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Enhanced Salmonella virulence instigated by bactivorous protozoa and investigation of putative protozoan G protein-coupled receptors associated with bacterial engulfment

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Enhanced *Salmonella* virulence instigated by bactivorous protozoa and investigation of putative protozoan G protein-coupled receptors associated with bacterial engulfment

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DEDICATION

This work is dedicated to my family. To my Mom, who taught me the value of education. To my Dad, who instilled in me a deep appreciation for the diversity of life and the scientific process used to investigate it. And to my wife, Courtney, who's encouragement and support has been essential.

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ABSTRACT

The protozoa are a diverse group of eukaryotic microorganisms that employ a variety of life strategies. Many protozoa are free-living while others are commensal organisms that thrive within the gut of vertebrates. In addition, a relatively small group of parasitic protozoa represent serious health threats to humans and animals. Certain free-living, commensal, and parasitic protozoa are bactivorous and rely on bacteria as a food source. However, some intracellular bacterial pathogens survive engulfment and thrive within these protozoa. This dissertation describes the investigation of *Salmonella* hypervirulence following bacterial engulfment and characterizes physiologic interactions between various protozoa and bacteria.

Initial studies entailed characterization of *in vitro* and *in vivo Salmonella* hypervirulence following engulfment by free-living and commensal bactivorous protozoa. This work revealed that *Salmonella enterica* serotype Typhimurium phagetype DT104 survive protozoan engulfment and use the intracellular environment as a venue for plasmid transfer and activation of virulence genes. In subsequent studies, receptors from ciliated protozoa were investigated with a strategy involving molecular cloning, RNA interference, and heterologous expression in yeast. These studies revealed the involvement of protozoan G protein-coupled receptors and a cyclic nucleotide binding protein in bacterial engulfment. The yeast expression system was then used to deorphanize a G protein-coupled receptor from *Entamoeba histolytica*, demonstrating that bacterial lipopolysaccharide is an agonist of this receptor. Engulfment assays demonstrated that *E. histolytica* trophozoites preferred to engulf bacteria with intact LPS and this process was sensitive to inhibition of G protein signaling. In summary, a variety of protozoa facilitate salmonellosis, G protein-coupled receptors play a role in bacterial

engulfment by protozoa, and these receptors represent novel drug targets worthy of further exploration.

CHAPTER 1: OVERVIEW

DISSERTATION ORGANIZATION

This dissertation describes work investigating the relevance of G protein-coupled receptor signaling in bacterial engulfment by protozoa. The first chapter describes the relevant organisms, pharmacology, and rationale for the research topic. Chapter 2 characterizes *Salmonella* hypervirulence following engulfment by various bactivorous protozoa *in vitro* and in animal models. Chapter 3 describes mitigation of bacterial engulfment and *Salmonella* hypervirulence with a protozoa-targeted RNAi approach. Chapter 4 details the identification and deorphanization of an intercellular signal-recognizing G protein-coupled receptor from the free-living ciliate *Tetrahymena.* Chapter 5 provides evidence that the human parasite *Entamoeba histolytica* engulfs bacteria based on deorphanization experiments revealing bacterial lipopolysaccharide activation of a G protein-coupled receptor. Chapter 6 summarizes the significance of the presented work and suggests avenues for future research. The appendices include related and unrelated work accomplished during the time of study.

PROTOZOAN BACTIVORY

In addition to predatory bacteria and bacteriophages, bacteria must survive predation by bactivorous protozoa. Bacteria encounter protozoa in the soil, in aquatic environments, and in the gastrointestinal tract of vertebrates. In these habitats, protozoa are significant regulators of bacterial populations due to their motility, chemosensory abilities, and voracious appetite for smaller microorganisms. For example, protozoa in the forestomach of ruminants can reduce rumen bacterial counts from 4.6×10^{10} to 2×10^9 [1]. Similarly, under controlled conditions,

protozoa are capable of clearing up to 90% of bacteria from a soil sample [2]. Because protozoa are such highly effective predators, their eating habits are prominent selective pressures in bacterial evolution.
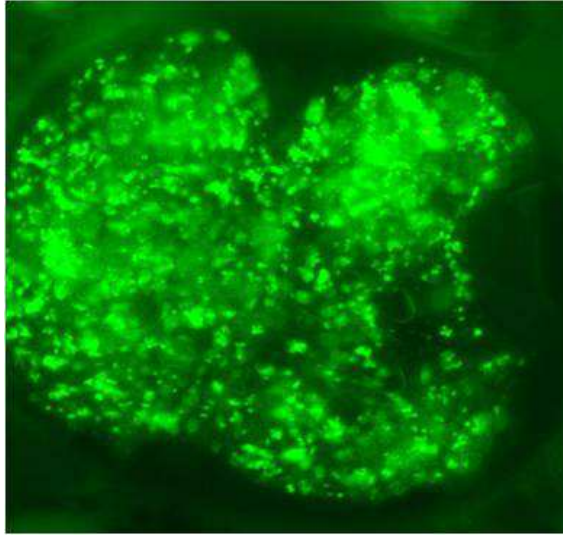
Figure 1.1. Fluorescently-labeled bacteria within ciliate protozoa

Bacterial mechanisms for aversion of predation act at various points in the predator-prey engulfment process. Many of these adaptations are designed to evade predation by thwarting pre-engulfment events such as chemosensation, contact, and phagocytosis. To avoid detection by protozoa, bacteria secrete soluble substances that inhibit chemosensation. Rapid bacterial motility can be utilized to escape: bacteria capable of traveling faster than 25μm/s are able to survive in the presence of bactivorous protozoa [3]. Large or filamentous bacteria are bulky and difficult for protozoa to ingest [4]. Similarly, forming biofilms or microcolonies allows bacteria reach a size that precludes them from protozoan engulfment. Biofilms are known to produce anti-protozoal toxins such as violacein and other cytotoxins which aid in bacterial

escape and survival [5]. In addition to these pre-engulfment mechanisms, some bacteria utilize post-engulfment mechanisms to adapt to predation. For example, bacteria may resist digestion in the phagolysosome; some species employing this technique have developed a commensal life style as they live within *Paramecium* [6].

ENHANCEMENT OF BACTERIAL PATHOGENESIS BY PROTOZOA

Given the potential lethal consequences of being ingested by a protozoan, it is not surprising that bacteria have developed numerous mechanisms for evasion of protozoan engulfment. Interestingly, the very mechanisms developed to avoid predation can be so exuberant that the bacteria become capable of virulence in humans or animals. For example, it is hypothesized that protozoan selection pressure has driven the evolution of the *Escherichia coli* Shiga toxin [7]. The type III secretion systems of *E. coli, Pseudomonas aeroginosa,* and *Chlamydia* are most well known for their roles in bacterial pathogenesis, however, these systems are also documented anti-predation mechanisms [8,9]. Biofilms, cell consortia that avoid protozoan bactivory, are also a major cause of morbidity and mortality as they form on biomedical implants and are more resistant to antimicrobials. Thus, traits that contribute to averting protozoan predation are also bacterial virulence factors.

Protozoa are capable of facilitating bacterial pathogenesis and virulence beyond their evolutionary influence on the development of bacterial defense mechanisms. Specifically, protozoa can act as a "Trojan horse" that engulf and protect pathogenic bacteria until an opportunity to infect a vertebrate arises. For example, *Mycobacterium tuberculosis* is capable of surviving within free-living protozoa [10]. A more often cited example is *Legionella pneumophilla* which does not reliably replicate extracellularly but is amplified within water-

born amoebae, thus forming the association between water-based cooling systems and
Legionnaire's disease [11]. In the field of water quality, there is interest in "Trojan horse"
protozoa as they protect bacteria from standard disinfectant procedures and may invalidate
assays for bacterial pathogens [12]. Interestingly, some species of protozoa, such a
Paramecium, have endosymbiont bacteria which require the intraprotozoal environment for
survival [13].
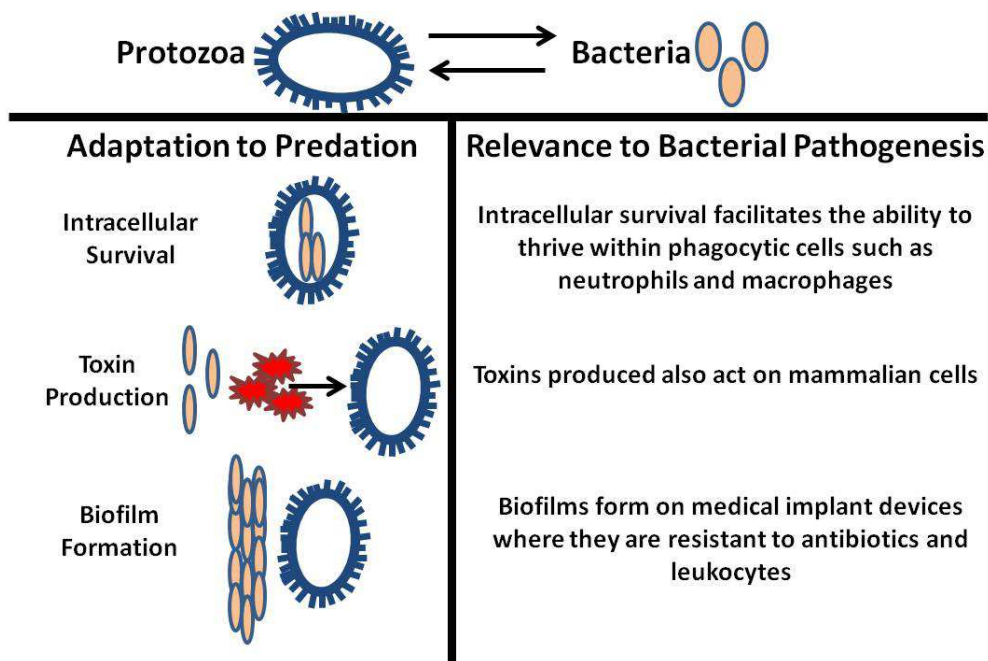
Figure 1.2. Summary of bacterial mechanisms for evading protozoa-mediated death and the
application of these mechanisms by bacterial pathogens.

While "Trojan horse" phenomena have been studied mostly in free-living protozoa, it is
also possible for protozoan parasites to harbor disease-causing pathogens. For example,
Trichomonas vaginalis, a venereally transmitted parasite, is capable of carrying *Mycoplasma
hominis* [14]. Since both free-living and pathogenic protozoa are capable of harboring

intracellular bacterial pathogens, a portion of the work presented investigates commensal protozoa which are found in the fore stomachs of ruminants.

SALMONELLA HYPERVIRULENCE INSTIGATED BY RUMEN PROTOZOA

Salmonella enterica is a Gram-negative, intracellular bacterial pathogen that causes food-borne illness throughout the world. While some serotypes of *S. enterica* are host-specific, *S. enterica* serotype Typhimurium exhibits a broad host range and is capable of causing disease in a variety of vertebrates. Within this serotype there are numerous subgroups designated by the adherence affinity of bacteriophage to a given strain, *i.e.*, the phagetype. *S. enterica* serotype Typhimurium phage type DT104 (DT104) is a pathogenic isolate which is especially virulent and causes severe gastroenteritis in both in both cattle and in humans. The virulence of DT104 is due to, at least in part, a chromosomal integron structure known as *Salmonella* genomic island 1 which bears multiple antibiotic resistance genes [15].

Systemic infection with DT104 requires invasion of the intestinal epithelium, however, DT104 is not inherently hyper-invasive [16]. Rasmussen *et al*. hypothesized that DT104 become hyper-invasive and subsequently more virulent following environmental signals from commensal protozoa present in the rumen (forestomach) of cattle. Testing this hypothesis revealed that DT104 was almost ten times more invasive in cell culture invasion assays following engulfment by rumen protozoa and this effect was recapitulated in a calf infection model [17]. These findings were consistently observed with DT104 and other isolates of *Salmonella* containing the *Salmonella* genomic island 1 integron. Carlson *et al.* investigated the genetic mechanisms underlying the rumen protozoa-instigated hypervirulence and found that SO13, a gene present on genomic island 1, was significantly up-regulated in DT104 following

engulfment by bovine rumen protozoa [18]. However, hyperinvasion could not be induced in *Salmonella* lacking the integron but expressing SO13, implying that other genes are needed to produce the hyperinvasive phenotype.

