Molecular characterization of the bacterial flora of the murine female genital tract
by Deirdre Kathleen McNamer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University

Abstract:
The female genital tract may harbor a potentially diverse microbial community that has not been thoroughly characterized, particularly in the murine system. A more complete knowledge of resident flora may enhance understanding of disease susceptibility and the persistence of infection which, in turn, may better guide research toward more efficacious treatments and protective measures. Organisms known to exist in this environment have largely been identified through cultivation. However, because the nutritional and environmental needs of previously uncultivated organisms cannot be anticipated, there are limitations to the use of this technique when attempting to characterize a community. Molecular techniques that take advantage of the conservation of ribosomal RNA have proven to be very useful for such efforts. The use of PCR in tandem with denaturing gradient gel electrophoresis (DGGE) allows for the characterization and comparison of microbial populations with relative ease and rapidity. This study applies these molecular techniques to the investigation of the microbial flora of the murine female genital tract. In addition, a comparison was made between cultivation and two different molecular techniques, DGGE and cloning. We found some overlap in the detection of organisms identified by the various methods, but some microbes were only detected by particular techniques. We further used DGGE to identify bacterial populations of this environment during the course of the natural estrous cycle, under the influence of exogenous hormones, following antibiotic therapy and in a Chlamydia trachomatis infection model. Our findings indicate that there is no change in the species present over the course of the estrous cycle or under the influence of administered hormones. However, because DGGE is not quantitative we were unable to determine if quantitative changes in the populations occurred. Our findings with the antibiotic treatment and Chlamydia infection model, though interesting, were not conclusive and require further investigation.

MOLECULAR CHARACTERIZATION OF THE BACTERIAL FLORA OF THE MURINE FEMALE GENITAL TRACT

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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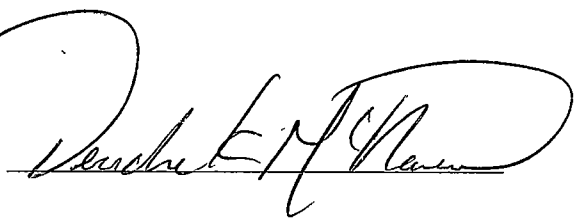
Signature 

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I dedicate the completion of this thesis and degree to my parents, William and Elizabeth McNamer, whose constant enthusiasm and pursuit of knowledge continues to amaze and inspire.

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ABSTRACT

The female genital tract may harbor a potentially diverse microbial community that has not been thoroughly characterized, particularly in the murine system. A more complete knowledge of resident flora may enhance understanding of disease susceptibility and the persistence of infection which, in turn, may better guide research toward more efficacious treatments and protective measures. Organisms known to exist in this environment have largely been identified through cultivation. However, because the nutritional and environmental needs of previously uncultivated organisms cannot be anticipated, there are limitations to the use of this technique when attempting to characterize a community. Molecular techniques that take advantage of the conservation of ribosomal RNA have proven to be very useful for such efforts. The use of PCR in tandem with denaturing gradient gel electrophoresis (DGGE) allows for the characterization and comparison of microbial populations with relative ease and rapidity. This study applies these molecular techniques to the investigation of the microbial flora of the murine female genital tract. In addition, a comparison was made between cultivation and two different molecular techniques, DGGE and cloning. We found some overlap in the detection of organisms identified by the various methods, but some microbes were only detected by particular techniques. We further used DGGE to identify bacterial populations of this environment during the course of the natural estrous cycle, under the influence of exogenous hormones, following antibiotic therapy and in a *Chlamydia trachomatis* infection model. Our findings indicate that there is no change in the species present over the course of the estrous cycle or under the influence of administered hormones. However, because DGGE is not quantitative we were unable to determine if quantitative changes in the populations occurred. Our findings with the antibiotic treatment and *Chlamydia* infection model, though interesting, were not conclusive and require further investigation.

INTRODUCTION

There are many compelling reasons to pursue studies of the microbial inhabitants of the female genital tract. First and foremost, the vagina is a critical portal of entry for disease. Through a better understanding of the natural, healthy state of the genital tract, greater awareness of disease susceptibility factors might be realized. Second, a strong understanding of the microbial community and the factors that influence population shifts in a non-diseased individual may elucidate the enigma of diseases that arise from commensal organisms such as candidiasis and bacterial vaginosis. Finally, study of these populations may help control the incidence of bacterial infections postpartum, post-Caesarean section, or following other surgical procedures involving the genital tract.

Historically, microbiologists have used cultivation to identify the bacteria of environmental samples including those of the female genital tract. However, many have speculated that pure-culture organisms may, in fact, represent less than 20% of the species that exist in natural environments (56). Molecular techniques that capitalize on the conservation of rRNA gene sequences have been instrumental in enhancing the ability to detect bacteria and other flora and to describe microbial communities more accurately. Microbial ecologists have successfully used these molecular approaches to understand a broad range of natural environments better. Molecular methods have been applied to the study of the female genital tract. However, they have been primarily limited to studies in which particular organisms, especially pathogens, rather than all potential organisms are targeted. The novelty of this study is that it attempts to broadly characterize the bacteria

of the female genital tract using molecular methods. Moreover, it compares traditional methods of cultivation and two different 16S rRNA-based molecular techniques.

Genital Tract Flora in Humans

The base of the genital tract ecosystem is the epithelium which is not a static entity and is subject to many different influences. This ecosystem is unique to the individual and can be affected by age, hormones, tissue composition, immune status and behavior. Behaviors such as sexual activity, method of birth control, tampon use, general hygiene and diet can dramatically affect the environment (12, 27). The microbial population is also individual. In addition, the organisms themselves will further determine biochemical parameters like pH, iron and oxygen availability and available nutrients such as amino acids and sugars.

In an attempt to examine some of these biochemical baselines, Larsen et. al. measured the total quantity of carbohydrates, proteins, and amino acids present in the human vagina. These measurements were chosen because they are the most likely sources of carbon and nitrogen to support bacterial growth (27). The investigators quantified these nutrients in the 44 women participating in the study and found carbohydrate levels to be 47.51 mg/g, protein 3.75mg/g and amino acid concentration to be .02086 mg/g – hypothetically enough to support significant microbial growth. Interestingly, all three nutrients were markedly reduced in the presence of colonizing yeasts. However, a direct correlation between the population densities of yeast and

bacteria could not be made. Other substances of import to microbial survival that have not bee well studied or quantified in the human female genital tract include vitamins and iron. Although vitamins are known to aid the growth of fastidious species and have been shown to be required for the growth of bacteria such as lactobacilli, little is known about the available vitamin content of the genital tract (27). Iron is known to be critical to many enzyme systems and to enhance the virulence of certain mucosal pathogens such as *N. gonorrhoeae* and *N. meningitides*. The degree of iron availability at times other than menses or compensatorial mechanisms employed by resident flora during these times is not well understood.

