

THE EFFECTS OF THE INTESTINAL ENVIRONMENT ON *SALMONELLA*
PATHOGENESIS AND MOLECULAR IDENTIFICATION OF MICROBES IN
THE CLINICAL LABORATORY

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Salmonella causes disease ranging from self-limiting enteritis to septicemia. To cause disease it must first invade the intestinal epithelium. To invade, it employs a type three secretion system and effector proteins encoded on *Salmonella* Pathogenicity Island 1 (SPI 1). In this work, we investigated the effects of the intestinal environment on *Salmonella* pathogenesis using an *in vivo* mouse model of infection as well as *in vitro* genetic studies.

In our *in vivo* studies, we characterized the intestinal environment in control, streptomycin-treated, *Salmonella* infected, and streptomycin-treated, *Salmonella* infected mice. Using 16S rDNA clone libraries we found that the microbiota in the ileum is different from that of the cecum and that streptomycin treatment alters the microbiota in both the ileum and cecum. Upon histopathological examination of the ileum, we also found that pretreatment with streptomycin prior to infection increased *Salmonella* pathology. We also defined the short chain fatty acids present in both the ileum and cecum using GC-MS and HPLC analysis and found that streptomycin treatment significantly decreased the fatty acid concentrations in the cecum and that this change correlated with an increase in pathology in the cecum.

Previous *in vitro* studies in our lab have shown that the short chain fatty acids propionate and butyrate repress *Salmonella* SPI 1 invasion genes. In this work, we further confirmed the effects of propionate and butyrate using concentrations of the fatty acids comparable to what we found in the cecum of the mouse. We also found that metabolism of propionate is necessary for the repressive effect on invasion genes and that the metabolic intermediate, propionyl-CoA, is important for this effect.

The molecular techniques used to define the microbiota of the intestinal environment are also applicable to the clinical laboratory setting. Use of ribosomal genes for species-level identification has shown promise for both bacteria and yeast. Therefore, we examined the usefulness of these techniques for yeast identification in the clinical veterinary laboratory and found that sequence analysis of the D1/D2 region of the yeast large ribosomal subunit is an effective method of identification.

BIOGRAPHICAL SKETCH

Cherilyn Garner graduated with a BA in Biology from Rhodes College in Memphis, TN. After finishing her undergraduate education, she spent a year in England and studied at Queen Mary and Westfield College in London. During this time, she developed an interest in Medical Microbiology. Upon her return to the United States, she obtained her post-baccalaureate certification in Clinical Laboratory Science from Tarleton State University in Fort Worth, TX. She then worked for 3 years in the Clinical Microbiology Laboratory before entering graduate school. Her interest in bacterial pathogenesis led her to Dr. Craig Altier's lab at North Carolina State University in Raleigh, NC where she began her doctoral studies. One year into her studies, Dr. Craig Altier accepted a position at Cornell University, so she then transferred to Cornell and finished her doctoral studies there.

For Lee and Roxie

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CHAPTER 1
INTRODUCTION

***Salmonella* significance for public health**

Salmonella enterica is a Gram negative bacterium that is a major cause of food-borne illness. It can cause disease with varying levels of severity causing a range of illnesses from self-limiting intestinal disease to septicemia. The majority of cases are resolved without treatment; however, more severe cases can occur, most commonly in infants, elderly populations, or people who are immunocompromised. Because most cases are resolved without treatment, it is difficult to quantify the number of cases that occur each year; however, the Centers for Disease Control and Prevention (CDC) estimate an incidence of 1.4 million cases per year resulting in approximately 400 deaths (1). *Salmonella* transmission usually occurs by eating contaminated food, and it is difficult to control transmission because it is commonly found in food animals such as chickens, cows, and pigs, along with contaminated produce.

It has now been determined that there are only two species of *Salmonella*, and *Salmonella enterica* is the more important of these, causing 99% of infections in humans and warm-blooded animals (13). However, in the species *enterica* there are over 2,400 named serotypes (13). Of these over 2,400 serotypes, six account for 61% of human infections, with serovars Typhimurium and Enteritidis accounting for almost 40% (15). Furthermore, of the enteric pathogens under surveillance in 2005, *Salmonella* had the highest incidence with an overall incidence of 14.55 per 100,000 people (15). Therefore, *Salmonella enterica* serovar Typhimurium (henceforth referred to as *Salmonella* Typhimurium) is a significant human pathogen.

***Salmonella* invasion of the intestinal tract**

In order for *Salmonella* to cause disease, it must first penetrate the intestinal epithelium. For this invasion process, *Salmonella* uses genes found in *Salmonella* pathogenicity island 1 (SPI 1), an island of genes located at centisome 63 in *S. Typhimurium* (11, 32, 34, 41, 52, 58, 64). These genes encode structural components of a type three secretion system and its effector proteins. The type three secretion system apparatus is encoded by the *inv/spa* operon and the *prg/org* operon, whereas the secreted effectors within the island are encoded in the *sic/sip* operon (reviewed by 2). The process of invasion involves this TTSS forming a “needle complex” (44) that delivers these effector proteins into the host cell cytoplasm, causing a change in the cytoskeleton of the host cell, leading to engulfment of the bacterium (reviewed by 31). It has been demonstrated that SPI 1 genes are required for both intestinal disease and systemic disease in mice (10, 32, 41, 73).

SPI 1 gene regulation

SPI 1 genes are controlled by a complex regulatory network of transcriptional and post-transcriptional regulators. Many of these regulators are located within SPI 1, however, it is thought that SPI 1 was obtained by horizontal transfer at some point in evolution (2, 31), so after its acquisition it came under the control of regulators outside the island as well. Regulators within the island are HilA, HilC, HilD, and InvF, with HilA characterized as the central regulator. HilA, a member of the OmpR/ToxR family, is a transcriptional activator that regulates transcription of the following genes in SPI 1: the *inv/spa* operons, the *prg/org* operons, and the *sic/sip* operon via the read-through transcript of the *inv/spa* operons (7, 20, 55). InvF is also a

transcriptional regulator of the AraC family (43). HilA is responsible for controlling gene expression of the TTSS apparatus, InvF is responsible for controlling secreted effectors outside of SPI 1, and together they control secreted effectors within SPI 1 (20, 26). InvF can be regulated by mechanisms independent of HilA, as well (3, 4, 62). HilA is controlled by HilC and HilD from within the island and RtsA and HilE outside SPI 1(27). Also, a recent study has shown that by an unknown mechanism, HilA can negatively regulate itself (22).

Several genes encoded outside of SPI 1 have been identified as important for control of HilA and InvF. Some examples include PhoP/PhoQ, BarA/SirA, and the Csr system. PhoP/PhoQ has been shown to negatively regulate invasion genes through HilA (8). This negative regulation is induced by low magnesium concentrations (68). The csr system both positively and negatively regulates SPI 1 invasion gene expression through changes in the level of CsrA (3). In *Salmonella* it consists of a small protein CsrA, and two untranslated RNAs, CsrB and CsrC, that control the level of CsrA (3, 29). The BarA/SirA regulatory system positively regulates invasion gene expression. BarA is a sensor kinase of the phosphorelay type, and SirA is its response regulator (4, 70). BarA/SirA induce invasion gene expression through their effect on expression of CsrB and CsrC (29, 49). It is thought that BarA/SirA induce expression of CsrB and CsrC, decreasing the levels of free CsrA, and inducing invasion gene expression (2).

Environmental control of invasion genes

Environmental factors mediate invasion gene expression through regulation of *hilA* expression (8). The conditions that positively regulate SPI 1

gene expression are low oxygen, log phase growth, high osmolarity, changes in DNA supercoiling, and slightly alkaline pH (8, 28, 33, 51, 65). Results from our lab also show that short chain fatty acid concentrations and pH are important for invasion gene expression. Acetate and formate induce invasion gene expression, whereas butyrate and propionate repress invasion gene expression and these short chain fatty acids are more effective at a pH of 6.7 rather than 8.0 (36, 49).

The intestinal environment

Studies have been done to better understand the intestinal environment, and many of the environmental conditions listed above can be found in the intestinal tract. There exists an axial oxygen gradient in the gut, with the lumen of the small intestine and colon being anaerobic, whereas the mucosal surface is microaerobic (21). The osmolality of the small intestine in humans is 250-425 mOsmol/kg depending on the location within the small intestine and the state of digestion (40, 46). Additionally, in various mammalian species, studies show that acetate, propionate, and butyrate are found in both the cecum and the colon, and that acetate, propionate, and formate have been found in the ileum (5, 12, 19, 47, 48, 57). Also, the pH throughout the distal ileum, cecum, and proximal colon is between 6.0 and 7.0 (6, 12). As there are differences in the fatty acids concentrations in the ileum and the large intestine, it seems likely that these conditions in the intestinal tract are signals for *Salmonella*, indicating a location for productive invasion. The conditions in the ileum, the area previously described as the location for invasion (14, 42), are conditions known to induce invasion genes (36, 49),

whereas the conditions of the cecum and colon may be more likely to repress invasion genes (49).

Microbiota of the intestinal tract

To understand the intestinal environment, it is important to consider the microbiota present there, as well. There are 10^{12} microbes/gram of contents in the distal intestinal tract, and it is estimated that there are 500-1,000 different species present (23, 75). However, of these species present, the majority belong to two phyla, either the Firmicutes or Bacteroidetes, and this is true for both mice and humans (25, 53). Understanding the components of the microbiota present is important, because protection from enteric pathogens has been attributed to the intestinal microbiota and termed “colonization resistance” (72). Also, the short chain fatty acids present in the intestinal tract are there because of bacterial fermentation of undigested carbohydrates such as pectins, cellulose, and hemicelluloses (19). Therefore, it is important to characterize the intestinal environment to better understand how the microbiota and their metabolic products interact with enteric pathogens, in particular *Salmonella*. Many studies have been performed to characterize the microbiota present in the large intestine (the cecum, colon, and feces) of both mice and humans (24, 25, 37, 45, 53, 67, 69, 71). In contrast with the wealth of information now available regarding the composition of the large intestinal microbiota, relatively little is known about that of the mouse small intestine. A comprehensive survey of the microbiota in the human small intestine has been completed with predominating groups consisting of the Firmicutes (Lachnospiraceae and Bacillus) and the Bacteroidetes (30). However, studies in mice have been more limited in their scope (9, 38, 63).

16S rDNA analysis

The majority of the studies characterizing the intestinal microbiota employed 16S rDNA analysis of genomic DNA isolated from the tissue or contents in this region of the intestinal tract. 16S rDNA analysis is useful because the 16S rDNA gene in bacteria has highly conserved regions with hypervariable regions in between (56, 66). The regions of high conservation among bacterial species allow for design of universal primers that will amplify the majority of bacterial species, whereas the hypervariable regions in between allow for identification of bacteria to the genus or species level (66). Therefore, 16S rDNA analysis provides a unique tool for characterization of bacterial populations.

16S rDNA sequence analysis in the clinical microbiology laboratory

16S rDNA sequence analysis can also be employed for bacterial identification in a clinical microbiology setting. Traditionally phenotypic methods have been utilized for bacterial identification in the clinical laboratory, however, recent studies of 16S rDNA analysis have shown success with implementation of 16S rDNA sequence analysis for identification of bacteria in the clinical laboratory setting (reviewed by 74). The method involves isolating bacterial DNA from a bacterial colony or a clinical sample and then amplifying the 16S rRNA gene using PCR. The PCR product is then sequenced and the sequence is identified using one of the various public or commercial sequence databases. This method is particularly advantageous for identification of unusual isolates, isolates with limited biochemical reactivity, and for isolates that do not grow well under *in vitro* conditions.

Sequence analysis of yeast in the clinical laboratory

In addition to molecular identification of bacteria using ribosomal genes, there has also been consideration of molecular identification of yeast using ribosomal genes. In yeast, the ribosomal RNA genes consist of the adjacent small subunit (18S rDNA), the internal transcribed spacer 1 (ITS1), the 5.8S, the internal transcribed spacer 2 (ITS2), and the large subunit, the last of which includes the D1/D2 region at its 5' end (reviewed by 59, and 60). For yeast identification, multiple ribosomal target genes have been considered, and of these regions, the ITS1, ITS2, and the D1/D2 region have shown to be the most useful for species-level identification, as a result of the variability within these regions (reviewed by 39, and 60). In fact, sequence analysis of these regions has shown great promise in the practice of clinical mycology (16-18, 35, 50, 54, 61). As with bacterial identification, this method provides advantages for identification of slow-growing, biochemically unreactive, and unusual yeast isolates.

Summary of results

In this work, we used an *in vivo* mouse model and *in vitro* molecular genetic techniques to examine the effects of the intestinal environment on *Salmonella* invasion. In chapter 2, we describe the intestinal environment of the mouse using 16S rDNA molecular techniques to define the microbiota present and HPLC or GC-MS to determine the short chain fatty acids present. We defined the normal conditions in the ileum and cecum and also determined the changes that occurred upon treatment with streptomycin, infection with *Salmonella*, or upon treatment with streptomycin prior to *Salmonella* infection. Also, we examined the ileum and cecum to determine any histopathological

changes in these locations as a result of the streptomycin treatment or *Salmonella* infection. We found that the microbiota found in the ileum is different from that of the cecum. We also found that streptomycin treatment altered the microbial populations in both locations and significantly decreased the short chain fatty acid concentrations in the cecum. Additionally, pretreatment with streptomycin prior to *Salmonella* infection increased the pathology observed in the ileum. The study in Chapter 3 examined the effects of propionate and butyrate on *Salmonella* invasion genes *in vitro*. In particular, we confirmed that propionate and butyrate repress invasion genes at concentrations comparable to that observed in the cecum of the mouse. We also found that metabolization of propionate is necessary for this effect and that production of the metabolic intermediate propionyl-CoA is important for this effect.

We also examined the effectiveness of using molecular techniques for microbial identification in the clinical veterinary laboratory setting. In particular, in Chapter 4 we investigated the usefulness of sequence analysis of the D1/D2 region of the large ribosomal subunit for yeast identification and found that it was an effective method of identifying yeast in the clinical veterinary laboratory.

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CHAPTER 2

Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a *Salmonella enterica* serovar Typhimurium murine model of infection*

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ABSTRACT

The small intestine is an important site of infection for many enteric bacterial pathogens, and murine models, including the streptomycin-treated mouse model of infection, are frequently used to study these infections. The environment of the mouse small intestine and the microbiota with which enteric pathogens are likely to interact, however, has not been well described. Therefore, we compared the microbiota and the concentrations of short chain fatty acids (SCFAs) present in the ileum and cecum of streptomycin-treated mice and untreated controls. We found that the microbiota in the ileum of untreated mice differed greatly from that of the cecum of the same mice, primarily among families of the phylum Firmicutes. Upon treatment with streptomycin, substantial changes in the microbial composition occurred, with a marked loss of population complexity. Characterization of the metabolic products of the microbiota, the SCFAs, showed that formate was present in the ileum but low or not detectable in the cecum while butyrate was present in the cecum but not the ileum. Treatment with streptomycin altered the SCFAs in the cecum, significantly decreasing the concentration of acetate, propionate, and butyrate. In this work, we also characterized the pathology of *Salmonella* infection in the ileum. Infection of streptomycin-treated mice with *Salmonella* was characterized by a significant increase in the relative and absolute levels of the pathogen and was associated with more severe ileal inflammation and pathology. Together these results provide a better understanding of the ileal environment in the mouse and the changes that occur upon streptomycin treatment.

INTRODUCTION

The small intestine serves as the site of colonization and attachment and the seat of pathogenesis for a number of important enteric bacterial pathogens of man and animals. Among the bacteria that cause diarrheal disease in the small bowel are *Vibrio cholerae*, which colonizes the small intestine and secretes toxins (64), and pathogenic forms of *Escherichia coli* including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC), all of which either adhere to or affect enterocytes in the small intestine (reviewed in 42). *Yersinia enterocolitica* and *Salmonella* have also both been shown to preferentially invade the ileum by targeting the Peyer's patches (9, 25). All of these species must thus survive within this region of the intestinal tract in competition with the resident microbiota, and must therefore express determinants necessary for virulence. Additionally, the small intestine is an important site for lesions associated with inflammatory bowel disease (IBD). Although the causes of IBD are complex and multiple, the microbiota of the small intestine is thought to be important to disease development. It has been alternatively theorized that IBD stems from an alteration in the host microbiota present, deficiencies in the host's response to and control of the microbiota, or changes in the function of a particular member of the microbiota (reviewed by 65, 72), the last of which supported by recent findings suggesting a connection between Crohn's disease and strains of *Escherichia coli* termed adherent and invasive *E. coli* (AIEC) (5, 19).

The expression of virulence determinants by bacterial pathogens often occurs in response to specific environmental cues. For enteric pathogens including EPEC, ETEC, DAEC, and *Y. enterocolitica*, a temperature near 37°C is important for virulence (13, 24, 28, 83). For EPEC, physiological

osmolarity, near neutral pH, and quorum sensing have all been implicated in pathogenesis (13, 92). Toxin production and hence virulence in ETEC is regulated by osmolarity and microaerophilic conditions (83) while Dr fimbriae production in DAEC is controlled by anaerobic conditions (24). Invasion by *Y. enterocolitica* is controlled by regulation of the expression of the invasin protein by an acidic pH (28). For *Salmonella*, low oxygen, log phase growth, high osmolarity, and slightly alkaline pH have been shown to positively affect expression of genes necessary for *Salmonella* invasion *in vitro* (2, 29, 31, 53, 73). Additionally, our previous work has shown that short chain fatty acid (SCFA) concentrations and pH are important for invasion gene expression. The SCFAs acetate and formate induce invasion gene expression, while butyrate and propionate repress these same genes at pH levels comparable to those of the mammalian intestinal tract (38, 52). Furthermore, previous *in vivo* studies have shown an association between decreased short chain fatty acids and susceptibility to *Salmonella* infection in a cecectomized mouse model (87).

Examination of the intestinal environment reveals that enteric pathogens are tuned to respond to cues naturally present in the gut. Host temperature is maintained close to 37°C. There exists an axial oxygen gradient in the gut, with the lumen of the small intestine and colon being anaerobic, whereas the mucosal surface is microaerobic (21). The osmolality of the small intestine in humans is 250-425 mOsmol/kg depending on the location within the small intestine and the state of digestion (41, 49). In various mammalian species, studies have shown that the SCFAs acetate and propionate are found in both the large and small intestine, while butyrate is present primarily in the cecum and colon and formate in the ileum (1, 6, 7, 18,

50, 51, 61, 67). Additionally, it is thought that the resident microbiota present in the intestinal tract produce quorum sensing molecules that may be important for interspecies communication (reviewed by 62).

Much of the environment of the intestinal tract is defined by the metabolic processes of the bacterial populations that reside within. There have been several recent comprehensive studies that reveal the large intestine (the cecum, colon, and feces) of mice and humans to be inhabited by a diverse population of bacteria (23, 27, 39, 47, 55, 76, 80, 84). There are 10^{12} microbes/gram of contents in the distal intestinal tract, and it is estimated that there are 500-1,000 different species present (22, 90). Of these species, the majority belong to two phyla, the Firmicutes and Bacteroidetes, this being true for both mice and humans (27, 55). Because the intestinal microbiota plays a critical role in protecting the host against enteric bacterial pathogens or “colonization resistance” (85), increased understanding of the composition of the intestinal microbiota is essential to defining bacterial pathogenesis. In contrast with the wealth of information now available regarding the composition of the large intestinal microbiota, relatively little is known about that of the mouse small intestine. A comprehensive survey of the microbiota in the human small intestine has been completed with predominating groups consisting of the Firmicutes (Lachnospiraceae and Bacillus) and the Bacteroidetes (30). However, studies in mice consist of a limited survey of the microbiota of the ileum (69), examination of bacteria present at the phylum level (40), and quantification of particular bacterial groups believed to be common inhabitants of the ileum (3).

