20, Y38, C197C, C221, and C198 (a strain obtained from a healthy bovine mammary gland and not associated with pathogenesis) (see Table S1 in the supplemental material) in TSBM medium {tryptic soy broth without dextrose [Difco Labs], supplemented with 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.6, and 20% [vol/vol] sterile skim milk}, using a microtiter plate biofilm formation assay (4). All isolates formed biofilms *in vitro*, with C198 forming the smallest biomass (Fig. 1).

A previously elucidated difference between strain C198 and all other strains tested is that C198 lacks the *hasAB* gene cluster and is, as a consequence, acapsular (J. Leigh, personal communication). In support of capsule production being involved in 0140J biofilm growth *in vitro*, *S. uberis* capsule size has been shown to increase in the presence of bovine milk-related constituents (12) and the addition of 20% (vol/vol) bovine milk to medium was found to promote 0140J biofilm formation *in vitro* (data not shown).

Differential proteomic analysis of *Streptococcus uberis* 0140J biofilm development. To determine factors involved in *S. uberis* biofilm growth and development, we compared the proteomes of 0140J planktonic cells (8 h) with those of biofilm cells (8 h), as well as comparing 8-h biofilm cells with 36-h biofilm cells.

Biofilms were grown in tube reactors (1) that were initially inoculated with *S. uberis* strain 0140J in TSBM for 1 h under no-flow conditions. Both biofilm and planktonic cells were grown at 37°C in TSB supplemented with 10% (vol/vol) lactose (46 g/liter). Protein extraction was carried out essentially as previously described (6). Protein samples (30 ng) were loaded onto 10% acrylamide gels (Protean III system; Bio-Rad) and stained (GelCode blue; Pierce) according to manufacturer's instructions. Gel lanes were cut into 13 to 15 equal-size gel

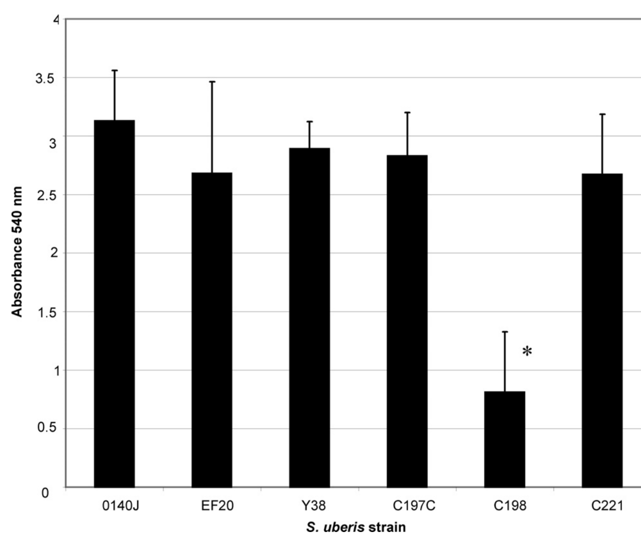

FIG. 1. Biofilm formation by *S. uberis* clinical isolates. Quantitative biofilm formation of *S. uberis* isolates after 24 h in TSBM. Strains 0140J, EF20, Y38, C197C, and C221 were isolated from diseased cows and form significantly more biofilm biomass than strain C198, isolated from a healthy cow. The asterisk denotes the statistical significance of biofilm formation between 0140J and C198 ($P \leq 0.05$).