The pH of the vagina is in the range of 4 to 4.7 and has been reported to fluctuate during the menstrual cycle (12, 27). Although the theory predominates that lactic acid produced by lactobacilli is largely responsible for vaginal pH, lactic acid is also produced during the conversion of glycogen by epithelial cells and glycogen concentration within cells increases with high levels of estrogen (12, 55). It is thought that low pH diminishes the growth of many organisms. However, this is based solely on the ability to cultivate organisms. As will be discussed, a variety of organisms have been recovered from the female genital tract despite this seemingly low pH. Many aspects of the biochemistry of this microbial habitat merit further study, but what is becoming clear is that the microbial inhabitants themselves may dictate many of these factors through their individual metabolic needs and by-products. Therefore it would benefit the overall state of research in this area to better catalog these populations.

Efforts have been made to describe the flora that reside in the female genital tract. Many species of bacteria are known to exist simultaneously in the vagina including gram-positive, gram-negative, anaerobic and facultative anaerobic species (27). However, our knowledge of genital tract flora is limited by biases inherent in the techniques used to discern microbial populations. Larsen and Monif recently summarized the findings of researchers investigating the female genital tract (31). Reports have included the following aerobic isolates with varying degrees of prevalence: Lactobacilli, *Staphylococcus* species (*S. aureus* and *S. epidermidis*), *Streptococcus* species, and Gram-negative rods including *E. coli, Klebsiella* and *Enterobacter* species. Anaerobic organisms isolated have included: *Bacteroides, Lactobacillus, Peptococcus, Peptostreptococcus, Veillonella, and Clostridium* species (31). Whether these finding are a complete representation of the species that colonize this environment remains to be decided, but they do provide a baseline from which to work.

The Hormone Cycle and Bacterial Populations

The female genital tract is an extremely dynamic environment. The question naturally arises regarding the impact of the hormone and menstrual cycle on genital tract flora. Menarche, pregnancy and menopause are all periods of unique hormonal constitution. Hormones are known to affect changes on the epithelium of the genital tract and the sloughing of the epithelial lining of the uterus during menstruation may have a dramatic effect on the resident flora. The most recent investigation charting the flora of

the vagina of healthy women over the course of the menstrual cycle has come from Eschenbach et. al. (12). This study included 89 women, 50 of whom were healthy and free of symptoms of bacterial vaginosis. Data was gathered from the women during three phases of the menstrual cycle (days 7-12, 19-24 and 1-5 of the next menstrual cycle) and included an analysis of the vaginal flora in addition to other parameters. The researchers found that over the course of the cycle there was a small rate of increase in the recovery of *Lactobacillus* species and *Bacteroides fragilis* while there was a decrease in the rate of recovery of non-*Lactobacillus* and *Prevotella* species (12).

These finding corroborate the findings of an earlier study by Johnson et. al.(23) but not those of Mehta (36). Johnson et. al. compared two phases of the menstrual cycle, menses and post-menstrual, in 34 women and found that *Gardnerella vaginalis* and aerobic *Lactobacillus* species increased in the post-menstrual phase while the majority of other organisms including *Staphylococcus* species, alpha and beta-hemolytic streptococci and *Peptostreptococcus* species declined (23). In contrast, Mehta in a study of 54 women, sampling at six time points, found no significant change in the prevalence of any of the same species (36). Although the data suggests that the menstrual cycle impacts resident flora, further investigation into this area is needed.

The Female Genital Tract and Disease

The incidence of sexually transmitted disease in the United States continues to grow. The CDC estimates that 15 million new infections develop every year (11). Many

of these diseases are transmitted more efficiently from men to women than from women to men (47). While sexual practices certainly play a large role in susceptibility, the extant flora may also influence the ability of an invading organism to colonize the genital tract. Interactions among established flora will determine population fluctuations and may act to inhibit or encourage the uptake of residency by new organisms, pathogenic or otherwise.

The antagonistic and synergistic effects that microbial species have on each other have long been recognized (27). Antagonistic effects may be direct or indirect. Bacteriocins are proteins produced by some bacteria that have direct bacteriacidal effects on other, closely related bacteria. The specific mechanism of action varies but in general they bind to cellular receptors and inhibit nucleic acid or protein synthesis (54). Gliotoxins with antibacterial properties such as those produced by *C. albicans* have been reported(27). Toxic by-products from bacteria have also been observed. Lactobacilli have been reported to produce significant amounts of H_2O_2 that can inhibit the growth of other bacterial species (22). As mentioned above, lactobacilli have been implicated in the regulation of environmental pH through the production of lactic acid. *Gardnerella vaginalis* is known to produce a hemolysin that is not directly bacteriacidal but active against leukocytes (27). Other antagonistic effects may be manifest indirectly via competition for common, essential nutritional resources or attatchment to surfaces. Limited supplies of oxygen, iron, amino acids, and carbohydrates may force the predominance of one species over another even in the absence of a single, specific antagonistic factor.

Synergistic effects encourage the cohabitation of species that benefit from one another's presence. Cross feeding may occur if one bacterial species produces a nutrient needed by another species (27). Alternatively, the by-products of one organism may affect other environmental factors that can create a favorable environment for a second species. Together, such growth encouraging or discouraging effects are likely to have a profound influence on the organisms that colonize the genital tract whether it is a commensal or a would be invader. Better understanding of the microbial flora, their relationship to one another and their part in facilitating disease would aid tremendously in developing prevention measures. Further, in depth knowledge will permit more effective treatments and will help avoid persistent infections.

The line delineating pathogenic organism from non-pathogenic organism has blurred considerably since the early work of Pasteur and Koch. The once simple framework presented by Koch for determining etiologic agents of disease is complicated by opportunistic pathogens, host responses and the fact that the presence of some, so-called pathogens does not necessarily lead to disease. Classical definitions of pathogenicity and virulence have relied on the organism's innate ability to cause disease in the host. The strictures imposed by this type of definition do not allow for the immune state of the host or instances of commensals moving from their natural environment within the host to novel or otherwise sterile areas. Nor does it account for damage or disease caused by the host's own immune response. More recently a classification scheme has been proposed that accounts for the significant factors of host response and opportunism (7). In this system, organisms are classified based on a damage-response

scale that accounts for both the disease caused directly by the organism as well as that of the host responding to that organism. The utility of such a system becomes obvious when studying a "classical" pathogen such as *Chlamydia trachomatis*, and is even more so when considering infection by organisms which can act as opportunists such as *Candida albicans.*

C. albicans is an example of an organism that may elude the classical definition of pathogen but is certainly known to be responsible for disease. Several studies have shown that the organism is present in the vagina of asymptomatic women and can be in stable association with the genital epithelium (31). Thus, the mere presence of the pathogenic organism does not constitute a disease state. It also presents an example of an organism whose prevalence and by extension whose ability to cause disease is subject to the physiology of the host and may be limited by other fellow microflora. Carriage of *C. albicans* is known to increase at puberty and during pregnancy (31) and an inverse relationship between certain bacterial isolates and the yeast has been shown (38). In addition the gliotoxin produced by *Candida* species can have antagonistic effects on other bacteria. The development of vaginal candidiasis is conditional and the factors that encourage disease will be better understood when a clear picture of the microbial population shifts associated with its colonization is attained.

