Vaginal microbial communities do not preclude colonization by *Staphylococcus aureus* with the gene for toxic shock syndrome toxin 1 (TSST-1)

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Introduction

The human body is host to more than ten times as many bacterial cells as human cells (Hooper and Gordon 2001). Virtually all sites of the body connected to the external environment are colonized by indigenous communities of bacteria that are exposed to microorganisms from the environment, some of which can be pathogenic. Thus, these indigenous communities represent the first line of defense against infectious disease and contribute in other ways to human health. Direct benefits of the indigenous microbial communities have been described for the microbial community in the intestinal environment and affect the efficiency in digesting and extracting nutrients from food that is consumed (Wostmann *et al.* 1983, Xu *et al.* 2003). The work of Wostmann et. al. (1983) shows how gnotobiotic rats need more calories to maintain the same weight as rats with normal microbial communities. Another study showed how obesity affects the microbial community, and suggested a role for the microbial community in improving health (Ley *et al.* 2005). In addition, there also appears to be indirect effects that are influenced by the indigenous microbial community, such as an apparent link between the normal intestinal microbial community and the development of allergies, such as atopic eczema (Kalliomaki and Isolauri 2003, Macdonald and Monteleone 2005). It is also thought that one of the indirect effects of the indigenous microbial community is the inhibition of colonization by pathogens. Characterizing the indigenous microbial communities in healthy individuals and those colonized by a pathogen may help us understand disease development.

Studies of the vagina, oral cavity, gut and other body sites have identified a vast number of bacterial at these body sites and shown that remarkable diversity exists between individuals (Figure 1, Table 1). Even though there is ongoing debate about which method is most appropriate for estimating species richness, these examples show that many bacterial species colonize the human body. Studies of the microbiology of the human body have historically focused on characterizing single species in pure cultures independent of the microbial

community from which they were isolated, and both pathogenic and indigenous microorganisms have been characterized in this way. This could be misleading because these species (populations) exist and function in the context of microbial communities wherein the phenotypes expressed are determined by the biotic interactions between distinct populations and with the host, and are influenced by the chemical and physical characteristics of the ecosystem in which they reside. Moreover, in the past investigators have largely relied on cultivation-dependent approaches in order to characterize the normal composition of these communities. The use of cultivation-dependent methods has resulted in numerically and ecologically important organisms being overlooked because we cannot yet culture all bacterial species. Equally important is that this resource intensive approach has precluded detailed longitudinal studies to examine natural fluctuations in community composition and function, or changes that occur in response to disturbances. Thus, little is known about the variation of normal compositions of indigenous microbial communities within individuals over time, and how they serve to maintain health.

Much of the history of medical microbiology has focused on identifying single pathogenic species; a focus that emanates from using the principles set forth by Koch's postulates (Grimes, 2006). These postulates require that a pathogen be isolated from all individuals who are affected by the disease then grown in pure culture, and innoculation of healthy individuals with the pure culture must then cause disease. Finally, the organism must be re-isolated from the individual made sick by the pathogen from pure culture. While this guide has proved useful for identifying numerous pathogenic organisms, some pathogens would not be accurately identified if these criteria were strictly adhered to. Koch himself found that cholera, one of the pathogens for which the postulates were developed, would not strictly meet these criteria as there are individuals that carry the pathogen but do not get sick (Grimes, 2006). Moreover, recent studies have provided evidence that some disease conditions are not caused by single infectious agents that can be specified by fulfilling Koch's postulates. Certain types of infectious disease

do not satisfy Koch's postulates and are classified as "polymicrobial infections" or result from changes in the relative abundances of autochthonous populations and may include those caused by opportunistic pathogens. Examples of these diseases include bacterial vaginosis (Reid and Bocking 2003), inflammatory bowel disease (Dai and Walker 1999, Linskens *et al.* 2001), and dental caries (Liljemark and Bloomquist 1996). To better characterize these disorders a different approach than Koch's postulates will need to be taken.

When examining the composition of the normal microbial communities of the human vagina there appear to be a limited number of community types that are present in healthy women, each of which exhibit dynamic equilibria in terms of their species composition. In contrast, bacterial vaginosis (BV) is characterized by an extraordinary change from the normal community structure (Eschenbach *et al.* 1988, Nugent *et al.* 1991). It is thought that these changes are often induced by certain behaviors, such as douching, having multiple sex partners, and frequent intercourse, that disrupt the homeostasis of an indigenous vaginal microbial community (Ness *et al.* 2002). In addition to problems that may be brought about by the changes to the community structure of indigenous microbial communities themselves, it is also thought that disturbances in an indigenous microbial community can facilitate colonization of pathogenic organisms (Guarner and Malagelada 2003, Eckburg *et al.* 2005). For example, it is known that women with BV are more likely to contract HIV and other sexually transmitted diseases than women without BV (Taha *et al.* 1998). This suggests that within the vaginal ecosystem, changes in the species composition and structure of the microbial community present will change the risk of acquiring disease after exposure to a pathogen. The relationship between the indigenous microbial community and colonization by a pathogen is a compelling hypothesis because it suggests that indigenous microbial communities are able to preclude pathogens with different levels of success.

It would be consistent with ecological theories of invasion if communities did possess differential abilities to exclude invasive species such as pathogens. For plants it has been shown that communities that differ in species composition vary in their ability to exclude invading exotic species (Sousa 1984, Hobbs and Huenneke 1992, Mack *et al.* 2000, Marvier *et al.* 2004). Understanding which communities are better at excluding a pathogen within the vaginal ecosystem would help to define risk to disease. As we learn more it is becoming apparent that indigenous microbial communities themselves may be among the risk factors that determine whether or not a person contracts a disease. An increased understanding of how indigenous microbial communities affect health will undoubtedly lead to the development of ways to manage these natural ecosystems to maintain the healthy dynamic equilibria of microbial communities that constitute the human microbiome.

Microbial Communities of the Human Vagina

Previous research has conclusively shown there are distinctive differences in the composition of vaginal communities in reproductive age women and a variety of community types can be considered normal and characteristic of health. (Sobel *et al.* 1981, Redondo-Lopez *et al.* 1990, Zhou *et al.* 2004, Hyman *et al.* 2005, Zhou *et al.* 2007). The studies done on vaginal community composition so far have largely employed a cross-sectional design wherein women were sampled at a single point in time. Because of the cross-sectional design too little is known about how community composition changes over time. It is difficult to understand the stability of different community compositions and how they respond to disturbance without performing longitudinal studies. The studies that have been performed over time have been done using microscopy or in conjunction with a treatment (Keane *et al.* 1997, Schwebke *et al.* 1999, Clarke *et al.* 2002). The available evidence suggests that there is variation in the relative abundances of populations over time, and that the magnitude of changes in community structure may differ

among women (R. Hickey, personal communication, Keane *et al.* 1997, Schwebke *et al.* 1999, Clarke *et al.* 2002). Understanding how the indigenous microbial community experiences temporal changes, either over the course of the menstrual cycle or over longer courses of time, will provide better information for assessing differences in risk to acquiring diseases.

Menstrual Toxic Shock (mTSS)

Between 1980 and 1981 1,047 otherwise healthy young women across the United States became ill and were eventually diagnosed with menstrual toxic shock syndrome (mTSS), a disease that had been described only two years earlier (Todd *et al.* 1978, Reingold *et al.* 1982, Todd 1988). Todd first published the connection that TSS cases were concurrent with colonization by *Staphylococcus aureus* (*S. aureus*). Toxic shock syndrome is characterized by a high fever, hypotension, desquamation and the involvement of multiple organ systems, and in some cases death. For the treatment of TSS intravenous immunoglobulin is used to inactivate the toxin that causes mTSS (Chesney 1983). In 1980 an association between the use of tampons, specifically super absorbent tampons, was postulated (Shands *et al.* 1980). Based on epidemiological data that linked the use of super absorbent tampons to mTSS these tampons were removed from the market and recommended practices of tampon usage were implemented. Subsequently the incidence of mTSS began to decrease from 6 in 100,000 women per year (Hajjeh *et al.* 1999). Current incidence of mTSS are still approximately 1 in 100,000 women per year, where ~2% of cases prove to be fatal (Hajjeh *et al.* 1999).

Toxic shock syndrome toxin 1 (TSST-1) was discovered in 1981 (Bergdoll *et al.* 1981, Schlievert *et al.* 1981) and appears to be responsible for mTSS. It is produced by strains of *S. aureus* with the TSST-1 gene (*tst*$^{+}$) and acts as a superantigen. Superantigens are proteins that bind T-cell receptors to MHC complexes of somatic cells without first being processed by the cell. Because they do not bind to the variable regions of T-cell receptors or MHC complexes,

superantigens initiate an immune response that is larger than the conventional immune response. Very little toxin is needed to elicit dramatic effects. Theoretically, as little as 10 picograms can stimulate upwards of 30% of an individual's T-cells. Work with rabbit models confirmed that the TSST-1 toxin is necessary and sufficient to induce toxic shock syndrome (Parsonnet *et al.* 1987). While the specific mechanisms that cause mTSS are understood, there are multiple steps involved in the development of the disease, most of which are poorly understood.

Development of mTSS: a multistep process

The development of disease in humans by organisms that produce toxins is a complex process that requires a confluence of events that involve not only the pathogen and host, but also the indigenous microorganisms of the human microbiome as well as physical and chemical characteristics of the anatomical site of infection. The circumstances that must exist in the stepwise events leading to disease can be illustrated by examining what occurs during the development of mTSS caused by *S. aureus* (see Figure 2). The initial event is colonization itself wherein an infectious dose of the pathogenic organism invades the vagina and retained by adherence to receptors expressed on epithelial cells or by some other means (step 1, Fig. 2). These organisms must then persist and avoid washout in vaginal secretions, and clearance by the host immune system. The environment of the vagina itself may help to prevent effective colonization by having a low pH that reduces the viability of *S. aureus tst*$^{+}$ cells (Whiting *et al.* 1996). If successfully introduced these organisms must then effectively compete with indigenous populations for nutrients and other resources that are required for bacterial cell growth and division. By doing so, the cell numbers of the pathogen will increase over time (step 2, Fig. 2). Often times invasion, persistence, and proliferation are lumped together and referred to simply as "colonization" when in fact they are three discrete events. Obviously the mere

presence of the pathogen is insufficient to cause disease in the absence of toxin expression; a process that occurs only under certain environmental conditions. Indeed, previous studies have shown that the expression of TSST-1 is subject to complex regulation by various factors that include, but are not limited to pH, oxygen, and carbon dioxide (Ross and Onderdonk 2000, Yarwood *et al.* 2000). Thus, for toxin expression to occur in women colonized by *S. aureus* the environmental conditions must be permissive to expression of the *tst* gene (step 3, Fig. 2). The conditions that prevail within the vagina are influenced by the metabolic activities of both the microbial community and host. In this regard it should be noted that the host has a major influence on microbial activities since all the nutrients metabolized by the bacterial populations present are derived from the host itself. The first three steps in the development of mTSS - colonization, proliferation and toxin production - appear to be the steps that are mostly influenced by the microbial community of the vagina. Once produced in the vagina, TSST-1 is subject to proteolysis. This is likely to occur to at least some extent given the high numbers of anaerobic bacteria typically present in vaginal communities; many of which are notoriously proteolytic. Thus it is feasible that even under conditions that favor expression of the *tst* gene, the extracellular levels of the toxin will be nil. Alternatively, the toxin once produced could be neutralized by mucosal antibodies. Thus, both proteolysis and the immune system could act to prevent persistence of the toxin in the vagina (step 4, Fig. 2). As described earlier, mTSS develops by eliciting a cytokine cascade triggered by T-cells in the circulatory system. To do so requires that the toxin cross the vaginal epithelium and enter the bloodstream (step 5, Fig. 2). The mechanism by which this occurs is not well understood, but the process is dose dependent and not very efficient (Davis *et al.* 2003). Once in the blood the toxin must productively interact with T-cells to elicit the exaggerated immune response that is characteristic of the disease (step 6, Fig. 2) before being neutralized by toxin-specific antibodies or otherwise cleared from the body. These last steps (4-6) are more likely influenced by host-specific factors rather than the

microbial community. This simple overview of the steps that must occur in the development of mTSS illustrates the complexity of the process. Three points should be emphasized. First, colonization, though necessary, is not sufficient to cause disease. The importance of this was illustrated by Parsonnet *et al.* (Parsonnet *et al.* 2005) who showed that 32 women of 3012 surveyed were vaginally colonized by *S. aureus tst*$^{+}$ yet did not have mTSS. Second, the development of disease can fail at any of the steps outlined above. Finally, the species composition and activities of indigenous bacterial communities could profoundly affect risk to mTSS.