Interestingly, virulence gene hyperexpression is not the only DT104 property augmented by rumen protozoa in cattle. The protozoa engulf and protect DT104 until they are digested in the abomasum and the bacteria are released. Pharmacologic defaunation of rumen protozoa from calves abrogated clinical disease and systemic bacterial burden [18]. In addition to protection in the harsh environment of the rumen, bovine rumen protozoa act as a venue for plasmid transfer. This phenomenon has been demonstrated *in vitro* as well as in animal models whereby recipient DT104 acquire antimicrobial resistance plasmids from donor bacteria in normal calves but not in defaunated animals [19].

Table 1.1. Summary of mechanisms by which bovine rumen protozoa facilitate *Salmonella* DT104.

PROPERTY	RELEVANCE TO DT104 PATHOGENESIS
Intracellular survival	DT104 passes safely through the rumen
Induction of SO13 Hyperexpression	Induction of hyperinvasive phenotype
Venue for plasmid transfer	Acquisition of antibiotic resistance plasmids

Rumen protozoa are fastidious and few investigators have been able to successfully propagate these microbes for extended periods of time. Therefore, relatively few studies of rumen protozoa taxonomy and genetics have been undertaken and there are no established genetic databases available for these microbes. This severely hampers the utility of bioinformatic and molecular biology approaches for the study of rumen protozoa. Recent metagenomic studies indicate that the major genera of rumen protozoa are ciliates, namely, *Entodinium*, *Epidinium*, *Dasytricha*, and *Isotricha* [20,21]. *Tetrahymena thermophila* is a related

free-living bactivorous ciliate that was selected as a model for several of the studies presented herein. *Tetrahymena* is easily cultured and there are numerous bioinformatic resources available for this organism. In addition, *Tetrahymena* is amenable to RNAi-based gene knockdown [22]. Studies in chapters 3 and 4 utilize this organism in the investigation of protozoan receptors associated with bacterial engulfment.

ENTAMOEBA HISTOLYTICA: A BACTIVOROUS PROTOZOAN PARASITE

While free-living or commensal protozoa contribute to bacterial pathogenesis, some protozoa are infectious parasites with the inherent ability to cause severe disease in humans and animals. Protozoan parasites represent diverse taxa and each has a preferred host tissue habitat. Depending on the particular organism, protozoan parasites can be found in blood, skeletal muscle, mucosal surfaces, or the gastrointestinal tract. *Entamoeba histolytica*, an anthroponotic amoeba, inhabits the intestine and is relevant to the work presented herein since it actively engulfs bacteria as a major source of nutrition [23].

E. histolytica is a cosmopolitan parasite that causes amebic colitis and systemic amebiasis. Amebiasis results in 100,000 deaths annually which is only second to malaria in terms of death by a protozoan [24]. *E. histolytica* is spread by a fecal-oral route and the life cycle consists of two distinct life cycle stages. Quadrinucleate cysts are shed in the feces and are the infective stage. Cysts survive the low pH of the stomach and excyst in the illeocecal region to form trophozoites that are pleomorphic, motile, and capable of reproduction by binary fission. Some trophozoites form the infective cyst stage following an unknown stimulus; they do not encyst *in vitro*. The amoebic galactose/N-acetylgalactosamine lectin binds to colonic epithelial cells; host cells are then killed by a combination of apoptosis induction and

insertion of pore-forming peptides [25]. Invasion of the mucosa and submucosal tissues results

in nearly pathognomonic flask-shaped tissue ulcers (Figure 1.3). Sequelae to amoebic colitis

include colonic perforation, ameboma, and rectovaginal fistulae. More rarely, trophozoites

gain access to the blood stream and cause liver or brain abscesses [26].

Figure 1.3. Characteristic flask shaped ulcer caused by *E. histolytica*. (Photo From: Stanley, 2003 [27])

E. histolytica has persisted throughout the world, in part due to the fact that there are

few pharmacologic agents available for the treatment of the disease. Several vaccines are

described in the literature, although no commercial vaccine is currently in use [28]. The main

class of drugs used for amebiasis are the nitroimidazoles, mainly metronidazole, which are

thought to bind and fragment DNA thus inhibiting DNA replication. These drugs are mutagenic

in bacterial systems and carcinogenic in animal models, therefore, they are considered

undesirable [29]. In addition, metronidazole elicits neurological side effects and is not for use in

pregnant individuals [29]. Drug resistance is also becoming an increasing concern since *E.*

histolytica possesses nitroimidazole reductases and P glycoprotein-like transporters that inactivate or efflux metronidazole, respectively [30,31]. Thus, the limitations of anti-amoebic therapy warrant investigation into new molecular targets that are unique in protozoa and could be exploited.

The hypothesis for this work presented in chapter 5 was that *E. histolytica* expresses a bacterial recognition receptor. The aim of identifying this receptor and its cognate ligand was to further understand the bacterial engulfment and to identify a unique amoebic receptor that could provide a new candidate for anti-amoebic therapy.

G PROTEIN-COUPLED RECPTORS

G protein-coupled receptors (GPCRs) are the largest known superfamily of transmembrane proteins and are broadly defined by their structure consisting of seven transmembrane-spanning α domains. Upon activation by a ligand, GPCRs convert an extracellular stimulus to an intracellular signal by activating cognate G proteins. G proteins are signal transduction proteins with the ability to bind guanosine triphosphate (GTP) and guanosine diphosphate (GDP). Some G proteins are small and consist of a single subunit whereas most GPCRs associate with heterotrimeric G proteins consisting of α, β, and γ subunits [32].

In the absence of a signal, the heterotrimeric G protein is associated with a GPCR and the α subunit is bound to GDP. Following ligand binding, GPCRs activate the G protein by inducing a conformational change resulting in the replacement of GDP with a GTP. The GTP-bound α subunit then disassociates and activates downstream signaling pathways that vary

depending on the G_α subtype. These signal transduction pathways lead to accumulation or depletion of second messenger molecules that instigates a biological effect [32,33].

The main families of G protein α subunits are $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$. $G\alpha_s$ and $G\alpha_i$ are named to indicate that they stimulate or inhibit adenylate cyclase, respectively, which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [32,34]. $G\alpha_q$ activates phospholipase C, leading to the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) that results in the production of diacyl glycerol and inositol 1,4,5-triphosphate (IP_3). IP_3 activates ion channels on endoplasmic reticulum which release Ca^{2+} into the cytosol [32,34]. $G\alpha_{12/13}$ is the least well characterized; it is thought that this family activates Rho GTPase nucleotide exchange factors (RhoGEFS) [35]. RhoGEFS catalyze the exchange of GDP for GTP by GTPase RhoA which ultimately leads to activation of Rho kinases (ROCK) and subsequent downstream biologic activity, including gene transcription and cytoskeletal movement [35]. Thus, seven transmembrane GPCRs are mostly associated with heterotrimeric G proteins but there is a growing number of receptors that interact with other cell signaling molecules.

GPCRs are expressed by all cells in vertebrates where they modulate a remarkable diversity of physiologic functions. GPCR ligands include catecholamines, peptides, purines, and even photons. Therefore, GPCRs mediate a variety of senses and homeostatic functions. It is not surprising then, given their importance, that 36% of the currently marketed drugs target GPCRs [36]. The central goal of the work presented herein was to characterize GPCRs from protozoa since these targets could be used to kill protozoan parasites or abrogate protozoan enhancement of bacterial pathogenesis.
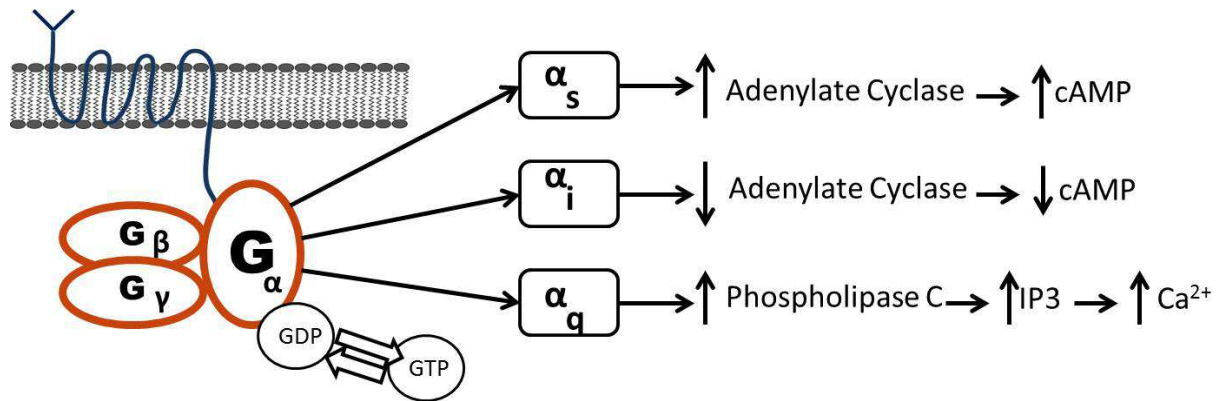
<u>Figure 1.4.</u> Overview of signal transduction pathways activated by GPCR signaling via Gα_s, Gα_i, and Gα_q.

HISTORY OF GPCR STUDIES IN PROTOZOA

GPCRs are expressed in animals, fungi, and bacteria. Protozoa represent vastly diverse taxa and the study of GPCRs in these organisms is only in its infancy. The first impediment to the study of protozoan GPCRs is that genomic resources and tools for studying these organisms have become available relatively recently compared to those for vertebrates. In addition, the biology of protozoa complicates their study when they are considered in light of scientific paradigms that are essentially formed solely based on the study of vertebrates. Protozoa utilize alternate codon usage (such as read-through stop codons), possess distinct unique organelles, and utilize a variety of chromosomal organizational schemes [37,38]. Therefore, progress in protozoan GPCR research has advanced slowly.

Several tiers of evidence supporting the existence and function of protozoan GPCRs exist. The most abundant, yet least convincing, evidence for GPCR signaling in protozoa is that protozoa exhibit physiological or behavioral responses to classical GPCR ligands. For example, *Tetrahymena* synthesizes biogenic amines and exhibits chemotactic responses when presented

with ligands for vertebrate GPCRs [39,40]. *Entamoeba invadens*, a parasite of reptiles, produces cAMP when stimulated with epinephrine and this phenomenon is sensitive to modulators of heterotrimeric G proteins such as pertussis toxin and cholera toxin [41]. Additional supportive indirect evidence is the identification of GPCR signal transduction machinery in protozoa. For example, heterotrimeric G proteins have been identified in *Dictyostelium*, *Trichomonas vaginalis*, *Entamoeba*, and other protozoa [42-44]. Database searches reveal GPCRs in the genomes of several protozoa and knock out mutants have suggested the function of some of these receptors. For example, knocking out GPCR6 from *Tetrahymena* resulted in decreased G protein activity and decreased chemotaxis, leading investigators to hypothesize that this receptor is a constitutively active GPCR [45]. Knocking-out GPCRs from the amoeba *Dictyostelium*, termed crl receptors, suggests that these receptors detect cAMP in the environment [46]. Picazarri *et al.* identified EhGPCR-1, a GPCR from *E. histolytica* with a putative role in phagocytosis [47]. This receptor was chosen as a subject for the presented studies with the hypothesis that EhGPCR-1 could bind bacterial ligands and induce bacterial engulfment.