A similar example of disease exists in the case of bacterial vaginosis. A clear etiologic agent for bacterial vaginosis has not emerged, but *Gardnerella vaginalis* has been implicated. Similar to *C. albicans, G. vaginalis* is commonly found in the vagina of healthy women. Characteristically, incidences of bacterial vaginosis are accompanied by

a rise in vaginal pH and a decline in the lactobacilli population. The theory has been put fourth that decreases in these populations cause the pH to shift which, in turn, allows for the overgrowth of other species of bacteria such as *G. vaginalis*. A clear cause and effect has not been demonstrated but again, this is an instance where the dynamics of native flora appear to play a significant role in the development of disease.

Chlamydia trachomatis is an example of an organism whose presence is associated with disease and that stimulates a host immune response that further contributes to manifestations of disease. *C. trachomatis* is an obligate, intracellular bacteria that is responsible for sexually transmitted disease as well as ocular infections world-wide. In this country the Center for Disease Control estimates that 3 million new sexually transmitted *Chlamydia* infections occur every year. These predictions are difficult to make accurately as the disease in approximately 75% of men and 50% of women is asymptomatic (11). Chlamydiae exist in two morphologies: as infectious elementary bodies (EB) or non-infectious, metabolically active reticulate bodies (RB). *C. trachomatis* species can be divided into three biovars; trachoma with serovars A-K, lymphogranuloma venereum (LGV) with serovars L1-3 and mouse pneumonitis (42). It is the latter of these three that is used in murine infection models such as the one used in this study.

C. trachomatis EBs primarily infect epithelial cells found on the mucous membranes of the urethra, endocervix, endometrium, and fallopian tubes in addition to those of the ano-rectum and conjunctivae. In women, if the disease remains untreated it can develop into salpingitis or pelvic inflammatory disease (PID). PID is an example of

host-response manifestations of disease and the tubal scarring that results is a common cause of infertility and potentially life-threatening ectopic pregnancies (11, 42). Impressive attempts have been made to illuminate the role of the host immune response in the resolution of *C. trachomatis* infection. Unfortunately, a clear picture has yet to emerge and thus attempts to develop effective vaccines or immunotherapies have been hindered. These lines of research should obviously be pursued, but other factors that may be of use in inhibiting infection also merit investigation. The study of the microbial composition of the vagina has the potential to make several contributions to this area of research. First, through an understanding of the composition of indigenous flora organisms that may inhibit or, conversely, aid infection can be identified. These types of studies may indicate whether or not probiotic therapies, or the introduction of organisms, would be useful in the treatment of *Chlamydia* infections. Such therapies have been attempted using H_2O_2 producing *Lactobacillus* sp. in the treatment of bacterial vaginosis though the results have been mixed (48) (5, 20). Second, by following the changes in the environment, during infection, circumstances that lead to disease progression, persistence, or resolution may be explained. Finally, the endogenous organisms that predominate during disease may be identified which could be used as biological markers of infection. Together, knowledge of these elements may contribute to the prevention and treatment of *C. trachomatis* infections.

The Mouse System

The mouse model has long served as a paradigm of the human response to treatment and disease. In using such a model it is imperative that the limitations of the system are understood. In analyzing how the mouse responds to disease, for example, it is important to note that while similarities do exist that allow reasonable conclusions, there may be critical differences that make extrapolation from the model invalid. When using mouse models for studying diseases that affect the female genital tract, factors such as anatomy, biochemistry, fertility cycle and the endogenous microbial community are critical. Our understanding of the microbial community of the female genital tract is limited. This is largely due to the limitations inherent in available techniques (namely cultivation) that permit only a selective view of the subject. A more thorough understanding of the composition of this community will allow for a better analysis of those factors that change the community and how changes occur.

Anatomy, Physiology and Microbiology of the Murine Female Genital Tract

The genital system of the female mouse extends inward from the external genitalia and includes the vagina, the external and internal cervix uterus, the oviducts and ovaries. The anatomy and biochemistry of the organs within the genital tract are unique and they undergo characteristic changes throughout the estrous cycle that will be

discussed. The anatomy and cellular composition are also unique. The vaginal orifice is sealed off with a membrane that opens at the time of initial ovulation (approx. 3 to 4 weeks) (17). The wall of the vagina is composed generally of a mucosal membrane, a muscular coat and a serous membrane. The mucosal membrane of the vagina is composed of stratified epithelial cells. These are flat, scale-like, nucleated cells that form the strata or layers that make up the epithelial lining and are subject to morphological changes during the estrous cycle. A transition occurs in the cervix where more columnar cells are present. In rodents the cervix can be divided into two distinct structures; the external cervix, which is positioned caudally, and the internal cervix that lies in the anterior portion of the genital tract. It, too, is composed of the mucosa-submucosa, muscularis and serosa. Mucosal goblet cells, a type columnar or tall, prismatic cell, of the cervix produce cervical mucus. This is primarily a glycoprotein substance. The production of mucus is largely under hormonal control and is most copious during the hormone cycle when estrogen is prevalent (19).

Another transition occurs between the cervix and the uterus. The uterus of the mouse is a duplex uterus (meaning it branches into two distinct uterine horns) beyond which lies the tubular oviducts and the ovaries. The walls of the uterus are composed of the endometrium and the myometrium. The epithelial lining of the lumen and a glandular subsurface comprise the endometrium while the myometrium is composed of the muscular surface of the uterine wall (19). The epithelial cells of the uterus are primarily columnar cells. These tall cells can be ciliated and secretory and exhibit gradations of

height that, again, are subject to the hormonal environment. These cells reach a maximum height and are secretory during periods of progesterone secretion (19).

The genital tract is considered to be a part of the common mucosal immune system (CMIS). It is inhabited by cells of the immune system and their accompanying secretory products. The presence of plasma cells of the IgA isotype has been reported in BALB/c mice, as has secretory IgA (S-IgA) (49) (60). Parr et. al. reported levels of S-IgA in the vaginal mucus at 154ug/ml. (49). IgG is also present with reported concentrations of 23 ug/ml (49), however, the origins (local vs. serum derived) of this antibody are unclear (49, 60). CD4+ and CD8+ T-cells are also present in the genital tract during the course of infection. In an uninfected state few of these cells are typically seen, and in BALB/c mice make up less than 1% of the total cell population (24, 41). Antigen presenting cells such as immature dendritic cells capable of interacting with both B and T cells can also be found though at low levels(4). Finally, neutrophils are found in the genital tract but this is largely dependent on the hormone state of the animal as will be discussed.