The work summarized in this thesis was designed to assess whether indigenous vaginal microbial communities influence colonization by *S. aureus tst*$^{+}$. Samples taken from healthy women not colonized by *S. aureus tst*$^{+}$ were compared to women colonized by *S. aureus tst*$^{+}$ using cultivation-independent methods based on the analysis of 16s rRNA gene sequences.

References

Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis. 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome Staphylococcus aureus isolates. *Lancet* **1**:1017-1021.

Chesney, P. J. 1983. Toxic-shock syndrome: A commentary and review of the characteristics of Staphylococcus aureus strains. *Infection* **11**:181-188.

Clarke, J. G., J. F. Peipert, S. L. Hillier, W. Heber, L. Boardman, T. R. Moench, and K. Mayer. 2002. Microflora changes with the use of a vaginal microbicide. *Sex Transm Dis* **29**:288-293.

Dai, D., and W. A. Walker. 1999. Protective nutrients and bacterial colonization in the immature human gut. *Adv Pediatr* **46**:353-382.

Davis, C. C., M. J. Kremer, P. M. Schlievert, and C. A. Squier. 2003. Penetration of toxic shock syndrome toxin-1 across porcine vaginal mucosa ex vivo: permeability characteristics, toxin distribution, and tissue damage. *Am J Obstet Gynecol* **189**:1785-1791.

Dethlefsen, L., M. McFall-Ngai, and D. A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* **449**:811-818.

Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* **308**:1635-1638.

Eschenbach, D. A., S. Hillier, C. Critchlow, C. Stevens, T. DeRouen, and K. K. Holmes. 1988. Diagnosis and clinical manifestations of bacterial vaginosis. *Am J Obstet Gynecol* **158**:819-828.

Grimes, D. J. 2006. Koch's Postulates—Then and Now. *Microbe* **1**:223-228.

Guarner, F., and J. R. Malagelada. 2003. Gut flora in health and disease. *Lancet* **361**:512-519.

Hajjeh, R. A., A. Reingold, A. Weil, K. Shutt, A. Schuchat, and B. A. Perkins. 1999. Toxic shock syndrome in the United States: surveillance update, 1979 1996. *Emerg Infect Dis* **5**:807-810.

Hobbs, R., and L. Huenneke. 1992. Disturbance, Diversity, and Invasion: Implications for Conservation. *Conservation Biology* **6**:324-337.

Hooper, L. V., and J. I. Gordon. 2001. Commensal host-bacterial relationships in the gut. *Science* **292**:1115-1118.

Hyman, R. W., M. Fukushima, L. Diamond, J. Kumm, L. C. Giudice, and R. W. Davis. 2005. Microbes on the human vaginal epithelium. *Proc Natl Acad Sci U S A* **102**:7952-7957.

Kalliomaki, M., and E. Isolauri. 2003. Role of intestinal flora in the development of allergy. *Curr Opin Allergy Clin Immunol* **3**:15-20.

Keane, F., C. Ison, and D. Taylor-Robinson. 1997. A longitudinal study of the vaginal flora over a menstrual cycle. *International Journal of STD \& AIDS* **8**:489.

Ley, R. E., F. Backhed, P. Turnbaugh, C. A. Lozupone, R. D. Knight, and J. I. Gordon. 2005. Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A* **102**:11070-11075.

Liljemark, W. F., and C. Bloomquist. 1996. Human oral microbial ecology and dental caries and periodontal diseases. *Crit Rev Oral Biol Med* **7**:180-198.

Linskens, R. K., X. W. Huijsdens, P. H. Savelkoul, C. M. Vandenbroucke-Grauls, and S. G. Meuwissen. 2001. The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. *Scand J Gastroenterol Suppl* 29-40.

Macdonald, T. T., and G. Monteleone. 2005. Immunity, inflammation, and allergy in the gut. *Science* **307**:1920-1925.

Mack, R., D. Simberloff, W. Lonsdale, H. Evans, M. Clout, and F. Bazzaz. 2000. Biotic invasions: Causes, epidemiology, global consequences, and control. *Ecological Applications* **10**:689-710.

Marvier, M., P. Kareiva, and M. G. Neubert. 2004. Habitat destruction, fragmentation, and disturbance promote invasion by habitat generalists in a multispecies metapopulation. *Risk Anal* **24**:869-878.

Ness, R. B., S. L. Hillier, H. E. Richter, D. E. Soper, C. Stamm, J. McGregor, D. C. Bass, R. L. Sweet, and P. Rice. 2002. Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina. *Obstet Gynecol* **100**:765.

Nugent, R. P., M. A. Krohn, and S. L. Hillier. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* **29**:297-301.

Parsonnet, J., Z. A. Gillis, A. G. Richter, and G. B. Pier. 1987. A rabbit model of toxic shock syndrome that uses a constant, subcutaneous infusion of toxic shock syndrome toxin 1. *Infect Immun* **55**:1070-1076.

Parsonnet, J., M. A. Hansmann, M. L. Delaney, P. A. Modern, A. M. Dubois, W. Wieland-Alter, K. W. Wissemann, J. E. Wild, M. B. Jones, J. L. Seymour, and A. B. Onderdonk. 2005. Prevalence of toxic shock syndrome toxin 1-producing Staphylococcus aureus and the presence of antibodies to this superantigen in menstruating women. *J Clin Microbiol* **43**:4628-4634.

Redondo-Lopez, V., R. L. Cook, and J. D. Sobel. 1990. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. *Rev Infect Dis* **12**:856-872.

Reid, G., and A. Bocking. 2003. The potential for probiotics to prevent bacterial vaginosis and preterm labor. *Am J Obstet Gynecol* **189**:1202-1208.

Reingold, A. L., N. T. Hargrett, K. N. Shands, B. B. Dan, G. P. Schmid, B. Y. Strickland, and C. V. Broome. 1982. Toxic shock syndrome surveillance in the United States, 1980 to 1981. *Ann Intern Med* **96**:875-880.

Ross, R. A., and A. B. Onderdonk. 2000. Production of toxic shock syndrome toxin 1 by Staphylococcus aureus requires both oxygen and carbon dioxide. *Infect Immun* **68**:5205-5209.

Schlievert, P. M., K. N. Shands, B. B. Dan, G. P. Schmid, and R. D. Nishimura. 1981. Identification and characterization of an exotoxin from Staphylococcus aureus associated with toxic-shock syndrome. *J Infect Dis* **143**:509-516.

Schwebke, J. R., C. M. Richey, and H. L. Weiss2. 1999. Correlation of behaviors with microbiological changes in vaginal flora. *J Infect Dis* **180**:1632-1636.

Shands, K. N., G. P. Schmid, B. B. Dan, D. Blum, RJ, Guidotti, NT, Hargrett, RL, Anderson, DL, Hill, CV, Broome, JD, Band, and others. 1980. Toxic-shock syndrome in menstruating women: association with tampon use and Staphylococcus aureus and clinical features in 52 cases. *New England Journal of Medicine* **303**:1436-1442.

Sobel, J. D., J. Schneider, D. Kaye, and M. E. Levison. 1981. Adherence of bacteria to vaginal epithelial cells at various times in the menstrual cycle. *Infect Immun* **32**:194-197.

Sousa, W. P. 1984. The Role of Disturbance in Natural Communities. *Annu Rev Ecol Syst* **15**:353-391.

Taha, T. E., D. R. Hoover, G. A. Dallabetta, N. I. Kumwenda, L. A. Mtimavalye, L. P. Yang, G. N. Liomba, R. L. Broadhead, J. D. Chiphangwi, and P. G. Miotti. 1998. Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. *AIDS* **12**:1699-1706.

Todd, J. 1988. Toxic shock syndrome. *Clin Microbiol Rev* **1**:432-446.

Todd, J., M. Fishaut, F. Kapral, and T. Welch. 1978. Toxic-shock syndrome associated with phage-group-I Staphylococci. *Lancet* **2**:1116-1118.

Whiting, R. C., S. Sackitey, S. Calderone, K. Morely, and J. G. Phillips. 1996. Model for the survival of Staphylococcus aureus in nongrowth environments. *Int J Food Microbiol* **31**:231-243.

Wostmann, B. S., C. Larkin, A. Moriarty, and E. Bruckner-Kardoss. 1983. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. *Lab Anim Sci* **33**:46-50.

Xu, J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper, and J. I. Gordon. 2003. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. *Science* **299**:2074-2076.

Yarwood, J. M., D. Y. Leung, and P. M. Schlievert. 2000. Evidence for the involvement of bacterial superantigens in psoriasis, atopic dermatitis, and Kawasaki syndrome. *FEMS Microbiol Lett* **192**:1-7.

Zhou, X., S. J. Bent, M. G. Schneider, C. C. Davis, M. R. Islam, and L. J. Forney. 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiology* **150**:2565-2573.

Zhou, X., C. J. Brown, Z. Abdo, C. C. Davis, M. A. Hansmann, P. Joyce, J. A. Foster, and L. J. Forney. 2007. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J* **1**:121-133.

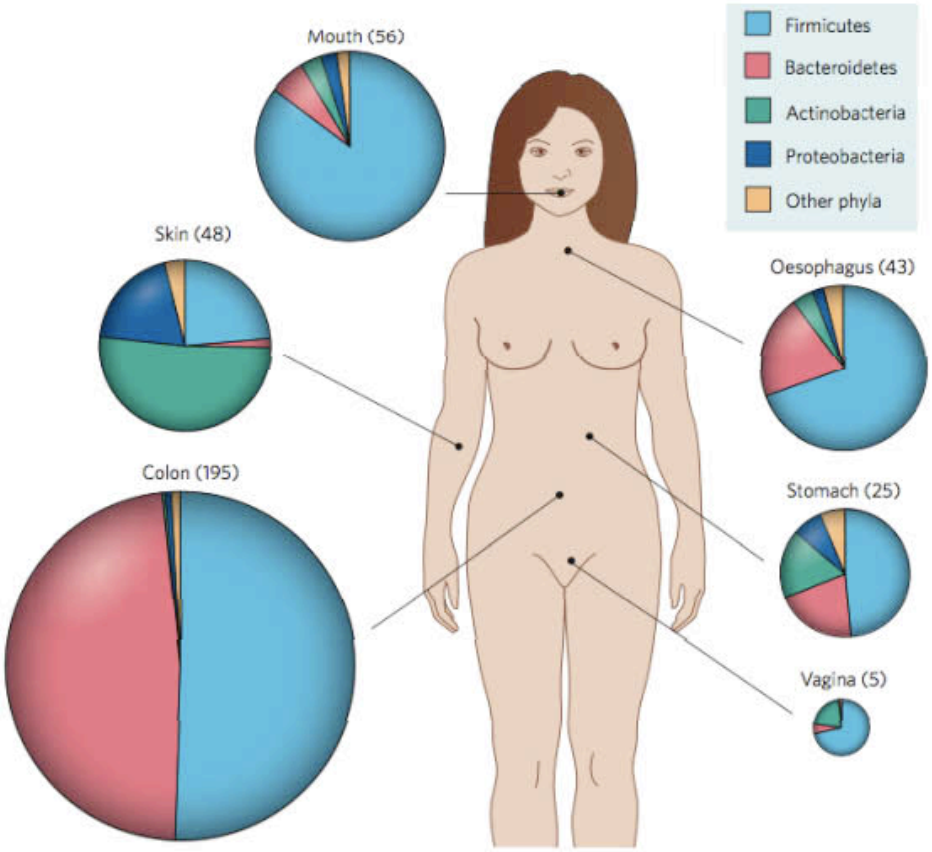

Figure 1. The human body is host to many different microbial communities. These communities are largely composed of species that are from four bacterial phyla, have characteristic community compositions, diversities, and abundances. This figure illustrates the mean species diversity in each of the sites of colonization, parenthetically noted, and the abundance of bacterial species is represented by the area of each pie graph (Dethlefsen *et al.* 2007).

