5-2016

Identifying genetic factors in Streptococcus uberis that enable evasion of the host immune response

Alexis Christine Burnham
aburnha1@vols.utk.edu

Identifying genetic factors in *Streptococcus uberis* that enable evasion of the host immune response

ALEXIS BURNHAM

DR. GINA M. PIGHETTI, ADVISOR

CHANCELLOR'S HONORS PROGRAM THESIS

DEPARTMENT OF ANIMAL SCIENCE
University of Tennessee, Knoxville

IDENTIFYING GENETIC FACTORS IN *STREPTOCOCCUS UBERIS* THAT ENABLE EVASION OF THE HOST IMMUNE RESPONSE

ABSTRACT

Mastitis—the inflammation of the mammary tissue—is a major endemic disease in dairy cattle that often results from intramammary infection, causing significant losses in the dairy industry in both profit and product quality. With the continued development of mastitis control programs, the incidence of infections associated with contagious pathogens in commercial dairies has declined, yet these measures have had limited success in controlling environmental mastitis pathogens, which remain an issue even in well-managed herds. The pathogen *Streptococcus uberis,* in particular, accounts for a substantial proportion of clinical and subclinical mastitis cases; furthermore, its various strains differ in terms of their virulence and resistance to the host immune response, often able to survive and proliferate despite substantial neutrophil activity, which normally would kill pathogens. To better understand the nature of this resistance, six mutated clones of the *S. uberis* clinical isolate UT888 were subjected to a bactericidal assay using fresh bovine neutrophils, and their growth at designated time points was compared, with the objective of identifying mutants that were susceptible to being killed by neutrophils. These mutants were selected from the previously generated mutant library based on the results of a preliminary MTT screening that indicated that the six chosen mutants produced significantly greater reactive oxygen species (ROS) in the presence of neutrophils (PMN+) than without (PMN-). As greater ROS production is considered an indicator of cell stress, it was expected that these mutants would be more susceptible to neutrophil killing and thus would exhibit less cell growth in PMN+ versus PMN- conditions in the bactericidal assay. However, five of the six mutants presented with a greater ratio of cell growth of PMN+: PMN-; a single mutant (12E2) exhibited a lower ratio and thus may present an area of interest for future investigations.

1. INTRODUCTION

Mastitis has long been regarded as the most economically devastating disease threatening the modern dairy industry, with an estimated 20-50% of all dairy cows affected (Wilson et al. 1997). The disease negatively impacts all levels of production from the cow to the consumer: mastitis is associated with increased cow morbidity and mortality within the herd, decreased farm efficiency and revenue, shorter milk shelf life, lower cheese-making ability, and poorer palatability of the final product. Since their

implementation in the 1960s, modern mastitis control protocols have had considerable success in the control of contagious pathogens, but they have been far less effective in limiting environmental pathogens, which remain an issue even in well-managed herds (Oliver and Mitchell 1984; Oliver 1988).

The pathogen *Streptococcus uberis,* in particular, is one of the most prevalent environmental pathogens and accounts for a substantial proportion of clinical and subclinical mastitis cases in both lactating and non-lactating cows (Oliver 1988; Phuektes et al. 2001). In comparison with the well-documented host response to the common mastitis pathogens *Escherichia coli* and *Staphylococcus aureus*, the physiological response to *S. uberis* infection is not yet thoroughly understood (Schukken et al. 2011). Furthermore, strains of *S. uberis* appear to differ in virulence following experimental challenge (Leigh et al. 2010; Hossain et al. 2015). Under normal circumstances, neutrophils act as one of the primary innate responders to bacterial infection; however, *S. uberis* has shown varying ability to evade phagocytic action (Leigh and Field 1994; Schukken et al. 2011). Studies have yet to identify the genes associated with evasion phenotypes in *S. uberis* (Field et al. 2003).

To better understand this unique ability of *S. uberis* to evade phagocytosis by neutrophils, mutant strains of the clinical isolate *S. uberis* were subjected to a bactericidal assay conducted with fresh bovine neutrophils. The levels of growth of the mutant strains at various time points in the presence of neutrophils and in their absence was compared, with the objective of identifying mutants that were susceptible to being killed by neutrophils.