The estrous cycle, analogous to the menstrual cycle in humans, lasts 4 to 5 days (6) (13). Depending on the criteria used this cycle can be divided into four distinct stages: proestrous, estrous, metestrous and diestrous (17). The periodicity of this cycle is directed by hormonal fluctuations in concert with ovarian activity. Both of these may, in turn, be subject to numerous environmental and behavioral factors. While a myriad of elements from the nervous, endocrine and reproductive systems have been described as participating in the estrous cycle, the major contributors are as follows: the gonadotropins

follicle stimulating hormone (FSH) and luteinizing hormone (LH), and the gonadal hormones estrogen and progesterone. The gonadotrophins are released by the anterior pituitary gland and are controlled via feedback mechanisms involving circulating levels of estrogen and progesterone. These steroid hormones act on the hypothalamus and are produced by the immature follicle and corpus luteum respectively as well as by extragonadal sources (17).

The proestrous and estrous stages are most similar to the follicular phase of the human menstrual cycle during which estrogen dominates. This portion of the cycle lasts approximately 2 days (24 and 28 hrs each). Metestrous and diestrous are comparable to the luteal phase of the human cycle and are marked by elevated progesterone levels and make up the remainder of the cycle (18 and 33 hrs each) (13). During early proestrous, low levels of estrogen produced by the immature follicle promote FSH release from the anterior pituitary. This further promotes follicle growth. As the follicle develops and produces more estrogen (in the theca interna cells) FSH release is inhibited and LH titers begin to rise. Small amounts of progesterone release also contribute to LH increases. Ovulation occurs at the end of estrous. Metestrous and diestrous ensue and are accompanied by increases in progesterone levels, a decrease in LH and estrogen. Finally, a resurgence of FSH marks the beginning of the next cycle (6) (17).

Rodents are considered to be spontaneous ovulators because it is an intrinsic rhythm of LH release that controls ovulation (6). However, it has been observed that when groups of female mice are housed together they become anestrous or may develop spontaneous psuedopregnancy due to suppression of FSH (57). The presence of male

primer pheremones can overcome this effect. These pheremones, present in the urine of the male mouse, stimulate gonadotropin production and secretion which, in turn, leads to estrous and ovulation. Serum progesterone decreases and serum estrogen levels rise following the introduction of the male. An LH surge occurs about 62 hours after initial exposure to the male. (58). Once initiated, estrous in grouped females tends to be synchronized. (6) (58).

In addition to certain behavioral modifications, the dynamic hormonal activity of the estrous cycle promotes a number of changes in cellular characteristics throughout the genital tract. These changes are reflected in the exfoliate cytology which is a useful tool for staging animals under study. Scanning electron microscope studies undertaken by Lamb et. al. (26) and Corbeil et. al.(9) have been useful for elucidating the unique changes in the uterus, cervix and vagina, and for drawing the correlation between cytology and stage.

Estrous is an anabolic stage and is therefore a time of active cell growth and secretion. Externally it can be identified by vaginal gaping, swelling of the vulva and behaviorally by the acceptance of a male for reproduction. Internally, the uterus is distended and displays prominent folds. SEM reveals that cells are densely covered with long microvilli that project into the lumen and glandular openings are apparent. In the cervix, cells are flatter and wider than those of the uterus but a clear transition from the uterus to the cervix is not obvious. In general, the cells of the internal cervix are smaller, and more uniform than those of the external cervix. The cells of this area show greater similarity to the cells of the vagina and some exfoliation is seen. Large, exfoliating cells

are found throughout the vagina during estrous. In addition, cells of this region are flat, keratinized and covered with microridges. They are polygonal with distinct borders and they lack a nucleus (9, 26).

Metestrous is the first of two catabolic stages of the estrous cycle. It can most easily be recognized by its state of cellular disorganization. Cells of the uterus are generally swollen or disrupted and denuded of microvilli. Free cells can be found in the lumen and neutrophils have been observed just below the basal lamina and migrating through the epithelial layer. The uterine glands are not as visible at this stage as they are in estrous. In the cervix desquamating surface cells can be found as well as bacteria, mucus and cellular debris. Cells in this region are generally flat and cornified. The cells of the internal cervix appear to be rounder and smaller than the external cervix. Unlike other stages of the cycle the junction between the vagina and cervix and uterus and cervix is clear. While the junction between cervix and vagina is clear at this stage, the two regions share similarities in cell morphology. Notably, a thin cornified layer can be found in the vagina and, like the cervix, there is an abundance of both bacteria and desquamating epithelial cells. These epithelial cells are less abundant at this stage than in estrous (9, 26).

During diestrous the appearance of an ordered epithelium returns. Although this too is a catabolic stage, cell composition does not appear as disorganized as metestrous. Cells throughout the genital tract have a uniform covering of microvilli (long and short depending on region). The cellular homogeneity of the uterus eventually gives way to a more mixed population in the cervix but the transition from uterus to cervix is difficult to

identify. The cells of the cervix are slightly rounded and appear smaller than in metestrous. In the vagina, mucus and neutrophils are found. In addition cells with irregular surfaces protrude into the lumen (9, 26).

Proestrous marks the beginning of the cycle and a return to an anabolic state. Proestrous bears some of the same markings of estrous. In the uterus, for example, folds and glandular openings become visible and cells have an even distribution of short microvilli. The flatter and wider cells are covered with both mirorugae and microvilli. During this stage, the vagina is characterized by mucified columnar cells overlying a thick layer of cornified squamous epithelium. Like the cells of uterus and cervix, cells of the vagina are covered with microvilli or ridges (9, 26).

Evans et. al. undertook a quantification study of genital tract cells by examining a number of parameters including a proliferative index and cell number (per organ). Their results corroborate the anabolic nature of proestrous and estrous and catabolic nature of metestrous and diestrous. In addition, they found that the dynamism of the uterus was much greater than that of the vagina and cervix. Differences in lowest to highest cell counts were four-fold in the uterus compared with two to three-fold changes in the vagina and cervix (13).

A number of biochemical variations occur in the genital tract throughout the estrous cycle. Koiter et. al. found that in addition to the changes in the numbers of bacteria and leukocytes in the vagina of cycling rats there were fluctuations in pH that also occured. The pH ranged from 6.9 in proestrous to 7.2 at estrous, to 7.7 at metestrous and 7.4 at diestrous (25). Mehrotra and Karkun examined the effect of hormones

(estrogen, progesterone and testosterone) on the biochemical constitution of the female rat cervix and vagina (35). While testosterone had little effect on the biochemistry, estrogen and progesterone treatments did impact levels of protein, glycogen, lactic acid, alkaline phosphatase and acid phosphatase. Estrogen increased protein, lactic acid levels and the activity of alkaline phosphatase in both the vagina and cervix. It also raised the glycogen content of the vagina but not the cervix. Progesterone enhanced protein and alkaline phosphatase activity in both the vagina and cervix but had a dichotomas effect in the two organs with regard to acid phosphatase activity and glycogen levels. Progesterone levels corresponded to a rise in glycogen levels in the vagina and a reduction in the cervix, while acid phosphatase activity rose in the cervix and declined in the vagina (35). Other researchers have observed the relationship between glycogen and estrogen levels. Bronson notes that glycogen content in the uterus is greatest during proestrous when high levels of estrogen are present (6).