A range of physiologic evidence supports GPCR existence and function in protozoa and numerous putative GPCR-encoding sequences can be found in genetic databases for protozoa. However, no studies have attempted to heterologously express these protozoan GPCRs in an attempt to reveal their cognate ligand. Therefore, a goal of this study was to utilize the yeast system described in the section below as it could offer a method for studying protozoan GPCRs.

GPCR DEORPHANIZATION AND A HETEROLOGOUS YEAST EXPRESSION SYSTEM

Initially, studies of GPCRs relied on the identification of a biological molecule that exerted a physiological response in cell cultures or tissue preparations. Following identification of a ligand, investigators would attempt to establish a cognate receptor. However, the advent of the polymerase chain reaction and molecular cloning significantly altered this method and established the more popular and successful modern practice of reverse pharmacology. Under this approach, a GPCR is identified on the basis of its DNA sequence. Therefore, the ligand is not initially known and the receptor is known as an orphan GPCR. Upon discovery of the ligand the receptor is considered deorphanized.

Reverse pharmacology typically begins with homology screening approaches and degenerate PCR used to clone GPCR-encoding sequences. The cloned receptors are then expressed in a heterologous cell system and interrogated with potential ligands. Receptors have been expressed in *Xenopus* oocytes and a variety of mammalian cell lines [33]. In these systems, GPCR activation is often assayed by fluorometric detection of calcium flux or measurement of other second messengers such as cAMP or inositol phosphates.

An interesting heterologous expression system has been designed that utilizes the common yeast, *Saccharomyces cerevisiae*. Knockout *S. cerevisiae* strains lack endogenous GPCRs and can be used for a convenient deorphanization platform [48]. In yeast, GPCRs bind pheromones from neighboring cells and activate the promoter Fus1. In these particular yeast strains, Fus1 controls the expression of His3 that confers the ability to synthesize histidine *de novo*. Therefore, activation of a heterologously expressed GPCR results in Fus1 activation, subsequent His3 expression, and the ability to grown in histidine-deficient media [48-50].

Therefore, receptor activation can be measured by a simple growth assay. The yeast has been further modified by replacing the unique yeast G protein G_{pa1} with amino acid residues from the C-terminus mammalian G_{α}. The resulting G protein is referred to as a "G_{α} transplant" which is promiscuous and exhibits high binding efficiency with non-yeast GPCRs [51].

Yeast heterologous expression was used to deorphanize the human β_2 adrenergic receptor as well as other mammalian GPCRs. More recently, the yeast expression system has been used to study the pharmacology of GPCRs from nematodes and arthropods [49,50]. A goal of the studies presented in this work was to utilize the yeast expression system as a tool to deorphanize putative GPCRs from protozoa.

PURPOSE

Rumen protozoa-mediated facilitation of salmonellosis warrants further investigation given the importance of DT104 in human and animal disease. It is not known if commensal protozoa from other ruminants are capable of facilitating DT104 in terms of activating the hyperinvasive phenotype or acting as a site of conjugal plasmid transfer. Because protozoa are so abundant in the environment and in the rumen of food animal species, the first specific aim of this work was to determine if commensal protozoa from other ruminants are capable of facilitating DT104.

The aim of the second phase of studies presented herein was to identify and deorphanize protozoan GPCRs. Characterization of protozoan GPCRs associated with protozoan bactivory are of broad interest in microbiology and evidence for GPCRs in protozoa is desirable in terms of general biological knowledge. In addition, GPCR characterization from protozoa may provide a means by which to ameliorate disease. First, targeting of GPCRs could be utilized

in the abrogation of rumen protozoa facilitated salmonellosis. Second, GPCRs from protozoan parasitic pathogens such as *E. histolytica* represent novel chemotherapeutic targets of interest in the development of new anti-protozoal agents. Revealing the ligands of putative protozoan GPCRs will help clarify their physiologic significance and reinforce interest in these receptors as drug targets.

REFERENCES

1. Belanche A, de la Fuente G, Pinloche E, Newbold CJ, Balcells J (2012) Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. J Anim Sci 90: 3924-3936.

2. Adiba S, Nizak C, van Baalen M, Denamur E, Depaulis F (2010) From Grazing Resistance to Pathogenesis: The Coincidental Evolution of Virulence Factors. Plos One 5.

3. Matz C, Jurgens K (2005) High motility reduces grazing mortality of planktonic bacteria. Appl Environ Microbiol 71: 921-929.

4. Hahn MW, Moore ERB, Hofle MG (1999) Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. Applied and Environmental Microbiology 65: 25-35.

5. Pukatzki S, Kessin RH, Mekalanos JJ (2002) The human pathogen Pseudomonas aeruginosa utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. Proceedings of the National Academy of Sciences of the United States of America 99: 3159-3164.

6. Fokin SI (2004) Bacterial endocytobionts of ciliophora and their interactions with the host cell. International Review of Cytology - a Survey of Cell Biology, Vol 236 236: 181-249.

7. Steinberg KM, Levin BR (2007) Grazing protozoa and the evolution of the Escherichia coli O157 : H7 Shiga toxin-encoding prophage. Proceedings of the Royal Society B-Biological Sciences 274: 1921-1929.

8. Matz C, Moreno AM, Alhede M, Manefield M, Hauser AR, et al. (2008) Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. Isme Journal 2: 843-852.

9. Erken M, Lutz C, McDougald D (2013) The Rise of Pathogens: Predation as a Factor Driving the Evolution of Human Pathogens in the Environment. Microbial Ecology 65: 860-868.

10. Medie FM, Ben Salah I, Henrissat B, Raoult D, Drancourt M (2011) Mycobacterium tuberculosis Complex Mycobacteria as Amoeba-Resistant Organisms. Plos One 6.

11. Rowbotham TJ (1980) Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. J Clin Pathol 33: 1179-1183.

12. King CH, Shotts EB, Wooley RE, Porter KG (1988) Survival of Coliforms and Bacterial Pathogens within Protozoa during Chlorination. Applied and Environmental Microbiology 54: 3023-3033.

13. Springer N, Ludwig W, Amann R, Schmidt HJ, Gortz HD, et al. (1993) Occurrence of fragmented 16S rRNA in an obligate bacterial endosymbiont of Paramecium caudatum. Proc Natl Acad Sci U S A 90: 9892-9895.

14. Dessi D, Delogu G, Emonte E, Catania MR, Fiori PL, et al. (2005) Long-term survival and intracellular replication of Mycoplasma hominis in Trichomonas vaginalis cells: potential role of the protozoon in transmitting bacterial infection. Infect Immun 73: 1180-1186.

15. Briggs CE, Fratamico PM (1999) Molecular characterization of an antibiotic resistance gene cluster of Salmonella typhimurium DT104. Antimicrob Agents Chemother 43: 846-849.

16. Carlson SA, Browning M, Ferris KE, Jones BD (2000) Identification of diminished tissue culture invasiveness among multiple antibiotic resistant Salmonella typhimurium DT104. Microbial Pathogenesis 28: 37-44.

17. Rasmussen MA, Carlson SA, Franklin SK, McCuddin ZP, Wu MT, et al. (2005) Exposure to rumen protozoa leads to enhancement of pathogenicity of and invasion by multiple-antibiotic-resistant Salmonella enterica bearing SGI1. Infect Immun 73: 4668-4675.

18. Carlson SA, Sharma VK, McCuddin ZP, Rasmussen MA, Franklin SK (2007) Involvement of a Salmonella genomic island 1 gene in the rumen protozoan-mediated enhancement of invasion for multiple-antibiotic-resistant Salmonella enterica serovar Typhimurium. Infect Immun 75: 792-800.

19. McCuddin Z, Carlson SA, Rasmussen MA, Franklin SK (2006) Klebsiella to Salmonella gene transfer within rumen protozoa: Implications for antibiotic resistance and rumen defaunation. Veterinary Microbiology 114: 275-284.

20. Shin EC, Cho KM, Lim WJ, Hong SY, An CL, et al. (2004) Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. J Appl Microbiol 97: 378-383.

21. Singh KM, Tripathi AK, Pandya PR, Rank DN, Kothari RK, et al. (2011) Dasytricha Dominance in Surti Buffalo Rumen Revealed by 18S rRNA Sequences and Real-Time PCR Assay. Current Microbiology 63: 281-288.

22. Howard-Till RA, Yao MC (2006) Induction of gene silencing by hairpin RNA expression in Tetrahymena thermophila reveals a second small RNA pathway. Mol Cell Biol 26: 8731-8742.

23. Mirelman D (1987) Ameba-Bacterium Relationship in Amebiasis. Microbiological Reviews 51: 272-284.

24. Petri WA, Haque R, Lyerly D, Vines RR (2000) Estimating the impact of amebiasis on health. Parasitology Today 16: 320-321.

25. Ralston KS, Petri WA (2011) Tissue destruction and invasion by Entamoeba histolytica. Trends in Parasitology 27: 253-262.

26. Petri WA, Jr., Singh U (1999) Diagnosis and management of amebiasis. Clin Infect Dis 29: 1117-1125.

27. Stanley SL (2003) Amoebiasis. Lancet 361: 1025-1034.

28. Petri WA (2002) Entamoeba histolytica: Clinical Update and Vaccine Prospects. Curr Infect Dis Rep 4: 124-129.

29. Lofmark S, Edlund C, Nord CE (2010) Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. Clinical Infectious Diseases 50: S16-S23.

30. Pal D, Banerjee S, Cui J, Schwartz A, Ghosh SK, et al. (2009) Giardia, Entamoeba, and Trichomonas Enzymes Activate Metronidazole (Nitroreductases) and Inactivate Metronidazole (Nitroimidazole Reductases). Antimicrobial Agents and Chemotherapy 53: 458-464.

31. Upcroft P, Upcroft JA (2001) Drug targets and mechanisms of resistance in the anaerobic protozoa. Clinical Microbiology Reviews 14: 150-+.

32. Rosenbaum DM, Rasmussen SGF, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. Nature 459: 356-363.

33. Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, et al. (2013) G protein-coupled receptor deorphanizations. Annu Rev Pharmacol Toxicol 53: 127-146.

34. Wettschureck N, Offermanns S (2005) Mammalian G proteins and their cell type specific functions. Physiological Reviews 85: 1159-1204.

35. Siehler S (2009) Regulation of RhoGEF proteins by G12/13-coupled receptors. Br J Pharmacol 158: 41-49.

36. Rask-Andersen M, Almen MS, Schioth HB (2011) Trends in the exploitation of novel drug targets. Nature Reviews Drug Discovery 10: 579-590.

37. Jukes TH, Osawa S (1993) Evolutionary Changes in the Genetic-Code. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 106: 489-494.

38. Vickerman K, Coombs GH (1999) Protozoan paradigms for cell biology. Journal of Cell Science 112: 2797-2798.

39. Goldman ME, Gundersen RE, Erickson CK, Thompson GA (1981) High-Performance Liquid-Chromatographic Analysis of Catecholamines in Growing and Non-Growing Tetrahymena-Pyriformis. Biochimica Et Biophysica Acta 676: 221-225.

40. Rodgers LF, Markle KL, Hennessey TM (2008) Responses of the ciliates Tetrahymena and Paramecium to vertebrate odorants and tastants. Journal of Eukaryotic Microbiology 55: 27-33.

41. Frederick J, Eichinger D (2004) Entamoeba invadens contains the components of a classical adrenergic signaling system. Molecular and Biochemical Parasitology 137: 339-343.

42. Bosch DE, Siderovski DP (2013) G protein signaling in the parasite Entamoeba histolytica. Experimental and Molecular Medicine 45.

43. Hirt RP, Lal K, Pinxteren J, Warwicker J, Healy B, et al. (2003) Biochemical and genetic evidence for a family of heterotrimeric G-proteins in Trichomonas vaginalis. Molecular and Biochemical Parasitology 129: 179-189.