The streptomycin-treated mouse model has proven to be an effective method to study enteric disease and has been used to study pathogens such

as *Vibrio cholerae*, enterohemorrhagic *E. coli* (EHEC), and *Salmonella* (4, 60, 64, 88). It is specifically useful for the study of *Salmonella* as untreated mice develop systemic salmonellosis without an enteric component, but pretreatment with oral streptomycin produces enterocolitis (4, 70). In this model of infection, the primary pathology occurs in the cecum, and studies have shown that changes in the cecal microbiota occur when mice are pretreated with streptomycin (4, 80). However, the effects of streptomycin treatment on the ileum have not been well characterized.

As the environment of the small intestine is poorly characterized in animals used as models of enteric infection, we have in this study characterized the bacterial populations, fatty acid composition, and histopathological changes of this region in untreated and streptomycin-treated mice, both uninfected and infected with *Salmonella enterica* serovar Typhimurium. We hypothesized that a unique bacterial population within the ileum, and the environment created by the population, would be important for the protection of the host against invading pathogens. We show here that there exists a stable bacterial community within the ileum that is different from that of the cecum, and that the microbial population associated with the ileal mucosa is different from that of the luminal contents. Additionally, we find that streptomycin treatment alters the microbial populations associated with both the ileum and cecum by decreasing species richness and changing the distribution of phylotypes present. We also find that streptomycin pretreatment allows *Salmonella* to overcome colonization resistance, increasing by two logs the number of *Salmonella* present at this intestinal site in treated animals, and thus alters the pathologic response of the ileum to *Salmonella* infection.

MATERIALS AND METHODS

Mouse experiments. For infection experiments, a spontaneous streptomycin-resistant isolate of *Salmonella enterica* serovar Typhimurium strain ATCC 14028s was used. Twenty-five milliliters of culture was grown overnight with aeration at 37°C in MOPS minimal media with 0.5% glucose (63). The bacteria were then pelleted, washed three times with PBS, and resuspended in 1 ml of PBS. Fifty microliters was then used to infect each mouse, equaling a dose of $\sim 10^9$ *Salmonella* per mouse. All mouse experiments were approved by the Institutional Care and Use Committee at Cornell University. Seven-week-old female C57BL/6 mice were obtained from Charles River Laboratories for all experiments and housed in a containment facility for pathogens. Mice were housed three to a cage and fed a standard laboratory diet. Mice were divided into two groups of twelve, with one group inoculated orally with 30 μ l of sterile water and the other with 30 μ l of sterile streptomycin sulfate solution (20 mg per mouse). Twenty-four hours later, six mice in each group of twelve were inoculated orally with 50 μ l of sterile PBS, and the remaining six from each group received 50 μ l of *Salmonella* at a total dosage of $\sim 10^9$ cfu. Forty-eight hours after infection with *Salmonella* mice were euthanized and tissues were collected from the ileum and the cecum of each mouse.

DNA isolation. Samples from the distal ileum and cecum containing both tissue and contents were collected and placed in bead tubes (MO BIO Laboratories, Inc., Carlsbad, CA), whereas samples of intestinal contents were carefully expressed into microcentrifuge tubes without scraping the mucosa. The mass was determined and the samples were then flash frozen with liquid nitrogen. Samples were stored at -80°C until they were processed. Total

DNA was isolated from samples using a modified version of the Qiagen DNeasy Blood and Tissue Kit (Qiagen). Briefly, 360 μ l of Buffer ATL was added to samples in their bead tubes and then homogenized for one minute using a Mini-Bead-Beater (BioSpec Products, Inc., Bartlesville, OK), a method of mechanical disruption that has been shown effective for the isolation of bacterial DNA from fecal samples (56). Then 40 μ l of proteinase K was added and samples were vortexed. Samples were then processed as described in the manufacturer's protocol with one change: prior to the addition of 200 μ l of 100% ethanol the samples were incubated at 70°C for 30 minutes. Samples of intestinal contents were processed similarly except that bead tubes were not used.

16S rRNA gene clone library construction and analysis. Clone libraries of 16S rRNA-encoding genes were constructed as previously described (91). Briefly, primers 8F and 1492R were used to amplify the 16S rRNA-encoding genes from the DNA samples (75). For cecal samples, 20 cycles were used for PCR, whereas 24 cycles were used for ileal samples due to the lower concentration of bacterial DNA present in the ileum. The increase in cycles allowed for amplification from all but 3 ileal samples among the various treatment groups. For the samples that did not amplify, there appeared to be a lower concentration of bacterial DNA leading to mispriming with mouse DNA, which interfered with clone library construction. Purified PCR products were then ligated into a T-tailed cloning vector (pCR 4-TOPO; Invitrogen) and used to transform competent cells. Ninety-four clones per library were sequenced by the Cornell University Sequencing Facility using the 8F primer. Sequences were then uploaded to the Ribosomal Database Project (RDP, <http://rdp.cme.msu.edu>) and the pipeline tool available via myRDP was used

for quality analysis and alignment (14). The RDP classifier (89), using the default 80% confidence threshold setting, and SeqMatch (14) were then used for taxonomic classification of the aligned sequences. Additionally, distance matrices were generated from the RDP-based sequence alignments and analyzed using the DOTUR program (74). Using a level of 97% sequence identity, DOTUR was used to assign sequences to OTUs, generate rarefaction curves, and calculate diversity indices. To determine the Bray-Curtis measure of β -diversity, the program EstimateS was utilized (15). All sequences generated in this study have been submitted to Genbank with accession numbers FJ834458-FJ838647.

Quantitative real-time PCR. The same DNA used for 16S rRNA gene clone library construction was used for quantitative real-time PCR. The 16S rRNA-encoding gene was used as the target to measure total bacteria and total *Salmonella* present in each sample as previously described (3), except for the bacterial strains used as assay standards. For assays of total bacteria, we used *E. coli* MG1655 as the standard, and for total *Salmonella* we used *Salmonella enterica* serovar Typhimurium ATCC 14028s. Standard curves were constructed using these strains to quantify the total number of 16S rRNA gene copies per sample, with R^2 values of ≥ 0.995 extending to 1,000 copies of 16S rRNA-encoding genes for total bacteria and 1 copy for *Salmonella*, defining the lower limits of the test. For each sample, copies of 16S rRNA-encoding genes per gram of tissue were calculated using the mass of the original sample.

Short chain fatty acid analysis and pH determination. Gas chromatography-mass spectrometry (GC-MS) was used to quantify short chain fatty acids (SCFAs) in the cecum. Intestinal contents from the cecum

were collected in 1% acidified water. The mass of samples was determined, and they were then flash frozen in liquid nitrogen. Samples were processed using a modified version of a previously published protocol (66). Samples were thawed at room temperature, vortexed for one minute, and then centrifuged. The supernatant was removed and placed in a 4 ml glass vial (National Scientific, Rockwood, TN) containing 50 μ l each of 20 mM stock isotopes, [$^2\text{H}_3$] – and [$1\text{-}^{13}\text{C}$]acetate (Cambridge Isotope Laboratories, Inc., Andover, MA), [$^2\text{H}_5$]propionate (Cambridge Isotope Laboratories, Inc., Andover, MA), and [$^{13}\text{C}_4$]butyrate (Sigma-Aldrich), which were used as internal standards for each sample. Samples were acidified with 10 μ l HCl and then extracted with 1 ml of diethyl ether four times. One ml of sample was placed in a separate tube containing 2.5 μ l of the derivatization reagent 1-tert-butyl-dimethyl-silyl-imidazole (Sigma-Aldrich) and heated at 60°C for 30 minutes. The derivatization step was performed on duplicate aliquots for each sample. Samples were then transferred to autosampler tubes, and analyses were performed on a Jeol GCMate II MS with an Agilent 6890N GC inlet equipped with a J&W Scientific DB-5MS column (30 m x 0.250 mm, 0.25 micron film thickness). A split injector was used with 250°C injector temperature and 50:1 split ratio. The initial oven temperature was 60°C held for 1 minute followed by a 5°C/min ramp to 120°C and a 25°C/min ramp to 270°C final temperature, which was held for 1 minute. Total run time was 20 minutes including a 3.5 min solvent delay. The derivatized acids were detected using unit-mass resolution selected ion monitoring (SIM) with magnetic field switching at 0.21 sec cycle time on "flat-top" mass peaks with fully open collector slit: formate (R.T. 3.98, SIM 3-4.5 min, m/z 75 and 103), acetate (R.T. 4.91/4.95, SIM 4.5-6.4 min, m/z 75, 117, and 121), propionate (R.T. 6.82/6.9,

SIM 6.4-7.5, m/z 75, 131, and 136), and butyrate (R.T. 9.07, SIM 7.5-20 min, m/z 75, 145, and 149). The timing and ions for the SIM program were selected based on 35-500 m/z full scans on standard samples. The isotopic internal standards were then used for quantification of the SCFAs in each sample. As a result of limitations with the protocol and the small quantity of intestinal contents in the small intestine of mice, we used HPLC to quantify all SCFAs in the ileum and formate in the cecum. HPLC and pH determinations were performed as previously described (38) except that a micro-combination pH probe (Microelectrodes, Inc., Bedford, NH) was used. For both GC-MS and HPLC analyses, sample preparation included steps to remove bacteria without lysis, and so SCFA concentrations reflect those of the intestinal milieu.

Tissue collection and histology. The distal ileum (~4.0 cm) and cecum were harvested and fixed in 10% neutral buffered formalin, embedded in paraffin, and the entire length was sectioned at 5 μ m thickness and stained with haematoxylin and eosin (H&E) for histopathological assessment. Sections of ileum were scored on a scale of 0 to 4 for the presence and distribution of polymorphonuclear leukocytes in five regions: (i) within the lamina propria of the intestinal mucosa, (ii) inside the intestinal crypts (cryptitis), (iii) at the periphery of Peyer's patches, (iv) within the interfollicular regions of Peyer's patches, and (v) within the subepithelial dome areas of Peyer's patches (0, none; 1, rare; 2, few scattered; 3, many groups; 4, large numbers) by a board-certified blinded veterinary pathologist. Ileitis severity was calculated as a sum of the scores for the five categorical parameters (maximum of 20). The scoring method is a modification of a method used previously (58), and histological designations for each scoring region are widely accepted and consistent with previously published work (10).

Statistical analysis. Analysis of short chain fatty acid concentrations was completed using the Wilcoxon rank-sum test. For qPCR and pH, the Kruskal-Wallis test was used to determine whether there was a significant difference among any of the groups, and then the Wilcoxon rank-sum test using a Bonferroni correction was utilized to determine significance between individual groups. Similarly, for the pathology scores, the Kruskal-Wallis test and then Dunn's post-test were performed. A p-value <0.05 was considered significant. Statistical analysis was performed using Jmp 7.0 software (SAS, Cary, NC) or GraphPad Prism version 5 (GraphPad Software, La Jolla, CA).

RESULTS

The mouse ileum harbors a defined microbial population. To increase our understanding of the mouse model of enteric infection and the potential interaction of pathogens with both the host and the resident microbiota in the small intestine, we first characterized the bacteria of the most distal segment, the ileum, using conventional mice and a culture-independent method. Ileal and cecal samples, including both tissue and contents, were taken from C57BL/6 mice, and 16S rRNA gene clone libraries were created from these samples. We analyzed a total of 536 partial rRNA-encoding gene sequences from the ileum and 533 from the cecum. The clone libraries of the cecum were examined to provide a point of reference for our characterization of the ileum, since much work has previously been done to characterize the microbiota of the cecum (47, 55, 80, 84). From these sequences, we determined that there exists a bacterial community within the ileum that consists primarily of the phyla Firmicutes (82%) and Bacteroidetes (16%). At the taxonomic level of family, the most predominant Firmicutes were of the

families Clostridiaceae (34%), Lactobacillaceae (24%), and Lachnospiraceae (17%) (Fig. 2.1A). Of the Clostridiaceae, the most predominant clones (32% of the total bacterial population) had high sequence identity with a bacterial group termed the segmented filamentous bacteria (SFB). The SFB have previously been described as unculturable bacteria that are closely associated with the ileal epithelium (20). Previous phylogenetic analysis has also shown the SFB to be most closely related to the genus *Clostridium*, a member of the family Clostridiaceae (78). Thus, SFB constituted a substantial portion of the ileal microbiota. In contrast to the bacterial composition of the ileum, the predominant family of the Firmicutes in the cecum was the Lachnospiraceae (52%), a member of the order Clostridiales, while merely 1% of the cecal microbiota consisted of the Lactobacillaceae and less than 1% of the Clostridiaceae (Fig. 2.1A). These differences between the bacterial composition of the ileum and that of the cecum at the family level suggest that there is a defined microbial community in the ileum that differs greatly from that found in the cecum.

Rarefaction analysis was next used both to identify differences in richness among the microbial populations of the cecum and ileum and to assess the efficacy of our sampling technique in obtaining representative samples from both of these sites (34, 36). This analysis revealed that the ileum had lower overall species richness compared to that of the cecum, with observed operational taxonomic units (OTUs) of 67 and 156, respectively (Table 2.1 and Fig. 2.2A). The analysis, however, also suggested an under-representation of the number of OTUs present at both sites, as shown by the positive slope of the curves in Fig. 2.2A and the Chao1 analysis of estimated richness (11, 12) (Table 2.1). This was nevertheless not surprising, as much

Figure 2.1. The microbial composition of the ileum. 16S rRNA gene clone libraries were created from the ileum and cecum of C57BL/6 mice. (A) Taxonomic analysis of the clone library sequences was performed using RDP Classifier and SeqMatch. Assignment to the phylum level for the sequences from the ileum and cecum of untreated mice is displayed with further distinction among the phylum Firmicutes to the family level. The total of the Firmicutes is shown by the arc extending around the pie chart. (B) Cluster analysis of the Bray-Curtis distance measure of diversity between the microbial communities for the ileum and cecum from untreated mice. For the Bray-Curtis distance measure an operational taxonomic unit (OTU) was defined as 97% sequence similarity. (C) 16S rRNA gene clone libraries were created from samples containing ileal tissue and contents or contents alone from untreated mice. Sequences were assigned to OTUs using a definition of 97% sequence similarity. A heatmap is used to show the relative abundance of OTUs, with specific OTUs detected in the sample oriented along the horizontal axis, and the dendrogram showing the distribution of OTUs. Darker coloring within the heatmap indicates greater representation of specific OTUs. Phyla are shown above the figure and highly represented OTUs are shown below. Numbers 1-6 and 16P-18P represent individual mice.

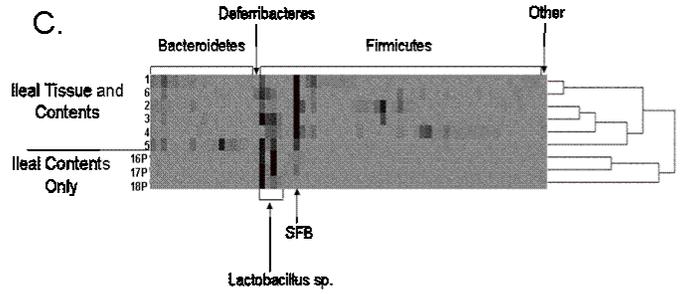
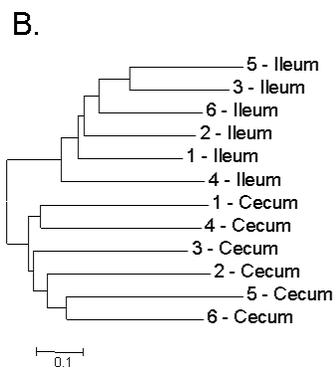
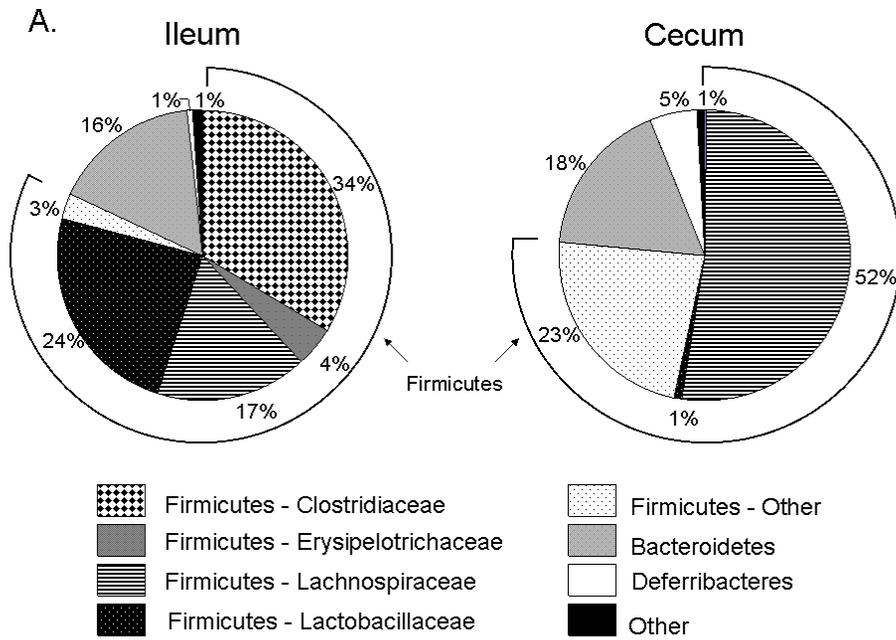


Table 2.1. Observed and estimated operational taxonomic units (OTUs)

Sample	Number of OTUs	Chao1 Richness Estimator (95% Confidence Interval)
Ileum	67	152 (101-282)
Ileal Contents	9	12 (9-32)
Ileum, Streptomycin-treated	49	62 (53-93)
Cecum	156	227 (194-288)
Cecum, Streptomycin-treated	97	138 (115-189)

is still unknown about the complete microbial richness of the intestinal tract. In addition to taxonomic analysis and determination of species richness, we next examined the diversity of the ileal and cecal samples using the Bray Curtis distance measure of diversity (57). Cluster analysis of the Bray Curtis distance using an OTU definition of 97% sequence similarity showed that the ileal samples clustered with each other, but separately from samples derived from the cecum (Fig. 2.1B). This analysis demonstrated that there were differences in the distribution of microbial OTUs in the ileal and cecal samples, and that the ileal sample taken from each mouse was more similar to other ileal samples than it was to the cecal sample taken from the same mouse. Together these analyses show that there is a unique microbial population associated with the ileum that is different from that of the cecum.