2. MATERIALS AND METHODS

2.1 Mutant library generation and preliminary screening

Mutant strains were obtained from a previously generated mutant library created through transposon mutagenesis from the clinical isolate *S. uberis* UT888, a strain characterized by its ability to evade neutrophils. A preliminary MTT screening was conducted in a previous experiment to measure reactive oxygen species (ROS) generation, commonly regarded as an indicator of cellular distress, for each mutant in the presence of neutrophils (PMN+) vs. without (PMN-) after one hour of growth (Kerro Dego et al. 2011). It was assumed that greater ROS production in PMN+ vs. PMN- conditions would indicate susceptibility to neutrophil killing. Six mutants—1H9, 4B2, 7E6, 8C4, 8H7, and 12E2—were identified on the basis of a significant increase in ROS production in the presence of neutrophils; this increase indicated that the transposon may have inserted into a gene related to the ability of UT888 to evade neutrophils.

2.2 Blood collection and neutrophil isolation

On the first day of each trial of the protocol, blood was collected from the jugular vein of a selected cow at the UT Little River Dairy after morning milking. Samples were collected in syringes containing the anti-coagulant Acid-Citrate-Dextrose (ACD) and placed on ice during transport. They were then analyzed using ABC VetCount before isolation, which consisted of a series of separations and lyses to achieve a concentrated sample of neutrophils with limited contamination from red blood cells. After the sample was centrifuged for 30 minutes at 4°C, 2000 RPM, the plasma and buffy coat were discarded and the red blood cells were removed by pipette to leave a final volume of approximately 5-10 mL. 20 mL ultrapure H_2O was added to lyse the sample, followed by 10 mL of 3x Roswell Park Memorial Institute medium (RPMI) to reestablish equilibrium. Hank's Balanced Salt Solution (HBSS) was added to reach a total volume of 50 mL, and the resulting sample was inverted and centrifuged for 10 minutes at 4°C, 1070 RPM. The supernatant was removed and the pellet re-suspended in 5 mL HBSS. The sample was lysed again with 10 mL ultrapure H_2O followed by the addition of 5mL 3x RPMI. HBSS was added to reach a total volume of 50 mL, and the resulting sample was inverted and centrifuged for 6 minutes at 4°C, 1070 RPM. The supernatant was removed and the pellet re-suspended in 5 mL HBSS before more HBSS was added to reach a total volume of 50 mL and the resulting solution was centrifuged for another 6 minutes at 4°C, 1070 RPM. Provided that the sample had attained a sufficient level of purity, it was re-suspended in 1-10 mL HBSS and enumerated by means of flow cytometry. The sample was then diluted in RPMI + 5% heat-inactivated bovine calf serum (BCS) to attain a concentration of 3,000,000 cells/mL. As shown in Fig.1, 500 μL of this diluted solution was added to the corresponding PMN+ wells in a 48-well plate, which was then incubated at 37°C, 5% CO_2.

2.3 Bactericidal assay

A bactericidal assay was performed to confirm that the mutants identified in the initial screening were more susceptible to PMN killing than the wild type *S. uberis* UT888. The protocol consisted of three days. Trials were completed on 1/29/16, 2/12/16, 2/19/16, 3/4/26, 3/11/16, and 3/18/16.

Day 1

During each round of the protocol, 1.5 mL aliquots of UT888 and the selected mutant strain(s) were thawed under the fume hood. Thawed aliquots were pipetted up and down five times, and a 200 μL sample from each was added to 2 mL media—Todd Hewitt Broth + 0.5% yeast extract (THBY) for UT888 and THBY + 1 μg/mL erythromycin for mutant strains—in their respective 15 mL tubes. Bacterial cultures were incubated at 37°C, 5% CO_2 for 18 hours.

Day 2

At approximately 3.5 hours before incubation was complete, the overnight bacterial cultures were removed and pipetted up and down five times before being transferred in 1.5 mL amounts to new corresponding, pre-warmed media. Resulting cultures were mixed thoroughly and expanded for an additional three hours.