44. Maeda M, Aubry L, Insall R, Gaskins C, Devreotes PN, et al. (1996) Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in Dictyostelium - Role of heterotrimeric G proteins. Journal of Biological Chemistry 271: 3351-3354.

45. Lampert TJ, Coleman KD, Hennessey TM (2011) A Knockout Mutation of a Constitutive GPCR in Tetrahymena Decreases Both G-Protein Activity and Chemoattraction. Plos One 6.

46. Raisley B, Zhang MH, Hereld D, Hadwiger JA (2004) A cAMP receptor-like G protein-coupled receptor with roles in growth regulation and development. Developmental Biology 265: 433-445.

47. Picazarri K, Luna-Arias JP, Carillo E, Orozco E, Rodriguez MA (2005) Entamoeba histolytica: Identification of EhGPCR-1, a novel putative G protein-coupled receptor that binds to EhRabB. Experimental Parasitology 110: 253-258.

48. Wang ZX, Broach JR, Peiper SC (2006) Functional expression of CXCR4 in Saccharomyces cerevisiae in the development of powerful tools for the pharmacological characterization of CXCR4. Methods Mol Biol 332: 115-127.

49. Gross AD, Kimber MJ, Day TA, Ribeiro P, Coats JR (2013) Quantitative Structure-Activity Relationships (QSARs) of Monoterpenoids at an Expressed American Cockroach Octopamine Receptor. Pest Management with Natural Products 1141: 97-110.

50. Kimber MJ, Sayegh L, El-Shehabi F, Song CZ, Zamanian M, et al. (2009) Identification of an Ascaris G protein-coupled acetylcholine receptor with atypical muscarinic pharmacology. International Journal for Parasitology 39: 1215-1222.

51. Brown AJ, Dyos SL, Whiteway MS, White JHM, Watson MAEA, et al. (2000) Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein alpha-subunit chimeras. Yeast 16: 11-22.

CHAPTER 2. COMPARISONS OF *SALMONELLA* CONJUGATION AND VIRULENCE GENE HYPEREXPRESSION MEDIATED BY RUMEN PROTOZOA FROM DOMESTIC AND EXOTIC RUMINANTS

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ABSTRACT

Recent studies have identified a phenomenon in which protozoa engulf *Salmonella* and the intra-protozoal environment hyperactivates virulence gene expression and provides a venue for conjugal transfer of antibiotic resistance plasmids. The former observation is relegated to *Salmonella* bearing the SGI1 multiresistance integron while the latter phenomenon appears to be a more generalized event for recipient *Salmonella*. Our previous studies assessed virulence gene hyperexpression only with protozoa from the bovine rumen while conjugal transfer has been demonstrated in rumen protozoa from cattle and goats. The present study examined virulence gene hyperexpression for *Salmonella* exposed to rumen protozoa obtained from cattle, sheep, goats, or two African ruminants (giraffe and bongo). Conjugal transfer was also assessed in these protozoa using *Salmonella* as the recipient. Virulence gene hyperexpression was only observed following exposure to the rumen protozoa from cattle and sheep while elevated virulence was also observed in these animals. Conjugal transfer events were, however, observed in all protozoa evaluated. It therefore appears that the protozoa-based hypervirulence is not universal to all ruminants while conjugal transfer is more ubiquitous.

INTRODUCTION

Salmonella enterica is a major cause of food borne illness throughout the world. Treatment of salmonellosis is becoming increasingly difficult as many stains are resistant to multiple antibiotics. This is especially true for *S. enterica* serotype Typhimurium phage type DT104 (DT104), which is resistant to five or more antibiotics as a result of the acquisition of an integron structure designated as *Salmonella* genomic island 1 [SGI1, [1]].

Recent studies revealed that rumen protozoa (RPz), commensal microbes inhabiting the forestomach of ruminants, provide a venue for conjugal transfer where *Salmonella* can acquire plasmid-borne antibiotic resistance genes [2]. Whereas many other bacteria are digested by RPz, *Salmonella* is an intracellular pathogen which is able to live, multiply, and accept conjugative plasmids within these microbes [2,3].

In addition to mediating antibiotic resistance gene transfer, RPz hyperactivate *Salmonella* virulence in strains bearing the SGI1 integron. In this model, RPz engulf *Salmonella* and induce overexpression of *hilA*, a transcriptional regulator of invasion [4]. HilA overexpression is in response to expression of SO13, an SGI1 gene with unknown function [5]. The resulting hyperinvasion leads to a faster onset of clinical signs, a greater recovery rate of the pathogen, and a poorer prognosis. This is especially evident in cattle where DT104 is 13 times more likely to die than calves infected with antibiotic-sensitive *S. enterica* serovar Typhimurium [6].

To assess this phenomenon in other ruminants, we assessed RPz isolated from goats, sheep, and two exotic ruminants (bongo and giraffe). RPz from these animals were evaluated

for the ability to induce virulence gene hyperexpression and foster plasmid conjugation in *Salmonella*.

MATERIALS AND METHODS

Summary of microbes used in this study

Bacterial strains are summarized in Table 2.1 with strain 98-420 serving as the model DT104 isolate and strain TH11 serving as the DT104 isostrain lacking SGI1 [7]. Bacteria were stored in cryopreservation tubes containing 50% glycerol:50% culture medium at -80°C and were grown in LB broth (Sigma) without antibiotics for TH11, with ampicillin plus chloramphenicol (32µg/mL each; Sigma) for DT104, or with ceftiofur (32µg/mL; Pfizer) for *Klebsiella*.

Rumen fluid was removed from fistulated animals as described previously [3]. Fluid was filtered to remove large particulate matter and mixed with an equal volume of Coleman's buffer D [8]. Protozoa were then allowed to settle for 2 h under CO_2. Settled protozoa were aspirated and washed twice with approximately 45ml Coleman's buffer D and then centrifuged for 20 s at 230 x *g*. Pelleted protozoa were resuspended in 30 mL Coleman's buffer D under CO_2. One milliliter was used for enumeration, and 3 mL (approximately 10^5 RPz) was used in each invasion assay.

RPz were isolated from individually housed giraffe (*Giraffa camelopardalis*) and bongo (*Tragelaphus eurycerus*) at the Blank Park Zoo in Des Moines, IA, USA. Regurgitated rumen contents were collected from the surface of watering tanks and suspended in 300 mL Coleman's buffer. This solution was immediately filtered through cheese cloth to remove

particulate matter and then centrifuged at 300 x g for 3 minutes. The pellet was resuspended

in 10 mL of Coleman's buffer and a hemocytometer was used to determine an estimated RPz

concentration of 10^5/mL. Trypan blue staining revealed approximately 90% viability of the RPz,

which is consistent with previous studies employing this method of RPz. Control studies

revealed that this sampling method yielded viable bovine RPz that facilitated plasmid transfer

and hyperinvasion (data not shown).

Salmonella invasion assays following survival within RPz

10^5 RPz were co-incubated with 2×10^8 CFUs *Salmonella* and agitated with a tube roller

for 24 h at room temperature in a 1.5mL microcentrifuge tube. Extracellular *Salmonella* were

then killed using 300µg/mL florfenicol (Schering-Plough). RPz were then lysed for 60 sec at

4,800 rpm using 2.5 mM glass beads and a mini-beadbeater (Biospec Products). The recovered

lysate was pelleted and resuspended in 350uL LB broth and 50 µL was plated on XLT agar

(Difco) to assess engulfment of *Salmonella*. The remaining bacteria were incubated with HEp-2

cell cultures in triplicate. Specifically, 100 µL was added to each tissue culture well containing

10^5 HEp-2 cells which were maintained in RPMI 1640 (GIBCO) containing 10% fetal bovine

serum at 37°C in a 5% CO_2 humidified incubator. The protozoa-derived bacteria were incubated

with HEp-2 cells for 1 h at 37°C and extracellular bacteria were then killed using 300µg/mL

florfenicol. HEp-2 cells were then lysed for 15 min at 37°C using 0.1% Triton-X. Dilutions of the

lysate were plated on *Salmonella* selective media and grown overnight at 37°C followed by

enumeration the next day. Percent invasion was calculated by dividing CFUs recovered by CFUs

added.

Conjugation Frequency Assay

Approximately 10^9 CFUs of both *S. enterica* serotype Typhimurium strain TH11 [7] and *K. pneumoniae* TCR2003 [2] were co-incubated with approximately 10^5 RPz in sealed glass tubes overnight on a rocker at 37°C. At the end of the incubation period, extracellular *Salmonella* were killed using 300μg/mL florfenicol. RPz were then lysed using a bead beater for 60 sec. The lysate was pelleted and resuspended in 200 mL Lennox L broth. Next, 50 μL was spread on Brilliant Green Agar (does not support the growth of *Klebsiella*; Difco) containing 32μg/mL ceftiofur. Plates were incubated at 37°C overnight and colonies were enumerated the next day. Conjugation frequency was determined by calculating CFUs of ceftiofur-resistant *Salmonella* recovered divided by CFUs of ceftiofur-sensitive *Salmonella* added to the assay.

In vivo infection experiments

10^6 CFUs/kg of DT104 was orally inoculated into 8-week old Holstein calves (n=6, approximately 70kg each, male), adult sheep (n=6, approximately 100kg each, female, mixed breeds), and goats (n=6, approximately 100kg each, female, mixed breeds) that harbored native populations of RPz. Animals were monitored for changes in appetite, stool consistency, and rectal temperature every 8 to 12 h. At the onset of signs related to systemic salmonellosis, animals were euthanized using xylazine (0.5 mg/kg intramuscularly; Phoenix Laboratories) and pentobarbital (1.2 mg/kg, intravenously; Fort Dodge Laboratories). Splenic samples, which are a reliable indicator of systemic burden [3,5], were collected following euthanasia.

Spleens were aseptically cut into small pieces, placed in an equal volume of LB broth, and vortexed vigorously for 2 min. Dilutions of the resulting homogenate were plated on XLT

agar containing ampicillin (32µg/mL) and chloramphenicol (32µg/mL) for enumeration.

Salmonella colonies were confirmed by PCR using primers specific for detection of the *sipB-sipC*

gene fragment as described previously [7].

Semi-Quantitative RT-PCR

RT-PCR was conducted to assess the expression of *hilA* and SO13. RNA was isolated

from bacteria using the RNeasy Mini Kit with enzymatic lysis for the initial cell disruption

(Qiagen). RT-PCR was carried out using the SuperScript One-Step RT-PCR with Platinum Taq kit

(Invitrogen) using the *hilA* or SO13 primers and PCR conditions described previously [5]. The

number of cycles in the PCR portion of the reaction was varied to empirically determine the

number of cycles required to produce a visible amplicon from the *Salmonella* RNA template

collected from each specimen. RT-PCR products were visualized on a 1.5% percent agarose gel

using ethidium bromide staining.

RESULTS

Assessment of Klebsiella to Salmonella conjugal transfer of antibiotic resistance plasmids within

RPz from various ruminants

Our previous studies revealed that *Salmonella* can conjugate and receive antibiotic

resistance plasmids while co-habitating in RPz obtained from cattle and goats [2]. To determine

if this phenomenon extends beyond these two domestic ruminants, a plasmid-bearing donor

strain of *Klebsiella* was co-incubated with recipient *Salmonella* in the presence of RPz obtained

from sheep and two African ruminants. The giraffe was chosen because a previous study

demonstrated that these ruminants have unique RPz [9]. The bongo was chosen since it is a member of the *Bovidae* family and thus their RPz may behave like cattle RPz.

Conjugation was measured by enumerating recovered ceftiofur-resistant *Salmonella* since the *Klebsiella* bears a plasmid encoding an extended-spectrum beta-lactamase [2]. As shown in Table 2.2, the *Klebsiella*-to-*Salmonella* plasmid transfer events were also observed in RPz obtained from sheep, giraffe and bongo. Conjugation frequencies were slightly higher in RPz obtained from the African ruminants although this difference may not be biologically significant.

