

Biofilm Production by *Streptococcus uberis* Associated with Intramammary
Infections

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Abstract

Mastitis is the inflammation of the mammary gland and is extremely problematic in the dairy industry, annually costing upwards of two billion dollars. *Streptococcus uberis* is one of the main infectious agents responsible for causing mastitis. We speculate that one of the reasons behind the prevalence of *S. uberis* infections is its ability to form biofilms. The objectives of this study were to determine in vitro slime production, biofilm formation and the presence of several genes associated with biofilm formation such as competence (*comEA*, *comEC*, *comX*) and quorum sensing *luxS*, in collected strains of *S. uberis*. Twenty seven strains of *S. uberis* isolated from mastitic cows, were tested by microtiter plate (MP), air-liquid interface (ALI), Congo Red agar (CRA) and PCR methods. The MP and ALI methods were optimized by adding different substrates (sucrose and lactose) to the culture media. A known biofilm former, *Staphylococcus epidermidis*, was used as a positive control. Of the 27 *S. uberis* strains, an average of 21 strains displayed a biofilm-phenotype when tested by MP and ALI. The strains were further classified as negative (4, 15 %), weak (2, 7%) and strong (21, 78%) biofilm formers. Substrates did not have an effect on biofilm production by *S. uberis*. When evaluated for slime (polysaccharide) production, all 27 strains were positive by the CRA method. We were able to amplify the competence and *luxS* genes by PCR in 96% of the strains. In summary, *S. uberis* is capable of forming biofilms in vitro on an abiotic surface. Furthermore, genes associated with biofilm formation were found to be highly conserved in the strains tested. The economic impact of mastitis on the dairy industry is reason alone to justify continued research in this area. Understanding the virulence factors that influence *S. uberis*'s ability to colonize and maintain infections will ultimately allow for effective and appropriate treatment protocols that could greatly decrease the impact of mastitis caused by this pathogen in the dairy industry.

Introduction

Mastitis is the most important disease affecting dairy cattle worldwide, costing the North American dairy industry approximately two billion dollars annually (National Mastitis Council, 1996). Mastitis generally encompasses clinical and subclinical infections of bacterial origin which may have a negative effect on the cow's milk production and quality, health care, and longevity in the herd (Melchior et al, 2005). The ability to cure and treat mastitis depends on many factors, including age, stage of lactation, position of the infected quarter, and somatic cell count at the time of treatment (Sol et al, 1997). In addition, recurrent mastitis infections can be responsible for up to 40% of infections (Hillerton and Kliem, 2002), making them the target of extensive research. The economic impact of this disease is considerable and thus any effort to reduce its prevalence is welcomed and beneficial.

Streptococcus uberis, a primary environmental pathogen, is a major cause of mastitis in dairy cattle. Chronic subclinical mastitis infections caused by *S. uberis* are extremely costly and difficult to treat (Steenefeld et al, 2007). *S. uberis* has several virulence factors including the ability to attach to a host's cell surface through the *S. uberis* adhesion molecule (SUAM) identified by Dr. S. P. Oliver's laboratory. Another potential virulence factor, possibly linked to *S. uberis*'s ability to adhere to cells, would be the formation of biofilms.

Many persistent and recurrent infections have been attributed to the formation of biofilms, or polymeric matrixes produced by bacterial colonies adhering to a biologic or abiotic surface (Costerton et al, 1999). A biofilm matrix is composed of microbial cells, polysaccharides, water, and other extra cellular products all of which allow the matrix to play host to numerous microenvironments and activities (Sutherland, 2001). Biofilms provide a sheltered and protected area for bacterial growth and allow them to be resistant to antibiotics; disinfectants and host defenses, thus the difficulties of treating recurrent infections may be related to the infecting

pathogens ability to produce biofilms (Melchior et al, 2005). In other species of mastitis-causing bacteria, such as *S. aureus*, up to 65% of the infections are associated with biofilm formation (Ymele-Leki and Ross, 2007). The ability of bacteria to form biofilms has been linked to numerous genes. As biofilm formation is an example of group behavior, it has been associated with the presence of quorum sensing and competence genes (Suntharalingam et al).