Approximately one hour before expansion was complete, the PMN 48-well plate was analyzed under the inverted microscope to confirm adhesion and to examine any morphological changes. Under the fume hood, the media was removed from each well containing neutrophils (PMN+) and replaced with 500 μL fresh RPMI + 5% heat-inactivated BCS. 500 μL of media was also added to the bacteria only (PMN-) wells as shown in Fig.1, before the 48-well plate was replaced in the incubator until bacterial expansion was complete.

Following the three hours of bacterial expansion, the cultures were centrifuged for one minute at 5000 RPM at room temperature. The supernatant was discarded and the pellet re-suspended in 15 mL RPMI + 5% heat-inactivated BCS. 500 μL of each re-suspended, expanded culture was transferred to their respective wells in the 48-well plate as shown in Fig. 1. The plate was returned to the incubator at 37°C, 5% CO_2 until the appropriate time points were reached.

At each time point—1, 2, 3, and 4 hours—the contents of the wells were removed and lysed in a 2% saponin solution in PBS. Serial dilutions in THBY were then performed for each culture for PMN+ and PMN- from 10^{-1} to 10^{-6}. For each strain, 90 μL of the final four dilutions—ranging from 10^{-3} to 10^{-6}—were used to inoculate pre-warmed agar plates made with their corresponding media (THBY for UT888 and THBY + 1 μg/mL erythromycin for mutant strains). Plates were allowed to dry for one minute and were placed in the incubator at 37°C, 5% CO_2 overnight.

Day 3

Colony counts were performed on the inoculated plates for each strain for PMN+ and PMN-, and the data was used to calculate CFU/mL for each treatment, mutant, and time point combination.

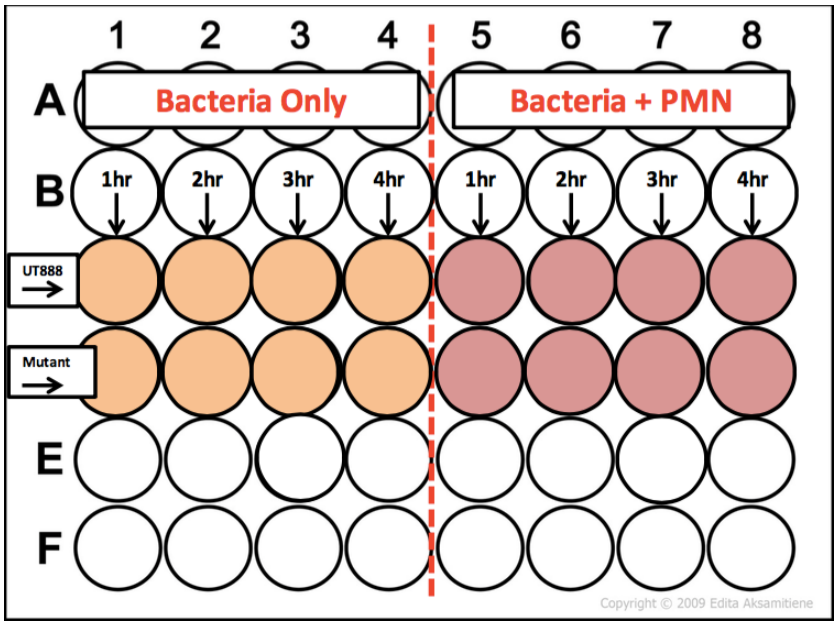

Figure 1. Arrangement of parent and mutant strains in the 48-well plate used during the bactericidal assay. Each strain has a well for each time point—1, 2, 3, and 4 hours—for cultures grown in the presence of neutrophils (PMN+) and without (PMN-). The depiction shown represents a trial testing a single mutant vs. the UT888 parent strain.

3. RESULTS

The CFU/mL data collected on day three of the protocol was used to calculate the ratio of cell growth for each of the strain in PMN+: PMN- conditions for each time point (Fig. 2 and 3). The four-hour time point appeared the most relevant because it allowed sufficient time for the bacteria to recover from the stress of changing media prior to co-culturing in the 48-well plate and because it consistently displayed the greatest growth. Cells depleted available nutrients by 24 hours in initial trials. The ratio of initial bacteria CFU preceding co-culture to neutrophil concentration was also calculated (Tables 1 and 2).