The estrous cycle has a quantitative effect on the microflora. Larsen et. al. quantified bacteria throughout the four stages of estrous. Their results showed that when gram-stained vaginal smears of rats were examined the greatest number of bacteria could be found during estrous while the fewest number were seen during metestrous. The same result held true when CFUs from vaginal lavages were examined (28). This result prompted the researchers to examine specifically the effect of ovarian estrogen on genital microflora. They found that the cyclic pattern of vaginal bacterial counts was abolished by ovariectomy but that treatment with estrogen led to a significant rise in counts three days post-treatment. Further, progesterone alone had no effect on these counts when

administered alone, but when given to ovariectomized rats in addition to estrogen it diminished estrogen's effects (30).

A thorough catalog of the bacteria of the murine vagina has not been done. Most likely this is due to the variation that can be expected among strains, breeders, housing and numerous other factors. Meysick and Garber obtained a baseline of vaginal flora in BALB/c mice for their study of *Trichomonas vaginalis* infection. In untreated mice they found that the predominant species was *Staphylococcus aureus* with an isolation frequency of 56% of mice as determined by cultivation, followed by *Enterococcus species* (32%), *Lactobacillus species* (16%), *Escherichia coli*, *Proteus mirabilis* and *Micrococcus luteus* (12%). The frequencies with which these isolates were found increased when the mice were treated with estrogen (37). Larsen et. al. attempted to inventory the bacteria of the rat genitalia including the differences in isolation between the cervix and the vagina. In general, they found the most common isolates to be alpha and non-hemolytic streptococci, *Pasteurella pneumotropica* and diphtheroid bacilli (28).

Current understanding of the anatomy of the mouse genital tract is fairly complete. What lacks is a strong understanding of the biochemistry and the composite bacterial flora. Although there have been studies to suggest the cyclic nature of expansion and decline in the populations, the studies are scant and those populations have not been well defined. What is needed is both an adequate catalog of the bacteria inhabiting the biological niche in question and the application of a means by which this can be done thoroughly.

Methods Used to Study The Microbial Flora

The field of medical microbiology has played a dominant role in forming approaches employed for the study of microbial ecology and bacterial physiology. It is important that deference to the techniques that have been pioneered in this field be shown but at the same time the significant limitations of such approaches must be recognized. Historically, various cultivation strategies have been employed for isolating the bacteria that inhabit a given environment, which could then be studied with respect to physiology. For major disease causing organisms that were not amenable to culture in laboratory settings, such as Treponema, microscopy and differential staining were employed. Certainly, the practical relevance of methods such as the Gram stain and their contribution to the treatment of disease cannot be denied. This general approach involving culture, microscopy and staining opened a world at the cellular and eventually molecular level that has contributed enormously to the basic understanding of life.

Yet, there are fundamental flaws to this traditional approach. For example cultivation imposes specific nutritional needs on the organisms within the niche in question, it assumes that natural conditions can be adequately imitated within the laboratory environment and it does not reflect the synergistic or antagonistic affects that organisms have on each other or other elements in their native environment. As a consequence, it excludes large portions of the microbial world. To use cultivation to describe the microbial community of an environment introduces major bias to a study,

and while it can be informative, it is best used in conjunction with cultivation-independent analyses.

Molecular Approaches in the study of Microbial Populations

The advent of the molecular age of microbiology has meant significant progress in the ability to characterize a microbial community. 16S rRNA analysis has proved to be an extremely useful tool for phylogenetic analysis. It is also demonstrating its utility for describing microbial communities and identifying organisms within particular environmental niches. The range of environments that can be studied is limitless; from hydrothermal vents (52), to hot spring cyanobacterial mats (56), to the gingival crevice (8), to the efforts of this study. Ribosomal RNA is the logical focus for the study of mixed microbial populations for a number of reasons. The nature of ribosomal RNA suggests that it be highly conserved from one organism to another because of the vital, fundamental function that it performs. Parts of the molecule are more or less conserved among the three domains, eubacteria, archaebacteria and eukaryotes, and thus make useful markers for taxonomy (18). Additional reasons to concentrate on ribosomal RNA are that the conservation of secondary structure extends to a conservation of primary sequence and the sequence length is long enough to provide statistically significant comparisons and therefore differentiation of species. Finally, its abundance in the cell also facilitates its use for study (46).

16S rRNA-based methods of community analysis including cloning and sequencing, oligonucleotide probes, and denaturing gradient gel electrophoresis (DGGE) have become common techniques employed by microbial ecologists. 16S rRNA genes can be cloned using DNA purified from a mixed microbial population sample. Individual clones can then be isolated and sequenced. Although this is a useful method, it can be expensive and time-consuming and should be done exhaustively in order to insure that all species are accurately represented (44). Oligonucleotide probes are also used for community analysis. They can be designed to target various conserved regions of the 16S rRNA and are useful for in situ, whole-cell analysis as well as the study of nucleic acid extracts. Fluorescent, enzymatic or radioactive labels can be attached to such probes depending on the constraints of the particular study. rRNA-targeted, whole-cell hybridizations can yield data on cell morphology, specific cell counts, and in situ distributions of organisms. In addition, the strength of the hybridization signal reflects the cellular rRNA content of individual cells that can be indicative of growth rate and activity (3).

The ease with which multiple, mixed-population samples can be compared has been advanced by the use of DGGE together with PCR amplification of select regions of the 16S rRNA gene. This is the primary method of community evaluation used in this study. The use of DGGE in analyzing nucleic acid sequences was first described by Fischer and Lerman (16). This technique capitalizes on the different melting characteristics of individual nucleic acid sequences. Molecules that are less stable migrate shorter distances or withstand less denaturant than those molecules that are more

stable, and even single base pair changes will impact the stability of a sequence and affect how it migrates through such a gel (16). More accurately, DGGE relies on the melting behavior of a molecule that is a result of the composite characteristics of its nucleic acid primary sequence and its helical structure. Specifically, the guanine and cytosine (G + C) content of the sequence and where those triple-bonded pairs lie within the molecule have a major impact on the stability of the molecule. Discrete segments within the molecule make up so-called melting domains. These domains are more or less subject to denaturant depending on their individual composition, and will "open" or lose their helical integrity as they migrate through a denaturing gel. This occurs in a stepwise rather than continuous fashion with the least stable domains "opening" first (1). The degree to which such a molecule is retarded as it migrates through the gel, then, is a function of the stability of its melting domains or the amount that they "open" under the given denaturing condition.

Because the migration patterns of DNA molecules through a denaturing gradient gel are ultimately a reflection of differences in sequence, this technique can be used to separate similarly sized sequences. The technique has proven particularly useful in analyzing pooled 16S rRNA gene segments from mixed microbial populations. First described by Muyzer et. al.(43) the application has been widely used to profile communities and to assess diversity and phylogenetic relationships in microbial communities. PCR is used to amplify 16S rRNA genes from DNA extracted from a natural environment and the resulting products are then separated based on melting characteristics by DGGE. Individual sequences or bands in the gel can be purified and

sequenced to identify populations within a community and rendering possible comparisons between different communities. In contrast to cultivation, this provides a comprehensive view of the community and does not require the researcher to make potentially invalid assumptions about the metabolic needs of the organisms inhabiting the environment in question.