Previous work in humans has shown that the microbial population associated with the feces is different from that of the colonic tissue (27). Thus,

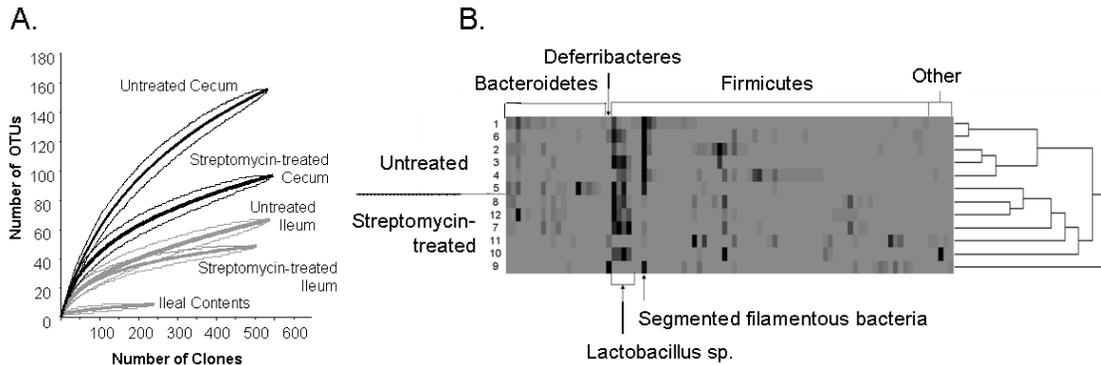


Figure 2.2. Streptomycin treatment alters the microbial composition of both the ileum and the cecum. (A) rarefaction analysis and 95% confidence intervals are shown for the 16S rRNA-encoding sequences obtained from each site in the untreated and streptomycin-treated mice. Central lines represent rarefaction analysis of each site with the surrounding lines representing the upper and lower 95% confidence intervals. For rarefaction analysis an operational taxonomic unit (OTU) was defined as 97% sequence similarity. (B) 16S rRNA gene clone libraries were created from samples containing ileal tissue and contents from untreated mice and those pretreated with streptomycin. Sequences were assigned to OTUs using a definition of 97% sequence similarity. A heatmap is used to show the relative abundance of OTUs, with specific OTUs detected in the sample oriented along the horizontal axis, and the dendrogram showing the distribution of OTUs. Darker coloring within the heatmap indicates greater representation of specific OTUs. Phyla are shown above the figure and highly represented OTUs are shown below. Untreated mice shown in this figure are the same as those shown in Fig. 1C.

we further characterized the ileum to determine whether differences existed between the population of bacteria present within the intestinal contents and that associated with the ileal tissue. Samples containing ileal contents but not the tissue of the ileum itself were taken from three C57BL/6 mice, and 238 16S rRNA gene clones were compared to those derived from ileal samples containing both tissue and contents. We found that there was decreased

species richness in the samples that contained only contents (Fig. 2.1C and Fig. 2.2A). The total number of OTUs observed was nine in the samples containing only contents, compared to 67 in the samples that included both tissue and contents. Additionally, analysis utilizing the Chao1 richness estimator further supported this difference in the richness of OTUs, with no overlap in the 95% confidence intervals (CIs) (Table 2.1). Furthermore, the Shannon–Weiner Diversity Index, an indicator of diversity within a population (57), was 2.87 in samples that included both tissue and contents compared to 0.98 in samples that contained only contents, indicating lower diversity within the bacterial community derived from the contents of the ileum. Analysis of the composition of OTUs present in the ileum (Fig. 2.1C) also showed that there was a larger number of OTUs present in the samples containing both tissue and contents, and that there was a difference in the distribution of these OTUs. Specifically, we found upon comparison that the relative abundance of the SFB was much lower in the samples containing only contents, $3\% \pm 2.6$ compared to $31\% \pm 13$ in samples with both tissue and contents. These results are consistent with previous reports of the SFB being closely associated with the ileal epithelium (20). Thus, these results show that there is greater bacterial diversity in the samples that contain tissue and contents when compared to those that contain contents alone and suggest the presence of a diverse bacterial population in close association with the intestinal mucosa.

Streptomycin alters the microbiota of the ileum. To better understand the streptomycin-treated mouse model of infection and specifically the potential changes that occur in the small intestine, we next characterized the microbiota of the ileum in streptomycin-treated mice using 16S rRNA gene clone libraries.

Mice were treated orally with streptomycin, and samples from the ileum and cecum were obtained 72 hours after that treatment. A total of 503 and 545 sequences were analyzed from the ileum and cecum of these mice, respectively. To allow comparisons with the untreated mice described in the previous section, we performed the two experiments simultaneously, using animals obtained as a single lot. We found that streptomycin treatment altered the composition of microbial communities in both the ileum and the cecum. Rarefaction analysis demonstrated that streptomycin treatment decreased species richness in both the ileum and cecum (Fig. 2.2A). In this case, the Chao1 estimator of richness supported this conclusion as well, as there was no overlap in the 95% CIs of the Chao1 of the treated sites when compared to the respective untreated sites (Table 2.1). We also examined the total number of 16S rRNA-encoding genes/gram in the ileum and cecum using qPCR. Interestingly, we found that there was a ten-fold decrease in bacterial numbers, from 10^9 to 10^8 16S rRNA gene copies/gram, in the ileum but that there was no significant change in the cecum. Additionally, the Bray-Curtis distance measure of diversity between the microbial communities showed that in both the ileum and the cecum the majority of samples from untreated mice clustered together but separately from the respective samples taken from streptomycin-treated mice (data not shown). Collectively, these results show that streptomycin treatment caused a decrease in the richness of the OTUs as well as altered the distribution of OTUs in the majority of mice. Some variation did occur within the groups. Specifically, for one mouse in the group that had not been treated with streptomycin, cluster analysis of both the ileum and cecum placed the composition of the microbiota between those of the other untreated mice and those of the streptomycin-treated group (not shown).

These results thus show variation among individual animals but demonstrate differences between the treated and untreated groups. Among ileal samples, we found that changes due to streptomycin treatment were not uniform among individual mice, with differing OTUs predominating after treatment with this antimicrobial. In particular, two mice had a single family of organisms predominate after treatment that was different from the other, in one case the family Lachnospiraceae and in the other the family Deferribacteraceae, whereas the remaining mice did not have a particular predominating group. Therefore, to further analyze these population changes we did not combine these libraries of sequences but instead characterized the changes in the ileum caused by streptomycin by examining the microbiota of individual mice for the presence and prevalence of specific OTUs (Fig. 2.2B; untreated mice shown in this figure are the same as those shown in Fig. 2.1C). Overall, there were changes in the distribution of OTUs as well as in the relative abundance of particular OTUs. More specifically, there was a decrease in the relative abundance of the SFB in the streptomycin-treated mice, $3\% \pm 4$ as compared to $31\% \pm 13$ in the ileum of untreated mice, consistent with previous reports that this group of bacteria is sensitive to streptomycin (43).

Streptomycin pretreatment enhances *Salmonella* infection of the ileum.

In the murine model of *Salmonella* infection, streptomycin treatment has been shown to enhance infection of the cecum (4, 80), but little is known about effects on the small intestine. We therefore next characterized the microbiota in the ileum of mice infected with *Salmonella* to determine whether infection was also altered in this region of the intestinal tract. C57BL/6 mice were pretreated orally with either sterile water or streptomycin, and 24 hours later both groups were orally infected with a streptomycin-resistant strain of

Salmonella. To examine the microbiota present in each of these groups, 16S rRNA gene clone libraries were created. A sufficient bacterial DNA concentration for clone library construction was obtained from 5 of 6 untreated mice and 4 of 6 streptomycin-treated mice, for a total of 452 and 362 sequences from the respective groups. Taxonomic analysis of these clone libraries showed that only one animal in the untreated group had detectable *Salmonella* in the ileum, with one *Salmonella* 16S rRNA containing clone identified among the 86 clones sequenced (not shown). In contrast, for mice pretreated with streptomycin prior to infection, 2-97% of the total bacterial population observed in the ileum consisted of *Salmonella*. To more precisely determine the extent of *Salmonella* infection of the ileum, we used qPCR to determine the number of *Salmonella*-specific 16S rRNA gene sequences present. We were able to detect *Salmonella* in all of the animals, whether or not they were pretreated with streptomycin, but found that streptomycin treatment significantly increased the number of *Salmonella* at this site (p-value<0.05). The number of copies of the *Salmonella* 16S rRNA gene per gram of tissue was increased in streptomycin-treated animals, with a median in pretreated mice of 4×10^6 , more than two logs greater than that of the untreated mice, having a median of 1×10^4 . There was, however, much variation among individual animals, with the number of *Salmonella* obtained from mice treated with streptomycin prior to infection ranging from 5×10^5 to 6×10^8 copies/gram of tissue (Fig. 2.3). One possible explanation for this variation could be varying levels of *Salmonella* infection in these mice. However, histopathological examination confirmed the presence of severe diffuse colitis similar to that previously reported in mice orally administered streptomycin prior to infection (4). Mucosal damage and inflammation ranged

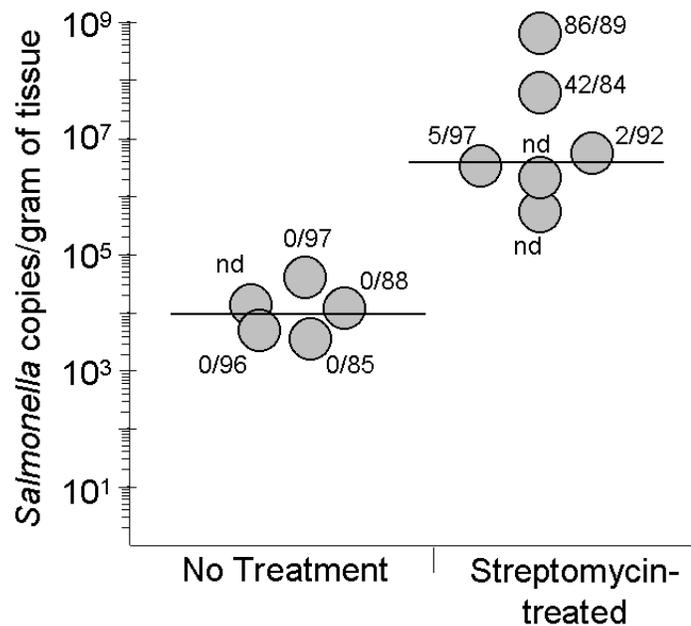


Figure 2.3. Streptomycin pretreatment increases *Salmonella* infection of the ileum. Real-time PCR was used to compare the number of *Salmonella* in the ilea of individual mice without additional treatment to those pretreated with oral streptomycin. The number of copies of the *Salmonella* 16S rRNA gene amplified using *Salmonella*-specific primers was determined and is shown relative to the mass of the ileal sample. The limit of detection for *Salmonella* was one copy/100 ng total DNA. Represented adjacent to the circles are the number of *Salmonella* 16S rRNA gene sequences identified over the total size of the clone library analyzed from this same animal. Circles with nd (not determined) show animals in which clone libraries could not be produced, primarily due to low bacterial DNA yields. Horizontal lines represent the median for each group.

from moderate and multifocal in one mouse to severe and focally extensive or diffuse in the other mice (not shown). Therefore, taken together these results show that, although variation exists in the number of *Salmonella* able to colonize the ileum, pretreatment with streptomycin significantly alters the

composition of the ileal microbiota and leads to enhanced survival and colonization of *Salmonella* in this important region of the intestine.

Streptomycin treatment alters the fatty acid composition of the intestine.

Since there are differences in the microbiota found in the ileum and cecum, we next considered whether these differences might manifest themselves as variations in the presence and concentrations of SCFAs in each intestinal location, suggesting a possible means by which *Salmonella* virulence might be modulated *in vivo*, as SCFAs have been shown to have differing effects on *Salmonella* invasion depending upon their composition and concentration (38, 52). We therefore characterized the SCFAs in these regions of the intestine using GC-MS and HPLC. As anticipated, there were significant differences in the SCFAs present in the ileum and cecum. We found that formate, previously identified as a positive signal for *Salmonella* invasion *in vitro*, was present in the ileum at a median concentration of 8.1 mM, but was low or below the level of detection (median concentration of 0 mM) in the cecum (Fig. 2.4). In contrast, butyrate, previously shown to be a negative signal for invasion, was not detectable in the ileum but was present in the cecum at a median concentration of 12.8 mM (Fig. 2.4). We also determined the pH of the intestinal lumen, as acidity affects the penetration of SCFAs into bacteria and thus their effects on gene expression. The median pH of the ileum was 6.68 and that of the cecum was 6.39, both at levels below neutrality, thus promoting increased bacterial uptake of SCFAs.

Since alterations in the microbiota occur with streptomycin treatment, and the microbiota produces SCFAs, we next determined whether changes occurred to the SCFA concentrations as a result of this treatment. We found

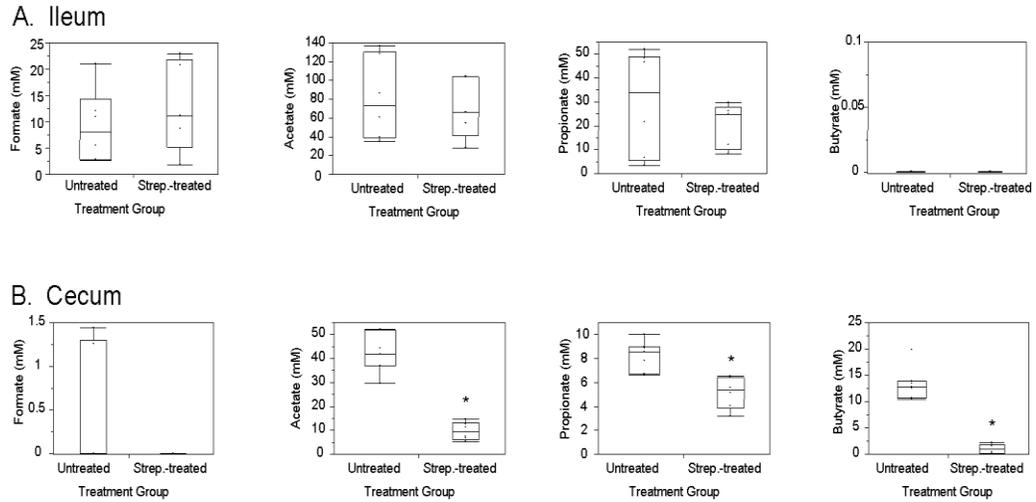


Figure 2.4. Short chain fatty acids of the untreated and streptomycin treated ileum and cecum. Intestinal contents were collected from the ileum and cecum of both untreated and streptomycin-treated mice 72 hours after treatment. SCFAs were quantified using (A) HPLC analysis for the ileum, or (B) GC-MS for the cecum. The box plots show the median with 25% and 75% quartiles. The bars in each box plot extend to the outermost points located within the quartile $\pm 1.5 \times$ the interquartile range. An asterisk (*) indicates significant difference at a p -value < 0.01 between the untreated mice and streptomycin-treated mice for short chain fatty acid concentrations.

that streptomycin treatment altered the SCFAs of the cecum, with a significant decrease in acetate, propionate, and butyrate, but did not significantly alter the SCFAs of the ileum or the pH of either site (Fig. 2.4 and not shown). The median acetate concentration was substantially reduced, from 42.0 mM to 9.4 mM, and the median butyrate concentration from 12.8 mM to 1.0 mM in the cecum, while the median propionate concentration was more modestly reduced from 8.5 mM to 5.4 mM. These results, taken with previous findings, show that SCFAs exist in concentrations capable of signaling the modulation of *Salmonella* virulence. Additionally, the results suggest that the ileal environment in normal mice is conducive to tissue invasion, while that of the

cecum represses invasion, but that treatment with streptomycin alters the environment of the cecum to reduce repressive signals, perhaps explaining the cecal pathology that is commonly observed during *Salmonella* infection of streptomycin-treated mice.

Streptomycin treatment prior to infection with *Salmonella* enhances ileal inflammation. Previous work on the streptomycin-treated mouse model of *Salmonella* infection primarily focused on development of severe colitis without detailed examination of the ileum (4). Therefore, we characterized the histopathological changes present in the ileum of control mice and mice infected with *Salmonella* with and without oral administration of streptomycin. When compared to mice infected with *Salmonella* without antibiotic treatment, streptomycin administration prior to infection with *Salmonella* enhanced the extent and degree of inflammation present in the distal ileum (Fig. 2.5 and 2.6). Five of the six mice administered streptomycin prior to *Salmonella* showed multifocal to segmental infiltration of the lamina propria at the base of intestinal crypts by clusters of polymorphonuclear neutrophils accompanied by epithelial cell damage and cryptitis (Fig. 2.5). Similarly, large numbers of neutrophils were present within interfollicular regions and at the periphery of Peyer's patches extending into the lamina propria of adjacent mucosa. In contrast, mice infected with *Salmonella* without streptomycin pretreatment had only few neutrophils admixed with few eosinophils at the periphery of Peyer's patches (6 of 6 mice) and interfollicular regions (2 of 6 mice). The subepithelial dome areas of Peyer's patches in 3 of 6 mice inoculated with *Salmonella* after oral streptomycin administration also were markedly expanded by large numbers of neutrophils, a change that was absent in mice from the other groups (Figs. 2.5 and 2.6). Moreover, the follicle-associated epithelium

Figure 2.5. Streptomycin administration prior to infection with *Salmonella* enhances the extent and degree of ileal inflammation in mice. Hematoxylin and eosin-stained ileum was obtained from mice pretreated with streptomycin and infected with *Salmonella*. A) Necrosis of ileal villi accompanied by focally extensive infiltration of lamina propria by large numbers of polymorphonuclear neutrophils extending along the base of intestinal crypts; 12X magnification. B) Infiltration of villous lamina propria by large numbers of polymorphonuclear neutrophils with segmental sloughing of intestinal epithelial cells; 20X magnification. C) Infiltration of ileal Peyer's patch subepithelial dome area by large numbers of polymorphonuclear neutrophils (asterisk) together with focal disruption of the follicle-associated epithelium (arrowhead) and transepithelial migration of neutrophils; 20X magnification.