The objectives of this research project were twofold: The first objective was to determine if *S. uberis* was able to form biofilms under in vitro conditions. The second objective was to compare phenotypic and genotypic characteristics of biofilm formation among different *S. uberis* strains.

Justification

The economic impact of mastitis on the dairy industry is reason alone to support research that could potentially provide a reason behind the chronic infections often seen in conjunction with *S. uberis* infections. Understanding the virulence factors that influence *S. uberis*'s ability to colonize and maintain infections will ultimately allow for effective and appropriate treatment protocols that could greatly decrease the impact of mastitis caused by *S. uberis* in the dairy industry.

Materials and Methods

Bacterial Strains and culture conditions: Twenty-six strains originally isolated from clinical cases of bovine mastitis and one ATCC (American Type Culture Collection) strain (O140J) of *S. uberis* were used in this study. The strains were provided by Dr. Steve Oliver (The University of Tennessee, 2640 Morgan Circle Drive, 59 McCord Hall, Knoxville, Tennessee 37996). A known biofilm former, *Staphylococcus epidermidis*, was used as a positive control. Unless otherwise

specified, Trypticase Soy Broth (TSB) or TS agar containing 5% sheep blood were used to propagate and/or culture the strains and the incubator was set at 37°C with 5% CO₂.

Congo Red Agar Method: *S. uberis* strains were screened for their ability to produce slime by plating them on Congo Red agar as previously described by Freeman et al. (1989). Briefly, Brain Heart Infusion (BHI) agar plates were prepared with 5% sucrose and Congo red stain (0.8g/L).

Plated individual strains were incubated for 24 to 72 hours at 37°C. Positive results were indicated by black colonies with a dry crystalline consistency. Colonies that darkened at the centers and/or without the dry crystalline morphology were considered non-biofilm formers.

Air-Liquid Interface Assay: Biofilm growth behavior by *S. uberis* was studied as previously described by Merritt et al (2005). Briefly, single colonies of each strain were inoculated into 5-ml of TSB, placed in a shaker incubator at 37°C and grown overnight to stationary phase. The following day bacterial cultures were diluted 1:100 in TSB media. Diluted cultures (300 µl) were carefully applied to the bottom half of a separate well of a sterile, 24-well plate that was secured at a 30° angle. Following a 48-hour incubation period, the media was aspirated; the plate was washed twice with 400µl of TSB to remove planktonic bacteria and 200 µl of TSB were added to each well to prevent drying of the biofilms. Biofilms were detected through a phase-contrast microscope. Strains were subjectively classified based on the density of biofilm formation as negative (<25% coverage), weakly positive (>25% coverage), moderately positive (>50% coverage) and strongly positive (>75% coverage). This experiment was repeated as described above with TSB supplemented with 5% sucrose.

Microtiter Plate Biofilm Assay: The ability of *S. uberis* strains to form biofilms in vitro on an abiotic surface was determined with a method previously described by others (Christensen et al 1985; Merritt, et al. 2005; Courtney HS 2009) with minor modifications. In brief, strains were

grown and diluted in TSB, as described under ALI. A sterile 96-well flat bottom polystyrene plate (Corning Costar) was inoculated with 100 μ l of the diluted culture and incubated for 48 hours. Planktonic bacteria were removed by washing the plate 4 times with 100 μ l of phosphate buffered saline (PBS) and any residual liquid was carefully aspirated. The plate was heat fixed for 1 hour at 60 °C and stained with 100 μ l Hucker's crystal violet solution for 2 minutes. The excess stain was removed by gentle shaking and washing with water until the water was clear. The plate was blotted dry and 100 μ l of a solution containing 10% methanol and 7.5% acetic acid was added, the plate was shaken for 1 minute and placed in a plate reader to record the absorbance at 563 nm. In addition, we studied the effect of different carbohydrates (sucrose and lactose) on biofilm formation by *S. uberis*. Experiments were performed as described above with the exception that media contained 5% sucrose or 0.5% lactose. Four wells in each plate containing uninoculated media (TSB with or without sucrose or lactose) were used as blanks. Likewise, each plate contained media inoculated with *S. epidermidis* as a positive control. Each strain was tested for biofilm production in quadruples and the experiment was repeated on 2 different occasions. The strains were categorized using a scale based on the average optical density of the blank wells plus 3 times the standard deviation of the mean. A strain was considered negative if the optical density was below the cutoff value and weakly positive if the OD was between the cutoff and 2 times this number. Any strain with an optical density greater than twice the cutoff value was categorized as strongly positive.