Although the use of molecular techniques circumvents many of the biases of cultivation, there are complications that can be introduced to DGGE at the level of PCR when amplifying the rRNA from mixed-cultures. It is important to identify these potential biases in order to avoid a skewed view of the populations that make up the community. These biases include: heteroduplex or hybrid formations of template DNA, preferential amplification of certain rRNA genes based on G+C content and other factors, the impact of gene copy number on amplification, and, of course, bias introduced by choice of primer and mutations generated or polymerase errors.

The formation of hybrid products (e.g. chimeras) can present a major confusion in trying to analyze DGGE profiles as a reflection of community diversity because they may be interpreted as additional or even novel microbial community members. For example, Liesack et.al. encountered this phenomenon while studying communities of barophilic bacteria. In analyzing the sequence and structure of six clones they found that one of their clones was actually composed of DNA from two closely related species that had been assembled during the PCR reaction – a hypothesis they confirmed by secondary structure analysis. Factors that can contribute to the formation of such chimera include; first, the presence of large regions of highly conserved sequence within the priming

region (as would be expected with closely related organisms); second, the availability of low molecular weight genomic DNA (4-6kb) or fragments of DNA such as those caused by physical shearing of the DNA. Problems introduced by such hybrids can be identified either through careful sequence or secondary structure analysis and in some cases may be avoided by creating cDNA from extracted RNA (32).

Preferential amplification of template DNA is also a potential problem and can occur for a number of reasons. In particular, the G+C content of the template may play a role in amplification under certain PCR conditions (51, 53). Lower G+C content sequences may be selectively amplified because the melting characteristics of such sequences lend themselves to more facile dissociation into single-stranded DNA. Therefore, there may be more primer hybridization with these molecules because they are more abundant in the PCR mixture (53). Further, the initial template proportions can impact the kinetics of the PCR reactions and the efficiency with which they take place such that bias is introduced. Differences in secondary structure may also impact the availability of priming sites or the polymerization reaction as can the choice of polymerase (53). One way in which some of these biases can be eliminated, namely dissociation of high G+C content molecules, is by subjecting reactions to more stringent conditions. This can be achieved by the addition of acetamide, alkali denaturation, or by the use of "hot start" PCRs (51).

16S rDNA copy number also impacts community analysis. This is particularly true if there is heterogeneity within the genes (50). The number of 16S rRNA genes can vary significantly among organisms. Gene numbers from 1 to 15 have been reported (34).

The gene copy number affects the amount of PCR product which can then alter perceptions of an organisms' prevalence within the community. Farrelly et. al. attempted to discern whether a clear relationship between gene copy number and PCR amplification could be established and then used for quantitative studies. Their conclusions based on the analysis of simple communities (two bacteria) suggested that such a relationship would be difficult to establish even if gene copy number was known let alone in instances where novel, uncharacterized bacteria were involved (14). Thus until a reliable technique is found, PCR amplification of 16S rRNA gene products cannot be considered quantitative.

Primer selection has a major impact on the type of organism that is targeted for amplification by PCR. Regions of ribosomal RNA are highly conserved among eukaryotes, eubacteria and archaebacteria. Other regions are semi-conserved and common only to members of a given domain, while variable regions are unique to individual organisms(59). This is an important consideration in primer design. The selection and use of PCR primers for the regions common to a domain, say eubacteria, will result in only eubacteria being amplified. Individual bacteria can then be recognized by differences in the variable sequence regions, which are similar in very closely related organisms. Obviously, the choice of 16S region that is amplified, its scope and variability, will greatly influence the organisms that are represented by amplification. Researchers can therefore use primers as a powerful tool to survey communities in as broad or as narrow a range as they choose. Further, properties of the primer itself can impact amplification. For instance, a 15-mer may have a different annealing efficiency

than an 18 or 20-mer primer(53). Three different primers were used in the course of this study representing three regions of conservation. One primer, "1070 forward" complements a region conserved among bacteria (3), while the "931 forward" primer complements a region conserved among archaea but also found in some bacteria (33). The final primer, "1392 reverse", represents the complement of a region universally conserved among all three domains (3).

While community analysis by PCR and DGGE appear to have some advantages over cultivation, they cannot be used to its exclusion. Due to the number of PCR amplification biases noted above and their non-quantitative nature, these molecular techniques fall short of presenting a complete picture. This is especially true of an environment such as the mouse vagina where population fluctuations have been observed and appear to be of importance. Molecular techniques are arguably more sensitive and comprehensive in their scope but the use of PCR and DGGE alone is not quantitative. Therefore it is best to consider the combined use of traditional and more quantitative molecular techniques in order to obtain a comprehensive understanding of community structure and function.

MATERIALS AND METHODS

Experimental Animals

Inbred female BALB/c mice 6 to 10 weeks old were purchased from the Animal Resource Center at Montana State University where they were maintained. In addition, two outbred strains of mice were purchased (Charles River Laboratories, Kingston, N.Y.) and maintained at Montana State University: 6 month old Swiss Webster mice and 6 to 8 week old CD-1 mice. Mice were housed in micro-isolater cages with filter tops. Bedding in all cages was autoclaved prior to use.

To induce natural cycling, BALB/c mice were transferred to Whitten cages (57) 1 to 2 weeks prior to the start of experimental protocols. Whitten cages are composed of two chambers within the same cage separated by mesh wiring. A male mouse is housed on one side of the divider and females on the other. Pheremones in the male urine pass through the divider to the females and induces regular, synchronized estrous cycling in females.

Sampling

All mice were euthanized with CO_2 prior to sampling. Three consecutive vaginal washes were performed with 50ul of sterile PBS. The PBS was prepared from sterile, nuclease-free water (Sigma). 20-30ul of the wash sample was set aside for cytology.

Mice were then swabbed using a cervicovaginal swab (Calgiswab; Spectrum Medical Industries, Los Angeles, CA.) and the swab placed in 0.2-0.3ml of sterile PBS. The vaginal and cervical epithelium were scraped in situ with a sterile inoculating loop and combined with the swab sample. Samples were frozen at –20° C or used immediately for culture. Wash and swab samples for each mouse were pooled prior to DNA extraction.