Assessment of DT104 hyperinvasion after exposure to RPz obtained from various ruminants

Our previous studies revealed that SGI1-bearing strains of *Salmonella* are hyperinvasive after exposure to RPz obtained from cattle. To determine if this phenomenon extends beyond cattle, SGI1-bearing *Salmonella* were evaluated for ovine and caprine RPz-mediated alterations in invasiveness using a HEp-2 tissue culture cell invasion assay. Additionally, we assessed this phenomenon in RPz obtained from the bongo and giraffe. The hyperinvasive state of DT104 was compared to that of TH11, a strain that lacks SGI1 and therefore is not capable of exhibiting the RPz-mediated hyperinvasive phenotype [3,5].

As shown in Figure 2.1, DT104 invasion was increased following recovery from bovine RPz which is consistent with previous data [3,5]. DT104 recovered from ovine RPz were also significantly more invasive when compared to that observed for TH11 although the increase in invasiveness was not as robust as the increase noted in the presence of bovine RPz. For all other RPz examined, invasiveness was indistinguishable for DT104 and TH11.

Assessment of RPz-mediated Salmonella hypervirulence in vivo

Previous studies indicated that RPz-mediated hyperinvasion is a predictor of hypervirulence *in vivo* [3,5]. In the present study we assessed clinical outcomes in calves, sheep, and goats infected with DT104 exposed to RPz. Studies involved monitoring the clinical signs and culturing splenic samples from infected animals. Control animals were infected with TH11 lacking SGI1 [3,5].

As depicted in Figure 2.2, significantly more DT104 were recovered from calves and sheep when compared to goats. Similarly, calves and sheep demonstrated pyrexia at 24-48 hr post infection whereas goat rectal temperatures did not differ from normal (Figure 2.3).

Assessment of SO13 and hilA expression in DT104 inoculated into cattle and sheep

Our previous studies indicated that RPz-mediated hypervirulence in DT104 is associated with enhanced expression of *hilA* and SO13 following protozoal engulfment [5]. In the present study, a semi-quantitative RT-PCR was used to determine relative expression levels of *hilA* and SO13 in DT104 and TH11 recovered from calves, sheep, and goats.

As shown in Figure 2.2, RPz-mediated hypervirulence was associated with increased expression of *hilA* and SO13 in DT104 recovered from calves and sheep. This was especially evident for the SO13 amplicon that could be amplified from DT104 recovered from calves and sheep in the primary RT-PCR reaction. In contrast, secondary and tertiary PCR reactions were required before a visible amplicon could be generated in TH11 recovered from all animals and from DT104 recovered from goats.

DISCUSSION

Previously we have demonstrated that DT104 exhibits hypervirulence following exposure to RPz obtained from cattle [3,5]. Additionally, bovine and caprine RPz can serve as venues for plasmid acquisition by *Salmonella* [2]. The study herein describes experiments addressing the hypothesis that these phenomena extend to an array of RPz obtained from domestic and exotic ruminants although the exotic ruminants were housed in a domestic environment.

Plasmid transfer studies revealed that all RPz tested could serve as facilitators for this event. This finding is consistent with our unpublished observations that free-living protozoa, such as *Tetrahymena* and *Acanthamoeba*, can mediate plasmid transfer into *Salmonella*. Additionally, our preliminary studies suggest that *Salmonella* can acquire antibiotic resistance genes while residing within pathogenic *Eimeria* and *Trichomonas* (not shown). That is, *Salmonella* appears to be able to conjugate with other bacteria in a wide array of protozoa. Based on our previous studies, it is unclear if this conjugation process can occur within mammalian cells that engulf or are passively invaded by *Salmonella* [2]. Differences in intracellular trafficking may account for this divergence.

The instigation of hypervirulence, however, is not as ubiquitous. Hyperinvasion and hypervirulence were noted only for *Salmonella* exposed to RPz obtained from cattle and sheep. Expression of SO13 and hyperexpression of *hilA* were associated with the augmented invasion and virulence observed in sheep in a manner similar to our previous and current findings with cattle. There does appear to be a difference in the clinical progression of hypervirulence in cattle versus sheep, while the disease course in goats was unremarkable. We hypothesize that

differences in RPz profiles account for the non-conserved nature of the hyperinvasion/hypervirulence. *Isotricha* is a putative non-engulfing RPz [3] that may be relatively less abundant in the bovine and ovine rumens. Alternatively, sheep and cattle rumens may contain unique RPz that engulf and modulate gene expression in *Salmonella*. The time of day, time after feeding, and diet are other factors influencing RPz profiles [11,12].

In summary, the phenomenon of RPz-mediated *Salmonella* gene transfer is viable in cattle, sheep, goats, bongo, and giraffe. The RPz-hypervirulence phenomenon is relegated to cattle and sheep. RPz profiles are the likely determinant of the divergence regarding the latter phenomenon.

REFERENCES

1. Briggs CE, Fratamico PM (1999) Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. Antimicrobial Agents and Chemotherapy 43: 846-849.

2. McCuddin Z, Carlson S, Rasmussen M, Franklin S (2006) Klebsiella to Salmonella gene transfer within rumen protozoa: implications for antibiotic resistance and rumen defaunation. Vet Microbiol 114: 275-284.

3. Rasmussen M, Carlson SA, Franklin SK, Wu MT, McCuddin ZP, et al. (2005) Exposure to rumen protozoa leads to enhancement of invasion and pathogenicity for multiple antibiotic resistant *Salmonella* bearing SGI-1. Infect Immun 73: 4668-4675.

4. Lee CA, Jones BD, Falkow S (1992) Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci USA 89: 1847-1851.

5. Carlson S, Sharma V, McCuddin Z, Rasmussen M, Franklin S (2007) Involvement of a Salmonella genomic island 1 gene in the rumen protozoan-mediated enhancement of invasion for multiple-antibiotic-resistant Salmonella enterica serovar Typhimurium. Infect Immun 75: 792-800.

6. Evans S, Davies R (1996) Case control study of multiple-resistant *Salmonella typhimurium* DT104 infection of cattle in Great Britain. Veterinary Record 139: 557-558.

7. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, et al. (1999) Detection of *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. Molecular and Cellular Probes 13: 213-222.

8. Coleman GS, Reynolds DJ (1982) The uptake of bacteria and amino acids by Ophryoscolex caudatus, Diploplastron affine and some other rumen Entodiniomorphid protozoa. J Appl Bacteriol 52: 135-144.

9. Dehority BA, Odenyo AA (2003) Influence of diet on the rumen protozoal fauna of indigenous African wild ruminants. Journal of Eukaryotic Microbiology 50: 220-223.

11. Ankrah P, Loerch SC, Dehority BA (1990) Sequestration, Migration and Lysis of Protozoa in the Rumen. Journal of General Microbiology 136: 1869-1875.

12. Purser DB, Moir RJ (1966) Dietary Effects Upon Concentrations of Protozoa in Rumen. Journal of Animal Science 25: 668-&.

Table 2.1. Summary of microbes used in this study.

Microbe	Relevant Characteristics	Reference
Salmonella enterica serotype Typhimurium DT104 strain 98-420	SGI1-bearing strain capable of exhibiting hypervirulence following exposure to RPz	[3]
Salmonella enterica serotype Typhimurium DT104 strain TH11	SGI1-free isostrain of DT104 incapable of hyperinvasion	[7]
Klebsiella pneumoniae TCR2003	Isolated from turtle feces in Iowa; bears a ceftiofur resistance plasmid	[2]

Table 2.2. Summary of *Klebsiella-Salmonella* gene transfer events observed *in vitro*. *K. pneumoniae* TCR2003 was used as the donor strain in all studies. Experiments used 10^9 potential recipients, 10^9 donors, and 10^4 RPz.