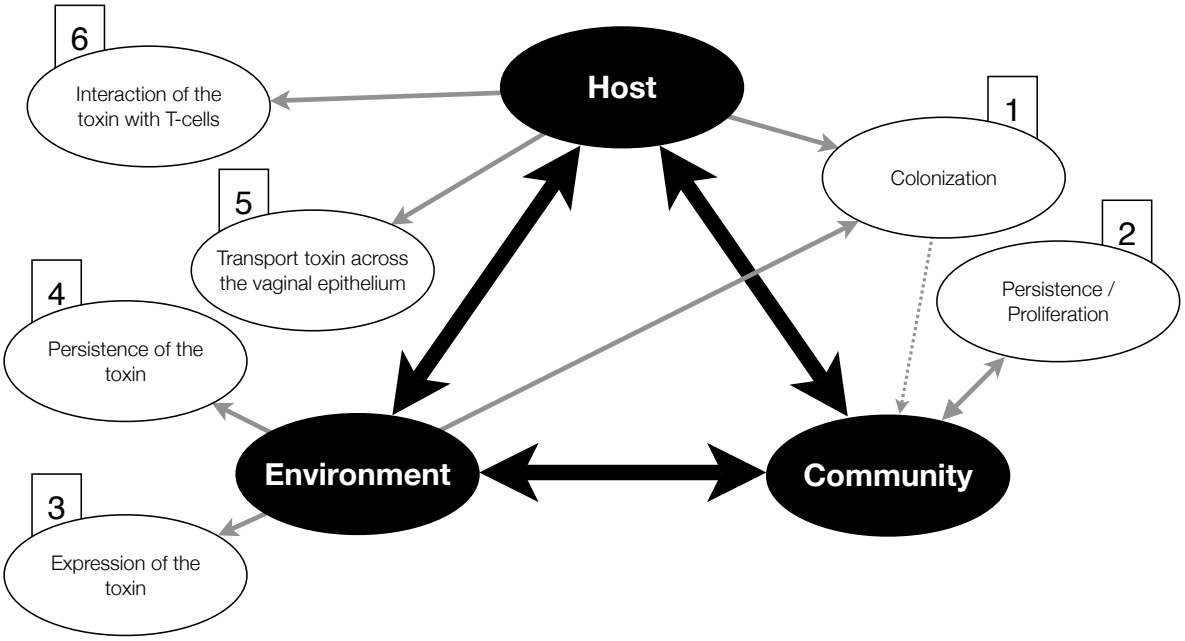

Figure 2. The network of ecological interactions that influences the development of mTSS. The three solid bubbles represent the different environmental variables present in the vagina, whereas the open bubbles represent the steps involved in mTSS development.

Table 1. Estimates of species abundance in different body locations based on 16S rRNA gene sequence data.

Location	Number of species present	Number of sequences	Number of subjects	References
Skin	~400	1,345	6	Gao et. al. 2007
Intestine	> 500	13,355	3	Eckburg *et. al*. 2005
Vagina	>275[a]	37,618	20	Hyman *et. a*l. 2005
Vagina	>75	6,259	52	Zhou *et. al.* 2007[b]
Mouth	~500	2,522	31	Paster *et. al*. 2001

a) As identified from the closest Genbank match in contrast to operational taxonomic units identified in the other studies.
b) Unpublished data.

CHAPTER 1

(to be submitted for publication)

Vaginal microbial communities do not preclude colonization by *Staphylococcus aureus* with the gene for toxic shock syndrome toxin 1 (TSST-1)

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Running title: mTSS and vaginal microbial communities

Key words: Toxic shock syndrome, mTSS, TSS, indigenous bacteria, vaginal microbial communities, community invasion, pathogen colonization.

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Abstract

The factors that determine a woman's risk to menstrual toxic shock syndrome (mTSS) are not well understood. Although not required for the diagnosis of mTSS, vaginal colonization by *Staphylococcus aureus* (*tst*$^{+}$) is surely a prerequisite for disease. Previous studies showed that diversity exists in the types of communities that are indigenous to the vagina, and these communities may contribute differently to the risk of colonization by *S. aureus*. This study was designed to assess whether or not the vaginal microbial community influences the colonization of toxigenic *S. aureus*. To this end samples were taken from women colonized or not colonized with *Staphylococcus aureus* that has the TSST-1 toxin gene. Differences in the vaginal communities of women in these two groups were assessed based on profiles of terminal restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes. Communities were classified into groups based on similarities in community profiles and the results showed there were no detectable differences in community type or rank abundance between the communities of women vaginally colonized with *S. aureus tst*$^{+}$ as compared to those that were not. Phylogenetic analysis of cloned 16S rRNA genes from vaginal communities of women colonized with *S. aureus tst*$^{+}$ showed that the predominant members were similar to those found in previous studies of vaginal microbial communities of healthy women. The analyses done did not reveal any unusual characteristics of vaginal communities from women colonized with S. aureus tst+ as compared to those without the organism. This suggests that vaginal microbial communities of the vagina do not preclude colonization of the vagina by *S. aureus tst*$^{+}$. Other factors that determine whether mTSS develops in women colonized with *S. aureus tst*$^{+}$ are discussed.

Introduction

Menstrual toxic shock syndrome (mTSS) is a rare disease characterized by several symptoms that occur concurrent with menses, including fever, rash, hypotension, desquamation, and the involvement of multiple organ systems (Todd *et al*. 1978). While rare, mTSS is a current health concern, with 1 in 100,000 menstruating women diagnosed with mTSS per year and ~2% of cases resulting in fatality (Hajjeh *et al*. 1999). Early investigations of the cause of mTSS identified toxic shock syndrome toxin 1 (TSST-1) as both necessary and sufficient to induce mTSS (Bergdoll *et al*. 1981, Schlievert *et al*. 1981, Bonventre *et al*. 1983, Cohen *et al*. 1983). It is thought that mTSS is associated with vaginal colonization by strains of *Staphylococcus aureus* that carry the gene for TSST-1 (*S. aureus tst*$^{+}$); though recovery of *S. aureus tst*$^{+}$ is not required for diagnosis of the disease (Reeves *et al*. 1984, Musser *et al*. 1990). TSST-1 is a superantigen that acts by binding to and cross-linking conserved regions in T-cell receptors and the major histocompatibility group II (MHC) proteins on antigen-presenting cells (Kotb 1995, Kotb 1998). Stimulation of the immune system by TSST-1 by this means is problematic because it bypasses the normal processing and presentation of antigens and results in activation of 5-30% of all T-cells, versus a conventional immune response, which typically results in 0.01% of T-cells being activated (Dinges *et al*. 2000, Llewelyn *et al*. 2004). The proliferation of T-cells that occurs is accompanied by a cytokine cascade that produces the symptoms of mTSS which can prove to be fatal (Callahan *et al*. 1990, Misfeldt 1990, Herman *et al*. 1991).

While the molecular mechanism of TSST-1 action is well understood, less is known about the steps leading to development of the disease. Previous work has suggested that three principle events are necessary for mTSS to occur (Parsonnet *et al*. 2005). First, *S. aureus tst*$^{+}$ must colonize and proliferate within the indigenous microbial community of the vagina. Second, sufficient TSST-1 must be produced and persist to cause disease. This requires that the

conditions that prevail in the vagina be conducive to expression of the *tst* gene, which is known to be regulated by environmental factors such as oxygen, carbon dioxide, and pH (Ross and Onderdonk 2000). Once expressed, the toxin must persist and avoid degradation by proteases (Blake *et al*. 1987, Dinges *et al*. 2000) or inactivation by IgM or IgG antibodies present in vaginal secretions (Kansal *et al*. 2007). Third, TSST-1 must penetrate the vaginal epithelium and enter circulation where it can bind to T-cell receptors and MHC proteins and elicit the extraordinary release of cytokines that has been observed in mTSS patients. mTSS will not occur if any of these steps are interrupted (Bonventre *et al*. 1988, Parsonnet *et al*. 2005).

Communities of indigenous microorganisms that reside in the vagina could also influence whether a woman is at risk to mTSS. One way is through the competitive exclusion of invasive species (including pathogens; Alpert *et al*. 2000, Hector *et al*. 2001). This could occur through competition for resources or by blocking adherence to epithelial cell receptors (Zarate and Nader-Macias 2006). In addition, the bacterial populations found in the vagina may change the environment in which they reside and create conditions that preclude successful colonization by non-indigenous species. This could include creation of a low environmental pH (Whiting *et al*. 1996) and the production by low molecular weight fatty acids that inhibit growth (Adams and Hall 1988). Finally, expression of TSST-1 is unlikely to occur unless the indigenous communities maintain a pH and concentrations of O_2 and CO_2 that permit expression of the staphylococcal superantigen TSST-1 (Schlievert and Blomster 1983). The ability of vaginal microbial communities to exclude *S. aureus tst*$^+$ or preclude the expression of TSST-1 might vary among individuals since previous studies by Zhou *et al*. (Zhou *et al*. 2004, Zhou *et al*. 2007) have shown that the composition of these communities varies in terms of the kinds and relative abundances of bacterial species present. Nothing is known about the communities indigenous to the vaginas of women colonized by *S. aureus tst*$^+$.

The present study was done to determine if there was an association between the composition and structure of vaginal microbial communities and the presence of *S. aureus tst*$^{+}$. The samples used for this study were obtained from women who were vaginally colonized with *S. aureus tst*$^{+}$ and a control group consisting of women who were not vaginally colonized with *S. aureus tst*$^{+}$. These samples had been collected as part of a survey of 3,012 healthy women to determine the prevalence of *S. aureus tst*$^{+}$ colonization. Of the women surveyed, 32 were vaginally colonized with *S. aureus tst*$^{+}$ but had not acquired mTSS (Parsonnet *et al*. 2005). The vaginal communities of women in these two groups were compared on the basis of terminal restriction fragment length polymorphism (T-RFLP) profiles of 16S rRNA genes, and the predominant members of vaginal communities of women colonized with S. aureus *tst*$^{+}$ were identified by phylogenetic analysis of cloned 16s rRNA genes.

Materials and Methods

Sample collection

The samples used in this study were collected as part of a study to determine the prevalence of *S. aureus* carriage in reproductive age women in North America (Parsonnet et al. 2005). Three thousand and twelve healthy, menstruating women between the ages of 13 and 40 were enrolled from five geographically separate sites in North America: Cincinnati, OH; East Brunswick, NJ; St. Petersburg, FL; Scottsdale, AZ; and Winnipeg, Manitoba, Canada. The demographics of the women sampled matched the racial profile of the 1990 United States census (White = 80%, Black = 12%, Hispanic = 5%, and Asian = 3%). Subjects were eligible for enrollment if they had regular menstrual cycles (21 – 35 days); used tampons at least occasionally; were able to read, write, and understand English; did not bathe or shower within the 2 hours prior to their scheduled visit; refrained from douching, vaginal medications, suppositories, feminine sprays, genital wipes, or contraceptive spermicides for 48 hours prior to their scheduled visit; and were willing to comply with all other protocol requirements. Subjects were not eligible if they were participating in another clinical study; were pregnant, actively trying to get pregnant or suspected they were pregnant; had a gynecological abnormality as judged by the study medical personnel; had a self-reported infection of the genitals within the past 6 weeks; had been medically diagnosed as having diabetes, kidney failure, hepatitis, AIDS (HIV positive) or toxic shock syndrome; or were using antimicrobial or antifungal drugs to treat a vaginal infection. Subjects completed a demographic questionnaire and classified themselves into one of four distinct racial groups: White, Black, Hispanic or Asian. Prior to sample collection informed consent was obtained from each subject enrolled in this study. Upon collection of the vaginal sample, the attending health practitioner noted any signs of possible genital abnormalities.

Presence of *S. aureus* was determined by culturing on selective media (Parsonnet et al. 2005). Of 3,012 women 268 were colonized vaginally by *S. aureus*. ELISA assays were done on cultivated strains to identify those that produced TSST-1. *S. aureus* strains that produced TSST-1 were found in 32 of the 268 swabs of women vaginally colonized by *S. aureus.*

Vaginal samples analyzed

Fifty-one vaginal swab samples were randomly selected from 4 groups (FemCare Division, Procter & Gamble., Cincinnati, Ohio). Twenty-one of the swabs were from women vaginally colonized with toxigenic S. aureus, while an additional thirty swabs were from women not vaginally colonized with toxigenic S. aureus that belonged to one of three sub-groups: 10 were from women not colonized vaginally, nasally or anally with S. aureus; 10 were from women colonized vaginally with non-toxigenic S. aureus, and 10 were from women whose nose or anus (but not the vagina) was colonized with toxigenic S. aureus. The samples were blinded while T-RFLP profiles of 16S rRNA genes were determined (as described below), but the blind was broken to select the 21 samples from individuals vaginally colonized with toxigenic S. aureus used for the construction of clone libraries.

Genomic DNA Isolation

Bacterial genomic DNA was isolated from vaginal swabs using previously described methods (Zhou *et al*. 2004).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

T-RFLP profiles of 16S rRNA genes were determined for each sample (Liu *et al*. 1997). Internal regions of 16s rRNA genes were amplified by PCR using the fluorescently labeled

primer pair, 8fm (AGAGTTTGATCMTGGCTCAG) and 926r (CCGTCAATTCCTTTRAGTTT) Invitrogen, Carlsbad, CA). Primer 8fm was labeled with VIC and 926r was labeled with 6-carboxy-flourescin (6-FAM; Applied Biosystems, Foster City, CA). The PCR reactions contained 1 μl of template DNA (~100 ng), 5 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.1 μM primer (8fm and 926r), 200 μM dNTP (GE Healthcare, Uppsala, Sweden), 5% by volume DMSO (Sigma-Aldrich, St. Louis, MO), 3 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), and 1x buffer (Applied Biosystems, Foster City, CA) in a final volume of 50 μl. The thermocycler (DNA Engine Dyad®, Bio-Rad, Hercules, CA) protocol was 5 min denaturation at 94ºC followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC, 2 min at 72ºC, and a hold period for 10 min at 72ºC.