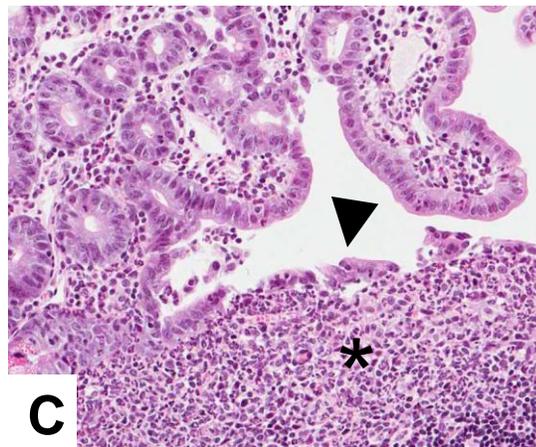
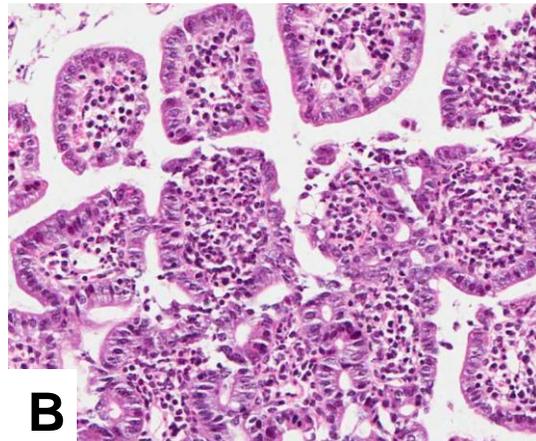
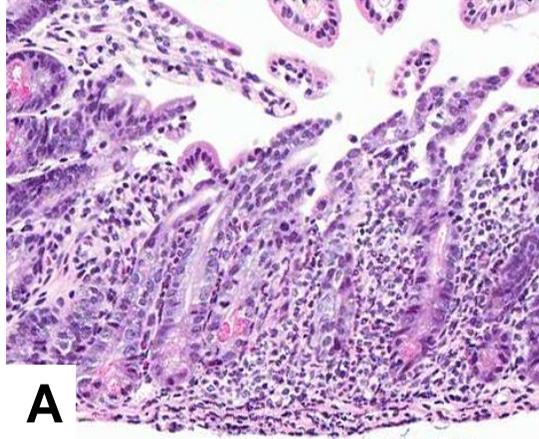
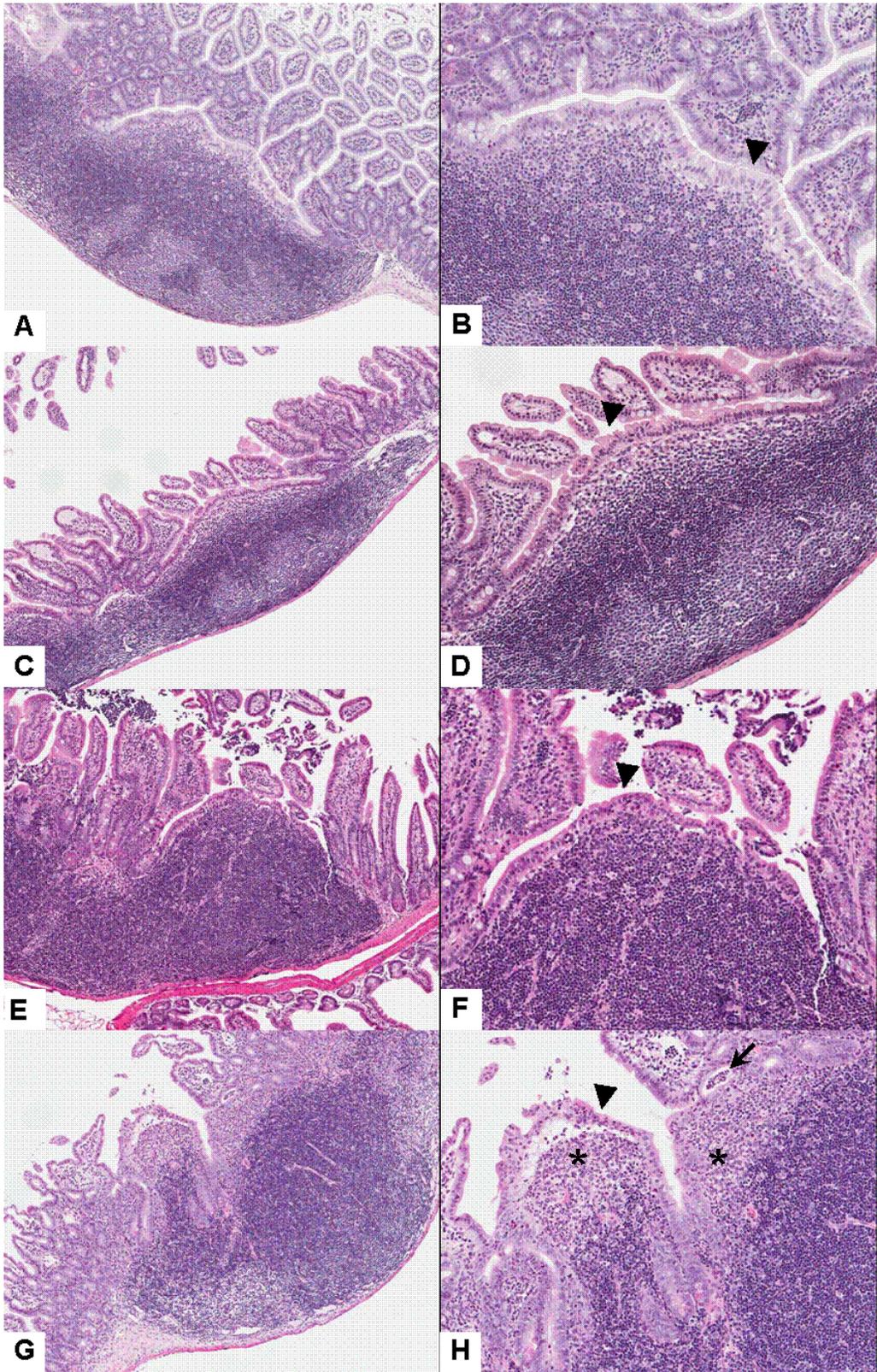


Figure 2.6. Streptomycin administration prior to infection with *Salmonella* is associated with ileal Peyer's patch inflammation in mice. Hematoxylin and eosin-stained ileal Peyer's patches of uninfected mice and mice infected with *Salmonella* with and without streptomycin pretreatment. (A-B) untreated and uninfected, (C-D) streptomycin-treated, (E-F) *Salmonella*-infected, (G-H) streptomycin-treated and *Salmonella*-infected. The subepithelial dome area is diffusely infiltrated by large numbers of polymorphonuclear neutrophils in the streptomycin-treated and *Salmonella*-infected mouse (G-H, asterisks), while the follicle-associated epithelium is markedly attenuated in mice infected with *Salmonella* with (H, arrowhead) or without (F, arrowhead) streptomycin compared with tall columnar epithelium in uninfected mice (B,D, arrowheads). The streptomycin-treated and *Salmonella*-infected mouse also displays focal disruption of the follicle-associated epithelium along with multifocal transepithelial migration of polymorphonuclear neutrophils and focal crypt abscess (H, arrow). (A,C,E and G, 6X magnification; B,D,F and H, 12X magnification).



overlying the dome areas of ileal Peyer's patches of mice receiving streptomycin with or without *Salmonella* was diffusely low cuboidal compared to tall columnar epithelium in untreated control mice (Fig. 2.6). To better quantify histopathological changes in the ileum of mice in each of the four treatment groups, lesions were scored on a 0 to 4 scale, and ileitis severity was calculated as the sum of the scores for the five categorical parameters described in Materials and Methods, with 20 representing maximum severity. Using this scoring system, the median score for untreated mice was 1, while those that had received both streptomycin and *Salmonella* and displaying ileitis had a median score of 15 (p-value<0.05). Slight increases in ileal pathology was present in groups that received *Salmonella* or streptomycin alone (medians of 4.5 and 7, respectively), but these differences were not significantly different from untreated mice. In addition to the ileal mucosal changes, histopathological examination of the ileum revealed a complete absence of long filamentous bacteria in the lumen of the ileum of mice treated with streptomycin with or without *Salmonella* infection (not shown). These results are thus consistent with the microbial ecology data demonstrating a decrease in the numbers of SFB in mice receiving streptomycin and show that antibiotic administration exacerbates the pathology of the ileum caused by *Salmonella*.

DISCUSSION

There has been much recent work describing the bacterial composition and chemical environment of the large intestine, including the cecum, colon, and feces, but relatively little is known about the comparable conditions of the small intestine. In this work we have characterized the ileum of the mouse,

the species used frequently as a model of infection for many of the enteric bacterial pathogens that cause disease in the small intestine, and have examined the effects on this organ of both antibiotic administration and infection with *Salmonella*. As an effective rodent model of human enteric disease requires an understanding of the types of bacteria that normally reside within the intestine, we first identified the constituents of the ileal microbiota. We found the microbiota of the mouse ileum to be quite different from that of the cecum, with the predominant Firmicutes present being the Clostridiaceae, Lactobacillaceae, and Lachnospiraceae, while in the cecum the predominant family was the Lachnospiraceae, with much lower relative abundance of the Lactobacillaceae and Clostridiaceae. These findings are consistent with previous results in humans which showed a similar higher relative abundance of the Lachnospiraceae in the large intestine and Lactobacillaceae in the small intestine (30). In contrast to those results, however, our findings also show a large relative abundance of the Clostridiaceae in the mouse ileum that was not observed in the human small intestine. In mice, 94% of the bacteria classified as Clostridiaceae belonged to a group of bacteria called the segmented filamentous bacteria (SFB). The SFB are a group of unculturable, Gram variable, long, segmented, and filamentous bacteria that have been shown to be closely associated with the ileal epithelium in mice and other vertebrates (20, 44). They have previously been observed in one human ileal sample (44), however, less is known about the prevalence in the normal human ileum. It is possible that they are not as abundant in the human ileum as in the mouse ileum or that more studies are necessary to look specifically for this group of bacteria in human samples.

Our characterization of the ileal microbiota also showed that there were differences in the microbial population associated with samples containing tissue and luminal contents when compared to those that contained only contents. In the samples containing only contents, there was lower species richness, with the majority of bacteria classified within the family Lactobacillaceae (Fig. 1C). This suggests that the great majority of the microbial diversity observed in the ileum arises from a population of bacteria intimately associated with the intestinal mucosa rather than within the ileal lumen. The increased relative abundance of the Lactobacillaceae in luminal samples could be attributed to several factors. Previous work using qPCR has shown that *Lactobacillus* is present in quantities between 10^7 and 10^9 in the distal small intestine (3) indicating that they are a predominant group in this location. Current data give conflicting reports on the ability of *Lactobacillus* species to adhere to the intestinal mucosa (35); therefore, one explanation for the relative abundance of Lactobacillaceae in intestinal contents is that the Lactobacillaceae may not be as closely associated with the ileal epithelium as are members of the Lachnospiraceae, Clostridiaceae, and Bacteroidaceae. Supporting this hypothesis is previous work showing that the SFB, members of the Clostridiaceae, are very closely associated with the ileal epithelium (20), which is consistent with our results showing the relative abundance of the SFB in samples containing tissue to be 10-fold greater than that in samples with intestinal contents alone. Additionally, *Lactobacillus* species have previously been shown to be abundant in the upper intestinal tract, including the stomach and upper small intestine (71, 82). Thus, the bacterial population in the ileal contents may be a transient population consisting primarily of bacteria shed from the upper intestinal tract. The disparity between the microbial

composition of the mucosa and that of the contents is important, because these results suggest that sampling the intestinal contents alone severely under-represents the diversity present in the ileum. Thus, to fully understand the interaction of invading pathogens with the host, it is important to characterize the bacteria associated with the mucosa as well as within the luminal contents.

Upon treatment of mice with streptomycin, we observed changes in the composition of the microbiota in both the ileum and cecum. Specifically, in the ileum there was a ten-fold decrease in the SFB. Previous work has suggested a role for the SFB in host pathogen interactions; specifically, they were shown to be important in providing protection against *Salmonella* and EPEC colonization of ileal surfaces (33, 37) and for stimulating the mucosal immune response in mice (46, 81). Correlated with this loss of microbiota was the enhanced infection of the ileum by *Salmonella* after treatment with streptomycin. We observed a significant increase in the number of *Salmonella* residing in the ileum in streptomycin-treated mice, suggesting that the administration of this antimicrobial alters the ileal environment sufficiently to allow improved colonization by the invading pathogen. In addition to the changes in the SFB observed with streptomycin-treatment, we also observed inflammation in the ileum of mice treated with streptomycin prior to infection with *Salmonella* that was not seen in animals infected with *Salmonella* without streptomycin treatment. In this study, mice were infected with *Salmonella* 24 hours after streptomycin treatment, while the characterization of the ileal environment and microbiota was conducted in streptomycin-treated mice 72 hours after administration of the antibiotic. A previous study has shown that recovery of the normal microbiota, as measured at the phylum level, occurred

five days after streptomycin treatment (80). Therefore, it is possible that antibiotic treatment elicited changes in the numbers of bacterial species not observed in this study that contributed to *Salmonella* colonization. However, even so, these results, along with those of previous studies, suggest a possible role for the SFB in preventing colonization and infection of the ileal surface of normal mice, either by physically blocking colonization or by creating an immune response and thus limiting *Salmonella* entry to the Peyer's patches and systemic disease. Although mice mono-associated with SFB exist (45), these mice would not allow a full understanding of the interaction between the SFB and surrounding commensals with the invading pathogen. Thus, further work examining the relationship between the SFB and *Salmonella* infection in the mouse is hampered by the intractable nature of this intestinal resident. In addition to the effects of the microbiota on *Salmonella* infection, the genetic background of the mouse might also play an important role. The strain used in this work, C57BL/6, is *Nramp1*^{-/-} and thus highly susceptible to *Salmonella* infection. A previous study noted greater ileal inflammation after *Salmonella* infection in untreated *Nramp1*^{+/+} strains in comparison to C57BL/6 mice (79), suggesting innate differences in their responses to infection. That work, however, did not examine the effects of streptomycin pretreatment on this pathology, and so the relative utility of mouse strains for this infection model remains to be investigated.

Short chain fatty acids (SCFAs) are produced by the intestinal microbiota and thus are largely responsible for the composition of the intestinal chemical environment. Furthermore, previous work has shown that SCFAs affect both viability and virulence gene expression in enteric pathogens (8, 32, 38, 48, 52, 54, 59, 77, reviewed by 86). Therefore, in addition to

characterizing the microbiota of the ileum and cecum, we also determined the SCFA concentrations in these regions to better understand the chemical cues likely to be sensed by pathogenic bacteria. We found that formate was present in the ileum, but was in low concentration or was undetectable in the cecum, and, conversely, that butyrate was present in the cecum but was not detectable in the ileum. These differences in SCFAs may be a result of the variation in the microbiota between these two locations. For example, the family Lachnospiraceae contains genera that are classified as butyrate-producers (16), and we found that there was a higher relative abundance of the Lachnospiraceae in the cecum (52%) when compared to that in the ileum (17%). Additionally, there is higher relative abundance of the Lactobacillaceae in the ileum (24%) when compared to the cecum (1%), and under certain conditions species of *Lactobacillus* have been shown to be heterofermentative, producing formate and acetate in addition to lactic acid (17, 68). Upon treatment with streptomycin, we observed significant changes in the SCFA concentrations in the cecum as was previously observed (67), however, we observed more substantial decreases in both the acetate and butyrate concentrations than were previously reported. It is likely that these changes in SCFAs occurred as a result of the changes in the microbiota in this region. Interestingly, at the family level, there did not appear to be a change in the relative abundance of the Lachnospiraceae, which contains several butyrate-producers. However, we did observe decreased species richness and a change in the distribution of phylotypes at the level of 97% sequence similarity in the cecum of streptomycin-treated mice. These results suggest changes at the species level; thus, it is possible that there were changes in the relative abundance of the butyrate-producing species present within the family

Lachnospiraceae leading to the changes in the butyrate concentration in the cecum. Another possibility is that streptomycin eliminates a particular group of bacteria important for acetate production. Previous work has shown that some butyrate-producers use exogenous acetate for butyrate production (26); therefore a decrease in the population of acetate producing bacteria could potentially lead to a decrease in both acetate and butyrate. Thus, it is possible that the changes in microbiota as a result of streptomycin treatment produced these changes in SCFA concentrations in the cecum.

On the basis of previous *in vitro* observations indicating modulation of *Salmonella* invasion gene expression by certain SCFAs, we hypothesized that alterations in the microbiota associated with streptomycin administration and the resulting changes in relative concentrations of SCFAs in the intestinal tract may affect the pathogenesis of *Salmonella* infection in the mouse model. Previous *in vitro* work from our laboratory and those of others has shown that specific SCFAs can positively or negatively affect the expression of *Salmonella* invasion genes. In particular, formate has been characterized as a positive signal and butyrate a negative signal (8, 32, 38, 52). Since in the mouse model of infection *Salmonella* invades primarily in the ileum, in particular the Peyer's patches, causing septicemia (9, 70), the observation that formate is detectable in the ileum and not the cecum suggests that it may be an important positive signal *in vivo* for *Salmonella* invasion gene expression. In contrast, the presence of butyrate in the cecum and not the ileum suggests that it may be an important negative signal *in vivo*. Therefore, these results, along with previously published results in cecectomized mice (87), indicate that the distribution and abundance of the SCFAs in the intestinal tract may play an important role in *Salmonella* virulence. Upon treatment with

streptomycin prior to *Salmonella* infection, there are changes in the intestinal pathology, with the appearance of pronounced cecal inflammation (4), and, as shown in this work, exacerbated pathology of the ileum as well. Changes in the ileum cannot be attributed to alterations in the concentrations of SCFAs present at this site, as untreated mice have a SCFA composition that is likely already conducive to bacterial invasion, and streptomycin treatment did not elicit a change in that composition. In contrast, the cecal contents of treated mice showed a significant reduction in SCFA concentrations, with butyrate being substantially reduced. These results thus suggest that streptomycin-treatment prior to infection decreases the negative signal provided by butyrate, allowing *Salmonella* invasion in the cecum and the cecal pathology observed in this infection model.

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CHAPTER 3

**Repression of *Salmonella* invasion by the intestinal fatty acid propionate
requires production of the high-energy intermediate propionyl-CoA**

ABSTRACT

Salmonella is a causative agent of food-borne illness. To cause disease, it must first invade the intestinal epithelium using genes encoded on *Salmonella* Pathogenicity Island 1 (SPI 1). Previous work in our lab has shown that the short chain fatty acids, propionate and butyrate, negatively regulate SPI 1 invasion gene expression *in vitro*. Additionally, our previous *in vivo* work also showed a correlation between a decrease in propionate and butyrate concentrations in the cecum with increased *Salmonella* pathology. Therefore, in this work, we further characterized the repressive effect of propionate and butyrate on SPI 1 invasion using *in vitro* studies. We found that concentrations of propionate and butyrate comparable to that seen in the normal mouse cecum repressed SPI 1 invasion genes and decreased invasion of HEP-2 cells. We also found that the negative effect on invasion genes was observed at a pH of 6.7 but not at a pH of 8.0, suggesting that fatty acids must enter the bacterial cytoplasm to have the repressive effect. Additionally, we found that it was necessary for propionate to be metabolized for repression of invasion and that the metabolic intermediate, propionyl-CoA, is important for this effect. We also found that propionate and butyrate cause a shift in the protein expression of flagellin. Thus, propionate and butyrate affect more than one virulence determinant important for intestinal pathogenesis.

INTRODUCTION

Salmonella enterica is a major cause of food-borne illness and can cause a range of illnesses from self-limiting intestinal disease to septicemia. For *Salmonella* to cause disease, it must first invade the epithelium of the intestinal tract. Invasion of the intestinal tract is mediated by a type three

secretion system (TTSS) which is encoded in *Salmonella* Pathogenicity Island 1 (SPI 1) (10, 29, 35, 43, 51, 57, 60) . The TTSS forms a “needle complex” (46) which is used to inject effector proteins into the host cell that cause changes in the host cell cytoskeleton leading to engulfment of the bacterium (reviewed by 28). The genes encoded in SPI 1 and the invasion process are required for both the intestinal and septicemic form of disease (9, 29, 43, 69).