In initial trails, frozen neutrophils from a single cow were used in order to limit variation between trials, but the extracted neutrophils were over-activated following the thawing process and prematurely exhausted themselves in the corresponding bactericidal assays. Viability decreased further with extended freezing time. To maintain the level of cell activity for the bactericidal assay, the trials shown used freshly isolated neutrophils.

A significant amount of variation was observed in the degree of susceptibility to neutrophil killing in the trials for the parent strain UT888, ranging from a low of 0.518 to a high of 2.406 (Fig. 2). The mean ratio for all UT888 trials was 1.152, with a standard deviation of 0.683.

Five of the six mutants displayed a greater ratio of cell growth of PMN+: PMN- in the first round of trials (Fig. 3). A single mutant, 12E2, displayed a lower ratio of growth in PMN+: PMN- in the initial trials, but repetition of this trial with a reduced inoculation amount yielded a greater ratio of growth. The mean ratio for all mutant trials was 1.303, with a standard deviation of 0.385.

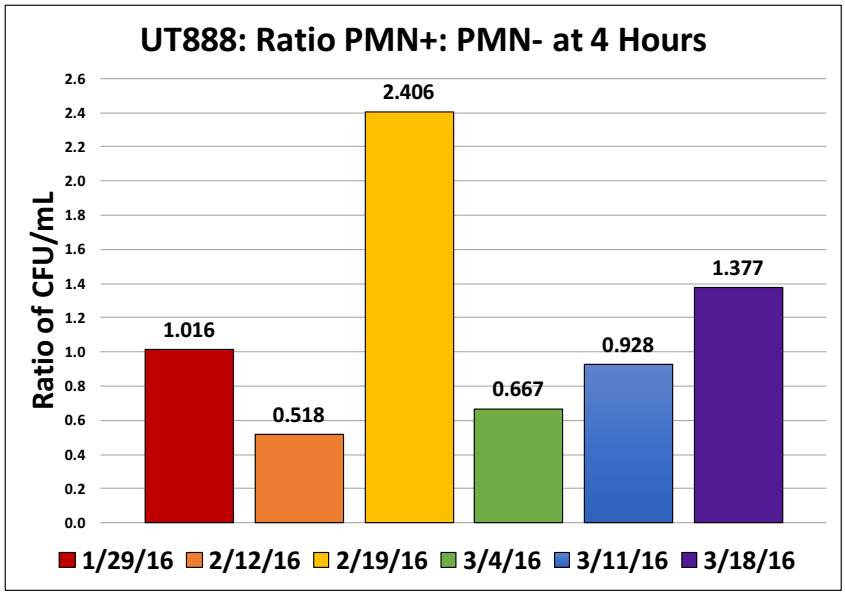

Figure 2. Ratio of CFU/mL in the PMN+ vs. PMN- samples taken at the 4-hour time point for each trial's respective UT888 culture. Each color corresponds to a different trial date.

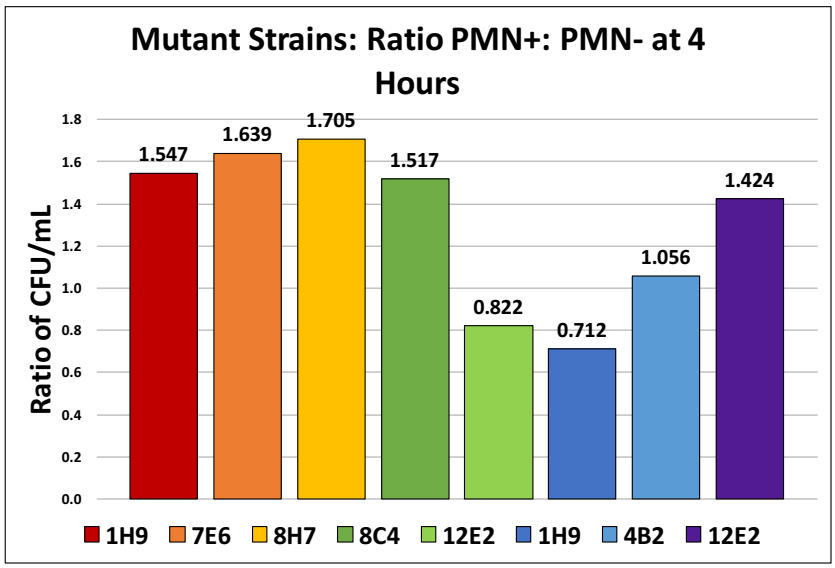

Figure 3. Ratio of CFU/mL in the PMN+ vs. PMN- samples taken at the 4-hour time point for each trial's respective mutant(s). Each color corresponds to a different trial date, which matches that of the UT888 trial performed on the same day. Trials performed with 2 mutants are represented by 2 different shades within the same color family.