The PCR amplicons from each sample were digested in two separate reactions, one of which included *Msp*I (C^CGG) and a second that included *Hae*III (GG^CC). Digestions were done as previously described (Zhou *et al*. 2004). For each sample, equal volumes of these digestions were combined and the resulting mixture had 4 fluorescently labeled terminal restriction fragments derived from each phylotype. *Msp*I and *Hae*III have been theoretically and empirically shown to resolve the bacterial populations likely to be found in vaginal samples.

T-RFLP profiles for each sample were obtained by capillary electrophoresis by using an ABI PRISM 3100 DNA Analyzer and GeneScan software (Applied Biosystems) as previously described (Zhou *et al*. 2004). CST ROX 25-1000 (BioVentures, Inc., Murfreesboro, Tenn.) was used as an internal size standard.

Statistical Analysis of T-RFLP Profiles

Cluster analysis of T-RFLP data was used to identify communities with similar numerically abundant populations using methods described by Abdo *et al.* (2006). Fisher's exact test was used to test the hypothesis that the distribution of community types found in

women colonized with *S. aureus tst*$^{+}$ was significantly different from the distribution of communities found in women not vaginally colonized by the organism (Ott and Longnecker 2000).

We conducted a power analysis to determine the number of samples that would be needed to identify a significant difference between the distributions of samples from the two groups of women (those vaginally colonized and not vaginally colonized by *S. aureus tst*$^{+}$). To perform this analysis we used an resampling algorithm implemented in R (R Team 2008). This analysis used the observed data (see Table 1). For both of the groups there were frequency distributions constructed using the observed community types in each group. We simulated drawing samples from both of the frequency distributions to test if there was a significant difference between the two sample sets. The process of drawing a set of samples from each distribution and comparing them was repeated 1,000 times. Significance was determined using a Fisher's exact test. If the frequency of correctly rejecting the null hypothesis based on 50 samples was less than 80%, the sample size was increased by 5, and the process was repeated. This was done until the frequency of correctly rejecting the null hypothesis exceeded 80% (Cohen 1988).

Correspondence analysis was used to determine if there was a relationship between vaginal colonization by *S. aureus tst*$^{+}$ and the presence and abundance of specific phylotypes in the samples (Greenacre 2007, Kirk 1995). The standardized T-RFLP data were used in conjunction with sample classification based on colonization status as identified by Parsonnet *et al.* (Parsonnet *et al*. 2005). An intraclass correlation was calculated for each DNA fragment category to determine if some DNA fragments are correlated with colonization or non-colonization.

16s rRNA gene clone library construction and sequence analysis

The 16S rRNA genes in each samples from women vaginally colonized by *S. aureus tst*$^{+}$ were amplified by PCR using 'universal' eubacterial bacterial primers 8fm and 926r, cloned, and sequenced to identify the numerically dominant bacterial populations in each sample. The PCR conditions were the same as for T-RFLP except the 8fm and 926r primers were not fluorescently labeled. The PCR product was cleaned with the QIAquick PCR Purification Kit (Qiagen, Fostercity, CA), eluted with 50 μl of water, and the DNA concentration was spectrophotometrically measured (ND-1000, NanoDrop, Wilmington, DE).

The 3'-A overhangs on the PCR products produced by AmpliTaq DNA polymerase that interfere with blunt end cloning were removed using T4 DNA polymerase (1 U) in 45 μl reactions that contained 5 μl of NeBuffer2 and 0.2 μl of 100 μM dNTP (New England Biolabs, Ipswich, MA) per 100 ng of PCR product. The mixtures were incubated for 15 min at 12ºC, then held at 4ºC while 4 μl of 125 mM EDTA, and heated to 75ºC for 20 min to inactivate the enzyme. The product was then kept at 4ºC until it was used in cloning reactions.

Prior to cloning the amplicons were cleaned with the QIAquick PCR Purification Kit (Qiagen, Fostercity, CA), eluted with 50 μl of water, and the DNA concentration was spectrophotometrically measured (ND-1000, NanoDrop, Wilmington, DE). The cleaned products were cloned using the Zero Blunt® TOPO® PCR cloning kit (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. For each reaction 1 μl of vector was added, 1 μl of 1:4 dilution of the salt solution, the appropriate volume of PCR product and water to a volume totaling 6 μl. The PCR product to vector ratio was ~8:1. The mixture was incubated at room temperature for 5 min., and 2 μl was used to transform 25 μl of electrocompetent *E. coli* cells that had been added to 25 μl of deionized nano-pure water. Electroporation was done at 2.5mV on a Gene Pulser® (Bio-Rad, Hercules, CA) in pre-chilled 1 mm cuvettes, and afterwards 250 μl of SOC medium was added to each cuvette. The cells were transferred to an Eppendorf tube that was incubated for 1 hour at 37ºC with agitation, then plated on Luria-Bertani (LB) plates

with 50 μg/ml of kanamycin (Fisher Scientific, Pittsburg, PA) and incubated overnight at 37ºC. Colonies that grew on the kanamycin plates were randomly picked and grown in deep-well microtiter plates with LB + 50 μg/ml of kanamycin for 24 hours at 37ºC with shaking. The cells in each well were pelleted by centrifugation at 6200 rpm for 45 min on a Sigma 4-15 laboratory centrifuge (Sigma, Germany), and washed twice with 0.9% NaCl. The washed cells were stored at -80ºC in 0.9% NaCl.

Plasmid DNA was isolated from 100 randomly chosen clones from each sample using QIAprep 96 Turbo BioRobot kits (Qiagen, Fostercity, CA) using the recommended protocol and stored at -80˚C. The cloned inserts were sequenced using 10 μl reactions that contained 2 μl of Big-Dye (Applied Biosystems, Foster City, CA), 0.5 μl of 3.2 μM 926r primer (Invitrogen, Carlsbad, CA) and 4 μl of template. The reactions were done in a thermocycler (DNA Engine Dyad®, Bio-Rad, Hercules, CA) using the following program: 1 min at 94ºC, 24 cycles of 95ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min with a final extension of 72ºC for 5 min. Sequencing products were cleaned by ethanol precipitation by adding 3 μl of 125 μM EDTA followed by 30 μl of 100% ethanol. The precipitated DNA was collected by centrifugation for 10 min at 1650 rpm at 4ºC using a Sigma 4-15 laboratory centrifuge (Sigma, Germany), and washed with 70% ethanol. The samples were dried at 55ºC then 10 μl of Hi Di™ formamide (Applied Biosystems, Foster City, CA) was added. The DNA sequences were determined using an ABI 3730 PRISM Genetic Analyzer using the standard protocol.

The sequence data was analyzed as previously described (Zhou et al. 2007) to verify that each sequence surpassed minimum standards for length and quality. Clones were assigned to phylotypes by comparing their 16S rRNA gene sequences to those of known organisms in the RDPII (Cole et al. 2003) using the BLAST algorithm (Altschul et al. 1990). The genus and species names were used if the sequence similarity to a type species was >97%; the genus only was used if the sequence similarity was <97%, but >90%. The uncultured bacterium was

described if the sequence similarity of clones to known organisms was < 90%. The sequences from vaginal communities and closely related organisms were aligned with ClustalX (Thompson *et al*. 1994) and used to construct a Neighbor Joining (N-J) tree based on Jukes-Cantor distances (Saitou and Nei 1987).

Results

The two primary goals of this study were to compare the vaginal microbial communities of women vaginally colonized with *S. aureus tst*$^{+}$ to those of women not vaginally colonized, and to identify the predominant species present in the communities of women colonized with *S. aureus tst*$^{+}$. The T-RFLP profiles provided the means to compare communities based on the numerically abundant members of the vaginal communities, while phylogenetic analysis of cloned 16s rRNA genes was used to identify predominant bacterial members of the vaginal communities sampled.

Comparison of community profiles

The T-RFLP profiles of 16S rRNA genes in samples from 21 women vaginally colonized with *S. aureus tst*$^{+}$ were compared to those of 30 women not vaginally colonized with the organism to determine if there were fundamental differences in the numerically abundant bacterial species present. Hierarchical clustering of the T-RFLP profiles of samples from all the women resulted in 4 significant clusters and 6 samples that did not cluster with 2 or more other samples (Figure 1). The *S. aureus tst*$^{+}$ samples were distributed among all the clusters found in this analysis. Statistical tests were used to determine if the observed distribution of sequences among clusters was significant. Using Fisher's exact test, communities colonized with *S. aureus tst*$^{+}$ were tested for a significant difference in the distribution among community types when compared to the communities that were not colonized. The cluster identity of each sample was used to construct a contingency table that contained the number of samples observed in each cluster and the expected number of samples that would be in each category if there was not a difference in colonization by *S. aureus tst*$^{+}$ (Table 1). The results of Fisher's Exact test showed that there was not a significant difference in the rank abundance of community types between the two groups resulting ($P = 0.7582$). Also, there was not a specific community type that

seemed to exclude *S. aureus tst*$^{+}$. While the scope of this conclusion is limited by the small number of available samples, this study provided no evidence to suggest a significant effect of microbial community structure on vaginal colonization with *S. aureus tst*$^{+}$.

A power analysis was performed to estimate the number of samples that would have been needed to detect a difference in the distribution of microbial communities from women vaginally colonized and not vaginally colonized with *S. aureus tst*$^{+}$. To do this we simulated sampling of the data using the abundance distribution of community types observed for women colonized with *S. aureus tst*$^{+}$ and women not vaginally colonized. The results suggest that 135 samples or more would be needed to detect a statistically significant difference between the distributions with 80% power.

To address whether or not there were specific populations present in the indigenous microbial community that encouraged or inhibited colonization by *S. aureus tst*$^{+}$ a correlation analysis was done. The analysis measured the correlation between T-RFLP profile data and colonization status. Figure 2 shows that no peak had a high correlation with colonization status. This analysis provides further evidence that there is not a predisposition to colonization by *S. aureus tst*$^{+}$ due to the numerically abundant members of the microbial community. None of the fragment sizes are strongly correlated with colonization status, indicating no abundant vaginal community member facilitates colonization by *S. aureus tst*$^{+}$ nor does an abundant community member inhibit colonization by *S. aureus tst*$^{+}$.

Composition of vaginal communities colonized with *S. aureus tst*$^{+}$

While the T-RFLP method is very useful for comparing communities on the basis of numerically abundant phylotypes, the method is limited in so far as a single DNA fragment may be composed of more than one phylotype. To confirm that this was not the case for these samples, libraries were constructed and the sequences of cloned 16S rRNA genes were

determined. This was only done on the samples from women colonized with *S. aureus tst*$^{+}$ because the composition of communities from normal healthy women have been previously described (Zhou *et al*. 2004, Zhou *et al*. 2007). The results of the analyses for women colonized with *S. aureus tst*$^{+}$ are shown in the supplemental Table 2. The results were unremarkable. The most common clone among all of the samples was by far *Lactobacillus iners*, accounting for over 40% of all clones sequenced. *Atopobium vaginae, Streptococcus* species, *L. crispatus, L. jensenii* and *L. gasseri* in addition to *L. iners* collectively account for more than 70% of all clones sequenced. The predominance of lactobacilli is consistent with the majority of work done previously (Masfari *et al*. 1986, Zhou *et al.* 2007). The presence of *Atopobium vaginae* and streptococci has also been found in more recent studies of healthy women (Zhou *et al*. 2004). Finally, it should be noted that no species were common to all the samples. These data show there were no differences in the composition of vaginal communities of women vaginally colonized with *S. aureus tst*$^{+}$ as compared to those that were not. .

Discussion

Here we sought to determine whether there were fundamental differences in the species composition of vaginal microbial communities of women colonized with *S. aureus tst*$^{+}$ as compared to women not vaginally colonized by the organism. Using data from T-RFLP profiles and the analysis of clone libraries, it was found that the vaginal community types in these two groups of women did not differ from one another. Women vaginally colonized by *S. aureus tst*$^{+}$ did not have unique community types nor were unexpected species present. There are several possible explanations for the inability to detect differences in the distribution of community types among these two groups of women. It could be that actual differences in the community composition were below the detection threshold of the techniques used in this study. Indeed, we know that there were differences in the community composition that weren't detected by the methods used because we did not detect *S. aureus tst*$^{+}$ in any of the samples tested. Since the PCR primers and methods used are known to amplify 16S rRNA genes from S. aureus, this suggests the organism was comparatively rare in these communities, and when present it constituted less than 1% of the total community. While the detection threshold may have limited our the ability to observe differences in less abundant community members, the results nonetheless indicate there were no significant differences in predominant members of vaginal communities in women of the two groups. Finally it is also possible that there were too few samples of women colonized by *S. aureus tst*$^{+}$ included in this study to detect a statistical difference in the distribution among community types. We were unable to include more samples of women colonized with *S. aureus tst*$^{+}$ because this is a rare event. Of 3012 women sampled, only 32 were colonized by *S. aureus tst*$^{+}$*.* The results of the power analysis suggest, using a conservative assumption that the observed distribution of community types was true, we would need samples from more than 135 women colonized by *S. aureus tst*$^{+}$ to detect a difference in the distribution of community types between women colonized and those not colonized, this

would require screening nearly 20,000 women. Alternatively, it could indeed be true that no significant differences exist between the vaginal communities of women colonized by *S. aureus tst*$^{+}$ as compared to women who are not colonized by the organism. This would imply that bacterial populations indigenous to the vagina are not an important determinant of whether colonization by *S. aureus tst*$^{+}$occurs.