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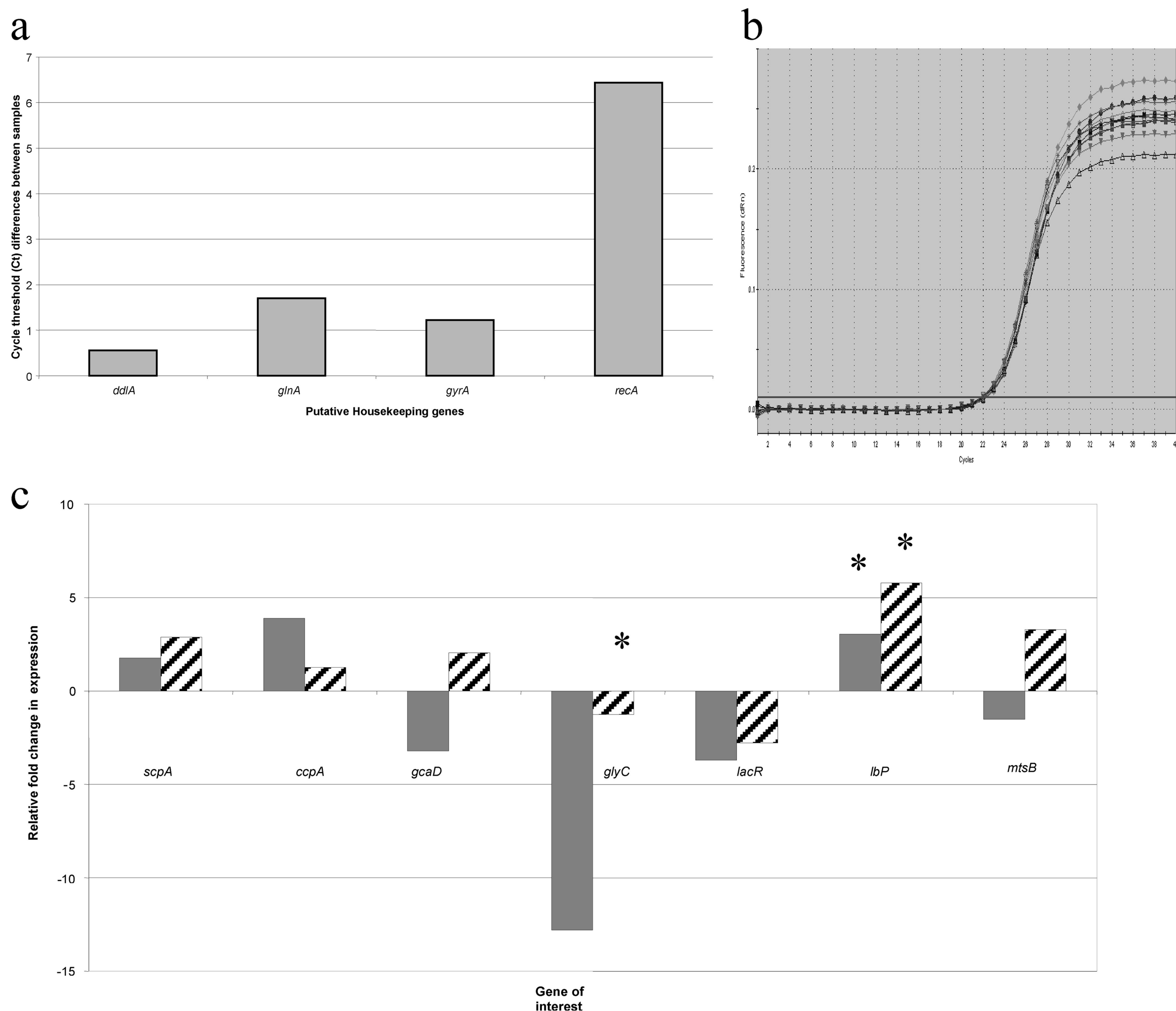

FIG. 2. Transcriptional analysis in 0140J planktonic and biofilm cells. (a) Difference in C_T values across all planktonic and biofilm cDNAs from reference gene candidates *ddlA*, *glnA*, *gyrA*, and *recA*. (b) Example of raw qRT-PCR data showing amplification curves of *ddlA* in 8-h planktonic, 8-h biofilm, and 36-h biofilm cDNA. (c) Relative fold change in expression of *scpA*, *ccpA*, *gcaD*, *glyC*, *lacR*, *lbp*, and *mtsB* in 8-h biofilm cells. The gray bars represent expression in early biofilm cells, in comparison to planktonic cells. The striped bars represent expression in early biofilm cells, in comparison to late biofilm cells. *glyC* and *lbp* show significant fold change between 8 h of planktonic growth and 8 h of biofilm growth (>9.06- and 2.72-fold change, respectively). *lbp* shows the only significant fold change in expression between 8-h and 36-h biofilm growth (>2.96-fold change). Asterisks denote statistical significance in difference in expression, as normalized by the reference gene *ddlA* ($P \leq 0.05$).