SPI 1 genes are controlled by a complex regulatory network of transcriptional and post-transcriptional regulators both within and outside SPI 1. Regulators within the island are HilA, HilC, HilD, and InvF, with HilA characterized as the central regulator. HilA, a member of the OmpR/ToxR family, is an activator that regulates transcription of the following genes in SPI 1: the *inv/spa* operons, the *prg/org* operons, and the *sic/sip* operon via the read-through transcript of the *inv/spa* operons (7, 18, 52). InvF is also a transcriptional regulator of the AraC family (45). HilA is responsible for controlling gene expression of the TTSS apparatus, InvF is responsible for controlling secreted effectors outside of SPI 1, and together they control secreted effectors within SPI 1 (18, 23). InvF can be regulated by mechanisms independent of HilA, as well (3, 4, 59). HilA is controlled by HilC and HilD from within the island and RtsA and HilE outside SPI 1 (24). Also, a recent study has shown that by an unknown mechanism, HilA can negatively regulate itself (20).

Several regulators encoded outside of SPI 1 have been identified as important for control of HilA and InvF, among them are PhoP/PhoQ, BarA/SirA, and the Csr system. PhoP/PhoQ has been shown to negatively regulate invasion genes through HilA (8). This negative regulation is induced by low magnesium concentrations (63). The csr system both positively and

negatively regulates SPI 1 invasion gene expression through changes in the level of CsrA (3). In *Salmonella* it consists of a small protein CsrA, and two untranslated RNAs, CsrB and CsrC, that control the level of CsrA (3, 26). The BarA/SirA regulatory system positively regulates invasion gene expression. BarA is a sensor kinase of the phosphorelay type, and SirA is its response regulator (4, 66). BarA/SirA induce invasion gene expression through their effect on expression of CsrB and CsrC (26, 49). It is thought that BarA/SirA induce expression of CsrB and CsrC, decreasing the levels of free CsrA, and inducing invasion gene expression (2).

Environmental factors mediate invasion gene expression through regulation of *hilA* expression (8). The conditions that positively regulate SPI 1 gene expression are low oxygen, log phase growth, high osmolarity, changes in DNA supercoiling, and slightly alkaline pH (8, 25, 30, 50, 61). It has also been shown that short chain fatty acids are important for both positive and negative regulation of invasion gene expression. Acetate and formate act as cytoplasmic signals to induce invasion gene expression, however acetate requires SirA for this effect, whereas formate does not (41, 49). For both of these fatty acids, the effects are observed at pH 6.7 but abrogated at pH 8.0, suggesting that the fatty acids must concentrate in the bacterial cytoplasm to have their effect (41, 49). In contrast, butyrate and propionate have been shown to repress invasion genes (12, 31, 49).

Studies have been performed to characterize the intestinal environment, and many of the environmental conditions listed above can be found in the intestinal tract. In particular, in various mammalian species, studies show that acetate, propionate, and butyrate are found in both the cecum and the colon, and that acetate, propionate, and formate have been

found in the ileum (5, 11, 16, 32, 47, 48, 55). Also, the pH throughout the distal ileum, cecum, and proximal colon is between 6.0 and 7.0 (6, 11, 32). The conditions in the ileum, the area previously described as the location for invasion (14, 44), are conditions known to induce invasion genes (41, 49), whereas the conditions of the cecum and colon may be more likely to repress invasion genes (49). Therefore, it seems likely that these conditions in the intestinal tract are signals for *Salmonella*, indicating a location for productive invasion. In fact, more than one *in vivo* study in mice has shown a correlation between a decrease in short chain fatty acid concentrations and increased susceptibility to *Salmonella* infection (32, 68).

Another determinant important to *Salmonella* virulence is the regulated expression of flagella. More specifically, flagellin has previously been shown to activate the host immune response (34). In *Salmonella*, there are two genes that encode flagellin, *fliC* and *fljB*. These two subunits are not expressed at the same time because of a mechanism called phase variation. Control of phase variation is achieved through the regulator FljA, with *fljA* being expressed as part of an operon with *fljB* (62). When this operon is expressed type 2 flagellin is produced, and FljA production negatively regulates *fliC*, preventing expression of type 1 flagellin (1, 70). Previous work has examined the importance of FliC in the mouse model of infection, finding that *fliC* expression occurred primarily in the Peyer's patches (17) and that it was important for induction of the host inflammatory response (reviewed by 34). Additionally, a previous study observed a decrease in *fliC* expression under conditions that represent intracellular conditions (17). Thus, as flagellin is important for the host immune response, it is possible that the environment of the host may affect which flagellin subunit is expressed.

In this work, we examined the repressive effects of propionic acid and butyric acid at the level of gene expression, secreted protein expression, and epithelial cell invasion and found that both fatty acids repressed invasion and that pH was important for the repressive effect. Additionally, as the pathways for propionate metabolism have previously been characterized in *Salmonella*, we further examined the repressive effect of propionic acid to understand the mechanism by which it affects invasion. We show here that metabolism of propionic acid is necessary for its repressive effect and that in particular the metabolic intermediate propionyl- CoA is important for this effect. Additionally, we found that propionic acid and butyric acid in the wild type caused a switch in the phase variation of flagellin present.

MATERIALS AND METHODS

Bacterial Strains. Bacterial strains and plasmids used in this study can be found in Table 1. All strains were isogenic to *Salmonella enterica* serovar Typhimurium strain ATCC 14028s. All gene deletions were created using a previously described one-step inactivation method (19). The chloramphenicol and kanamycin markers from pKD3 and pKD4 respectively were used. Primers at the 5' end included 40 bp of homology to the region surrounding the start and stop codon of the gene to be deleted and the 3' end of the primers contained sequence homologous to the plasmids. The resultant PCR product was then transformed into *Salmonella enterica* serovar Typhimurium strain ATCC 14028s containing the pKD46 plasmid which contains the λ Red recombinase which allows for allelic exchange. All deletion mutants were checked for the loss of the gene using PCR. P22 transduction was used to move marked deletions to create multiple mutations in one strain. Also, when

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
Strains		
ATCC 14028s	Wild type	American Type Culture Collection
CA412	<i>sipC::lacZY</i>	8
CA1272	Δ <i>ackA</i>	This study
CA1274	<i>sipC::lacZY</i> Δ <i>ackA</i>	This study
CA1828	<i>sipC::lacZY</i> Δ <i>ackA</i> , <i>tdcD</i>	This study
CA1857	<i>sipC::lacZY</i> Δ <i>ackA</i> , <i>tdcD</i> , <i>pduW</i>	This study
CA1861	<i>sipC::lacZY</i> Δ <i>ackA</i> , <i>tdcD</i> , <i>pduW</i> , <i>prpE</i> , <i>acs</i>	This study
CA1831	<i>sipC::lacZY</i> Δ <i>ackA</i> , <i>tdcD</i> , <i>pduW</i> , <i>pta</i>	This study
CA1872	<i>sipC::lacZY</i> Δ <i>prpE</i> , <i>acs</i>	This study
CA1889	Δ <i>ackA</i> , <i>tdcD</i> , <i>pduW</i>	This study
CA1512	<i>sipC::lacZY</i> Δ <i>prpC</i>	This study
CA1796	<i>sipC::lacZY</i> Δ <i>acs</i> , <i>prpE</i> , <i>pta</i>	This study
CA1821	<i>sipC::lacZY</i> Δ <i>acs</i> , <i>prpE</i> , <i>pta</i> , <i>pf1B</i> , <i>tdcE</i>	This study
Plasmid		
pCAYC177	vector only control	15
pCA171	<i>ackA</i> ORF on pCAYC177	This study

necessary for strains with multiple mutations or where there might be a question of polarity, the FLP recombinase was used to remove markers (19).

The complementation plasmid was created by amplifying the *ackA* open reading frame (ORF) including enough upstream sequence to include the ribosome binding site. The PCR product was then cloned into the kanamycin gene in the cloning vector pACYC177.

β -galactosidase assays. Cultures were grown overnight standing at 37°C in LB/100 mM MOPS pH 6.7 containing either no additive, 10 mM propionic acid, or 10 mM butyric acid. For experiments where a pH of 8.0 was necessary, 100 mM HEPES pH 8.0 was used in place of the MOPS. All cultures were grown in triplicate and β -galactosidase activity was measured as previously described (56).

HEp-2 invasion assays. The invasion assay was performed as previously described (4), except for upon infection plates were centrifuged at 100 x g. Also, for this assay, cultures were grown overnight standing at 37°C in LB/100 mM MOPS pH 6.7 with no additive, 10 mM propionic acid, or 10 mM butyric acid. Quadruplicates were tested for each strain in each condition.

Analysis of secreted proteins. Secreted proteins were isolated and analyzed as previously described (4), with the exception that cultures were grown in LB/100 mM MOPS pH 6.7, with either no additive, 10 mM propionic acid, or 10 mM butyric acid. To identify unknown proteins, bands were cut from the gel and submitted to the Cornell University Proteomics and Mass Spectrometry Core Facility for matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) analysis.

Statistical Analysis. Results from β -galactosidase assays and invasion assays was analyzed using a one-way analysis of variance to determine if the mean of at least one strain or condition differed from any of the others. The Tukey-Kramer HSD multiple comparison test was then used to determine

which means were statistically different. A p-value <0.05 was considered significant. Statistical analysis was performed using Jmp 8.0 software (SAS, Cary, NC).

RESULTS

Propionate and butyrate repress SPI 1 invasion gene expression. Fatty acids are the predominant metabolic product of the anaerobic intestinal microbiota and so largely define the environment of the mammalian intestinal tract. Previous work in our lab showed that propionate and butyrate, major constituents of the large intestine, reduced SPI 1 invasion gene expression *in vitro* when supplied at physiologically relevant concentrations (32, 49). Therefore, we sought to understand how these fatty acids repress invasion genes. *In vitro* studies of the effects of these fatty acids on invasion genes have used varying concentrations of the fatty acids (12, 21, 31, 49). However, our recent *in vivo* work using a mouse model of infection showed the median propionate and butyrate concentrations in the cecum to be 8.5 mM and 12.8 mM respectively (32); therefore, for this work we proceeded with 10mM concentrations of the fatty acids. Using media buffered to pH 6.7 and 10 mM concentrations of either propionic acid or butyric acid, we used *lacZY* reporter fusions to the SPI 1 invasion genes, *sipC*, encoding a secreted effector protein, and *hilA* and *invF*, both transcriptional regulators. Using these strains, we found that there was a significant decrease in gene expression for each, more than 8-fold for *sipC* and 5-fold each for *invF* and *hilA*, in the presence of either propionic acid or butyric acid (Fig. 3.1 and data not shown). Additionally, previous work in our lab has shown that pH is important for the

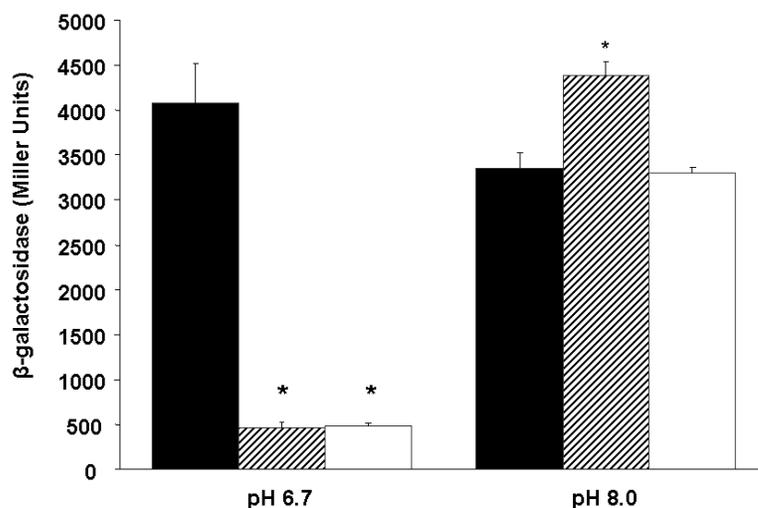


Figure 3.1. Propionate and butyrate repress *sipC* invasion gene expression. Cultures of wild type with the *sipC::lacZY* reporter fusion were grown overnight standing in LB with either 100 mM MOPS pH 6.7 or 100 mM HEPES pH 8.0, containing no additive (black bars), 10 mM propionic acid (striped bars), or 10 mM butyric acid (white bars). β -galactosidase assays were used to assess *sipC* expression. An asterisk (*) indicates a statistically significant difference from wild type with no additive at $p < 0.01$.

effects of acetate and formate on invasion gene expression, suggesting that accumulation of the fatty acid in the bacterial cytoplasm was necessary for the observed effects (41, 49). Thus, we also examined whether pH, and by extension, the ability of the fatty acid to enter the cytoplasm was necessary for the repressive effects of propionate and butyrate. In contrast to the repression observed when the medium was maintained at pH 6.7, using a pH of 8.0 we found that propionate and butyrate failed to have repressive effects on *sipC*, *invF*, or *hilA*. Instead, there was a slight increase in expression in the presence of propionate (Fig. 3.1 and not shown). Thus, these results show that pH is important for the repressive effects of propionate and butyrate and

suggest that these fatty acids must enter the bacterial cytoplasm to have their negative effects.

Since SPI 1 invasion genes are repressed in the presence of propionate and butyrate, we next examined whether these fatty acids had any effect on invasion of epithelial cells *in vitro*. Using wild type *Salmonella* grown overnight in media buffered to pH 6.7 with either no additive, 10 mM propionic acid, or 10 mM butyric acid, we found that propionic acid and butyric acid significantly decreased invasion of HEP-2 cells, by 2-fold and 5-fold, respectively (Fig 3.2). Therefore, these results, consistent with those of other studies (12, 22, 67), confirm that propionate and butyrate not only affect expression of invasion genes, but also decrease invasion of epithelial cells.

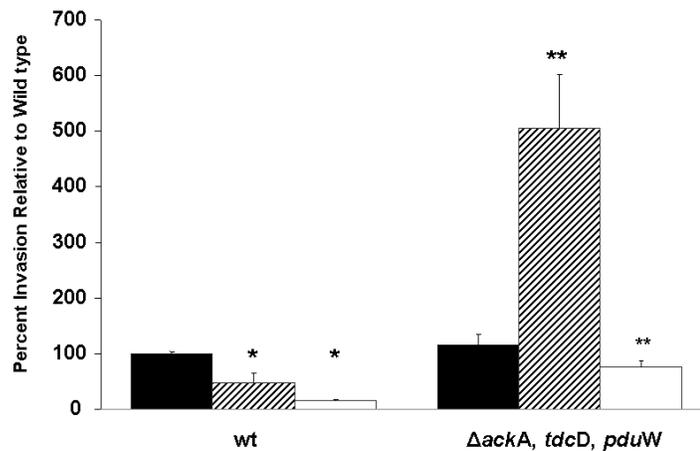


Figure 3.2. Propionate and butyrate significantly decrease invasion of HEP-2 cells in wild type but not in an *ackA*, *tdcD*, *pduW* mutant. Strains were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive (black bars), 10 mM propionic acid (striped bars), or 10 mM butyric acid (white bars). Invasion of HEP-2 cells was assessed using a gentamicin protection assay. Invasion of all strains and the various conditions are shown relative to wild type invasion in no additive which was set to 100%. An asterisk (*) indicates a statistically significant difference from wild type with no additive at $p < 0.01$. Two asterisks (**) indicates a statistically significant difference from wild type with propionate or butyrate $p < 0.01$.

Metabolism of propionate is necessary for its repressive effect on SPI 1 invasion genes. As pH and the ability of propionate and butyrate to enter the bacterial cytoplasm were important for their repressive effects on SPI 1 invasion genes, we next asked whether the fatty acids acted directly on invasion genes or whether they must be first converted to a metabolic product to have an effect. In enteric organisms, little is known about the metabolism of butyrate; however, the pathways for propionate metabolism have been well-characterized in *Salmonella* and *E. coli* (36-40, 58)(Fig 3.3).

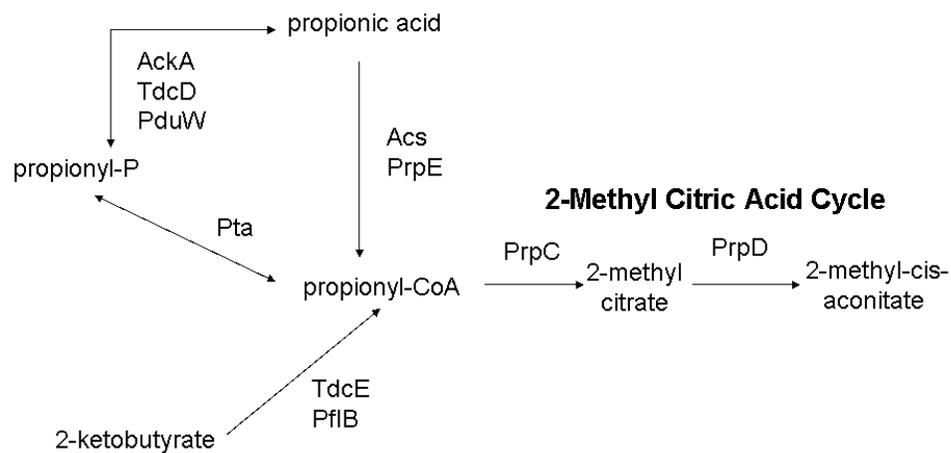
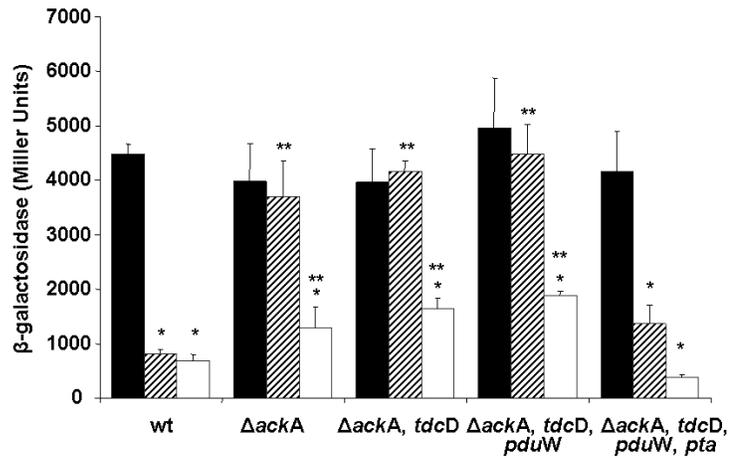


Figure 3.3. Diagram of known pathways and genes required for propionate metabolism

Therefore, we examined the genes in the propionate metabolic pathways to determine whether they were important for the repressive effects of propionate on SPI 1 invasion. There are two characterized pathways for the initial steps

of propionate metabolism, the propionate-kinase phosphotransacetylase pathway and the acyl CoA synthetase pathway, that both lead to the intermediate propionyl-CoA which then feeds into the methyl citric acid cycle (58). The propionate-kinase phosphotransacetylase pathway utilizes three genes, *ackA* which is an acetate kinase, and *tdcD* and *pduW* which are propionate kinases, that first metabolize propionic acid to propionyl phosphate (58). The propionyl phosphate is then metabolized to propionyl-CoA by a phosphotransacetylase encoded by *pta*, which is expressed in an operon with *ackA* (58). The acyl CoA synthetase pathway metabolizes propionate directly to propionyl-CoA without the propionyl phosphate intermediate, using two additional genes, *acs* and *prpE*, which encode acetyl CoA and propionyl-CoA synthetases (39). To determine whether metabolism of propionate was important for its repressive effect on invasion, we first examined the propionate-kinase phosphotransacetylase pathway. Using the *sipC::lacZY* reporter strain, we tested a non-polar null mutant of *ackA* and found that the mutation prevented the repression of *sipC* normally seen in the presence of propionate. There was a significant, nearly 4-fold, increase in gene expression when compared to wild type in the presence of propionic acid, and the mutation restored invasion gene expression to the level seen in the mutant with no additive (Fig. 3.4A). Additionally, the effect seen was specific for propionic acid, because invasion gene expression in the presence of butyrate was significantly increased but never reached the complete restoration observed with propionic acid (Fig. 3.4A). Thus, these results demonstrate that metabolism of propionate is necessary for the repressive effect on invasion genes.