The samples for this study were collected from women that had not, and were not experiencing, mTSS and they could be viewed as 'carriers' of the organism. The work of Parsonnet *et al.* (2005) showed that approximately 1 in 100 women are colonized with *S. aureus tst*$^{+}$, suggesting that the incidence of carriage is common, while the incidence of disease is very low (approximately 1 case in 100,000 women per year). This implies that although a fair number of women are colonized by the pathogen, the conditions required for the expression of virulence are insufficient, or that TSST-1 is neutralized or degraded before it can stimulate a catastrophic cytokine cascade. The abundance of *S. aureus tst*$^{+}$ in these 'carriers' was not determined, nor is it known if the women were persistently colonized since the samples were taken as part of a cross sectional study with one sampling event. Nonetheless, the results of this study and that of Parsonnet *et al.* (2005) indicate that vaginal colonization by *S. aureus tst*$^{+}$ is a poor predictor of risk to mTSS.

The reasons why mTSS does not occur in women vaginally colonized with *S. aureus tst*$^{+}$ are unknown. It has previously been postulated that serum antibodies that bind the TSST-1 toxin and prevent it from binding to MHC proteins are critical to preventing the disease (Bonventre *et al.* 1983, Schlievert and Blomster 1983). Although this is largely consistent with studies on antibody titers against TSST-1 in reproductive age women, direct evidence to support this is lacking. . These studies have shown that toxin specific antibodies are absent in women who contracted mTSS, which suggests that circulating antibodies are important for protection (Bonventre *et al.* 1984, Christensson and Hedstrom 1985, Stolz *et al*. 1985, Solino Noleto *et al.*

1986). In addition, the finding that TSST-1 specific antibodies inhibit the development of TSS in rabbit models seems to confirm the importance of specific antibodies (Best *et al.* 1988, Bonventre *et al.* 1988). Moreover two recent studies show there were high titers of antibodies specific to TSST-1 present in healthy women who were colonized by S. aureus tst+ (Parsonnet et al. 2005, Kansal et al. 2007) but had not contracted mTSS, suggesting these antibodies were protective. In a study of 39 healthy individuals, Kansal et al. (Kansal et al. 2007) showed that 35 of the 39, including all 20 who were colonized with S. aureus tst+, had sufficient levels of inactivating antibody to neutralize TSST-1. The data from this study suggests that high levels of neutralizing antibodies are common, and may be protective against mTSS. Importantly, however, there are instances of healthy women who have antibody titers that are low and comparable to those of women that have experienced mTSS. This suggests that perhaps antibody titer alone cannot explain why some women colonized with S. aureus tst^+ do not develop mTSS. A more careful examination of the probable steps involved in the development of mTSS is probably warranted to identify those that may be important in reducing risk to the disease.

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References

Abdo, Z., U. M. Schuette, S. J. Bent, C. J. Williams, L. J. Forney, and P. Joyce. 2006. Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environ Microbiol* **8**:929-938.

Adams, M. R., and C. J. Hall. 1988. Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. *Int J Food Sci Technol* **23**:287-292.

Alpert, P., E. Bone, and H. C. 2000. Invasiveness, invasibility and the role of environmental stress in the spread of non-native plants. *Perspectives in Plant Ecology, Evolution and Systematics* **3**:52-66.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J Mol Biol* **215**:403-410.

Best, G. K., D. F. Scott, J. M. Kling, M. R. Thompson, L. E. Adinolfi, and P. F. Bonventre. 1988. Protection of rabbits in an infection model of toxic shock syndrome (TSS) by a TSS toxin-1-specific monoclonal antibody. *Infect Immun* **56**:998-999.

Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis. 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome Staphylococcus aureus isolates. *Lancet* **1**:1017-1021.

Blake, E., C. Cook Jr, and J. Bashinski. 1987. Evidence that" vaginal peptidase" is a bacterial gene product. *J Forensic Sci* **32**:888-99.

Bonventre, P. F., C. Linnemann, L. S. Weckbach, J. L. Staneck, C. R. Buncher, E. Vigdorth, H. Ritz, D. Archer, and B. Smith. 1984. Antibody responses to toxic-shock-syndrome (TSS) toxin by patients with TSS and by healthy staphylococcal carriers. **J Infect Dis** **150**:662-666.

Bonventre, P. F., M. R. Thompson, L. E. Adinolfi, Z. A. Gillis, and J. Parsonnet. 1988. Neutralization of toxic shock syndrome toxin-1 by monoclonal antibodies in vitro and in vivo. *Infect Immun* **56**:135-141.

Bonventre, P. F., L. Weckbach, J. Staneck, P. M. Schlievert, and M. Thompson. 1983. Production of staphylococcal enterotoxin F and pyrogenic exotoxin C by Staphylococcus aureus isolates from toxic shock syndrome-associated sources. *Infect Immun* **40**:1023-1029.

Boskey, E. R., K. M. Telsch, K. J. Whaley, T. R. Moench, and R. A. Cone. 1999. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. *Infect Immun* **67**:5170-5175.

Callahan, J. E., A. Herman, J. W. Kappler, and P. Marrack. 1990. Stimulation of B10.BR T cells with superantigenic staphylococcal toxins. *J Immunol* **144**:2473-2479.

Cash, H. L., C. V. Whitham, C. L. Behrendt, and L. V. Hooper. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* **313**:1126-1130.

Christensson, B., and S. A. Hedstrom. 1985. Serological response to toxic shock syndrome toxin in Staphylococcus aureus infected patients and healthy controls. *Acta Pathol Microbiol Immunol Scand* [B] **93**:87-90.

Cohen, J. 1988. Statistical Power Analysis for the Behavioral Sciences. Erlbaum, Hillsdale, NJ.

Cohen, M. L., L. M. Graves, P. S. Hayes, R. J. Gibson, J. K. Rasheed, and J. C. Feeley. 1983. Toxic shock syndrome: modification and comparison of methods for detecting marker proteins in Staphylococcus aureus. *J Clin Microbiol* **18**:372-375.

Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**:442-443.

Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of Staphylococcus aureus. *Clin Microbiol Rev* **13**:16-34, table of contents.

Farage, M., and H. Maibach. 2006. Lifetime changes in the vulva and vagina. *Arch Gynecol Obstet* **273**:195-202.

Fernandez, D., A. Valdivia, J. Irazusta, C. Ochoa, and C. L. 2002. Peptidase activities in human semen. *Peptides* **23**:461-8.

Greenacre, M. J. 2007. Correspondence analysis in practice. Chapman & Hall/CRC, Boca Raton.

Hajjeh, R. A., A. Reingold, A. Weil, K. Shutt, A. Schuchat, and B. A. Perkins. 1999. Toxic shock syndrome in the United States: surveillance update, 1979 1996. *Emerg Infect Dis* **5**:807-810.

Hector, A., K. Dobson, A. Minns, E. Bazeley-White, and H. L. J. 2001. Community diversity and invasion resistance: An experimental test in a grassland ecosystem and a review of comparable studies. *Ecological Research* **16**:819-831.

Herman, A., J. W. Kappler, P. Marrack, and A. M. Pullen. 1991. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu Rev Immunol* **9**:745-772.

Johnson, D. 1998. Applied Multivariate Methods for Data Analysts. Duxbury Press, Belmont, CA.

Kansal, R., C. Davis, M. Hansmann, J. Seymour, J, Parsonnet, P, Modern, S, Gilbert, and K. M. 2007. Structural and Functional Properties of Antibodies to the Superantigen TSST-1 and Their Relationship to Menstrual Toxic Shock Syndrome. *Journal of Clinical Immunology* **27**:327-338.

Kirk, R. E. 1995. Experimental Design: Procedures for the Behavioral Sciences. Brooks/ Cole, Pacific Grove, CA.

Kotb, M. 1995. Bacterial pyrogenic exotoxins as superantigens. *Clinical Microbiology Reviews* **8**:411-426.

Kotb, M. 1998. Superantigens of Gram-positive bacteria: structure—function analyses and their implications for biological activity. *Current Opinion in Microbiology* **1**:56-65.

Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* **63**:4516-4522.

Llewelyn, M., S. Sriskandan, M. Peakman, D. R. Ambrozak, D. C. Douek, W. W. Kwok, Cohen, J. and D. M. Altmann. 2004. HLA class II polymorphisms determine responses to bacterial superantigens. *J Immunol* **172**:1719-1726.

Masfari, A. N., B. I. Duerden, and G. R. Kinghorn. 1986. Quantitative studies of vaginal bacteria. *Genitourin Med* **62**:256-263.

Miller, T., J. Kneitel, and B. J. 2002. Effect of community structure on invasion success and rate. *Ecology* **83**:898-905.

Misfeldt, M. L. 1990. Microbial "superantigens". *Infect Immun* **58**:2409-2413.

Musser, J. M., P. M. Schlievert, A. W. Chow, P. Ewan, B. N. Kreiswirth, V. T. Rosdahl, A. S. Naidu, W. Witte, and R. K. Selander. 1990. A single clone of Staphylococcus aureus causes the majority of cases of toxic shock syndrome. *Proc Natl Acad Sci U S A* **87**:225-229.

Ott, R., and M. Longnecker. 2000. An Introduction to Statistical Methods and Data Analysis. Duxbury Press, Belmont, CA.

Parsonnet, J., M. A. Hansmann, M. L. Delaney, P. A. Modern, A. M. Dubois, W. Wieland-Alter, K. W. Wissemann, J. E. Wild, M. B. Jones, J. L. Seymour, and A. B. Onderdonk. 2005. Prevalence of toxic shock syndrome toxin 1-producing Staphylococcus aureus and the presence of antibodies to this superantigen in menstruating women. *J Clin Microbiol* **43**:4628-4634.

R Development Core Team 2008. R: A Language and Environment for Statistical Computing.

Reeves, M. W., L. Pine, J. C. Feeley, and D. E. Wells. 1984. Presence of toxic shock toxin in toxic shock and other clinical strains of Staphylococcus aureus. *Infect Immun* **46**:590-597.

Ross, R. A., and A. B. Onderdonk. 2000. Production of toxic shock syndrome toxin 1 by Staphylococcus aureus requires both oxygen and carbon dioxide. *Infect Immun* **68**:5205-5209.

Royce, R. A., T. P. Jackson, J. M. J. Thorp, S. L. Hillier, L. K. Rabe, L. M. Pastore, and D. A. Savitz. 1999. Race/ethnicity, vaginal flora patterns, and pH during pregnancy. *Sex Transm Dis* **26**:96-102.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**:406-425.

Schlievert, P. M., and D. A. Blomster. 1983. Production of staphylococcal pyrogenic exotoxin type C: influence of physical and chemical factors. *J Infect Dis* **147**:236-242.

Schlievert, P. M., K. N. Shands, B. B. Dan, G. P. Schmid, and R. D. Nishimura. 1981. Identification and characterization of an exotoxin from Staphylococcus aureus associated with toxic-shock syndrome. *J Infect Dis* **143**:509-516.

Schütte, U. M. E., Z. Abdo, S. J. Bent, C. Shyu, C. J. Williams, J. D. Pierson, and L. J. Forney. 2008. Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Appl Microbiol Biotechnol* doi 10.1007/s00253-008-1565-4

Solino Noleto, A. L., E. da Costa Cesar, and M. S. Bergdoll. 1986. Antibodies to staphylococcal enterotoxins and toxic shock syndrome toxin 1 in sera of patients and healthy people in Rio de Janeiro, Brazil. *J Clin Microbiol* **24**:809-811.

Stolz, S. J., J. P. Davis, J. M. Vergeront, B. A. Crass, P. J. Chesney, P. J. Wand, and M. S. Bergdoll. 1985. Development of serum antibody to toxic shock toxin among individuals with toxic shock syndrome in Wisconsin. *J Infect Dis* **151**:883-889.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**:4673-4680.

Todd, J., M. Fishaut, F. Kapral, and T. Welch. 1978. Toxic-shock syndrome associated with phage-group-I Staphylococci. *Lancet* **2**:1116-1118.