slices, and each slice was processed as previously described (19). Capsular supernatant fractions were directly processed (13). Peptides were resuspended and separated (3, 18). Eluted peptides were analyzed by tandem mass spectrometry (LTQ ion trap mass/LTQ Orbitrap FT-MS; ThermoScientific), and spectra (*m/z* range, 350 to 1,800) were searched against the annotated *S. uberis* 0140J genome (23) (SEQUEST, BioWorks v3.3; Thermo Scientific).

The transition from 8-h planktonic growth to 8-h biofilm growth in 0140J correlated with an increase in proteins predicted to be involved in adhesion (SUB0837 and SUB1152), glutamine transport (SUB1152), internalization (SUB1212), and sugar metabolism (SUB0135, SUB0235, SUB0161, and SUB0750) (see Tables S2 and S3 in the supplemental material). This is of interest with regard to pathogenesis of infection as such processes have been implicated in virulence and/or biofilm growth in other streptococcal species (7, 8, 16). For example, a glutamine transport *glnP* mutant of *Streptococcus pneumoniae* showed decreased adherence to human pharyngeal epithelial cells (9).

The development of 0140J biofilms correlated with the downregulation of a relatively large number of proteins (i.e., 34) involved in metabolism, cell wall synthesis, and cell division, suggestive of a decrease in growth rate/metabolic activity

as a biofilm ages (from 8 to 36 h) (see Tables S4 and S5 in the supplemental material).

Although GeLC-MS/MS alone is not a quantitative technique, it was noticeable that some proteins, although expressed in planktonic and biofilm cells, showed marked differences in peptide coverage between these growth types, which may indicate a quantitative difference in abundance of the protein. Quantitative reverse transcription (qRT-PCR) was carried out on these candidates to further assess and quantify putative differential expression at the transcriptional level.

Quantitative verification of proteomic analyses during 0140J biofilm growth. RNA isolation and cDNA synthesis were carried out as previously described (21) (see Table S1 in the supplemental material). qRT-PCR was normalized to *ddlA* (gene 7391862; SUB1257) due to *ddlA* showing the least variation in expression across all samples (Fig. 2a and b). Changes in transcriptional expression between 8-h planktonic cells and 8-h biofilm cells as well as 8-h biofilm and 36-h biofilm cells were calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method (15, 21) and plotted as the fold change in mRNA expression levels.

The transition from 8-h planktonic to 8-h biofilm growth was accompanied by a significant increase in *lbp* (SUB0145) expression (>2.7-fold) (Fig. 2c) and decrease in *glyC* (>9-fold). Mutation of SUB0145 has recently been shown to impair virulence for the bovine lactating gland (11). In addition to iron, lactoferrin (Lf) is also capable of binding other divalent metal ions (such as manganese, which has been shown to be involved in *S. uberis* virulence via the *mtu* operon [20]). Intriguingly, the bovine Lf concentration is greatest during the dry period, which is also the period in which the highest recorded rates of *S. uberis* infections occur (22). However, *lbp* expression significantly decreased (2.9-fold) in 36-h biofilms in comparison to 8-h biofilms (Fig. 2c). As *glyC* has been shown to be an osmoprotectant (14), our data suggest that 8-h biofilm cells are not under greater osmotic stress than their planktonic counterparts. However, it is interesting that a >9-fold decrease in mRNA levels of *glyC* in 8-h biofilms, in comparison to 8-h planktonic cells, still resulted in GlyC detection by GeLC-MS/MS.

In conclusion, we present evidence of *S. uberis* biofilm formation in which clinical isolates had greater biofilm biomass than a strain obtained from a healthy cow. Differential expression analysis at the translational level during 0140J biofilm growth suggest that glutamine transport, adherence interactions, and sugar metabolism are important processes in early biofilm growth, while at the transcriptional level, an increase in mRNA levels of *lbp* was apparent.

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