Salmonella recipient	RPz source	Replicates	Number of ceftiofur- resistant *Salmonella* recovered/10^9 recipients (mean $\pm$ sem)
TH11	Bovine	12	408 ± 85
TH11	Caprine	12	387 ± 115
TH11	Ovine	12	298 ± 37
TH11	Bongo	12	877 ± 83
TH11	Giraffe	12	1094 ± 27
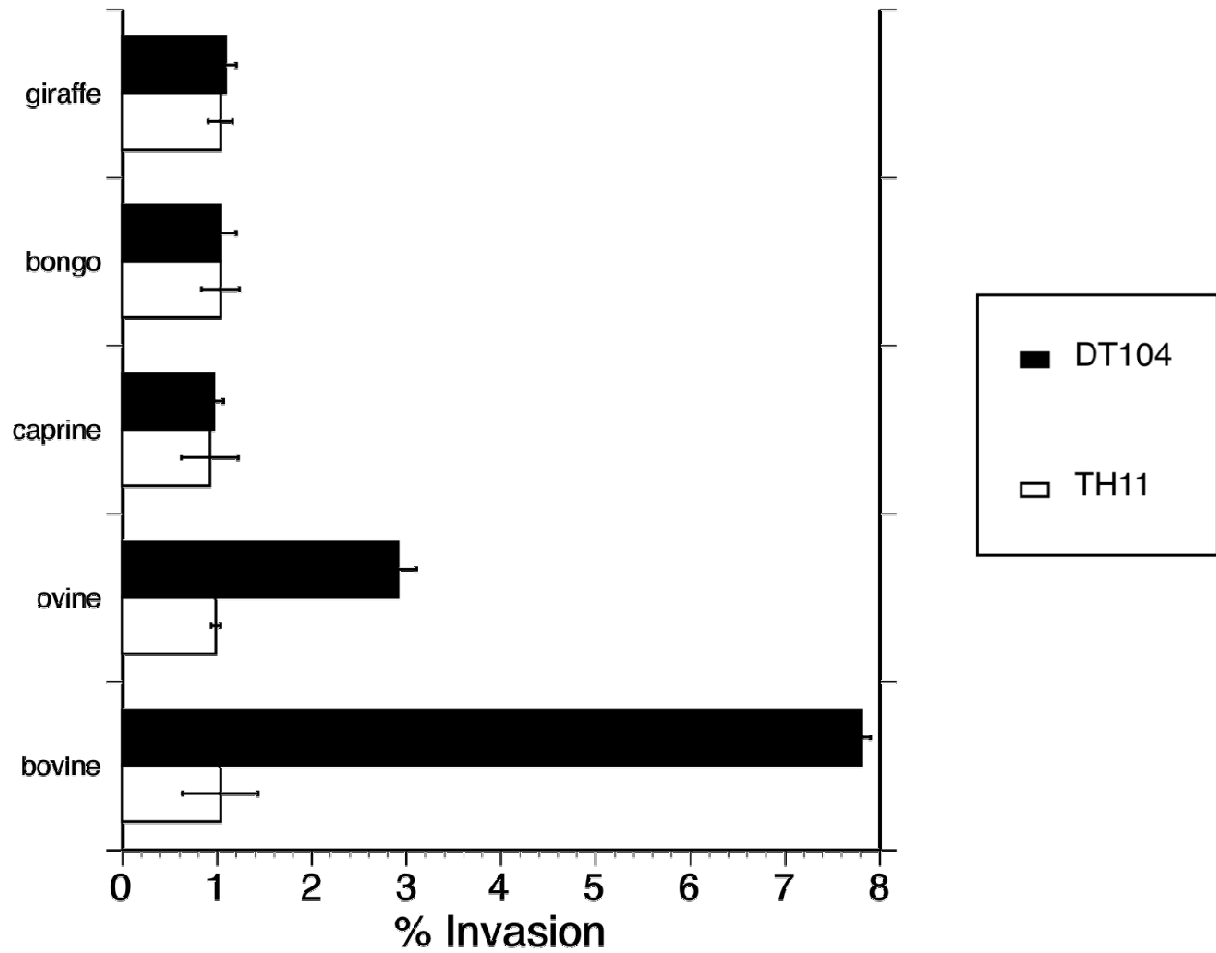
Figure 2.1. Assessment of HEp-2 cell invasion by SGI1-bearing (DT104) or SGI1-free (TH11) *Salmonella* recovered from RPz. The source of the RPz is indicated on the *y*-axis. Each bar represents the mean ± standard error for 12 replicates.
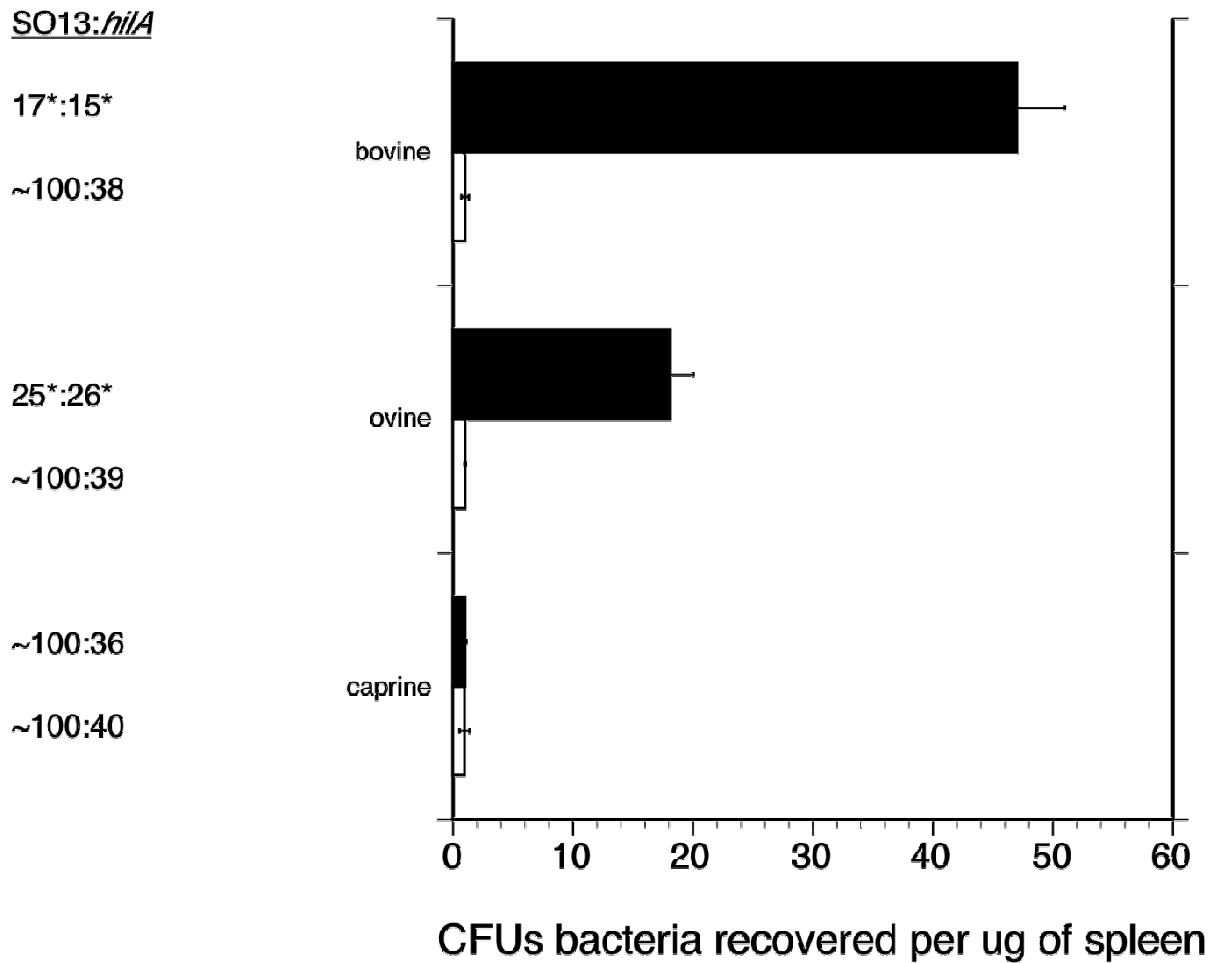
Figure 2.2. Assessment of bacterial loads and semi-quantitative analysis of virulence gene expression for *Salmonella* DT104 or TH11 exposed to RPz in sheep, calves, and goats. Left side, virulence gene expression of *hilA* and SO13 in DT104 (upper sets of numbers) and TH11 (lower sets of numbers) recovered from calves, sheep, and goats. Values indicate the number of PCR cycles necessary to generate a visible SO13 or *hilA* amplicon following an RT-PCR assay. Right side, quantitation of DT104 (filled bars) or TH11 (open bars) recovered from splenic samples of animals infected with the pathogens. Each bar represents the mean ± sem for 6 animals.* Indicates hyperexpression.
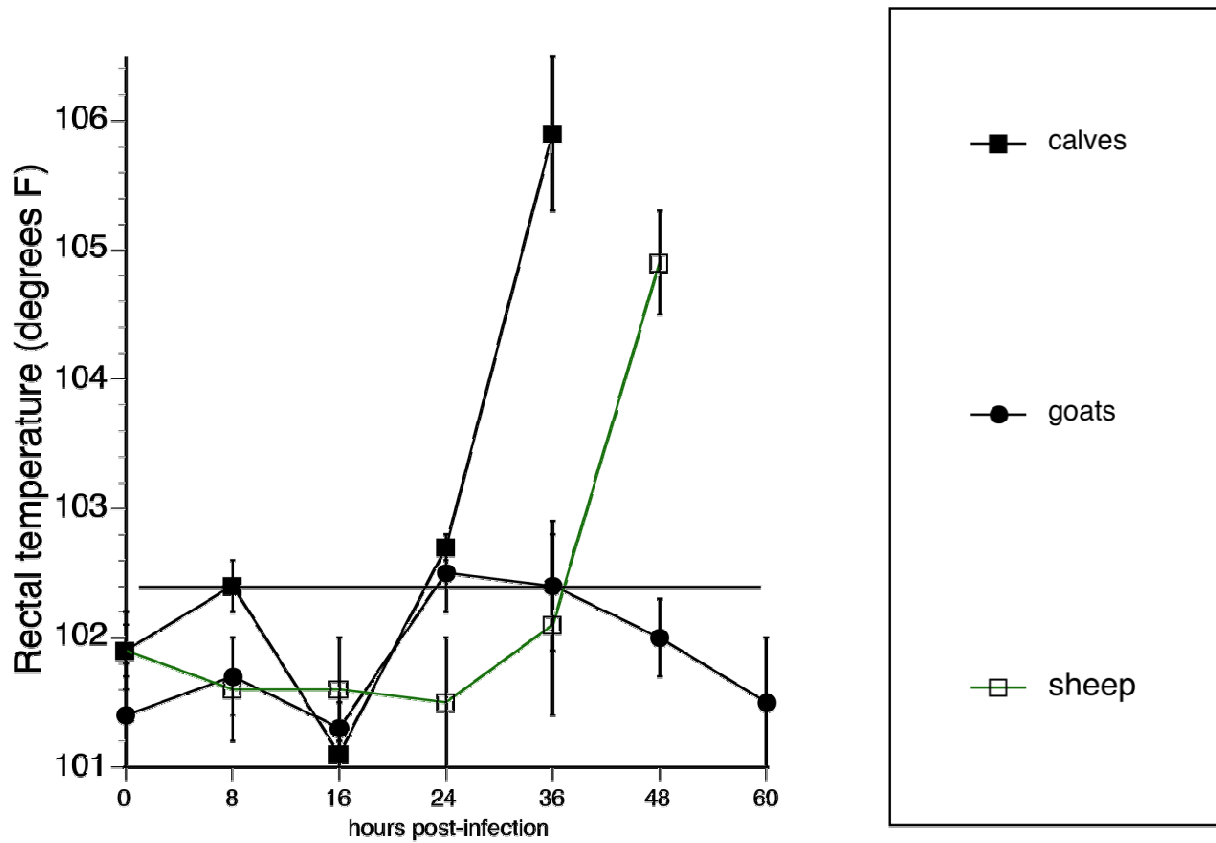
Figure 2.3. Onset of pyrexia in calves, sheep, and goats orally infected with DT104. Rectal temperatures were determined every 8 to 12 hours. The upper limit for normal bovine rectal temperature (~102.5^{o}F) is indicated by the horizontal line. Each point represents the mean ± sem for 6 animals.

CHAPTER 3. RNAi-MEDIATED INHIBITION OF *SALMONELLA* HYPERVIRULENCE INSTIGATED BY *TETRAHYMENA*

Matt T. Brewer and Steve A. Carlson

ABSTRACT

Recent studies have identified a phenomenon in which rumen protozoa engulf *Salmonella* and the harsh intracellular environment induces virulence gene expression and a hyperinvasive phenotype. In the present study, we investigate the hyperinvasive behavior of *Salmonella* DT104 following engulfment by *Tetrahymena thermophila*, a free-living ciliate. We evaluate the use of siRNA designed to target the expression *Tetrahymena* genes with a potential role in bacterial engulfment. Protozoa-mediated *Salmonella* hypervirulence, as measured by an *in vitro* cell invasion assay and an *in vivo* calf infection study, was observed following engulfment by *Tetrahymena.* Electroporation of *Tetrahymena* with siRNA targeting expression of a putative cyclic nucleotide binding protein designated TetCNB-1 resulted in decreased expression of *CNB1*. Decreased *CNB1* expression was associated with inhibition of bacterial engulfment and inability to induce the DT104 hyperinvasive phenotype. These data suggest that TetCNB-1 is associated with the engulfment and intracellular processing of bacteria. In conjunction with an RNAi-based approach, *Tetrahymena* may offer a useful model for the study of rumen protozoa-mediated *Salmonella* hypervirulence.

INTRODUCTION

Protozoa are predators that readily consume bacteria as a nutrient source. However, several bacterial taxa are capable of resisting degradation by protozoa and thriving within these

cells [1]. This observation is relevant to intracellular bacterial pathogens since intra-protozoal survival implicates protozoa as environmental reservoirs for these organisms [2,3]. Such symbioses are not restricted to environmental protozoa. In the case of *Salmonella enterica* serotype Typhimurium phagetype DT104 (DT104), bacteria survive engulfment by commensal rumen protozoa located in the foregut of ruminants. Here, DT104 is protected from the harsh environment of the rumen until they are transported to the abomasum where the protozoa are degraded and DT104 is released [4,5].

In addition to their protective role, rumen protozoa (RPz) enhance the virulence of DT104. First, they provide a site of conjugal plasmid transfer where *Salmonella* can acquire antibiotic resistance genes [6,7]. Second, engulfment of DT104 by RPz stimulates hyperexpression of bacterial virulence genes leading to the induction of a hyper-invasive phenotype that results in faster onset of clinical signs and a greater recovery rate of the pathogen [4,5]. Conjugal plasmid transfer is observed in a variety of commensal and free-living protozoa while protozoa-mediated hypervirulence is initiated only in RPz from sheep and cattle [4-6]. Protozoa-mediated hypervirulence is restricted to DT104 strains bearing a virulence gene cassette designated *Salmonella* genomic island 1 (SGI1) [4,5]. Other taxa of protozoa also hyperactivate SGI1-bearing strains of DT104. For example, the amoeboid protozoan *Acanthamoeba* potentiates salmonellosis in both cattle and swine [4,8].

The protozoal receptors and cellular processes governing bacterial recognition, engulfment, and activation of the DT104 hyperinvasive phenotype are not well-characterized. This is partially due to the difficulty of maintaining rumen protozoa in axenic culture in addition to the fact that there are no genomic databases or tools available for these organisms. On the

other hand, *Tetrahymena thermophila* is a widely utilized model ciliate that is a known predator of bacteria. *Tetrahymena* is amenable to axenic culture and has readily available genomic database resources. Therefore, *Tetrahymena* may be a potentially useful model for the study of protozoal processes related to rumen protozoa-mediated *Salmonella* hypervirulence.