A.



B.

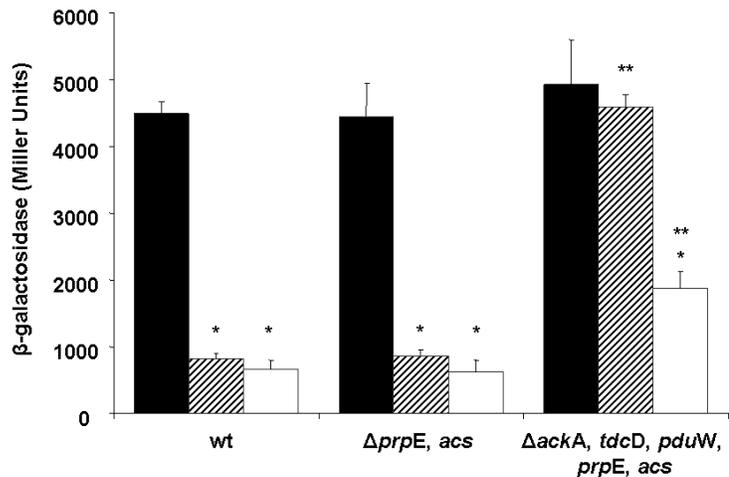


Figure 3.4. Metabolism of propionate is necessary for its repressive effect on invasion gene expression. Wild type and mutant strains with the *sipC::lacZY* reporter fusion were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive (black bars), 10 mM propionic acid (striped bars), or 10 mM butyric acid (white bars). β -galactosidase assays were used to assess *sipC* expression. A) Mutants containing deletions of genes in the propionate-kinase phosphotransacetylase pathway, B) Mutant containing deletions of the genes in the acyl CoA synthetase pathway and a mutant containing deletions of genes in both pathways. An asterisk (*) indicates a statistically significant difference from each respective strain with propionate or butyrate compared to no additive at $p < 0.01$. Two asterisks (**) indicates a statistically significant difference of a mutant strain grown in propionate compared to wild type with propionate or mutant strain grown in butyrate compared to wild type with butyrate at $p < 0.01$.

Although the loss of *ackA* appeared to prevent the repression of *sipC* expression by propionate, and this mutant was designed to be non-polar, *ackA* resides immediately upstream from *pta* in an operon. To determine whether the observed phenotype could be attributed solely to *ackA*, we created a complementation plasmid containing the *ackA* ORF and tested this plasmid in the *ackA* mutant containing the *sipC::lacZY* fusion. As expected, the *ackA* mutation with and without the pACYC177 cloning vector in the *sipC::lacZY* background restored invasion gene expression in the presence of propionic acid (Fig. 3.5).

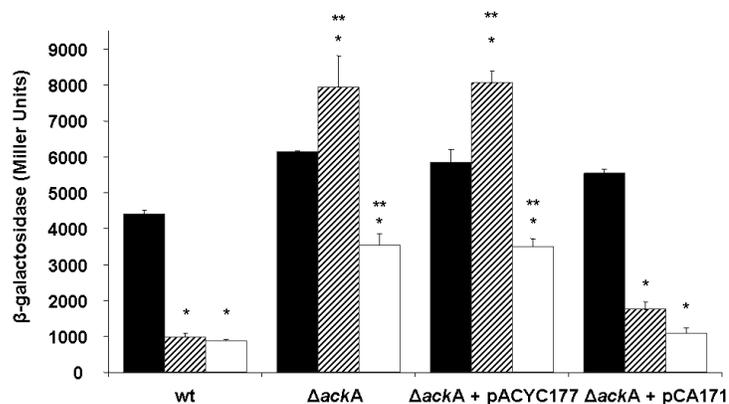


Figure 3.5. Complementation with *ackA* restores repression of invasion genes in the presence of propionate. A complementation plasmid containing the *ackA* ORF was created (pCA171) and it and the vector only control (pACYC177) were moved separately into the *ackA* mutant. These strains were tested along with the *ackA* mutant and wild type. All strains had the *sipC::lacZY* reporter fusion and were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive (black bars), 10 mM propionic acid (striped bars), or 10 mM butyric acid (white bars). β -galactosidase assays were used to assess *sipC* expression. An asterisk (*) indicates a statistically significant difference from each respective strain with propionate or butyrate compared to no additive at $p < 0.01$. Two asterisks (**) indicates a statistically significant difference of a mutant strain grown in propionate compared to wild type with propionate or mutant strain grown in butyrate compared to wild type with butyrate at $p < 0.01$.

The levels of expression observed in this figure for the mutant with no additive, with propionate, and with butyrate are slightly higher than that seen in Fig. 3.4A showing some variation in the results, however, even with the variation observed there is still a significant increase with butyrate and the complete restoration of gene expression in the presence of propionate in the *ackA* mutant as previously seen. Upon addition of the complementation plasmid containing the *ackA* ORF, however, we found that the repressive effects of propionic acid were restored (Fig. 3.5). These results thus show that *ackA* is necessary and sufficient for the negative effect of propionic acid.

As there are two additional genes, *tdcD* and *pduW*, that encode proteins that can metabolize propionic acid to propionyl phosphate, we next asked whether they were also important for the repressive effect of propionic acid. The increase in invasion gene expression with the double or triple mutant when compared to the single mutant was not statistically significant (Fig. 3.4A), and so these genes had slight additive effects on the restoration of expression in the presence of propionic acid at best. However, in contrast to the results seen at the level of gene expression, we found using a HEp-2 invasion assay, that invasion in the *ackA*, *tdcD*, *pduW* mutant was not only restored in the presence of propionic acid, but, in fact, was increased 10-fold when compared to the wild type with propionic acid and 5-fold when compared to the wild type with no additive (Fig. 3.2). These results show that metabolism of propionate is necessary for the repressive effect on invasion and suggest that propionate itself may affect other factors important for cell invasion in addition to the effect observed on invasion gene expression *in vitro*.

As we had shown that the acetate/propionate-kinase genes were important for the repressive effect of propionate on SPI 1 invasion genes, we next asked whether the genes in the acyl CoA synthetase pathway of propionate metabolism were necessary as well. We found that the loss of this pathway through null mutations in both necessary genes, *acs* and *prpE* (Fig. 3.3), did not alter the negative effect of propionic acid on invasion genes (Fig. 3.4B). To further address this question, we also examined the effects of deleting all the known genes necessary for propionate metabolism, *ackA*, *tdcD*, *pduW*, *acs*, and *prpE*, and found there was not a significant increase in restoration when compared with the *ackA* single mutant, the *ackA*, *tdcD* double, or the *ackA*, *tdcD*, *pduW* triple mutants (Fig. 3.4). These results thus show that the metabolic pathway leading through propionyl phosphate is the important pathway for the effects on SPI 1 invasion genes.

Propionyl phosphate is not the important intermediate for the repressive effect of propionate. SPI 1 invasion gene expression was restored in the *ackA*, *tdcD*, *pduW* mutant in the presence of propionic acid, suggesting that the metabolic intermediate created by these protein products, propionyl phosphate, might be important for this negative effect. To determine the importance of propionyl phosphate for the repressive effect of propionate, we examined invasion gene expression using a strain with all the known pathways to propionyl phosphate deleted (Fig. 3.3), an *ackA*, *tdcD*, *pduW*, *pta* mutant, using a *sipC::lacZY* fusion. If propionyl phosphate negatively affected invasion genes, this strain would be expected to have a phenotype identical to that of the *ackA*, *tdcD*, *pduW* mutant. Surprisingly, we found that in the presence of propionic acid invasion gene expression in the *ackA*, *tdcD*, *pduW*, *pta* mutant was significantly lower than in the *ackA*, *tdcD*, *pduW* mutant under the same

conditions and also lower than in the *ackA*, *tdcD*, *pduW*, *pta* without the addition of propionic acid. Furthermore, invasion gene expression in this strain was not significantly higher than wild type with propionic acid (Fig. 3.4A). Thus, the addition of the *pta* mutation restored the repressive effect of propionate on invasion genes, demonstrating that propionyl phosphate is not the important intermediate for the negative effect on invasion genes.

The intermediate propionyl-CoA is important for the repressive effect of propionate on SPI 1 invasion. Previous work suggests that entry into the 2-methyl citric acid cycle and the intermediate 2-methyl-citrate are important for the toxic effects of propionate on growth of *Salmonella* (37) (Fig. 3.3). Therefore, we hypothesized that this might also be the case for its effect on SPI 1 invasion gene expression. Entry into the 2-methyl citric acid cycle, as well as production of 2-methyl citrate, requires the gene *prpC* (40). Additionally, *gltA* has been proposed to play a lesser role to *prpC* in the production of 2-methyl citrate from propionyl-CoA (37). To test our hypothesis, we examined null mutations of *prpC* and *gltA* alone, and *prpC* and *gltA* together using the *sipC::lacZY* fusion strain. We found that invasion gene expression in the presence of propionic acid was the same as that seen in wild type for all three strains (Fig. 3.6 and not shown). To further confirm that the 2-methyl citric acid cycle and 2-methyl-citrate production were not necessary for the repressive effect of propionate on invasion gene expression, we also examined a null mutation of *prpD*, the next gene in the cycle, required for conversion of 2-methyl citrate to 2-methyl-cis-aconitate (38). However, once more, invasion gene expression was the same in the *prpD* mutant as in wild type (not shown). Thus, these results show that neither entry into the 2-methyl

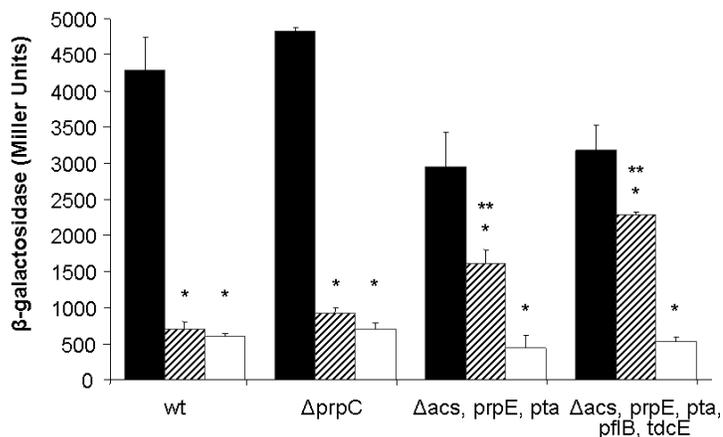


Figure 3.6. Production of the intermediate propionyl-CoA is important for the repressive effect of propionate. Wild type and mutant strains with the *sipC::lacZY* reporter fusion were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive (black bars), 10 mM propionic acid (striped bars), or 10 mM butyric acid (white bars). β -galactosidase assays were used to assess *sipC* expression. An asterisk (*) indicates a statistically significant difference from each respective strain with propionate or butyrate compared to no additive at $p < 0.01$. Two asterisks (**) indicates a statistically significant difference of a mutant strain grown in propionate compared to wild type with propionate or mutant strain grown in butyrate compared to wild type with butyrate at $p < 0.01$.

citric acid cycle nor the intermediate 2-methyl-citrate is required for the repressive effect of propionate.

Since propionyl phosphate and the 2-methyl citric acid cycle were not important for the repressive effect of propionate on invasion genes, we hypothesized that propionyl-CoA may be important for the negative effect of propionate. Propionyl-CoA is an intermediate in the propionate metabolic pathway and can be produced from propionate directly by the products of the genes *acs* and *prpE*, or indirectly from propionyl phosphate through *pta* (39, 58) (Fig. 3.3). Therefore, to address the question of whether propionyl-CoA production was important for the negative effect of propionate, we next created

null mutants of *prpE*, *acs*, and *pta* in the *sipC::lacZY* fusion strain. Using this strain, we found a 2-fold increase in invasion gene expression in the presence of propionic acid when compared to wild type with propionic acid (Fig. 3.6). However, there are previous reports that *tdcE* and *pfl* can produce propionyl-CoA from endogenous sources (36). Thus, we created a mutant that deleted all known pathways for the production of propionyl-CoA from endogenous and exogenous sources (a *prpE*, *acs*, *pta*, *pflB*, *tdcE* mutant). We found that invasion gene expression was increased 3-fold from the wild type level in the presence of propionic acid (Fig. 3.6). Additionally, for both the *prpE*, *acs*, *pta* mutant and the *prpE*, *acs*, *pta*, *pflB*, *tdcE* mutant, the increase in invasion gene expression seen in the presence of propionic acid was specific for propionic acid, as the same effect was not observed with butyric acid. Therefore, these results suggest that production of propionyl-CoA from propionate is necessary for the negative effect of propionate.

Effect of propionic acid and butyric acid on phase variation of flagellin.

SPI 1 not only encodes genes necessary to produce a type three secretion system (TTSS), it also encodes several of the effector proteins secreted by the TTSS (53). Thus, since propionate and butyrate repressed SPI 1 invasion gene expression, we hypothesized that it would also decrease the expression of the secreted effector proteins. To test this hypothesis we examined secreted proteins of *Salmonella* grown in media buffered to pH 6.7 with either: no additive, 10 mM propionic acid, or 10 mM butyric acid. Using an SDS PAGE we found that propionate and butyrate mildly decreased the secreted effector proteins, but more interestingly, there were changes in two unknown proteins upon treatment with either fatty acid (Fig. 3.7). One protein found just below 50 kDa was heavy in wild type with no additive, however, upon

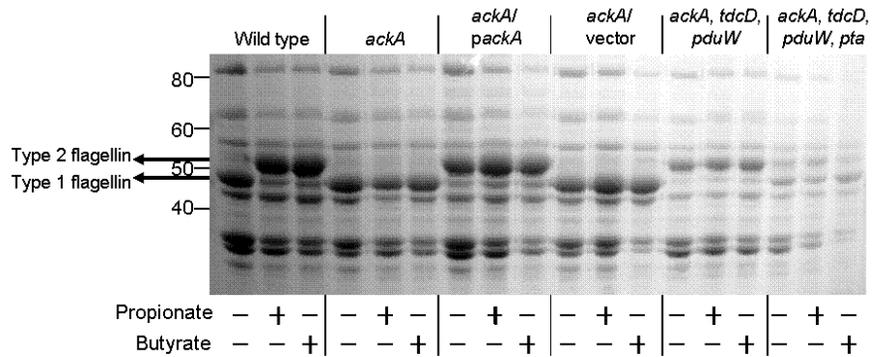


Figure 3.7. Effect of propionate and butyrate on secreted proteins. Wild type and mutant strains were grown overnight in LB with 100 mM MOPS pH 6.7 containing either no additive, 10 mM propionic acid, or 10 mM butyric acid. Secreted proteins were isolated and separated by 10% SDS PAGE. Strains are listed on the horizontal axis above the gel and growth conditions are shown on the horizontal axis below the gel. Molecular weights in kDa are shown along the left vertical axis of the gel.

treatment with either 10 mM propionic acid or 10 mM butyric acid the intensity of this band was decreased and a distinct band appeared just above 50 kDa. To determine the identity of the proteins represented by these bands, MALDI-TOF analysis was utilized. Upon comparison of the sequence obtained by MALDI-TOF, we found that proteins of both molecular weights were flagellin. The protein with the apparent molecular weight of 56 kDa in the samples treated with fatty acids was identified as type 2 flagellin, encoded in *Salmonella* by *fljB*, while the protein with an apparent molecular weight of 52 kDa, more abundant in cultures with no additive, was identified as type 1 flagellin, encoded by *fliC*. Therefore, these results suggest that these fatty acids may cause a change in flagellar phase variation between type 1 and type 2 flagellin.

DISCUSSION

For *Salmonella* to cause disease it must first invade the intestinal epithelium and to do so it requires genes encoded in SPI 1. In this work, we show that propionic acid and butyric acid, at a pH and with concentrations comparable to those seen in the murine intestinal tract (32), repress SPI 1 invasion genes and reduce invasion of epithelial cells. These results are consistent with previous studies examining varying concentrations of these fatty acids as well as various pH levels (12, 22, 31, 67). Therefore, we sought to further understand the mechanism by which these fatty acids repress invasion and since little was known about butyric acid metabolism in *Salmonella*, we concentrated on characterizing the effect of propionic acid. We found that deletion of the genes necessary for production of the propionyl phosphate intermediate from propionate restored invasion gene expression in the presence of propionate whereas deletion of the genes necessary for directly metabolizing propionate to propionyl-CoA did not. These results thus implicated two intermediates of propionate metabolism, propionyl phosphate and propionyl-CoA, as possibly important for the repressive effect of propionate. Upon deletion of *pta*, the phosphotransacetylase that can reversibly interconvert propionyl phosphate and propionyl-CoA, however, the restoration of invasion gene expression was abrogated. One possible explanation for these results is that for metabolism of propionate in the conditions used for this study, the propionyl-kinase phosphotransacetylase pathway is the major route for propionate metabolism. Therefore, when this pathway is no longer present, propionyl phosphate is not being produced by the normal pathway. However, the bacterium may use the acyl-CoA synthetase pathway alternatively to metabolize propionate. When this occurs,

since propionyl phosphate is not being produced by its normal pathway, it is possible that the *pta* gene may utilize propionyl-CoA for production of propionyl phosphate thus decreasing the repressive effect of propionyl-CoA. However, when *pta* is deleted, propionyl-CoA may accumulate due to the loss of the pathways by which it is metabolized, causing the repressive effect on invasion gene expression. Further evidence for the involvement of propionyl-CoA in repression of invasion gene expression is the fact that upon deletion of the genes necessary for metabolism of exogenous propionate, there is a two-fold increase in invasion gene expression in the presence of propionate when compared to wild type in the presence of propionate. Also, a mutant that has genes deleted in the known pathways for production of propionyl-CoA from both endogenous and exogenous sources, a *prpE*, *acs*, *pta*, *pflB*, *tdcE* mutant, has a 3-fold increase in invasion gene expression in the presence of propionate when compared to wild type with propionate. However, there is not full restoration in either of these mutants, suggesting that there may be an as yet uncharacterized propionyl synthetase enzyme that is able to metabolize propionate to propionyl-CoA, thus preventing complete restoration of invasion gene expression. Further evidence to support this hypothesis is that a mutant that blocks the known pathways of propionate metabolism (*ackA*, *tdcD*, *pduW*, *acs*, *prpE*) has restored invasion gene expression in the presence of propionate (Fig. 3.4B). However, further mutation of *pta* in this mutant restores repression of invasion gene expression (not shown), suggesting that propionyl-CoA can still be produced from propionate in this strain. Additionally, if entrance into the 2-methyl citric acid cycle is blocked, propionic acid still has a repressive effect on invasion genes, suggesting that propionyl-CoA or some derivative of it is responsible for the observed effect.