Whiting, R. C., S. Sackitey, S. Calderone, K. Morely, and J. G. Phillips. 1996. Model for the survival of Staphylococcus aureus in nongrowth environments. *Int J Food Microbiol* **31**:231-243.

Zarate, G., and M. E. Nader-Macias. 2006. Influence of probiotic vaginal lactobacilli on in vitro adhesion of urogenital pathogens to vaginal epithelial cells. *Lett Appl Microbiol* **43**:174-180.

Zhou, X., S. J. Bent, M. G. Schneider, C. C. Davis, M. R. Islam, and L. J. Forney. 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiology* **150**:2565-2573.

Zhou, X., C. J. Brown, Z. Abdo, C. C. Davis, M. A. Hansmann, P. Joyce, J. A. Foster, and L. J. Forney. 2007. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J* **1**:121-133.

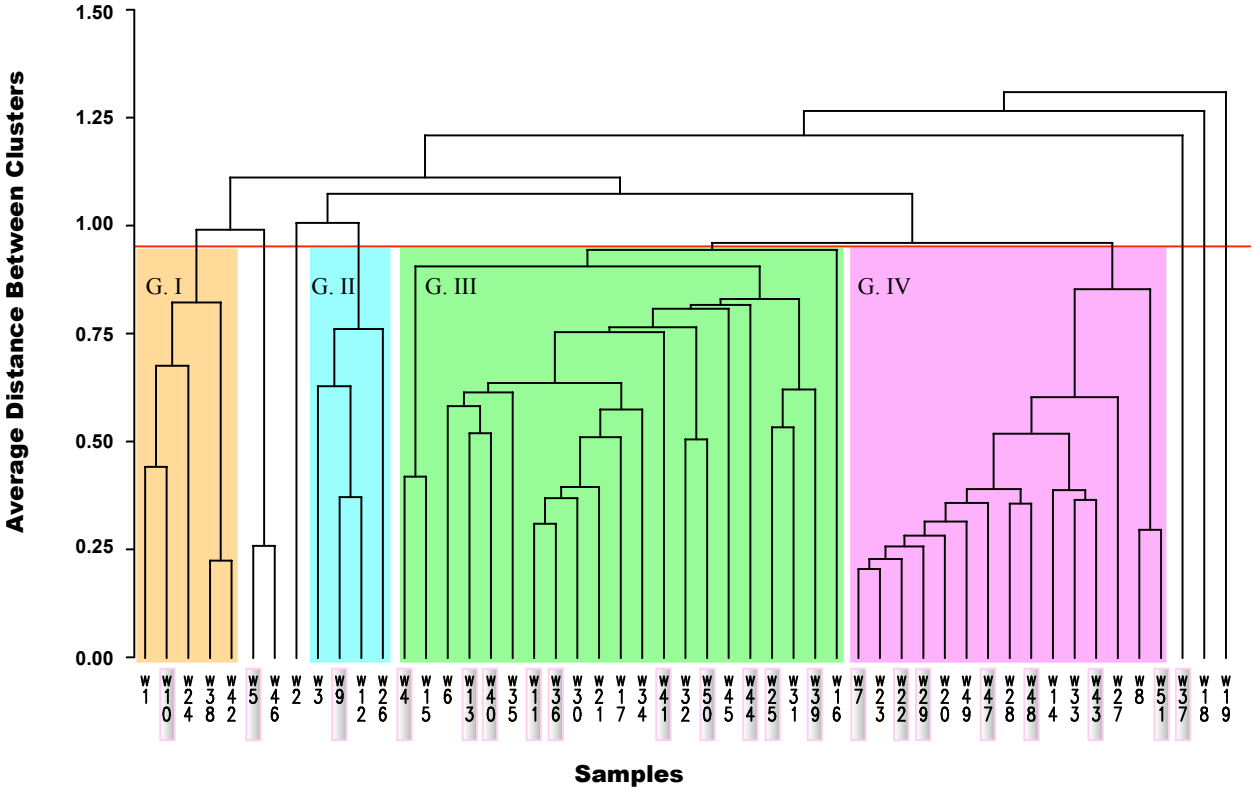

Figure 1. A dendrogram showing the similarity of vaginal microbial communities from women vaginally colonized and those not vaginally colonized by *S. aureus tst*$^{+}$. Samples highlighted in the grey boxes represent vaginal community profiles from women who were vaginally colonized with *S. aureus tst*$^{+}$.

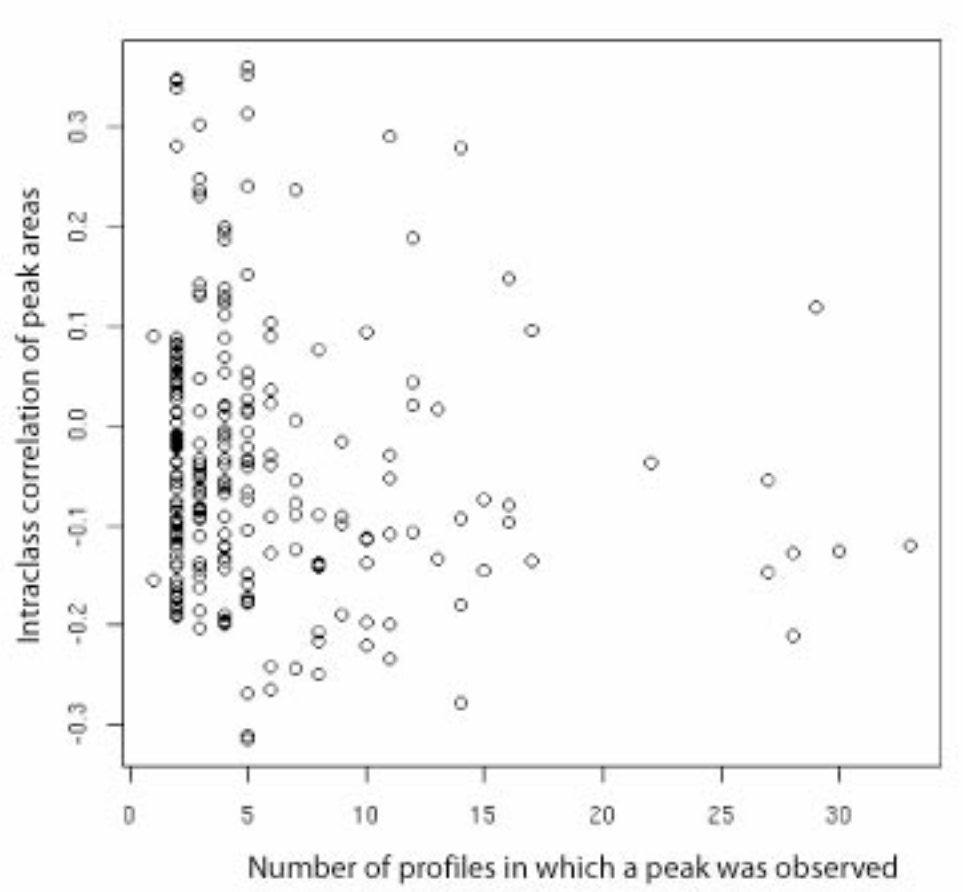

Figure 2. Intraclass correlation between DNA fragment size and colonization status to identify DNA fragments in T-RFLP profiles of 16S rRNA genes that are correlated with colonization by *S. aureus tst+*. Each circle shows the number of samples in which the fragment occurred and how well the fragment was correlated to colonization status.

Table 1. The Fisher's exact test was used to determine if there was a significant difference between the frequency that samples occurred in each of the groups

Origin of sample	Women vaginally colonized with *S. aureus tst+*		Women without vaginal *S. aureus tst+*		Total samples per group
	observations [a]	expected frequency [b]	observations	expected frequency	
Group 1	1	2.0588	4	2.9712	5
Group 2	1	1.6471	3	2.3529	4
Group 3	10	8.6471	11	12.3529	21
Group 4	7	6.1765	8	8.8235	15
Other	2	2.4705	4	3.5294	6
Total number of samples analyzed	21		30		51

a) The observations were counted by identifying the number of samples of a particular colonization status within each group identified in the cluster analysis.
b) The expected frequencies are calculated by multiplying the total number of samples with the same colonization status by the number of samples identified in the corresponding group (figure 1) and then divided by the total number of samples.

APPENDIX I

Construction of 16S rRNA gene libraries for the analysis of microbial communities

Background

Research done on the ecology of microbial communities is often focused on determining how communities differ from one another in terms of species composition and rank abundance. To achieve this end the populations found in any given community are characterized using cultivation independent methods that rely on DNA sequence polymorphisms in 16S ribosomal RNA genes. Research done in our laboratory on the bacterial communities found in the human vagina have largely relied on two methods. In the first of these, community "fingerprints" are obtained by assessing the patterns of terminal restriction fragment length polymorphisms (T-RFLP) of 16S ribosomal RNA genes that have been amplified from the numerically dominant bacterial populations in a community. Because of its ease of use and low cost, T-RFLP is useful in studies done to compare large numbers of different communities. While this method is a facile means to assess differences in the numerically abundant members that comprise a community, it cannot be used to identify the populations present in a community, and there is a risk of false negatives (i.e., two communities that actually differ in species composition or evenness may appear to be the same based on their T-RFLP profiles.) A second commonly used approach is to identify and quantify populations in a community by phylogenetic analysis of cloned 16S ribosomal RNA gene sequences derived from a microbial community. Because this method entails the construction of cloned 16S rRNA gene libraries, and sequencing large numbers of cloned inserts, it is somewhat more expensive in terms of time and resources. These two approaches are useful for addressing different kinds of research

questions. Community fingerprints based on T-RFLP profiles are a simple and straightforward way to monitor changes in the numerically dominant phylotypes (or operational taxonomic units, OTUs) in different communities. In contrast, phylogenetic analysis of cloned ribosomal RNA genes provides detailed information about the identity of the populations present, but requires more sophisticated data analysis steps, and introduces an additional bias due to preferential cloning of genes. When used in combination the two methods are complementary, and can be used to identify differences or changes in community composition and identify predominant members in the communities being studied.

The methods used in the laboratory to construct libraries of cloned 16S ribosomal RNA genes have historically given inconsistent results in terms of the number of transformants obtained from a given sample. For this reason, experiments were done to examine various steps in this process in an effort to develop a protocol that consistently yielded high numbers of clones and was more time efficient.

Methodologies previously used

Work done previously by Forney and coworkers has used clone libraries of 16S ribosomal RNA genes to characterize the composition of microbial communities in various kinds of environmental samples. In some projects investigators have sought to intensively analyze the species composition of microbial communities by sampling upwards of 1000 cloned 16S ribosomal RNA genes, while in other studies fewer clones per sample were needed. One way to improve the procedure for identifying the microbial species present in these communities would be to increase the efficiency of producing clone libraries.

The protocol for cloning 16S ribosomal RNA genes used in previous studies employed the pCR®2.1 vector of the Topo® TA® kit (Invitrogen, Carlsbad, CA) that relies upon the 3'-A overhang left by PCR amplification by Taq polymerase as a "sticky end" for ligation of an

amplicon into the vector. The insertion site for cloning fragments into the pCR®2.1 plasmid is the lacZα gene, and transformants are selected based on their resistance to kanamycin (Fisher Scientific, Pittsburgh, PA). Colonies that appear white on media containing X-gal (Research Products International Corp., Mt. Prospect, IL) are presumed to have arisen from a cell transformed by a plasmid with an insert because cloned DNA fragments interrupt expression of the gene encoding LacZα. While this sounds straightforward, it is not always possible to discern white colonies from blue colonies because the screen is “leaky” and some colonies appear pale blue. As a result investigators are left to make choices that often result in the selection of clones with plasmids that lack inserts. One objective of the work described here was to explore the usefulness of other cloning vectors that rely on alternative methods for distinguishing colonies that carry plasmids with cloned inserts from those that do not.

Two changes to the cloning protocol were evaluated in this study: (a) a comparison of transforming chemically competent cells, and transformation via electroporation, and (b) a comparison of the TOPO® TA® and TOPO® Zero Blunt® kits that are based on different cloning vectors. In addition, we attempted to optimize the ratio of vector to insert DNA in the cloning reactions.