Protozoan predation of bacteria involves chemical detection of prey, followed by predator-prey interactions that initiate cytoskeletal rearrangement and subsequent phagocytosis [9]. Chemosensory feeding behavior may be driven by G protein-coupled receptors (GPCRs) which bind to bacterial components [10]. GPCR ligands can also have indirect stimulatory or inhibitory effects on phagocytosis in *Tetrahymena* [11,12]. Alternatively, ciliates such as *Tetrahymena* possess an oral groove where food items are swept indiscriminately [13]. Therefore, cyclic nucleotide binding proteins such as protein kinases may be more pertinent to the phagocytic process [14].

The present study was undertaken to assess the hyperinvasive behavior of *Salmonella* DT104 following engulfment by *Tetrahymena*. In addition, RNAi-based studies were employed to identify protozoal genes related to bacterial engulfment and induction of the *Salmonella* hyperinvasive phenotype.

MATERIALS AND METHODS

Cell Cultures

Cell strains and plasmids used are summarized in Table 3.1. Bacteria were stored in cryopreservation tubes containing 50% glycerol:50% culture medium at -70°C and grown in LB broth at 37°C with antibiotics such as ampicillin (50 μg/mL, Sigma), minocycline (50 μg/mL,

Sigma), and cefepime (50 µg/mL, Bristol-Myers Squib). Wild-type *Tetrahymena thermophilia* were obtained from ATCC and axenically grown in proteose peptone medium (5.0 g proteose peptone, 5.0 g tryptone, 0.2 g K_2HPO_4, 1 L distilled water) at $25^{o}C$. Media was replaced every four days and cells were diluted 1:50 in fresh media. HEp-2 cells were maintained in RPMI1640 (Gibco) containing 10% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator.

RNAi Experiments

Bovine rumen protozoa were obtained from a fistulated cow and total RNA was extracted using an RNA mini kit (Qiagen). cDNA was then constructed by performing a RT-PCR reaction utilizing the Superscript One-Step RT-PCR kit (Qiagen) and random hexamers and polyT oligomers primers. PCR products were cloned into pCRXL and sequenced by the Iowa State University DNA facility. A BLAST analysis was conducted using GenBank to identify *Tetrahymena* homologs with a putative role in phagocytosis and three genes were selected. Two of the genes encode putative g protein-coupled receptors and the third encodes a putative cyclic nucleotide binding protein (XM_001021012.2, XM_001010055.2, and XM_001027519.2, respectively). siRNA engineered to silence these genes was designed using the Invitrogen webportal. The sequences CAGAAGTGCATTGAAGGAATA, TGGCTCAGTGTAAGTGACTTAATAT, and GCTGATTCATTTAATAGCCTTGCTT were deemed to be appropriate targets.

Tetrahymena were grown in 1 mL of media to a concentration of 10^4 cells/mL and were harvested by centrifugation at 3,000xg for 10 min. The pellet was washed twice, resuspended in 100µL, and transferred to a 0.2cm cuvette. Cells were then electroporated (0.2 cm electrode gap, 10µF, 5 ms) with 0.5nM siRNA. Post-transformation viability was assessed using trypan

blue staining 24 hours after electroporation. Transfected *Tetrahmena* were used in *in vitro* engulfment and invasion assays as well as an *in vivo* calf infection study.

Tetrahymena gene expression was assessed by RT-PCR following RNA extraction with the RNeasy minikit (Qiagen). The number of cycles in the PCR portion of the reaction was varied to determine the number of cycles needed to produce a visible amplicon from wild-type and knock-down strains. RT-PCR products were visualized on a 1.5% agarose gel using ethidium bromide staining.

Engulfment and Invasion Assays

10^4 siRNA-transfected *Tetrahymena* were co-incubated with 10^9 CFUs *Salmonella* and gently rolled for 18 hr at room temperature. To kill extracellular bacteria, 100μg/mL cefepime (Maxipime, Bristol-Myers Squib) was added and the mixture was rolled for 1 hr at room temperature. *Tetrahymena* cells were lysed for 60 s at 4,800 RPM using 0.5 mm glass beads and a mini-beadbeater (Biospec products). The lysate was centrifuged at 3,000 RPM for 2 min and resuspended in 300μL LB broth (Sigma). 100μL aliquots were plated on LB agar (Sigma) in triplicate for enumeration.

Tetrahymena and *Salmonella* were co-incubated as described above and the *Salmonella*-containing lysate was used in a HEp-2 cell invasion assay. 100μL aliquots of lysate were added in triplicate to cell culture wells containing approximately 10^5 HEp-2 cells and incubated for 1 hr at 37ºC in a 5% CO_2 humidified incubator. Extracellular bacteria were then killed using 100μg/mL cefepime during a 1 hr incubation at 37^oC in a 5% CO_2 humidified incubator. HEp-2 cells were lysed using 1% Triton X at 37^oC and the lysate was plated on

selective media and grown overnight at 37°C. Colonies were enumerated and percent invasion

was calculated by dividing CFU recovered by CFU added.

In vivo infection study

Salmonella were co-incubated with RNAi-transfected *Tetrahymena* as described above

except that each strain was paired with a *Salmonella* DT104 isolate with a unique antibiogram

(Table 3.1). Neonatal Holstein calves (n=3) were orally inoculated with 9mL of a cocktail

containing 3mL of each *Salmonella*-laden strain of *Tetrahymena*.

Animals were monitored for changes in appetite, stool consistency, and rectal

temperature every 8 to 12 hrs. At the onset of signs related to systemic salmonellosis, calves

were euthanized with xylazine (0.5mg/Kg intramuscularly; Phoenix Laboratories) and

pentobarbital (1.2mg/Kg, intravenously; Fort Dodge Laboratories). Splenic samples, which are a

reliable indicator of systemic burden, were collected following euthanasia.

Splenic tissue was aseptically cut into 0.1cm pieces, placed in an equal volume of PBS,

and vortexed for 2 min. Dilutions of the resulting homogenate were plated on BGA agar

containing either ampicillin (50 μg/mL), minocycline (50 μg/mL), or cefepime (50 μg/mL) for

enumeration. *Salmonella* colonies were confirmed by PCR using primers specific for detection

of the *sipB-sipC* gene fragment as described previously [15].

RESULTS

Engulfment by Tetrahymena induces the hyperinvasive phenotype of DT104

Previous studies indicate that a variety of commensal rumen protozoa engulf, protect,

and induce a hyperinvasive phenotype in *Salmonella* DT104. We hypothesized that

Tetrahymena, a free-living ciliate, would also induce the hyperinvasive phenotype. In the present student, *Salmonella* liberated from *Tetrahymena* were nearly 7 times more invasive in a HEp-2 cell invasion assay (Figure 3.1). In support of these *in vitro* findings, oral inoculation of Holstein calves with *Salmonella*-laden *Tetrahymena* resulted in illness consistent with protozoa mediated hypervirulence (rapid onset of pyrexia, diarrhea, dehydration, depression; data not shown) and an increased systemic load of bacterial (Figure 3.2).

RNAi inhibition of bacterial engulfment and protozoa-mediated hypervirulence in vitro

To determine if siRNA can interfere with expression of protozoal genes associated with bacterial engulfment, *Tetrahymena* were electroporated with siRNA and assayed for their ability to engulf *Salmonella* DT104. Next, bacteria recovered from protozoa were evaluated for invasive behavior in a HEp-2 cell invasion assay [4,5].

Transfection of *Tetrahymena* with *CNB1*-targeting siRNA was associated with inhibition of bacterial engulfment by *Tetrahymena* and we observed a 5 fold decrease in the number of bacteria engulfed by the protozoan (Figure 3.3). In addition, knockdown of *CNB1* had an inhibitory effect on protozoa-mediated hypervirulence *in vitro*. As shown in Figure 3.1, the invasive ability of *Salmonella* recovered from this line of knockdown protozoa was comparable to bacteria not exposed to protozoa. Transfection of *Tetrahymena* with GPCR-targeting siRNA did not alter bacterial engulfment or protozoa-mediated hypervirulence. RT-PCR revealed that electroporation with RNAi targeting *CNB1* was successful (Figure 3.1) while RNAi targeting *Tetrahymena* G protein-coupled receptors was unsuccessful in achieving knockdown of gene expression (data not shown).

RNAi inhibition of protozoa-mediated hypervirulence in vivo

In vivo infection studies consisted of orally inoculating Holstein calves with *Salmonella*-laden RNAi-transfected strains of *Tetrahymena*. Each transfected strain contained a *Salmonella* isolate with a unique antibiogram and each calf received all three strains. Thus, the relative virulence of bacteria originating from each *Tetrahymena* strain could be determined by comparing the systemic burden of *Salmonella* bearing a particular antibiogram.

At the time of necropsy, selective media containing antibiotics was used to isolate *Salmonella* from splenic tissue. While *Salmonella* originating from two of the *Tetrahymena* strains was abundant (approximately 3×10^7 CFU/gm spleen), *Salmonella* originating from *CNB1*-knockdown *Tetrahymena* only accounted for approximately 0.03% of the total systemic burden (Figure 3.2). All *Salmonella* isolates were confirmed by PCR amplification of the *sipBC* locus [15].

DISCUSSION

Previous studies in our laboratory have demonstrated that DT104 exhibits a hyperinvasive phenotype following engulfment by rumen protozoa [4-6]. The present study assessed the ability of *Tetrahymena*, a free-living ciliate, to mediate these changes. In addition, we assessed the impact of siRNA on bacterial engulfment and the ability to induce the hyperinvasive phenotype of DT104.

In vitro cell invasion assays and *in vivo* calf infection studies revealed that DT104 is hyperinvasive following engulfment by *Tetrahymena*. Consistent with our previous studies, this phenomenon occurred in DT104 bearing SGI1 [4-6]. *Tetrahymena* and other free-living ciliates may represent an important reservoir for *Salmonella* and other intracellular pathogens. While

some taxa of protozoa provide refuge for bacteria, some species preferentially predate and kill pathogenic bacteria [16]. Further research is needed to understand the significance of *Salmonella* and other pathogens harbored within environmental protozoa such as *Tetrahymena*.

Protozoa require the ability to detect and engulf bacterial prey items in the environment. However, the receptors mediating these feeding behaviors are not well known. In the present study, we electroporated *Tetrahymena* with siRNA targeting expression of putative GPCRs and Tet-CNB1, a putative cyclic nucleotide binding protein. While we did not observe phenotypic effects associated with transfection with siRNA targeting G protein-coupled receptors, knockdown of *CNB1* inhibited bacterial engulfment by *Tetrahymena*. Interestingly, knockdown of *CNB1* also ameliorated protozoa mediated *Salmonella* hypervirulence. That is, these *Tetrahymena* were not capable of activating the hyperinvasive phenotype *in vitro* or in a calf model.

The calf model in this study is novel and presents a platform for further studies investigating RPz-mediated salmonellosis. Protozoa that were allowed to feed on DT104 with a unique antibiogram were orally inoculated into Holstein calves. All calves received all *Tetrahymena* knockdown strains and we could then detect the relative systemic burden of DT104 originating from a particular *Tetrahymena* by growth on selective media. This approach could be utilized to determine the relative contributions of different RPz genera to hypervirulence since it is unknown which RPz are the most significant inducers of the DT104 hyperinvasive phenotype.

Cyclic nucleotide binding proteins have a variety of functions including their role as enzymes and transcription factors [17]. Since knockdown of Tet-CNB1 had effects on both engulfment and ability to invoke the hyperinvasive phenotype, it is possible that this is a transcription factor that regulates multiple systems within the cell. Alternatively, TetCNB-1 could be a kinase involved in intracellular vesicle trafficking. The physiologic role of *Tetrahymena* cyclic nucleotide binding proteins, in particular TetCNB-1, is unclear at this point and warrants further investigation.