Therefore, we next asked how propionyl-CoA could have this negative effect on invasion genes. One possible model of how propionyl-CoA represses invasion is by the inactivation of proteins through the addition of a propionyl moiety donated by propionyl-CoA. Propionyl-CoA has previously been reported to inactivate PrpE via N-Lysine propionylation (33). For this to occur, Pat (Protein Acetyltransferase) transfers a propionyl group from propionyl-CoA to a lysine residue on PrpE, which contains the motif PXXXXGK, thus inactivating the protein (33, 64). Therefore, we hypothesized that propionyl-CoA might repress invasion genes by protein inactivation. Previous work has shown that the Fad system, involved in long chain fatty acid oxidation, regulates invasion genes in *Salmonella* (54). FadD is an acyl CoA synthetase that is also necessary for transport of long chain fatty acids into the cell and FadR is a transcriptional regulator of the *fad* (fatty acid degradation) and *fab* (fatty acid biosynthesis) genes (27). Additionally FadK has recently been identified as an anaerobic acyl CoA synthetase (13). Upon examining the protein sequence of both FadD and FadK, we found that both had the PXXXXGK motif important for propionylation. Thus, we examined a *pat* mutant, single mutants of *fadD*, *fadK*, and *fadR*, and double mutants of *fadD,fadK* and *fadD,fadR* in the presence of propionic acid and found that invasion gene expression was still repressed in all the strains (not shown). Therefore, from these results we concluded that propionylation by *pat* was not important for the repressive effect of propionate on invasion genes nor were proteins known to be required for fatty acid transport. There still remains the possibility however, that propionyl-CoA exerts its repressive effect by modification of other, uncharacterized proteins.

Although our results suggest that propionate must be metabolized to propionyl-CoA to have the observed repressive effect on invasion genes, one other possible model to explain our results that cannot be completely ruled out is that it is acetyl-CoA rather than propionyl-CoA that is responsible for the negative effect on invasion genes. Many of the genes necessary for propionate metabolism are also involved in acetate metabolism. Acetate kinase, the product of *ackA* can reversibly interconvert acetate to acetyl phosphate, while phosphotransacetylase, encoded by *pta*, reversibly interconverts acetyl phosphate to acetyl-CoA. Acetyl-CoA synthetase, encoded by *acs*, can metabolize acetate directly to acetyl-CoA (65). Thus, it is possible that the effect we observe in our deletion mutant of *ackA* could be a result of changes in the metabolism of acetyl-CoA to acetyl phosphate. However, if the effect we observe was a result of changes in acetate metabolism, it would be expected that the same effect would be observed with acetate as seen with propionate; previous results show that this is not the case (49). Also, the only known pathway for metabolism of propionate to acetyl-CoA is through the 2-methyl citric acid cycle. The results in this paper show that deletion of *prpC*, the gene necessary for entry into the 2-methyl citric acid cycle, does not affect the repressive effect of propionate on invasion genes. Thus, though we cannot completely rule out this model, our results suggest that propionyl-CoA, and not acetyl-CoA, is the important metabolic intermediate.

Using a HEP-2 invasion assay, we also found that propionate significantly decreased invasion of epithelial cells consistent with the results observed for invasion gene expression. For invasion genes, we had found that metabolism of propionate was important for the repressive effect, with

invasion gene expression restored in the presence of propionic acid for an *ackA*, *tdcD*, *pduW* deletion mutant. Thus, we examined this same mutant using a HEP-2 invasion assay and found that in the presence of propionic acid that invasion was not only restored to the level of wild type without propionic acid but was actually increased 5-fold and that in the presence of butyric acid that invasion was restored to the level of wild type. These results were surprising as the same level of increase was not observed in the assays examining invasion gene expression; however, the invasion assay measures more than just invasion genes. In *Salmonella*, the invasion process is complex involving multiple virulence factors (42), so it is possible that propionate affects other factors important for invasion. In fact, data suggest that propionate affects flagellin production and motility (not shown) as well. Thus, it is possible that the increase in invasion is a result of the effect on invasion genes in combination with one or more other virulence factors.

In this work, we also found that propionate and butyrate caused changes in flagellin expression. In *Salmonella*, there are two genes, *fliC* and *fliB*, that encode flagellin and they are not expressed at the same time as a result of phase variation. In this work, we found that there was a shift from type 1 flagellin (encoded by *fliC*) to type 2 flagellin (encoded by *fliB*) in the presence of both propionate and butyrate. These results suggest that environmental conditions may be important for determining which type of flagellin is expressed. Consistent with these results, previous work examining *fliC* expression found that *fliC* expression is decreased under *in vitro* conditions resembling the intracellular environment whereas expression is present in the Peyer's Patches of mice *in vivo* (17). Therefore, these results suggest that the bacterium may use phase variation to alter its flagellin

expression in response to environmental signals in the intestinal tract. However, further work is necessary to characterize this phenomenon.

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CHAPTER 4

Molecular identification of veterinary yeast isolates using sequence-based analysis of the D1/D2 region of the large ribosomal subunit*

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ABSTRACT

Conventional methods of yeast identification are often time-consuming and difficult; however, recent studies of sequence-based identification methods have shown promise. Additionally, little is known about the diversity of yeast identified from various animal species in veterinary diagnostic laboratories. Therefore, in this study, we examined three methods of identification using 109 yeast isolated during a one year period from veterinary clinical samples. Comparison of the three methods, traditional substrate assimilation, fatty acid profile analysis, and sequence-based analysis of the D1/D2 region of the large ribosomal subunit showed that sequence analysis provided the highest percent identification among the three. Sequence analysis identified 87% of isolates to the species level whereas substrate assimilation and fatty acid profile analysis identified only 52% and 47% respectively. Less stringent criteria for identification increased the percent of isolates identified with each method to 98% for sequence analysis, 61% for substrate assimilation, and 55% for fatty acid profile analysis. We also found that sequence analysis of the ITS2 region provided further identification for 36% of yeast not identified to the species level by D1/D2 sequence analysis. Additionally, we identified a large variety of yeast from animal sources, with at least 30 different species among the isolates tested, and with the majority not belonging to the common *Candida* sp. such as *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* group. Thus, we determined that sequence analysis of the D1/D2 region was the best method for identification of the variety of yeast found in a veterinary population.

INTRODUCTION

In both veterinary and human diagnostic laboratories, correct identification of yeasts is important for the care of patients. Traditionally, yeast identification has been performed using biochemical analysis, substrate assimilation methods, morphological examination, or by various combinations of the three. To increase the ease of identification, commercial tests have been created that use these methods. For example, the Yeast API 20C AUX strip (bioMérieux) primarily uses substrate assimilation, Uni-Yeast-Tek (Remel) combines substrate assimilation, biochemicals, and morphology, and API Candida (bioMérieux) utilizes biochemical reactions for identification. Even with the convenience provided by these methods, identification of yeasts by these conventional applications can still be time-consuming and difficult. In addition, considerable variability in the efficacy of these methods has been reported for identification of clinically important yeast (11, reviewed by 12, reviewed by 32, 36, 42). The variation in ability to identify yeast isolates correctly can be attributed to the limitations of the databases used for the comparison of clinical isolates, as well as the subjectivity involved in interpretation of results. In addition to traditional biochemical and assimilation methods, analysis of fatty acids using gas chromatography (GC) has also been investigated as a method of yeast identification. One particular system, the Sherlock Microbial Identification System (MIDI), determines the profile of fatty acids produced by the yeast and compares that profile to an existing database to determine the identification. This method decreases the subjectivity in interpreting the results and decreases turn-around time; however, previous studies have reported limitations with the database, as well as variability in identification depending upon the growth medium (9, 17, 18).

Recent studies have examined the effectiveness of various molecular identification methods for yeast using ribosomal RNA genes, such as sequence analysis, multiplex PCR, real-time PCR, microarray technology, PCR-based fragment length polymorphisms and restriction fragment length polymorphisms (reviewed by 32). Ribosomal RNA genes are used as the targets in these tests because they contain both regions of DNA sequence that are conserved among yeast genera and those that vary among species. For yeast, the ribosomal RNA genes consist of the adjacent small subunit, the internal transcribed spacer 1 (ITS1), the 5.8S, the internal transcribed spacer 2 (ITS2), and the large subunit, the last of which includes the D1/D2 region at its 5' end (reviewed by 31, and 32). Of these regions, the ITS1, ITS2, and the D1/D2 region have been shown to be the most useful for species-level identification of yeast, as a result of the variability within these regions (reviewed by 14, and 32). In particular, sequence analysis of these regions has shown great promise in the practice of clinical mycology, with several large-scale studies showing these regions to differentiate clinical yeast isolates obtained from humans to the species level (3, 4, 6, 13, 24, 26, 33). Advantages of sequencing include increased objectivity of result interpretation, shorter turn-around times, and a better characterization of unusual organisms as a result of the phylogenetic information that molecular methods provide.

In human medicine the species of yeasts cultured from patients is limited, with *Candida albicans* being the predominant species isolated (19, 30). In contrast, veterinary yeast isolates can be cultured from a wide variety of animal species, allowing for the possibility of more diversity among the isolates identified. Though much work has been performed examining phenotypic and genotypic methods of yeast identification from veterinary sources, the majority

of these studies have concentrated either on a single species of animal or yeast (2, 15, 16, 28, 29). To our knowledge, there has been only one large-scale examination of multiple yeast and animal species to assess yeast identification (5). That study used conventional phenotypic tests for identification of these organisms; no studies examining sequence-based analyses have been performed.

Therefore, in this work, we examined the variety of yeast seen in a veterinary diagnostic laboratory during a one year time period, and determined the feasibility and effectiveness of identification by a traditional phenotypic method, a method analyzing fatty acid profiles, and a sequence-based molecular method. We found that sequence analysis could provide identification to the genus and species level for a much higher percentage of the isolates tested than either of the phenotypic methods. We also found that there is great diversity in the yeast identified from veterinary sources, with at least 30 different species identified among the 109 isolates tested and only 48% belonging to the more common *Candida* sp. such as *C. albicans*, *C. parapsilosis* group, *C. glabrata*, and *C. tropicalis*. Additionally, seven percent of the isolates were identified as *Arxula adenivorans*, and this more unusual isolate was found only in horses and dolphins.

MATERIALS AND METHODS

Selection of yeasts and growth conditions. Yeasts used in this study were obtained from the culture of clinical veterinary samples during the year 2007 by the Animal Health Diagnostic Center of Cornell University. All isolates that could be retrieved as pure cultures from frozen archived stocks were used, constituting 91% of the total isolates from that year. Isolates were plated

initially onto Sabouraud Dextrose agar from which an isolated colony was sub-cultured onto the same medium for 24 hours at 28°C. Cultures were then harvested for the identifications methods described below.

Identification by nutrient assimilation. Sufficient quantities of yeasts were harvested from agar medium to prepare a suspension in water equivalent to a McFarland 2 standard. Then, 100 µl of this suspension was transferred to an ampule of API C Medium and samples were inoculated into the cupules of an API 20 C AUX test strip (bioMerieux, Durham, NC) and incubated for 72 hours at 29°C. The test strips were visually interpreted at 48 and 72 hours post inoculation according to the recommendations of the manufacturer, with each strip containing a cupule lacking nutrients to serve as the negative control. Positive tests were defined by turbidity greater than that of the control when examined at either time point. As a positive control, all samples were tested in conjunction with *Candida guilliermondii* ATCC 6260. This test produced a numerical profile that was then compared to a database provided by the manufacturer (<https://apiweb.biomerieux.com>).

Fatty acid-based identification. Yeast isolates were harvested from the second and third quadrants of agar medium to obtain organisms in the log phase of growth. Fatty acid methyl esters were produced and then extracted using the protocol recommended by the manufacturer (MIDI, Inc., Newark, DE). Fatty acid analysis was performed using an Agilent Technologies 6890N gas chromatograph system. The Sherlock MIS YEAST28/YSTCLN software version 6.0 was used for analysis. Samples were accompanied by the reference strain *Candida albicans* ATCC 14053 as the positive control, and a reagent blank as the negative control. The system was calibrated using a calibration mix supplied by the manufacturer (MIS; MIDI Inc., No. 1200-A

Calibration Standards Kit). This method generated a Similarity Index (SI), with an SI of 1.000 indicating complete identity.

Sequence-based identification. A small portion of a single yeast colony was placed in 5 µl of Lyse and Go reagent (Pierce Protein Research Products, Rockford, IL) and placed in the thermocycler using the settings recommended by the manufacturer for genomic DNA. Upon completion, 45 µl of a PCR master mix containing 300 nM concentration of each primer was added to the Lyse and Go reaction. For amplification of the D1/D2 region of the large ribosomal subunit, primers NL1 and NL4 were used (20). For amplification of the ITS region, primers ITS5 and ITS4 were used (44). Samples were then placed in the thermocycler using the following settings: 94°C for 2 min, then 30 cycles of 94°C for 30 sec, 52°C (NL1 and NL4) or 54°C (ITS5 and ITS4) for 45 sec, and 72°C for 90 sec, with a final elongation step of 72°C for 10 min. PCR reactions were then visualized using a 1% agarose gel. PCR products were prepared for sequencing using ExoSAP-IT (USB, Cleveland, OH), a product which removes remaining primers and dNTPs using a combination of Exonuclease I and Shrimp Alkaline Phosphatase. Sequencing was then performed by the Cornell University Sequencing Center using primer Seq-NL1, ATCAATAAGCGGAGGAAAAG, or ITS3 (44). This protocol was used for all yeasts in this study, except for one isolate eventually identified as *C.*

lusitaniae. As a result of previously reported polymorphisms in the D1/D2 region (22), Seq-NL1 did not provide sequence long enough to be used for identification, so the NL4 reverse primer was used to sequence this isolate. Sequences were then uploaded to the Ribosomal Database Project (RDP) Pipeline (8) for sequence editing and quality analysis and were then downloaded and further edited manually if necessary. Sequences were then

compared to the non-redundant NCBI database using BLASTN, with the default settings used to find the most similar sequence and were sorted by the E score. To be considered a good identification to the species level, the identification had to meet the following criteria: i) sequence identity $\geq 99.0\%$, ii) $\geq 1.0\%$ separation from the next closest species, iii) $\geq 90.0\%$ sequence coverage for the matching sequence, and iv) matching sequence published in a peer reviewed journal article or submitted by the ATCC. For identification to the genus level, all of the above criteria were used, except that a sequence identity of $\geq 97.0\%$ was used and 1.0% separation from the next species was not required. NCBI and the doctor fungus website (<http://www.doctorfungus.org>) were used to determine whether various yeast names were anamorphs, teleomorphs, or synonyms. Additionally, as different naming schemes exist for various yeast species, we used the following rules to remain consistent among the three identification methods: i) isolates of *C. albicans* and *C. africana* were always called *C. albicans*, ii) isolates of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* were all called *C. parapsilosis* group, and iii) all variants of *Cryptococcus neoformans* were called *C. neoformans*.

Creation of the phylogenetic tree. A representative sequence from each species or isolate, in the cases where species-level identification was not reached, was aligned using CLUSTAL W2 for multiple alignment with the default settings (23) (<http://www.ebi.ac.uk/Tools/clustalw2>). The multiple alignment file was then used to create a neighbor-joining phylogram with CLUSTAL W2. The *C. lusitaniae* sequence was excluded from the tree, since the sequence was obtained using the reverse primer and therefore would not align properly with the other sequences.

RESULTS

Sequence analysis of the D1/D2 region provides better identification of veterinary yeast isolates than either substrate assimilation or fatty acid profile analysis. In this study, we included 109 yeasts isolated during a one year period in the Cornell University College of Veterinary Medicine Animal Health Diagnostic Center, and representing 91% of the yeasts isolated from clinical cases submitted to the laboratory for that year. Yeast isolates were obtained from a variety of animals and sources with representatives from mammals, reptiles, amphibians, and avians (Table 4.1).

Table 1. Animals included in study and most common yeast species isolated from each animal group

Animals (n)	No. of different yeast species isolated	Most common yeast species isolated (% of total)
Avians (10)	7	<i>Candida albicans</i> (40%)
Amphibians (1)	1	<i>Debaryomyces nepalensis</i> (100%)
Mammals (84):		
Bovines (8)	4	<i>Candida glabrata</i> (50%)
Canines (10)	4	<i>Candida albicans</i> (70%)
Dolphins (8)	3	<i>Arxula adenivorans</i> (50%)
Equines (42)	20	<i>Candida parapsilosis</i> group (14%)
Felines (7)	3	<i>Candida parapsilosis</i> group (57%)
Other (9)	6	<i>Candida albicans</i> (44%)
Reptiles (14)	11	<i>Candida tropicalis</i> (21%)

The class of mammals, which comprised the majority of the isolates (77%), was further subdivided into bovines, canines, dolphins, equines, and other species, this last group being comprised of animal species represented only once in this study.

To determine the most effective method of identification for yeasts in the veterinary clinical laboratory, we used three methods of identification, comparing two commercially available phenotypic methods to sequence analysis of the D1/D2 region of the large subunit rRNA gene. The first commercial method was a traditional phenotypic method of substrate assimilation that determines the ability of the organism to grow in cupules containing one of 19 different carbohydrate sources (API 20 C AUX). Using this method, a 7-digit code was generated based upon the presence or absence of growth, and the code was then compared to a database provided by the manufacturer. We considered an identification classified by the manufacturer as acceptable or better to the species level to be a reliable identification. The second commercial phenotypic method was fatty acid profile analysis (Sherlock Microbial Identification System). This method uses GC analysis to determine the fatty acid profile of the yeast. The software provided then compares the profile to a database and provides a similarity index, with the confidence in the identification based on the degree of similarity to known organisms within the database. For this method, based on the manufacturer's recommendations, a similarity index of ≥ 0.500 with 0.100 separation from the next match was considered a good identification. For sequence analysis, we sequenced the D1/D2 region of the large ribosomal subunit and used the CLSI recommendations for bacterial sequence analysis to assist in interpretation of our results (7). Thus, for sequence analysis an

identification of $\geq 99\%$ sequence similarity with at least 1% separation from the next closest species was considered a good identification. Using these methods and interpretive criteria, we found that 52% of the isolates tested using substrate assimilation, 47% using fatty acid profile analysis, and 87% using D1/D2 sequence analysis could be identified to the species level (Table 4.2). These results show that sequence analysis provided the highest percent identification to the species level of the three methods.

Table 2. Percent Identification of yeast isolates by all methods tested

	% ID (species level*)	% ID (genus level or lower stringency**)
Substrate Assimilation	52	61
Fatty Acid Profile Analysis	47	55
D1/D2 Sequence Analysis	87	98
ITS2 Sequence Analysis	79	90

*Substrate assimilation-acceptable ID or greater to species level, fatty acid profile analysis- ≥ 0.500 similarity index with 0.100 separation between species, and sequence analysis- $\geq 99\%$ sequence identity with $\geq 1\%$ separation between species.

**Substrate assimilation-acceptable ID or greater to species or genus, fatty acid profile analysis- ≥ 0.300 similarity index with 0.100 separation between species, and sequence analysis- $\geq 97\%$ sequence identity without the requirement for 1% separation if the two most similar sequences are the same genus.