Isolation and amplification of genomic DNA: Genomic DNA was extracted from each *S. uberis* strain using a commercially available kit by Promega[®]. Primer sets for the amplification of *comEC*, *comEA*, *comX* and *luxS* were designed from the published KEGG sequence of the *S. uberis* genome using Primer BLAST (Table 1). A 50 μ l reaction volume consisted of 25 μ l

GoTaq[®], 1.5 µl of each primer, 20 ng of template DNA. Thirty cycles of amplification, each consisting of denaturation at 95°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min; with a final extension at 72°C for 7 min were performed in a BioRad Thermocycler. The amplified PCR products were confirmed by electrophoresis on 2% agarose gels.

Results and Discussion

Streptococcus uberis was able to form biofilms under in vitro conditions. All of the *S. uberis* strains evaluated consistently produced slime as demonstrated by the CRA assay. The CRA results were highly dependent on the contents of the media. CRA plates supplemented with 5% sucrose displayed black crystalline morphology within 24-48 hours (27 of 27); whereas non-supplemented plates displayed smooth, pink colonies even after 48 hours of incubation (Fig. 1). We evaluated the biofilm forming behavior by the ALI assay and found considerable variation among the different strains tested. Of the 27 strains, 5 were determined to be negative, 6 were weakly positive, 9 were moderately positive, and 7 were strongly positive. Supplementation of the media with a carbohydrate source such as 5% sucrose did not have an effect on biofilm formation as tested. In both experiments, bacteria grew as planktonic organisms on the liquid media but the ability to form biofilms and their density was not consistent among strains (Fig. 2). In the standard media (TSB) Microtiter-Plate Assay, 23 of 27 strains of *S. uberis* were considered positive, with 78% being strong biofilm formers, 7% being weak formers, and 15% having no formation (OD₅₆₃ range 0.095-0.191). It was hypothesized that the addition of substrates would increase the prevalence of biofilm formation; however, this was not the case. With the addition of 5% Sucrose, 67% were strong formers, 15% weak formers, and 18% displayed no formation (OD₅₆₃ range 0.079-0.159). With the addition of 0.5% Lactose, 74% of

the strains were strong formers, 15% weak formers, and 11% showed no formation (OD₅₆₃ range 0.078-0.156) (Table 2, Graph 1). The vast majority of the strains (26, 96%) tested in this study had the genes for biofilm production as demonstrated by positive amplification of the quorum sensing *luxS* gene as well as the competence genes (*comX*, *comEA* and *comEC*) (Fig. 3). We were not able to amplify the competence genes in one strain despite numerous attempts.

Slime production by *S. uberis* strains occurred in all strains and within the same time frame as *S. epidermidis*. In contrast, some slime-positive *S. aureus* isolates may be relatively slower than *S. epidermidis*, taking longer than 24 hrs and up to 72 hours. Slime production has been associated with pathogenicity in *S. aureus* isolates associated with mastitis in the bovine (Vasudevan et al., 2002). Therefore, the ability of *S. uberis* to produce slime might be a desirable virulence factor during colonization of the udder. It has been shown that slime production is important, allowing the bacteria to aggregate and form biofilms (Arciola CR et al., 2002).

Biofilm behavior varies dramatically among *S. uberis* strains. These results are consistent with other studies where biofilm formation by some pathogens was only elicited under special conditions (Olson et al., 2002). It is possible that those *S. uberis* strains that displayed a negative or weak phenotype for biofilm formation may behave differently if culture conditions were optimized or a different biofilm assay was used. We did not perform alternative assays or time-course assessment on these strains; however, it is something that we may pursue in the near future.