Materials and Methods

PCR amplification of 16S ribosomal RNA genes

The "universal" eubacterial bacterial primers 8fm (5'-AGAGTTTGATCMTGGCTCAG-3', Invitrogen, Carlsbad, CA) and 926r (5'-CCGTCAATTCCTTTRAGTTT-3', Invitrogen) were used to amplify 16s rRNA genes from genomic DNA isolated for each of the 3 samples: pure cultures of *S. aureus*, *E. coli*, and a mixture of the two. PCR reactions contained 1μl of template DNA (~100 ng), 5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.1 μM of each primer (8fm and 926r), 200 μM dNTP (GE Healthcare, Uppsala, Sweden), 5% by volume DMSO (Sigma-Aldrich, St. Louis, MO), 3 mM $MgCL_2$ (Applied Biosystems), and 1X Buffer (Applied Biosystems). The PCR reaction was brought to a final volume of 50 μl with HyPure Molecular grade water (HyClone Laboratories Inc., Logan, UT). Reactions with the Pfx polymerase (Invitrogen) contained 1 μl of template DNA (~100 ng), 1U of Pfx DNA polymerase , 0.1 μM out each primer (8fm and 926r), 200 μM dNTP (GE Healthcare, Uppsala, Sweden), 5% by volume DMSO (Sigma-Aldrich, St. Louis, MO), and 1x AccuPrime buffer (Invitrogen) in a final volume of 50 μl. The thermocycler (DNA Engine Dyad®, Bio-Rad, Hercules, CA) protocol used was: 5 min denature at 94ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and finished with 10 min at 72ºC. The size of the PCR products were estimated following agarose gel electrophoresis and staining with ethidium bromide. The PCR product was then cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) as instructed by the manufacturer. The DNA concentration was estimated spectrophotometrically with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

T4 polishing

The 3'-A overhangs on PCR products produced by AmpliTaq DNA polymerase (Applied Biosystems) that interfere with blunt end cloning were removed using 1 U T4 DNA polymerase

(New England Biolabs, Ipswich, MA) in 45 μl reactions that contained 5 μl of NeBuffer2 (New England Biolabs) and 0.2 μl of 100 μM dNTP (GE Healthcare) per 100 ng of PCR product. The mixtures were incubated for 15 min at 12ºC, then held at 4ºC while 4 μl of 125 mM EDTA (Fisher Scientific), and heated to 75ºC for 20 min to inactivate the enzyme. The product of T4 polishing was then cleaned with the QIAquick PCR Purification Kit (Qiagen) as instructed by the manufacturer. The DNA concentration was estimated spectrophotometrically with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Ligation and transformation

Protocols recommended by the manufacturer (Invitrogen) were used for ligation of PCR products into either the pCR®2.1 vector of the TOPO TA kit or the pCR®-Blunt II-TOPO® vector of the TOPO® Zero Blunt® kit. The DNA concentrations, as measured by the NanoDrop 1000, was adjusted so that the insert to vector ratios were 3:1 or 8:1. For each reaction 1 μl of vector was added to 1 μl of the salt solution provided in the TOPO® Zero Blunt® kit, the appropriate volume of PCR product, and water (provided in the kit) were added to a final reaction volume of 6 μl, then the mixtures were incubated at room temperature for 5 min. The transformation step used 2 μl of each ligation reaction, while the remainder of the reactions were kept on ice until transformation was complete then stored at 4ºC.

The transformation of chemically competent *E. coli* cells was performed as recommended by the manufacturer (Invitrogen). Cells were thawed on ice then 2 μl of the ligation reaction was added to 50 μl of chemically competent *E. coli* cells (Invitrogen). The cells were heat shocked at 42˚C for 30 sec and then placed on ice for 5 min. Once all samples had been shocked and placed on ice, 250 μl of S.O.C. medium (provided in the kit) was added to each sample and transferred to a screw top Eppendorf tube.

Transformation of *E. coli* cells by electroporation was done in half reactions; 2 μl of the ligation reaction was added to 25 μl of water (provided in the kit), which was then added to 25 μl of electrocompetent cells (Invitrogen). The 1 mm cuvettes were stored at -20ºC before the mixture of cells, ligation product and water were gently added, and the cuvettes were placed on ice. The cuvettes with the cells and ligation product were electroporated at 2.5mV on a Gene Pulser (Bio-Rad, Hercules, CA) and returned to ice. When the samples arced additional attempts at electroporation were made. Once all samples had been electroporated, 250 μl of S.O.C. medium (provided in the kit) was added to each cuvette and then transferred to a screw top Eppendorf tube.

The Eppendorf tubes of all transformations were placed in a shaking incubator for 1 hour at 37ºC to allow for the expression of the antibiotic resistance gene encoded on the vector. To grow colonies 10, 20 and 30 μl of each transformation were plated on Luria-Bertani (LB) plates containing 50 μg/ml of kanamycin (Fisher Scientific) and incubated overnight at 37ºC. Plates for cells transformed with the the pCR®2.1 vector also contained 40 μg/ml of X-gal (Research Products International Corp.).

Screening of clones was not necessary for the blunt end samples as the pCR®-Blunt II-TOPO® vector employs the *ccd*B gene for selection that is lethal to *E. coli* if the gene is not interrupted by the insertion of PCR product. Thus, all colonies that grew were presumed to have a cloned insert. For transformations done with the pCR®2.1 vector, white colonies were selected. From each transformation 16 colonies were selected and grown in 1.5 ml-deep well plates that contained LB + 50 μg/ml of kanamycin (Fisher Scientific). The plates were incubated for 24 hours at 37ºC with shaking. The cells in each well were pelleted at 6200 rpm in a Sigma 4-15 laboratory centrifuge (Sigma, Germany) for 15 min and washed with phosphate buffered saline (PBS; NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 x 7 H_20 2.72 g/L, KH_2PO_4 0.24 g/L adjusted to pH 7.4), or saline twice. Cell pellets were resuspended in 40 μl of PBS and stored at -80ºC.

The presence of cloned inserts in plasmids was verified by PCR and digestion of the cloned DNA with restriction enzymes. The PCR reagent reaction mix was constructed in 2 parts: the first half of the reaction mix contained about half of the $MgCl_2$, buffer, and HyPure Molecular grade water, (HyClone Laboratories Inc.), the second half of the reaction mix contained the remaining $MgCl_2$, buffer, and HyPure Molecular grade water, as well as the primers M13 forward (5'-GTAAAACGACGGCCAG-3', Invitrogen) and M13 reverse (5'-CAGGAAACAGCTATGAC-3', Invitrogen), polymerase, dNTPs, and DMSO (Table 1). To perform the PCR, 2 μl of the cells presumed to contain plasmids with inserts were added the reaction mix that only contained $MgCl_2$, buffer, and HyPure Molecular grade water. These mixtures were heated to 99˚C for 10 min after which it was cooled and held at 4˚C while the remainder of the reaction mix was added. Otherwise the thermocycler protocol was the same as that described above. After PCR amplification *Hae*III (Invitrogen) was used to digest the PCR product and produced a restriction profile for each of the inserts. Due to differences in the 16S rRNA gene sequences of *S. aureus* and *E. coli,* each produce different restriction patterns when using *Hae*III. Thus, we were able to verify the origin of the insert based on the restriction patterns of *S. aureus* and *E. coli* 16S rRNA genes and determine whether the cloning frequency between these two species was the same.

Results

To increase the efficiency of cloning PCR amplified 16S ribosomal RNA genes, two different steps in the procedure were optimized. First, the efficiencies of cloning using two different vectors were compared. One vector was the pCR®2.1 vector of the TOPO® TA® Kit, which had been extensively used by Forney and coworkers, and a second vector was the pCR®-Blunt II-TOPO® vector of the TOPO® Zero Blunt® Kit. The former relies on so called 'blue-white' screening as a means to distinguish colonies that carry cloned genes from those that do not, while the latter employs a system wherein transformation of *E. coli* cells with plasmids lacking a cloned insert is a lethal event. Next, the method used to transform the ligation mixture into *E. coli* cells was evaluated. The standard method previously used in the laboratory relied upon transformation of chemically competent *E. coli* cells with a ligation mixture prepared using the pCR®2.1 vector. In contrast, cloning protocols using the pCR®-Blunt II-TOPO® vector typically relies upon electroporation of *E. coli* cells. Both vectors were tested with both transformation methods. We also evaluated two vector-to-insert (PCR amplicon) ratios to determine which yielded more transformants.

Two different operators (Jacob Pierson and Ursel Schütte) performed the comparison in parallel and achieved similar results; representative data are shown in Table 2. The transformation method specific for each kit produced more transformants with cloned inserts: chemical transformation with the pCR®2.1 vector, and electroporation with the pCR®-Blunt II-TOPO® vector. Also the 8:1 insert to vector ratio was superior for pCR®-Blunt II-TOPO®, but the insert to vector ratio had little effect on the number of colonies obtained with pCR®2.1. The pCR®-Blunt II-TOPO® proved to produce more colonies. Neither of the methods had a significant bias for inserting either the *E. coli* or *S. aureus* fragments.

The vector pCR®-Blunt II-TOPO® requires blunt-ended PCR products for ligation. For this experiment, we used Pfx polymerase, a polymerase that yields PCR products with blunt ends. After testing the protocol, we found that amplification with this polymerase produced additional products of unexpected size that were not present in the products obtained using Taq polymerase. This bias in amplification was unacceptable because of the possible cloning of artifacts it might introduce. To use Taq polymerase for PCR amplification of 16S ribosomal RNA genes coupled with the pCR®-Blunt II-TOPO® vector, a T4-polymerase polishing step was added to remove the 3'-A overhang from the PCR products to produce blunt ends. The use of Taq polymerase and T4 polishing did not effect the number of clones obtained.

Conclusions

The new protocol using the pCR®-Blunt II-TOPO® vector and transformation by electroporation was shown to be an efficient way to clone 16S ribosomal RNA genes amplified from genomic DNA. It was also preferable to using the pCR®2.1 vector because it obviated the need for blue-white screening, thereby reducing the incidence of false positives, and the number of transformants obtained was markedly increased. Moreover, we have preserved the ability to compare the results of future studies to those done in the past by minimizing changes in the PCR protocol used.

TABLE 1: PCR reaction mixture for colony PCR

Reagent	Stock Concentration	Volume per reaction (µl)	Final Concentration
PCR grade H_2O	---	33.65	---
Buffer	10 x	5	1 x
$MgCl_2$	25 mM	6	3 mM
DMSO	---	2.5	5 %
dNTP	25 mM	0.4	200 µM
M13 F Primer	20 µM	0.125	0.05 µM
M13 R Primer	20 µM	0.125	0.05 µM
Taq Polymerase	5 U/µl	0.2	5 U

TABLE 2. Comparison of the cloning efficiency observed for the pCR-Blunt2-TOPO vector and the pCR 2.1 vector using chemically transformation and electroporation.

Template to vector ratio	Volume plated (µl)	Number of clones observed per plate					
		pCR 2.1 Vector				pCR-Blunt2-TOPO Vector	
		Chemical Transformation		Electroporation		Chemical Transformation	Electroporation
		Blue [a]	White [b]	Blue	White		
3:1							
	10	3	107	4	45	3	67
	20	10	187	17	101	7	159
	30	19	291	13	192	10	299
8:1							
	10	5	59	6	22	18	201
	20	3	80	13	74	19	834
	30	5	138	35	211	32	TNTC[c]

[a] Blue colonies indicate that plasmid does not contain an insert.

[b] White colonies indicate that the plasmid contains an insert; these are the colonies of interest.

[c] The colonies on this plate were too numerous to count.

APPENDIX II

Development of an improved method for the isolation of bacterial genomic DNA from vaginal microbial communities

Background

A technical challenge in conducting long-term or expansive studies of microbial communities is the consistent application of methods. It is particularly important for the isolation of genomic DNA from samples since any bias introduced at this step will be magnified at each subsequent step. The goal of the research described below was to develop a robust and facile means to isolate genomic DNA that provides consistent results when used by different investigators.

Investigators in the laboratory have previously relied on the method reported by Coolen *et al.* to isolate genomic DNA in studies of vaginal microbial ecology (Zhou *et al.* 2004, Coolen *et al.* 2005). This method suffered at least three shortcomings. First, the isolation method required a large amount of starting material, leaving little sample in reserve that might be needed in case of a catastrophic error or event. Second, the method was sensitive to slight variations in how the isolations were performed, causing inconsistent results to be obtained within sets of samples processed by the same analyst, and disparate outcomes when different analysts processed the same sample. Finally, the method was time consuming and limited the number of samples that could be processed each day. To overcome these problems we sought to develop a new, more robust DNA isolation method.

Results

It was decided *a priori* that the method should (a) retain the enzymatic lysis step that employs lysozyme, mutanolysin, and staphylolysin to weaken the cell walls of gram-positive bacteria, which are particularly difficult to lyse; (b) include bead beating as a method for the physical lysis of bacterial cells; and (c) employ the UltraClean™ Fecal DNA Isolation Kit (MO BIO™ laboratories, Inc., Carlsbad, CA) because it already included a bead beating cell lysis step and used filtration (through columns containing a silica-based matrix) rather than DNA precipitation and centrifugation steps for purification of genomic DNA. The latter would reduce the time and labor required to process samples.