Table 1. Initial ratio of CFU to PMN before co-culturing in the 48-well plate for the UT888 trials.

Strain	Date	Initial Ratio CFU:PMN
UT888	1/29/16	144.667
UT888	2/12/16	211.000
UT888	2/19/16	148.000
UT888	3/4/16	15.200
UT888	3/11/16	9.867
UT888	3/18/16	13.800

Table 2. Initial ratio of CFU to PMN before co-culturing in the 48-well plate for the mutant strain trials. The final trial shown (3/18/16) used a reduced initial inoculation amount equivalent to one third of the original.

Strain	Date	Initial Ratio CFU:PMN
1H9	1/29/16	1826.667
7E6	2/12/16	870.000
8H7	2/19/16	1813.333
8C4	3/4/16	109.333
12E2	3/4/16	89.333
1H9	3/11/16	181.3333333
4B2	3/11/16	22.933
12E2	3/18/16	76.800

4. DISCUSSION

The objective of this study was to verify the results of the preliminary MTT screening, which determined that the selected mutants of the clinical isolate *S. uberis* UT888—1H9, 4B2, 7E6, 8C4, 8H7, and 12E2—had greater ROS production in the presence of neutrophils than without. This trend was interpreted to indicate that the transposon may have inserted into a gene related to the ability of *S. uberis* UT888 to evade neutrophils.

A surprising level of variation was observed in the growth of UT888. As shown in Figure 2, the ratio of cell growth in PMN+: PMN- conditions ranged from a low of 0.518 to a high of 2.406 in the six trials shown. As UT888 is a clinical isolate that was previously shown to induce experimental infection and is characterized by its ability to evade neutrophils (Rambeaud et al. 2003), the degree of variation in this

study was unexpected, in that a lower ratio of growth would suggest susceptibility to neutrophils. However, this variation in resistance to neutrophils could be attributed to the use of freshly extracted neutrophils as opposed to frozen neutrophils, which were not viable in initial trials. Due to the nature of a dairy operation and animal handling regulations, blood was collected from a different cow for each week's trial, introducing the possibility that the viability and killing ability of neutrophils differed due to lactation stage, health status, or other cow specific variables.

Five of the six mutant strains tested displayed a greater ratio of cell growth in PMN+: PMN- conditions in the first round of trials, as depicted in Figure 3. This greater ratio of growth suggests that these strains were not only able to evade neutrophil action but also able to proliferate. Interestingly, this finding appears to contradict the results of the earlier screening, which indicated that the selected mutants had significantly greater ROS generation in the presence of neutrophils and would appear to suggest that these strains were susceptible to neutrophil killing. However, this finding introduces the possibility that the increased ROS generation observed in the initial MTT assay were due to the rapid growth of bacteria and consequent depletion of nutrients, rather than to the susceptibility to neutrophils as initially assumed.

A single mutant—12E2—displayed a lower ratio of cell growth in PMN+: PMN- conditions in the first round of trials (Fig. 3), which denotes susceptibility to neutrophils. This lower ratio was interpreted to indicate that the transposon had inserted into a gene related to the ability of *S. uberis* UT888 to evade neutrophils. In order to determine if this observed lower growth ratio was consistent, the 12E2 assay was repeated on 3/18/16. Given the large growth ratios observed in the early trials, the initial inoculation amount on day two of the bactericidal assay was reduced to one-third the original amount (e.g., reduced from 1.5 mL to 0.5 mL) for this final trial in order to investigate the possibility that the neutrophils were being overwhelmed by the disproportionately large number of bacteria. Under these conditions, 12E2 displayed less susceptibility to neutrophil killing, as demonstrated by an increase in the ratio of growth in PMN+: PMN- conditions (Fig. 3).