This study looked for the presence of the *luxS*, *comEA*, *comEC*, and *comX* genes. The *luxS* gene is an example of a quorum-sensing gene and is highly conserved in most species. *ComX* is an alternate sigma factor and acts as the link between the *luxS* and other competence genes. *ComEA* and *comEC* are both competence genes allowing for the transformation of

genomic DNA through the uptake of a free-floating DNA strand. *ComEA* acts as a receptor for the DNA and then passes the DNA to *comEC*, which is a channel protein that regulates DNA uptake. The majority of the strains evaluated in this study contained the genes mentioned above. The presence of these genes is necessary for the formation of biofilms. Only one *S. uberis* strain failed to amplify the competence genes. Although this could be attributed to human error, it is possible that this strain did not have the competence genes or that the primers that we used were not complementary to this strain's genomic sequence.

In summary, *S. uberis* forms biofilms in vitro on abiotic surface. The substrates did not appear to have an effect on its ability to form biofilms under the conditions and assays that we used. Future work will concentrate in associating *S. uberis*' ability to form biofilms in vivo and intramammary infection. This will allow for development of strategies to better manage and prevent mastitis caused by this pathogen.

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Table 1 – Primers used to amplify genes related to biofilm formation.

Gene	Primers	Product Size
<i>luxS</i>	5'-TTTGATGTTTCGCTTGGTTCA-3' 5'-AGTTTTGCCCATTCTTTTGC-3'	317 bp
<i>comX</i>	5'-GATTGGTTACAAGAAGGCCG-3' 5'-TTCGTTTTTCGGAAAGTTTGG-3'	732 bp
<i>comEA</i>	5'-GCTCAAACGATAGGGAGGA-3' 5'-CCTTCTGATCCCTTTGTCCA-3'	304 bp
<i>comEC</i>	5'-GCGGAGTCTTGTCCTTTGTC-3' 5'-ATGACTTTGCCACCACTTCC-3'	288 bp

Fig 1 – Congo Red Agar Method ± Sucrose: Ability to produce slime by *S. uberis*. (a) Positive control *S. epidermidis* (+), (b) *S. uberis* UT888.

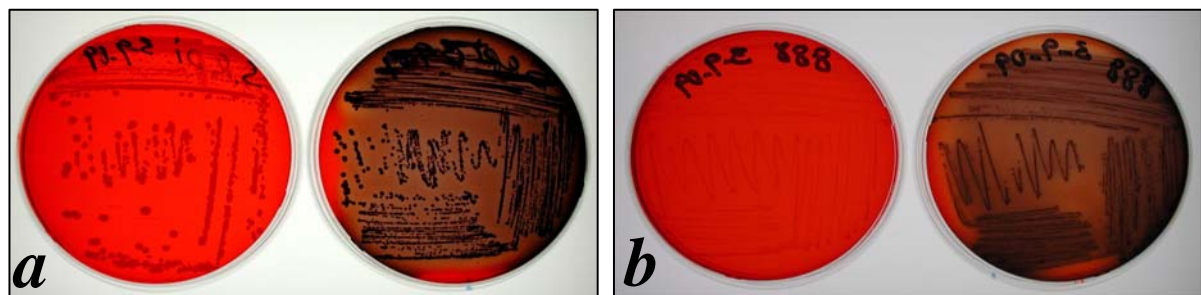


Fig. 2 – Biofilm behavior of different *S. uberis* strains as determined by Air-liquid Interface Assay. Each picture is labeled with the strain number. Blank and positive control are included.