The isolation efficiency of the new protocol was tested using two species. *Staphylococcus aureus* was chosen because it is difficult to lyse and so it can be difficult to obtain good yields of DNA. In contrast, *Escherichia coli* was chosen because it relatively easy to lyse and we wanted to verify that the method did not excessively shear the DNA from easily ruptured cells. *S. aureus* and *E. coli*, were grown in Tryptic soy media at 37˚C overnight. The optical density of the cultures was measured spectrophotometrically at 600 nm, and standardized to 0.343 OD with phosphate buffered saline (PBS; NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 x 7 H_20 2.72 g/L, KH_2PO_4 0.24 g/L adjusted to pH 7.4), and the actual number of colony forming units was determined by serial dilution and plating on tryptic soy agar.

We used the QIAamp DNA Mini Kit (Qiagen Inc, Valencia, CA) with the addition of enzymatic lysis and bead beating to isolate genomic DNA from the two test species. A schematic of the complete protocol is shown in (Fig. 1). To begin, 700 µl of TE 50 (10mM EDTA in 50mM Tris); pH ...) was added to a 500 µl aliquot of sample, and the mixture was kept on ice. The remainder of the sample was archived at -80˚C as a reserve. An enzyme cocktail consisting of 500 µg of lysozyme (Sigma-Aldrich, St. Louis, MO), 150 U of mutanolysin (Sigma-Aldrich) and 12 U of lysostaphin (Sigma-Aldrich) dissolved in 100 µl TE was added, and the

mixture was incubated at 37˚C in a dry heat block (Analog Heatblock, VWR,West Chester, PA) for 1 hour. After incubation, ~160 mg of 0.1 mm zirconium beads (Biospec Products, Inc., Bartlesville, OK) were added, and the samples were shaken (Mini-Beadbeater-96, Biospec Products, Inc.) for 1 min. After briefly centrifuging the sample to remove liquid from the lid and to settle the beads, 200 µl of the supernatant was transferred to a clean microfuge tube. The remainder was archived at -80˚C. To the 200 µl subsample, 20 µl of proteinase K and 200 µl of Buffer AL (of the QIAamp DNA Mini Kit) were added. The solution was vortexed for 15 sec and incubated at 56˚C for 10 min in a dry heat block. Afterwards, the samples were briefly centrifuged, 8 µl of 3M acetate and 200 µl of ethanol (96-100%) were added, the mixtures were vortexed for 15 sec, and then briefly centrifuged again.

Genomic DNA was isolated using QIAamp columns following the protocol divided by the manufacturer. Samples were added to individual columns on a vacuum manifold and suction was applied to remove the liquid. Next the columns were washed with 750 µl of the AW1 solution (QIAamp DNA Mini Kit) and the liquid was again removed under suction. After adding 750 µl of the AW2 solution (QIAamp DNA Mini Kit) the columns were placed in clean 2 ml tubes and centrifuged. After emptying the liquid from the tubes, the columns in tubes were centrifuged again. Genomic DNA was eluted from the columns by adding 100 µl of the AE solution (QIAamp DNA Mini Kit) by placing each column in a clean 1.5 ml microfuge tube, and centrifuging for 1 min. This process was then repeated once.

PCR was performed on all of the samples to see if the method was reliable. For each sample 5 µl of PCR product was added to 2 µl of 6X loading dye and this entire mixture was loaded into a well. A 1kb Plus DNA Ladder size standard (Invitrogen, Carlsbad, CA) flanked each set of isolations and the Low Mass ladder (Invitrogen) was used to separate the samples from the *E. coli* and *S. aureus* DNA isolations. Electrophoresis was performed at 98 volts for 30 min. The gel was stained for 30 min in a solution that contained 5 ppm ethidium bromide, then

de-stained for 10 min. in distilled water. The gels were then imaged using an AlphaImager (Alpha Innotech Corporation, San Leandro, CA) and DNA concentrations were estimated by comparing the sample to the Low Mass ladder and the DNA concentration was also spectrophotometrically measured (ND-1000, NanoDrop, Wilmington, DE).

To assess the reproducibility of this new protocol, a trial was done in which seven different analysts used the protocol to isolate genomic DNA from identical samples. The results showed that analysts could independently obtain similar results (Figure 2). The results also showed the importance of using two separate elution steps, because the amount of DNA extracted from the first and second elution steps varied from one analyst to the next. Moreover, even when performed by a single analyst neither the first nor second elution consistently yielded more DNA than the other. An additional experiment was done determine if the recovery of DNA was improved by increasing the amount of AE solution (the elution buffer) used. The yield of DNA was not noticeably different (data not shown).

Summary

A new DNA isolation protocol was developed that (a) included bead beating as a physical means for disrupting bacterial cells, (b) did not require all of the sample and yielded subsamples that could be used in the event of catastrophic loss of materials, (c) was reproducible with low inter-operator variability, and (d) could be done in less time. In addition, the yield of DNA was roughly four-fold higher than the method previously used in the laboratory.

References

Coolen, M. J. L., E. Post, C. C. Davis, and L. J. Forney. 2005. Characterization of Microbial Communities Found in the Human Vagina by Analysis of Terminal. Applied and Environmental Microbiology

Zhou, X., S. J. Bent, M. G. Schneider, C. C. Davis, M. R. Islam, and L. J. Forney. 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology 150:2565-2573.

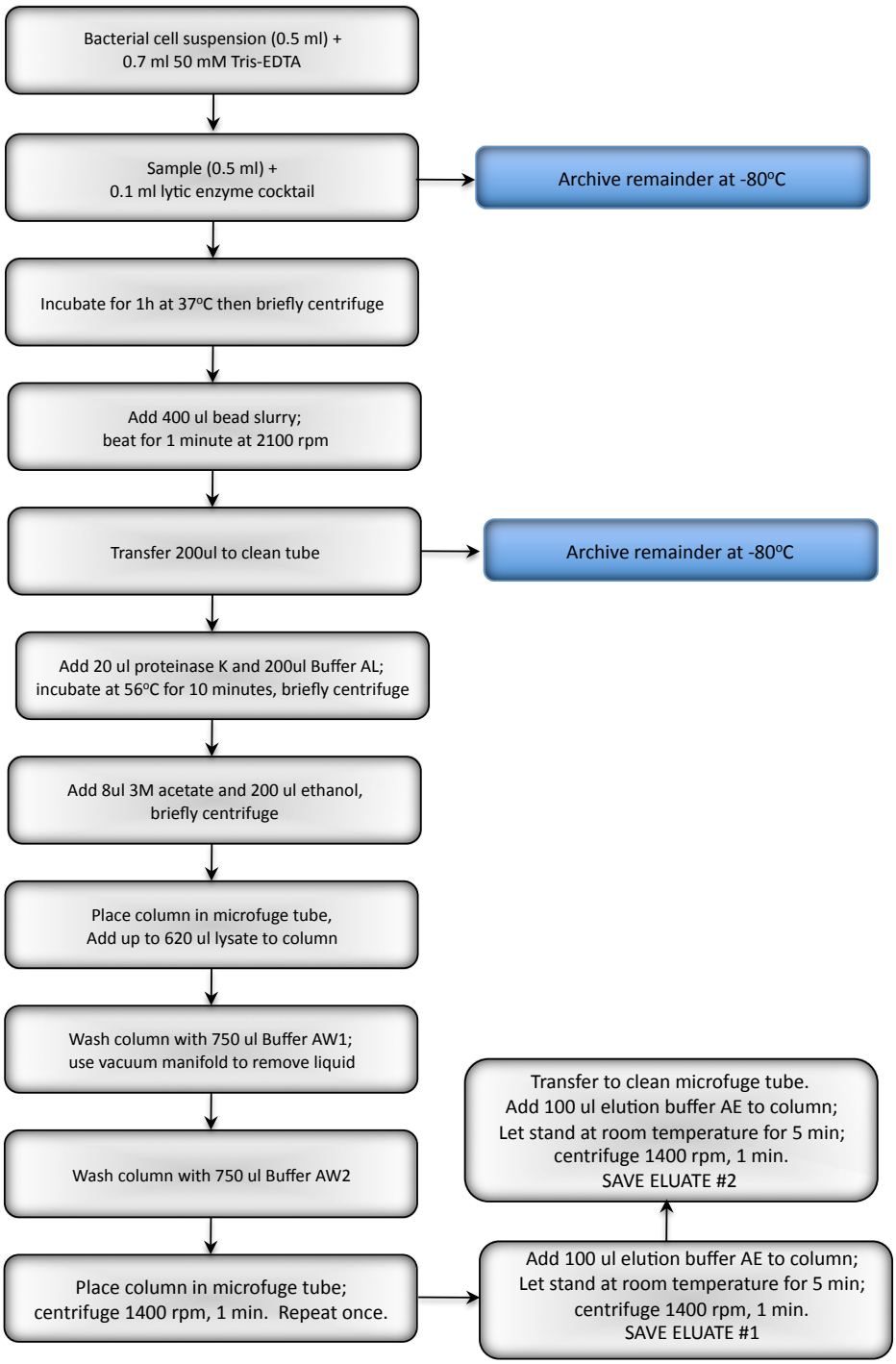

Figure 1. Summary of the new genomic DNA isolation procedure.

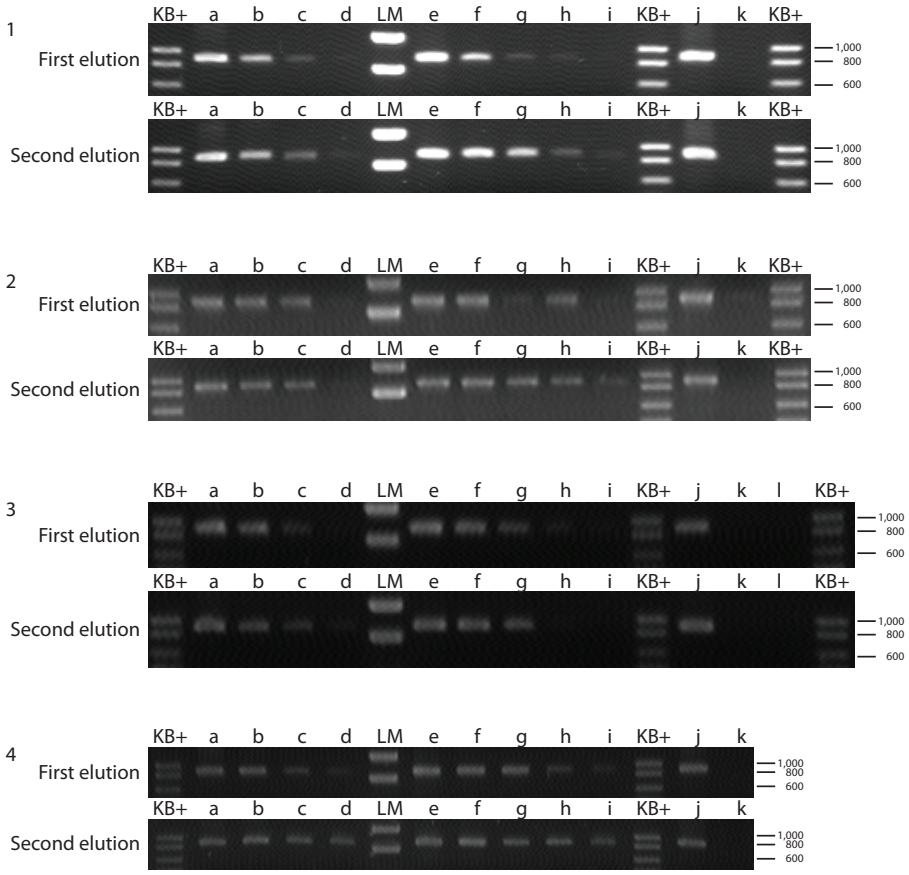

Figure 2. Reproducibility of procedure used for the isolation of genomic DNA. Four analysts isolated genomic DNA from the same identical cell preparations. PCR was performed using 1 μl of template DNA from both the first and second elutions and the results for these eight sets of samples following gel electrophoresis and staining with ethidium bromide are shown. Lanes marked KB+ are the 1kb plus ladder, while those marked LM are the low mass ladder (Invitrogen, Carlsbad, CA). Lanes *a, b, c*, and *d* show the results obtained using *E. coli* cell suspensions that contained approximately 10^8, 10^7, 10^6, and 10^5 cells per ml , respectively. Similarly *e, f, g,* and *h* show the results obtained using *S. aureus* cell suspensions that contained approximately 10^8, 10^7, 10^6, and 10^5 cells per ml. Lane *i* is the negative control in which no cells were present in the sample. Lanes *j* and *k* were the positive and negative controls for the PCR reaction in which previously isolated genomic DNA from *E. coli* and no template DNA were used, respectively. Lane *l* was intentionally left empty. Fragment sizes for the 1kb plus ladder (in bp) are shown on the right.