5. CONCLUSION

This study provides insight into the genetic basis of the ability of *S. uberis* to evade the host immune response. Given the degree of variation observed in this study, it is difficult to arrive at many concrete conclusions. However, these findings suggest that the increase in ROS generation observed in preliminary MTT assays could be tied to increased cell growth and the consequent depletion of nutrients

as opposed to the previously assumed susceptibility to neutrophils. Results should be validated in future studies with a greater number of repetitions per strain. In addition, it is recommended that the inoculation amount be adjusted to standardize the initial ratio of CFU: PMN in order to exclude the possibility that the neutrophils could be overwhelmed by the number of bacteria. A better understanding of the genetic basis of the ability of *S. uberis* to resist neutrophil killing will provide much needed insight into the interactions of environmental pathogens with the bovine immune system and support the development of more effective mastitis prevention and control methods in the dairy industry.

ACKNOWLEDGMENTS

I am enormously thankful for the guidance of my research advisor, Dr. Gina Pighetti, who was willing to take me on as a student after my initial plans went awry and without whom I would have been lost. Special thanks goes to Lydia Siebert, a Ph.D. candidate in Dr. Pighetti's lab who walked me through every step of this project and answered all of my many questions. Thanks also go to Leszek Wojakiewicz for his help in the lab and experimental design.

REFERENCES

Field, T.R., P.N. Ward, L.H. Pedersen, J.A. Leigh. 2003. The hyaluronic acid capsule of *Streptococcus uberis* is not required for the development of of infection and clinical mastitis. Infect. Immun. 71: 132-9.

Hossain, M., S.A. Egan, T. Coffey, P.N. Ward, R. Wilson, J.A. Leigh, R.D. Emes. 2015. Virulence related sequences; insights provided by comparative genomics of *Streptococcus uberis* of differing virulence. BMC Genomics 16: 334.

Kerro Dego, O., M.E. Prado, X. Chen, D.A. Luther, R.A. Almeida, and S.P. Oliver. 2011. PGh9:ISS1 transpositional mutations in Streptococcus uberis UT888 causes reduced bacterial adherence to and internalization into bovine mammary epithelial cells. Vet. Microbiol. 151: 379- 385.

Leigh, J.A., S.A. Egan, P.N. Ward, T.R. Field, T.J. Coffey. 2010. Sortase anchored proteins of *Streptococcus uberis* play major roles in the pathogenesis of bovine mastitis in dairy cattle. Vet. Res. 41: 63-71.

Leigh, J.A., T.R. Field. 1994. Streptococcus uberis resists the bactericidal action of bovine neutrophils despite the presence of bound immunoglobulin. Infect. Immun. 62(5): 1854-9.

Oliver, S.P. 1988. Frequency of isolation of environmental mastitis-causing pathogens and incidence of new intrammamary infection during the nonlactating period. Am. J. Vet. Res. 49: 1789-93.

Oliver, S.P., B.A. Mitchell. 1984. Prevalence of mastitis pathogens in herds participating in a mastitis control pathogen. J. Dairy Sci. 67: 2436-2440.

Phuektes, P., P.D. Mansel, S.D. Rodney, N.D. Hooper, J.S. Dick, G.F. Browning. 2001. Molecular epidemiology of *Streptococcus uberis* isolates from dairy cows with mastitis. J. Clin. Microbiol. 39: 1460-6.

Rambeaud, M., R.A. Almeida, G.M. Pighetti, S.P. Oliver. 2003. Dynamics of leukocytes and cytokines during experimentally induced *Streptococcus uberis* mastitis. Vet. Immunol. Immunopathol. 96: 193-205.

Schukken, Y.H., J. Günter, J. Fitzpatrick, M.C. Fontaine, L. Goetze, O. Holst, J. Leigh, W Petzl, H.-J. Schuberth, A. Sipka, D.G.E. Smith, R. Quesnell, J. Watts, R. Yancey, H. Zerbe, A. Gurjar, R. N. Zadoks, H.-M. Seyfert. 2011. Host-response patterns of intrammamary infections in dairy cows. Vet. Immunol. Immunopathol. 144: 270-89.

Wilson, D.J., R. N. Gonzalez, H. H. Das. 1997. Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. J. Dairy Sci. 80: 2592-2598.