Cytology

Approximately 20ul of wash sample was applied to a clean microscope slide and allowed to air dry. Slides were then stained using the Hema 3 Stain Set (Biochemical Sciences Inc. distributed by Fisher Scientific) for staining characteristic of Wright-Giemsa stains. The primary components of this Romanowsky stain are azure B, a basic dye, and eosin y, an acidic dye. Azure B binds to anionic molecules of the cell such as DNA while the eosin Y binds to cationic sites on proteins. This stain is typically used for differentiation among erythrocytes, leukocytes and platelets. In this the case it was used to differentiate nucleated from non-nucleated epithelial cells and to indicate the presence of granulocytes (primarily neutrophils). Cycle phase was determined using the following criteria: estrous was marked by the presence of non-nucleated, cornified epithelial cells which in some cases formed aggregate-like masses; metestrous was characterized by the influx of leukocytes, primarily neutrophils, and the continued presence of cornified epithelial cells; diestrous was distinguished from metestrous by the predominance of leukocytes with few if any epithelial cells; proestrous was determined by the presence of

single or aggregate rounded, nucleated epithelial cells and by the general paucity of cells in the sample (Figure 1).

DNA extraction

Cell lysis and DNA extraction protocols were based on those of Moré et al. (39). Cells (0.2-0.3 ml samples) were treated with 0.26 ml of 10% sodium lauryl sulfate solution (0.5 M Tris/HCl, 0.1 M NaCl, pH adjusted to 8) and added to 1.88g of Zirconium beads (Biospec) and 120 mM sodium phosphate buffer (pH=8) (approx. 0.8 ml). Cells were lysed by mechanical disruption at 6.5m/s for 45 s using a beadbeater (Savant Instruments, Bio 101) at room temperature. Crude cell lysates were then precipitated with 0.4 volume of 7.5 M ammonium acetate. The DNA in the resulting supernatant was precipitated overnight at –20° C with 0.7 volume of isopropanol and recovered by centrifugation in a standard table top centrifuge at 14,000 rpm for 30 min at 4°C. DNA pellets were resuspended in 50ul of nuclease-free water (Sigma).

PCR

PCR conditions and primers were based on those previously described (15). Primers were designed to complement conserved regions of bacterial 16S rDNA genes. The nucleotide sequence of the primers are as follows: primer 1 (*E. coli* positions 1055 to 1070) 5'-ATGGCTGTCGTCAGCT-3'; primer 2 (E. coli positions 1392 to 1406 with the addition of a 40-base GC clamp)5'CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCC

CCCGCCCCACGGGCGGTGTGTAC-3'. In addition, a primer specific for archaea was also used for some experiments (*E.coli* positions 915 to 934) 5'AAGGAATTGGCGGGG GAGCA-3' in combination with the 1392 primer with the GC clamp [Amman, 1995]. PCRs were performed in a 50ul reaction mixture composed of the following: 5ul 10X reaction buffer with $MgCl_2$ (Fisher brand T*aq* buffer "A" - 100mM Tris-HCL pH=9.0, 500mM KCl, 15mM $MgCl_2$, 1% Triton X-100), 1ul PCR nucleotide mix with 10mM of each dNTP (Promega), 12.5-25uM of forward and reverse primer (described above), T*aq* DNA polymerase (Fisher) at 1.25 units in 0.25 ul, templated DNA and nuclease-free water (Sigma) to final volume. Optimal template concentrations were determined empirically. Generally, a 1:50 dilution of template was found to work best. The temperature cycle for the PCR was 1 min of denaturation at 94° C, 1 min of annealing at 55° C, and 1 min of primer extension at 72° C. This was repeated for 29 cycles. An initial denaturation step was used in which PCR mixture without polymerase was raised to 94° C for 5 min prior to the addition of polymerase. A final primer extension step of 10 min was also used. PCR products were analyzed using ethidium-bromide stained agarose gels. Those reaction mixtures yielding products of the expected size were used for DGGE analysis.

DGGE

DGGE conditions were similar to those used by Ferris et. al. (15) with the addition of a concentration gradient of acrylamide (6-11%) along with the denaturant gradient (10). Acrylamide gels were run with 0.5X TAE buffer (1XTAE is 0.04 M Tris

base, 0.02 M sodium acetate, and 1.0 mM EDTA; pH adjusted to 7.4) A Hoeffer SE 600 gel electrophoresis unit was used with glass plates (16 by 18 cm), 0.75mm spacers and 1-cm-wide loading wells. A 10-gallon (40 L) aquarium served as the lower buffer chamber. DGGE gels contained a 40 to 70% gradient of urea and formamide (UF) solution increasing in the direction of the electrophoresis (100% UF solution is 40% (vol/vol) formamide plus 7.0 M urea). DGGE was conducted at a constant voltage of 70V at 60° C for 17 h. Gels were stained with syber-green and photographed.

DGGE bands were purified for sequence analysis by gently pressing a disposable 10 µl plastic, filter pipette tip (Molecular Bio-Products, San Diego, CA) into the center of a band and immersing the tip into a series of previously prepared PCR mixtures containing all reagents except template DNA. Band purity was confirmed by analyzing the resulting PCR products on a DGGE gel in comparison to the sample from which the band was obtained. Bidirectional sequencing reactions of DGGE bands were performed using primers 1114f (*E.coli* positions 1099 to 1114) 5' GCAACGAGCGCAACC C 3' and 1392r (described above) and an ABI Prism 310 Genetic Analyzer with a 47 cm capillary (PE Applied Biosystems, Foster City, CA) and Prism BigDyè terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA). Sequencing reactions were prepared in a 20 ul reaction mix composed of the following: 4 ul Terminator Ready Reaction Mix (PE Biosystems), 2 ul 5X buffer (400mM Tris pH=9.0, 10mM $MgCl_2$), 3-10 ng of PCR template (volume determined by template quantification on gel), 3.2 pM of primer in 1ul and nuclease-free water to 20ul final volume. The sequences were aligned using the Sequencher 3.1.1 software program (Gene Codes

Corporation, Ann Arbor, Mich.) and compared to sequences deposited in GenBank by performing a BLAST search (2).

Cloning

For cloning, a PCR product of the expected length was cloned into a vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA.). PCR product generated from DNA extracts of mouse 2 (see figure 4) were ligated into a pCR-TOPO vector in a 5ul reaction using 1ul of PCR product. The reaction was gently mixed and allowed to sit at room temperature for 5 min. Cloning reaction was then transformed into competent cells (provided by manufacurer) by gentle mixing followed by incubation on ice for 30 min. Cells were then heatshocked for 30 sec at 42° C followed by a 2 min incubation on ice. 250ul of SOC medium (with kanamycin) was then added to the cells and they were incubated an additional 1 h at 37° C with shaking. Following incubation, 50ul of cells was used to inoculate LB/kanamycin plates containing X-GAL and incubated overnight at 37° C. From these plates white colonies were selected and cultured in LB medium with 50 ug/ml of kanamycin overnight at 37°C. Plasmid DNA was isolated using a QIAprep Miniprep Kit (Qiagen, Hilden, Germany). This kit employs the use of cell lysis under alkaline conditions as a means of separating plasmid DNA from chromosomal DNA and other cellular components. Cell wall-bound chromosomal DNA coprecipitates with cellular and other insoluble material while the plasmid DNA remains in solution. The plasmid DNA is then neutralized and renatured under high salt conditions. It is then

purified on a silica column. Sequences were analyzed as described above with the following changes: sequencing reactions were performed using only the 1392r primer.