We next examined whether the use of less stringent identification criteria would increase the percent identification for any of the methods. For

substrate assimilation, we considered identification defined by the manufacturer as acceptable to either genus or species level, for fatty acid analysis we used a similarity index ≥ 0.300 with 0.100 separation, and for sequence analysis we expanded the criteria to include sequence identity of $\geq 97\%$ without the requirement of $\geq 1\%$ separation if both the most similar and the second most similar sequence were of the same genus. Using these less stringent criteria, we found an increase in the number of yeast isolates identified by both substrate assimilation and fatty acid analysis, to 61% and 55% identification, respectively (Table 4.2). However, for D1/D2 sequence analysis we found that we could identify almost all isolates to the genus level using this criteria with only two isolates not identified (Table 4.2). These results thus show both that D1/D2 sequence analysis is the better method for identification of veterinary yeast isolates to the level of species and that the great majority of such isolates can be identified to at least the genus level.

Comparison of yeast identifications derived from each method tested.

To further examine the three identification methods, we next compared the identification determined by each method. Since sequence analysis provided the identification to species level for the greatest number of these isolates, we used sequencing as the reference method and compared the identifications determined by the other methods to the sequencing results using isolates that had been identified to the species level by D1/D2 sequencing. For this analysis, we used yeasts for which we had identified at least two isolates for any given species, a total of 82 isolates. We found that substrate assimilation identified only 50% of the isolates to the species level correctly. Of members of the genus *Candida* identified in this study, the most common of the genera, it identified correctly to the species level 14 of the 18 *Candida albicans* isolates,

12 of 13 *Candida parapsilosis* group isolates, 10 of 12 *Candida glabrata* isolates, and 3 of 9 *Candida tropicalis* isolates (Fig. 4.1).

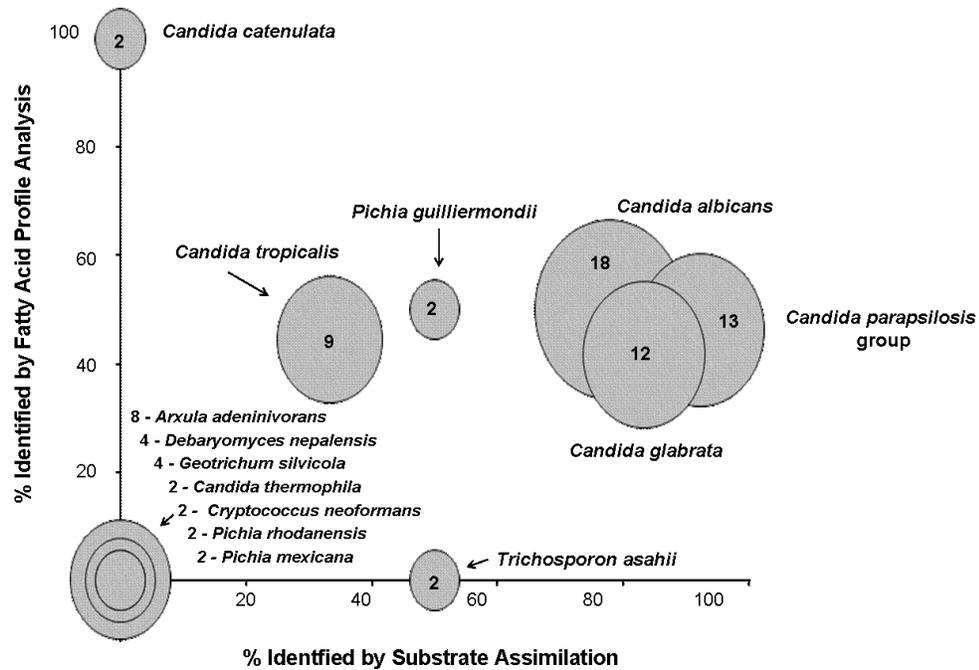


Figure 4.1. Comparison of identifications provided by substrate assimilation and fatty acid profile analysis to D1/D2 sequencing results. The x-axis and y-axis shows the % of isolates for each species that substrate assimilation and fatty acid profile analysis identified concordantly with the D1/D2 sequencing results respectively. The size of the bubbles is proportional to the number of yeast isolates for each species and the numbers inside the bubbles or next to the bubbles gives the exact number. Only isolates with a good identification to the species level by D1/D2 sequence analysis and where n=2 were included in this comparison for a total of 82 isolates.

For *Trichosporon asahii* and *Pichia guilliermondii* we found that nutrient assimilation could identify 1 of 2 isolates correctly for each species. All of the remaining 26 isolates identified by sequencing failed to be identified correctly by the nutrient assimilation method. These species ranged in prevalence from eight isolates (10%) for *Arxula adenivorans* to two isolates for each of *Candida thermophila*, *Cryptococcus neoformans*, *Pichia mexicana*, and *Pichia rhodanensis* species (Fig. 4.1). In comparison to DNA sequencing, fatty acid profile analysis identified 33% of the isolates correctly to the species level (Fig. 4.1). Only one species, *Candida catenulata*, was correctly identified in all cases, with both of two isolates being identified (Fig. 4.1). However, for all other species, none was correctly identified more than 50% of the time. More specifically, correct identifications were obtained for 9 of 18 *Candida albicans* isolates, 6 of 13 *Candida parapsilosis* group isolates, 5 of 12 *Candida glabrata* isolates, 4 of 9 *Candida tropicalis* isolates, and 1 of 2 *Pichia guilliermondii* isolates (Fig. 4.1). The rest of the isolates, 26 of 82, were not identified correctly. These results thus suggest that, although phenotypic methods may provide a species identification, such identifications may frequently be incorrect, as we found that 50% of those defined by nutrient assimilation and 67% by fatty acid analysis failed to agree with that determined by sequencing.

Sequence analysis of the ITS2 region of yeast isolates. As we found that the identity of 13% of the yeast isolates could not be determined to the species level by sequence analysis of the D1/D2 region, we next examined whether sequence analysis using the ITS2 region would be useful for further identification of these isolates. We used a subset of the isolates described in this study, with 90 yeast isolates being selected, including the 14 isolates not previously identified to the species level by sequencing of the D1/D2 region.

The entire ITS region was amplified and the ITS2 region was sequenced. Using the same criteria for identification as previously described for D1/D2 analysis, we found that sequence analysis of the ITS2 region could identify 79% of the 90 yeast isolates analyzed to the species level and 90% to the genus level (Table 4.2). We also found that sequence analysis of the ITS2 region could further identify 5 of the 14 yeast not previously identified by D1/D2 analysis to the species level. Upon comparison, we found that the two methods showed agreement on the identification of isolates 91% of the time. In addition, the 9% that did not agree consisted of only two species: *Geotrichum silvicola* and *Debaryomyces nepalensis* identified by analysis of the D1/D2 region were identified respectively as *Galactomyces geotrichum* and *Debaryomyces hansenii* using the ITS2 region. For the *Geotrichum* isolates, one explanation for the disagreement may be that at the time of analysis there were no ITS2 sequences available for *Geotrichum silvicola*, suggesting that the D1/D2 identification may be the correct one. For the *Debaryomyces* isolates, the disagreement in identification may occur because these species are very closely related, and previous work has shown that it is difficult to differentiate between the two with either the D1/D2 or the ITS region (27).

The diversity of yeast in a veterinary population. In this study, we also sought to examine the diversity of yeast observed in a veterinary diagnostic laboratory setting. Using the results from both the D1/D2 and ITS2 sequence analysis, we identified 30 different species of yeast among 109 isolates, which may underestimate the total, as it does not include the 9 isolates that could not be identified to the species level. These results thus show that there is great variety in the yeasts identified from veterinary sources. In contrast to yeast

identified in human hospitals, only 17% of isolates were identified as *Candida albicans*. Additionally, less than 50% of the total belonged to the species *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and the *Candida parapsilosis* group. However, one point to note is that 39% of the yeasts were isolated from horses, and *C. albicans* was not isolated from any sample obtained from a horse in this study. In contrast, *C. albicans* was the predominant species identified in avians, canines, and in other mammals (Table 4.1). Additionally, one yeast species that comprised 7% of the total yeast isolates was *Arxula adenivorans*. Interestingly, it was isolated only from horses and dolphins, and for dolphins it was the predominant species identified (Table 4.1).

Even with use of sequence analysis of the D1/D2 region and the ITS2 region, there were still two isolates that could not be identified to the genus level using the criteria implemented in this study. The first (Unknown 1) was isolated from a horse and had 96% sequence identity to *Candida peoriaensis* and the second (Unknown 2) was isolated from a reptile and had 83% sequence identity to *Candida ghanaensis*. These percent identities were both based upon D1/D2 sequence analysis; in both cases, the percent identities for the ITS2 region were lower and to different organisms. These results suggest in both cases that these two isolates may represent uncharacterized species, showing that this method can be used to identify new and unusual yeast isolates. To better understand how these yeasts were related, we examined the phylogeny of representative species or isolates (in the cases where species identification was not made) identified in this study using sequences derived from the D1/D2 region. As seen in Figure 4.2, Unknown 1 falls into a group that contains various *Ogataea* sp., *Pichia* sp., and *Candida* sp.;

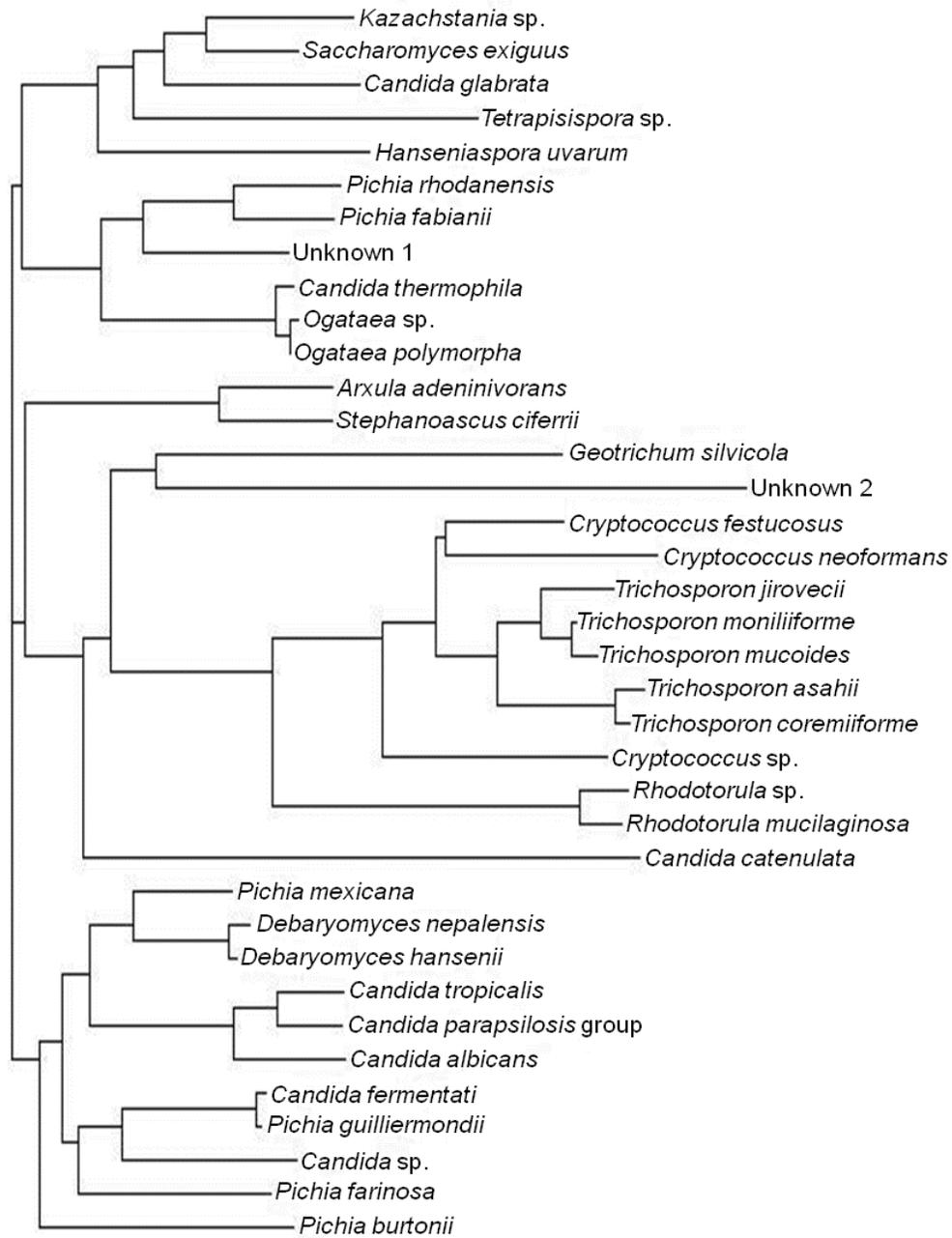


Figure 4.2. Phylogenetic tree of yeast isolates. A neighbor-joining phylogram of representative D1/D2 sequences was created using Clustal W2 multiple alignment tool.

interestingly all of the isolates in this particular grouping, including Unknown 1, came from equine sources. Unknown 2 falls in a group with *Geotrichum silvicola*, but the length of the branch indicates a distant relationship, consistent with the low percent identity to any known D1/D2 sequence (Fig. 4.2). These results therefore provide a phylogenetic framework that may be useful to the clinician when an exact identification is not possible.

DISCUSSION

In this study, we examined the usefulness of sequence analysis in comparison to more traditional phenotypic methods for identification of veterinary yeast isolates and found that sequence analysis provided more effective identification of the yeast isolates than either of the phenotypic methods tested. Substrate assimilation identified only 52% of the isolates to the species level and 61% to the genus level. These results are not completely surprising since there are reports in the literature of 59.6% - 99.3% identification for the API 20 C (11, 36, 42). For fatty acid profile analysis, we could identify 47% of the isolates to the species level and 55% using lower stringency for identification. These results are lower than previously reported, however, depending on the growth conditions, previous studies showed species level identification of 49%-71% (9, 17, 18). Thus, our results for the phenotypic methods were slightly lower than previously reported, but this may be a result of the diversity of veterinary yeast isolates. Significant to note is that the percentage of identifications described above represent all of those considered to be acceptable to the species level by each method; they do not take into account whether the identification was correct. Upon comparison of these methods to the isolates that had good identifications to the species level

by sequence analysis, we found that only 50% and 33% of the identifications made by substrate assimilation and fatty acid profile analysis respectively were consistent with the identification obtained using sequence analysis. These results thus suggest that for veterinary isolates identifications using these methods may frequently be incorrect.

Though sequence analysis of either the D1/D2 region or the ITS2 region provided higher percent identification than either of the phenotypic methods, there were still limitations with each of these methods. Using sequence analysis of the D1/D2 or ITS2 region provided identification of only 87% and 79% of the isolates to the species level, respectively. The percent identification for both the D1/D2 region and ITS regions are lower than previous reports for human clinical isolates (6, 13, 24, 26, 33, 34). However, this may be attributed to the variety of yeast isolated from animal sources, as well the number of uncommon isolates found in a veterinary population. For example, in this work we found that *Candida albicans* accounted for only 17% of the isolates and that other common *Candida* sp. such as *Candida tropicalis*, *Candida glabrata*, and the *Candida parapsilosis* group only accounted for 31%. Thus, the majority of yeasts isolated from veterinary sources do not belong to the species most commonly isolated from human sources (30, 39). In fact, one yeast that comprised 7% of the total yeast isolates was *Arxula adenivorans*, also known as *Blastobotrys adenivorans* (21). It has previously been characterized as an anamorphic, nonpathogenic, halotolerant, and dimorphic yeast isolated from environmental sources such as soil, maize silage, and wood hydrolysates, and has been studied to determine its usefulness in biotechnological applications (reviewed by 43). As mentioned above, it is not thought to be a pathogen, though recently a case of

Blastobotrys proliferans was reported as the causative agent of peritonitis in a human (35). However, currently the clinical significance of these isolates is not known, but the ability to identify them may allow for future understanding of their relevance to veterinary medicine.

Of the isolates that could not be identified to the species level using sequencing of the D1/D2 region, the lack of adequate separation between the two most similar species in the database was the most frequent cause. This may occur with closely related species, and previous reports suggest that the differentiation of closely related species may require sequence analysis of the ITS region (10, 38). However, in our study, we found that sequencing the ITS2 region provided identification of only 36% of the isolates not previously identified to the species level by D1/D2. Additionally, for the majority of isolates that could not be identified to the species level using the ITS2 region, the inability to identify the organism could be attributed to low sequence identity to the sequences present in the NCBI database that met the criteria described in Materials and Methods. Therefore, in contrast to the D1/D2 region, where difficulty in identification was a result of lack of adequate separation between the two most similar species, the difficulty with identification using the ITS2 region was a result of lack of available ITS2 sequences in the database for some of the species isolated in this study. Thus, based on the current database available, the best identification method for veterinary isolates is sequence analysis of the D1/D2 region of the large ribosomal subunit.

Another complication of sequence analysis is that the NCBI database is constantly growing and is not highly curated, and as such includes sequences that may not be well-supported. Additionally, naming conventions for yeast

are constantly changing. In addition to the confusion caused by certain yeasts having both anamorphic and teleomorphic names, there are also many yeasts that have synonymous names. Furthermore, with the advent of molecular phylogenetic analysis there have been several new species suggested as subdivisions of traditional species. For example, a recent study has suggested that the species *Candida africana* may be a separate species from *C. albicans* (41), but there is still uncertainty about this distinction (1, 37). Similarly, recent work has shown that *Candida parapsilosis* groups II and III are actually separate species, *Candida orthopsilosis* and *Candida metapsilosis*, respectively (40), and that *Cryptococcus neoformans* has been described to have variants (25). These changes in naming conventions present significant obstacles to the correct identification of yeast isolates in the clinical setting. As all of the methods tested here rely upon comparisons to databases for proper identification, the maintenance of those databases to accurately reflect the currently accepted nomenclature is essential. Therefore, as the limitations involved in all methods, including sequencing, are a result of database maintenance, one way to address this issue may be to create databases of sequences derived from the NCBI collection within individual laboratories. By creating an in-house database, a laboratory might control the quality of the sequences and use the sequences of isolates most commonly seen in the particular laboratory setting. In this case, sequences from this study could be used to create a database specific for yeast commonly isolated from veterinary sources.

One consideration for the implementation of sequence analysis in the clinical laboratory is cost-effectiveness. Examination of the three methods described in this work showed that consumables cost \$12 for substrate

assimilation using the API 20 C AUX kit, \$3 for fatty acid profile analysis, and \$5-6 for sequence analysis. However, other important cost considerations include a large capital investment in the GC for fatty acid profile analysis, and for sequence analysis access to a reliable sequencing center that can provide the service at a reasonable cost. Additional considerations for all three methods include the turn-around time for each test. Using fatty acid analysis and sequence analysis a turn-around time of 1-2 days is possible, whereas use of substrate assimilation requires 4-5 days. Therefore, individual laboratories must take into account the volume of testing, the need for expedience, and the availability of equipment or sequencing services for their individual labs to determine the best identification option. However, based on the results presented here and our analysis of costs, we found that sequence analysis of the D1/D2 region of the large ribosomal subunit was the most accurate and cost-efficient method of yeast identification for this veterinary diagnostic laboratory.

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