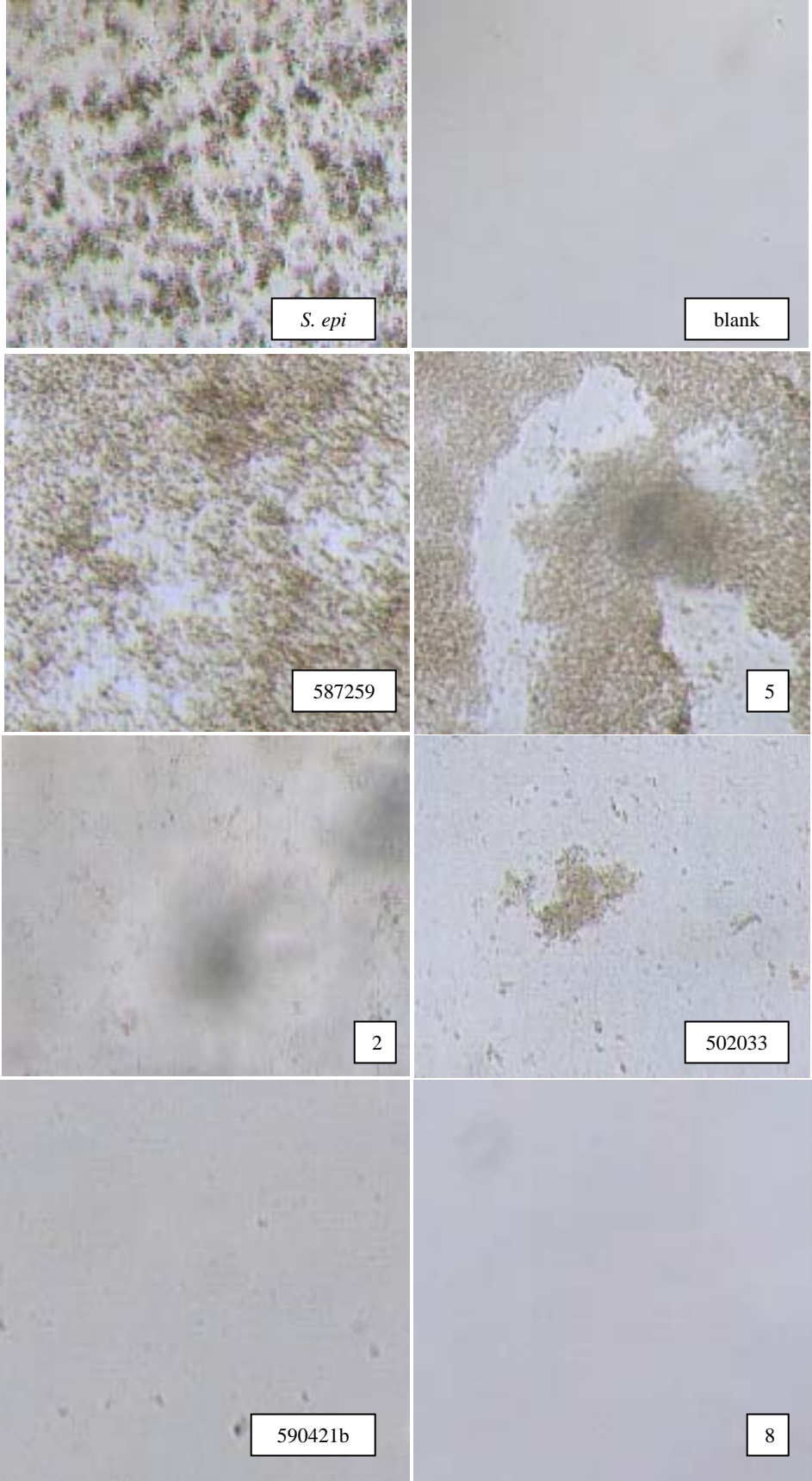
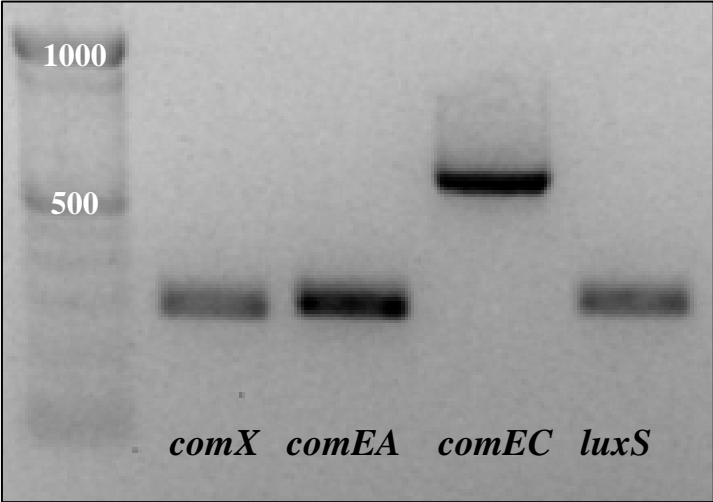
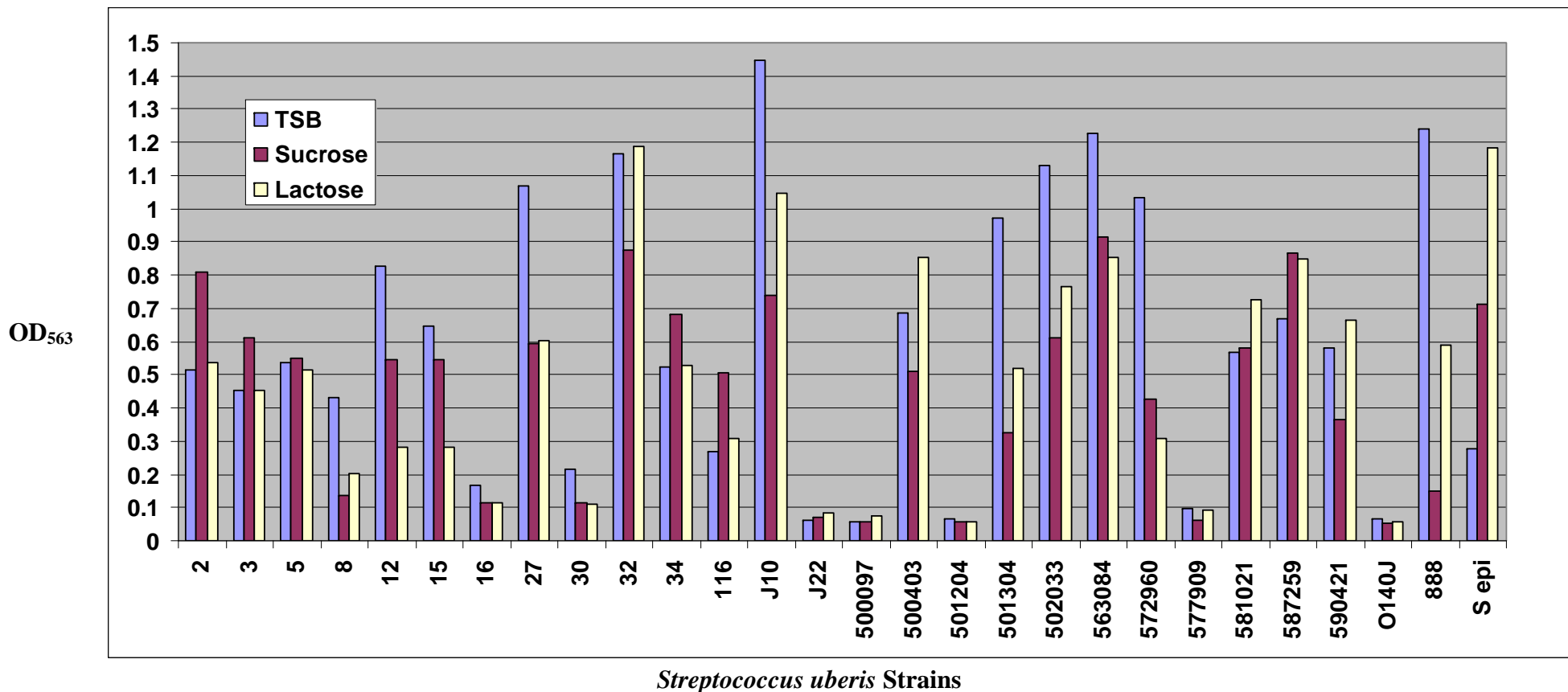


Fig 3 – 2% Agar Gel Electrophoresis depicting amplified PCR products from *S. uberis* genes involved in biofilm formation.



Graph 1 – Microtiter Plate Assay: Influence of substrate (sucrose or lactose) on biofilm formation by different *Streptococcus uberis* strains.



	TSB Only	TSB + 5% Sucrose	TSB + 0.5% Lactose
Positive	21	18	20
Weak Positive	2	4	4
Negative	4	5	3
Total	27	27	27

Table 2 – Classification of *S. uberis* strains based on effect of substrate on biofilm formation as determined by Microtiter Plate Assay.