Culturing

For culturing samples, wash samples were immediately added to the swab/scrape sample and approx. 20ul was removed for Gram staining. The sample was then used to inoculate blood agar plates (Columbia blood agar base containing 5% sheep red blood cells), chocolate agar plates (blood agar with lysed red blood cells) or PRAS Thioglycollate medium pH=7 +/- 0.3 (Anaerobe Systems, Morgan Hill, CA.). Plates were then incubated under aerobic or CO_2 (candle jar) conditions at 37° C for 24 to 48 h. The Thioglycollate cultures were incubated under anaerobic conditions for 24 h at which time growth from the broth was used to inoculate blood and chocolate agar plates for an additional 24h of growth under aerobic and anaerobic conditions. If necessary, colony isolates were obtained by transfer to fresh plates and additional overnight incubation. DNA from isolates was extracted, PCR amplified and sequenced as described above.

Hormone Treatment

In order to further observe the effects of hormones and estrous cycling on the bacterial community within the vagina we applied a hormone treatment to induce specific stages of the estrous cycle. These protocols were based on those of Han et. al (21) and

Morrison et.al. (40). Three days prior to sampling, each mouse received a single 0.5 mg subcutaneous (s.c.) injection of estradiol valerate (Sigma) suspended in sterile sesame oil (Sigma) to induce pseudoestrous or a 2.5 mg dose s.c of Depo-provera (medroxy-progesterone) to induce pseudodiestrous. The induction of the respective stages were confirmed by cytology. Mice were sampled and the bacterial community analyzed by DGGE. This protocol was performed in duplicate with 5 total mice in each cage with one untreated mouse per cage.

Antibiotic Treatment

Mice were treated with a panel of antibiotics in order to ablate commensal vaginal bacteria. Mice were injected intraperitoneally (i.p.) twice daily with the following antibacterials suspended (and mixed) in sterile water: Gentamicin, 0.0625 mg per mouse per dose; Vancomycin, 0.375 mg per mouse per dose; Clindamycin, 0.5 mg per mouse per dose. Mice were sampled prior to and immediately following treatment, and at 24 h, 4days and 12 days post-treatment and samples analyzed by DGGE. This protocol was performed in triplicate and included untreated control mice in each cage that were sampled at days 0 and 12.

Genital Tract Infection with *Chlamydia trachomatis*

The infection protocol was based on that of Morrison et. al. (40). Mice were treated with Depo-provera (medroxy-progesterone acetate) three days prior to infection. Mice were infected with 1,500 IFU (100 ID_{50}) of *Chlamydia trachomatis* MoPn in SPG (250mM sucrose, 10mM sodium phosphate, 5mM D-glutamic acid; pH adjusted to 7.2) in 5ul injected into the vaginal vault. Groups were divided and sampled as follows; five infected mice and one mock infected were sampled at day 3, one group at 7 and one at 35 post-infection. Infection was confirmed by swabbing the vaginal vault of 2 control mice at day 7, placing it in 0.5ml SPG and then enumerating IFUs by isolation onto HeLa cell monolayers in a 48 well plate. The swabs were brought up to 1.0 ml in SPG. Sterilized glass beads were added to the samples and they were vortexed vigorously. Samples were then diluted 1:10, 1:100 and 1:1000. The confluent HeLa cell monolayers were treated with 0.5ml of 1x DEAE and incubated for 15 min. Following incubation, wells were washed twice with PBS and 0.3 ml of the mouse sample dilutions were added to the wells in triplicate. The plate was then spun at 2000 RPM, 37° C for 1 hr. The inoculum was removed and replaced with DMEM containing 10 ug/ml cyclohexamide. Cells were incubated at 35° C in a humidified 5% CO_2 incubator for 24 hrs. The media was then aspirated and wells were washed twice with PBS. Cells were then fixed by the addition of 0.5 ml of methanol to the wells followed by a 10 min incubation. Inclusions were visualized by indirect immunofluorescence. MoPn-specific anti-major outer membrane protein (MOMP) Mab MP-33b was added at 1 ug/well in PBS containing 2% BSA and incubated for 1 hr. Plates were washed twice and fluorescein-tagged goat anti-mouse IgG (Sigma) was added at 1:300 dilution in PBS/BSA and incubated for 1 hr.

In addition, mice from the day 35 sample group were bled and their serum antibody responses were assayed by enzyme-linked immunosorbent assay (ELISA). Wells were coated overnight 10 ug/ml of formalin-fixed *Chlamydia trachomatis* MoPn elementary bodies in 100 ul of Tris buffer (50mM Tris, 150mM NaCl, pH=7.5). Wells were washed three times with wash buffer (12mM Tris HCL, 0.5% Tween-20) and blocked with 200 ul blocking buffer (12mM Tris HCL, 0.2% BSA) for 1 hr 15 min at 35° C. Wells were washed three times and mouse sera was added at 1:50 and 1:250 dilutions in 100 ul blocking buffer before incubating the plate an additional 1 hr 15 min at 35° C. Wells were washed five times and the secondary antibody, alkaline-phosphatase conjugated goat anti-mouse IgG2A (Southern Biotech Associates, Inc., Birmingham, AL.), was diluted 1:250 in blocking buffer and 100 ul was added to each well. The plate was incubated 1 hr 15 min at 35° C. Wells were again subjected to a five time wash. Wells were developed by adding 100 ul of p-nitrophenyl phostate in Sigma-fast Tris buffer (Sigma). The plate was incubated at room temperature for 30 min at which time the reaction was terminated using 50 ul of 5N NaOH. Absorbance was read at 405nm using a BioRad Model 550 plate reader.

RESULTS

Bacterial Community in Naturally Cycling Mice

Five to six week old female BALB/c mice were placed in Whitten cages for two weeks in order to induce natural, synchronized estrous cycling through the presence of male pheremones. Following synchronization of the cycles, mice were sacrificed at staggered time points in order to achieve a panel of samples representative of the four major stages of the estrous cycle. Mice were sampled by a series of vaginal washes followed by a swab and scrape of the vaginal lumen. Cytology of the vaginal wash was employed in order to determine the stage of estrous (Figure 1). All stages of the estrous cycle were represented in both cages used in the experiment. DNA was then extracted from the samples. Bacterial DNA was PCR amplified with primers targeted to highly conserved regions of the 16S rDNA gene. PCR products were then run on a denaturing gradient gel (DGGE) (Figure 2).

The DGGE profile revealed eight distinct bands (Figure 2); a doublet at the top (bands 1 and 2) followed by a series of three bands (bands 4, 5 and 6), one band below this (bands 7) and, finally, another doublet at the bottom of the gradient (bands 8 and 9). The identity of individual bands was determined (Table 1). The bacteria represented included: *Pasteurella pneumotropica*, a *Psuedomonas* species, *Nocardioides simplex* and a species most closely related to a *Phyllobacterium* species. DGGE banding patterns or