Tetrahymena possesses the cellular machinery for RNAi and investigators have achieved knockdown of gene expression with hairpin structures [18]. Electroporation may offer a viable tool for delivery of dsRNA molecules. In the present study, the decrease in mRNA levels of TetCNB-1 was transient, lasting approximately 1 week. This is presumably because transfected and non-transfected cells were present in the cultures and after several matings normal gene expression returned. Electroporation of individual cells may offer an option for generating *Tetrahymena* knockdown strains; however, this work is beyond the scope of the present study. Trypan blue staining assays and growth kinetic studies revealed that RNAi did not have an effect on the viability of the *Tetrahymena* cells (data not shown).

In summary, this study demonstrated that *Salmonella* DT104 bearing SGI1 are hyperinvasive following engulfment by *Tetrahymena*, a free-living ciliate. Electroporation of *Tetrahymena* with siRNA targeting the knockdown of Tet-CNB1, a cyclic nucleotide binding protein, perturbed both bacterial engulfment and the protozoan's ability to invoke the hyperinvasive *Salmonella* phenotype. Additional work is required to clarify the role of cyclic nucleotide binding proteins in *Tetrahymena*. In conjunction with an RNAi-based approach,

Tetrahymena may provide a useful model for the study of protozoa-mediated *Salmonella*

hypervirulence.

REFERENCES

1. Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. Clinical
   Microbiology Reviews 17: 413-+.

2. Barker J, Humphrey TJ, Brown MWR (1999) Survival of Escherichia coli O157 in a soil
   protozoan: implications for disease. Fems Microbiology Letters 173: 291-295.

3. Whan L, Grant IR, Rowe MT (2006) Interaction between Mycobacterium avium subsp
   paratuberculosis and environmental protozoa. Bmc Microbiology 6.

4. Carlson SA, Sharma VK, McCuddin ZP, Rasmussen MA, Franklin SK (2007) Involvement of a
   Salmonella genomic island 1 gene in the rumen protozoan-mediated enhancement of
   invasion for multiple-antibiotic-resistant Salmonella enterica serovar Typhimurium.
   Infect Immun 75: 792-800.

5. Rasmussen MA, Carlson SA, Franklin SK, McCuddin ZP, Wu MT, et al. (2005) Exposure to
   rumen protozoa leads to enhancement of pathogenicity of and invasion by multiple-
   antibiotic-resistant Salmonella enterica bearing SGI1. Infect Immun 73: 4668-4675.

6. Brewer MT, Xiong NL, Dier JD, Anderson KL, Rasmussen MA, et al. (2011) Comparisons of
   Salmonella conjugation and virulence gene hyperexpression mediated by rumen
   protozoa from domestic and exotic ruminants. Veterinary Microbiology 151: 301-306.

7. McCuddin Z, Carlson SA, Rasmussen MA, Franklin SK (2006) Klebsiella to Salmonella gene
   transfer within rumen protozoa: Implications for antibiotic resistance and rumen
   defaunation. Veterinary Microbiology 114: 275-284.

8. Xiong N, Brewer MT, Day TA, Kimber MJ, Barnhill AE, et al. (2010) Evaluation of the
   pathogenicity and virulence of three strains of Salmonella organisms in calves and pigs.
   Am J Vet Res 71: 1170-1177.

9. Roberts EC, Legrand C, Steinke M, Wootton EC (2011) Mechanisms underlying chemical
   interactions between predatory planktonic protists and their prey. Journal of Plankton
   Research 33: 833-841.

10. Leick V, Lindemose S (2007) Chemokinesis by Tetrahymena in response to bacterial oligopeptides. Journal of Eukaryotic Microbiology 54: 271-274.

11. Chiesa R, Silva WI, Renaud FL (1993) Pharmacological Characterization of an Opioid Receptor in the Ciliate Tetrahymena. Journal of Eukaryotic Microbiology 40: 800-804.

12. Quinonesmaldonado V, Renaud FL (1987) Effect of Biogenic-Amines on Phagocytosis in Tetrahymena-Thermophila. Journal of Protozoology 34: 435-438.

13. Smith HE (1982) Oral-Apparatus Structure in the Carnivorous Macrostomal Form of Tetrahymena-Vorax. Journal of Protozoology 29: 616-627.

14. Hartz AJ, Sherr BF, Sherr EB (2008) Using inhibitors to investigate the involvement of cell signaling in predation by marine phagotrophic protists. Journal of Eukaryotic Microbiology 55: 18-21.

15. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, et al. (1999) Detection of multiresistant Salmonella typhimurium DT104 using multiplex and fluorogenic PCR. Molecular and Cellular Probes 13: 213-222.

16. Ravva SV, Sarreal CZ, Mandrell RE (2010) Identification of Protozoa in Dairy Lagoon Wastewater that Consume Escherichia coli O157:H7 Preferentially. Plos One 5.

17. McCue LA, McDonough KA, Lawrence CE (2000) Functional classification of cNMP-binding proteins and nucleotide cyclases with implications for novel regulatory pathways in Mycobacterium tuberculosis. Genome Research 10: 204-219.

18. Howard-Till RA, Yao MC (2006) Induction of gene silencing by hairpin RNA expression in Tetrahymena thermophila reveals a second small RNA pathway. Molecular and Cellular Biology 26: 8731-8742.

Table 3.1. Summary of microbes and nucleic acids used in this study.

Identity	Details	Reference/Comments
Salmonella typhimurium DT104	Hyperinvasive after exposure to protozoa	Rasmussen et al., 2005
Salmonella typhimurium TH11	Not hyperinvasive protozoa exposure	Carlson et al., 1999
Hep-2 Cells	Mammalian cell line	
pCRXL	Confers zeocin resistance	Commercially Available
pMinocycline	Confers minocycline resistance	Xiong et al.,2011
pCeftriaxone	Confers ceftriaxone resistance	McCuddin et al., 2006
RNAi1	AUAUUAAGUCACUUACACUGAGCCA	Targets GPCR
RNAi2	GCUGAUUCAUUUAAUAGCCUUGCUU	Targets GPCR
RNAi3	CAGAAGTGCATTGAAGGAATA	Targets Tet-CNB1
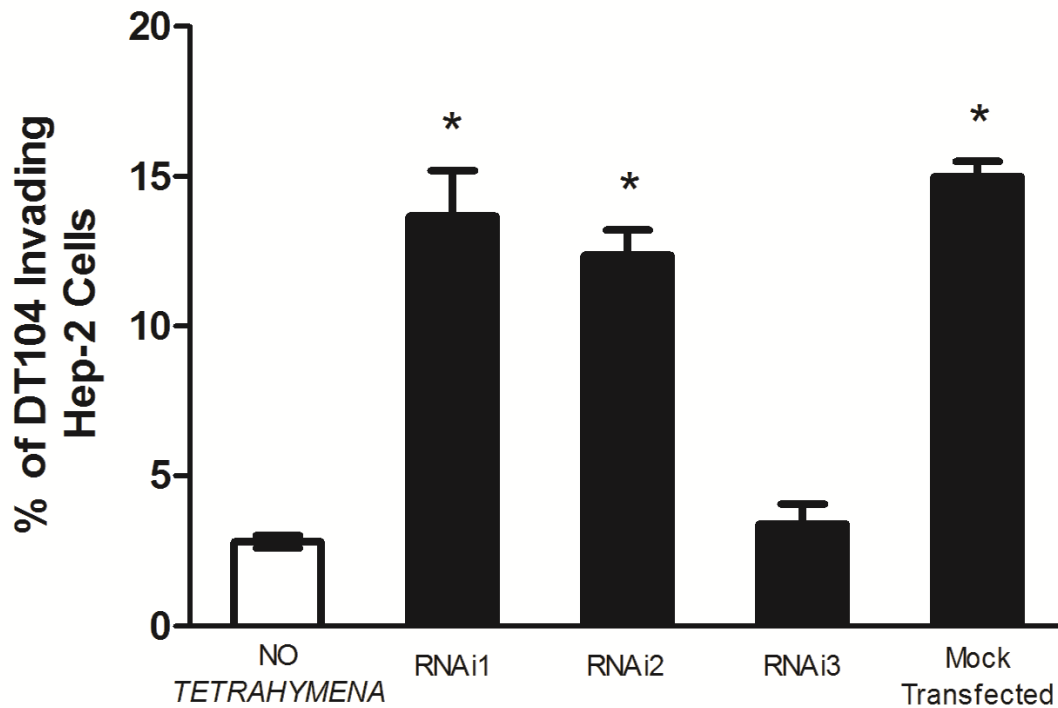
Figure 3.1. Invasion of HEp-2 cells by *Salmonella* DT104 following engulfment by *Tetrahymena*. *Tetrahymena* were elecroporated with siRNA targeting G protein coupled receptors (RNAi1 and RNAi2) or TetCNB-1(CNBP). The open bar represents the invasion of DT104 without any exposure to *Tetrahymena.* Percent invasion is calculated by dividing the number of bacteria recovered from within cells by the number of bacteria added. Data represented are the mean ± sem for three independent experiments performed in triplicate. *p<0.05 versus DT104 invasion in the absence of protozoa.
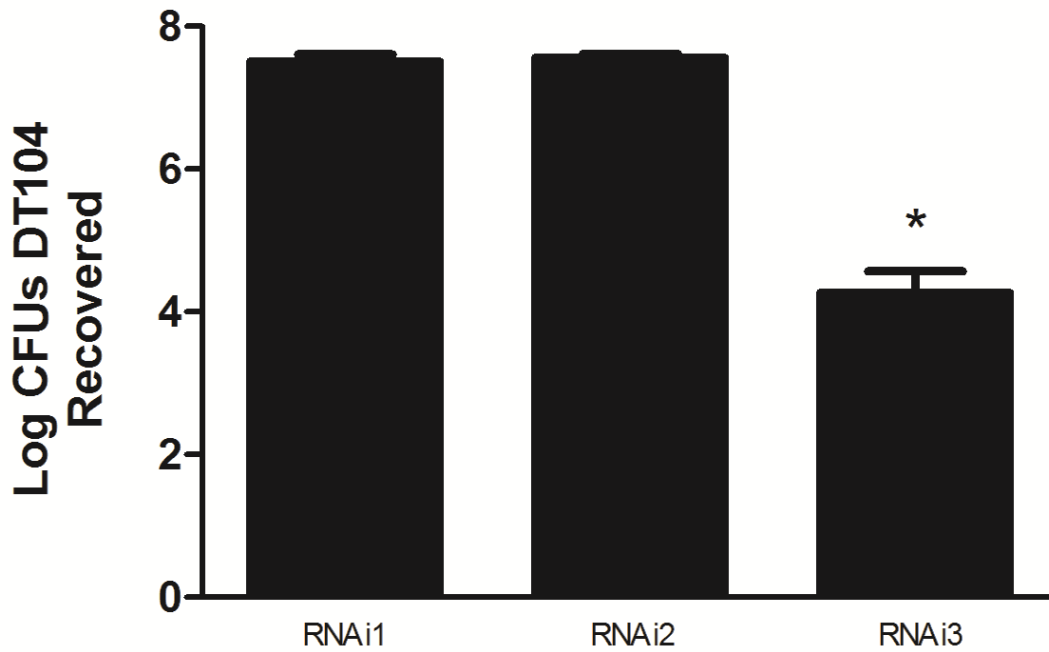
Figure 3.2. Recovery of *Salmonella* DT104 from spleens of Holstein calves orally inoculated with bacteria-containing *Tetrahymena*. *Tetrahymena* strains were electroporated with RNAi and paired with DT104 containing a unique antibiogram and all 3 calves received an equal amount of all three knockdown strains. RNAi1 and 2 were designed to target GPCRs while RNAi4 targeted TetCNB-1. Bars represent the average CFU/gram of spleen from each of three calves plated in triplicate. *p<0.05 versus other knockdown strains
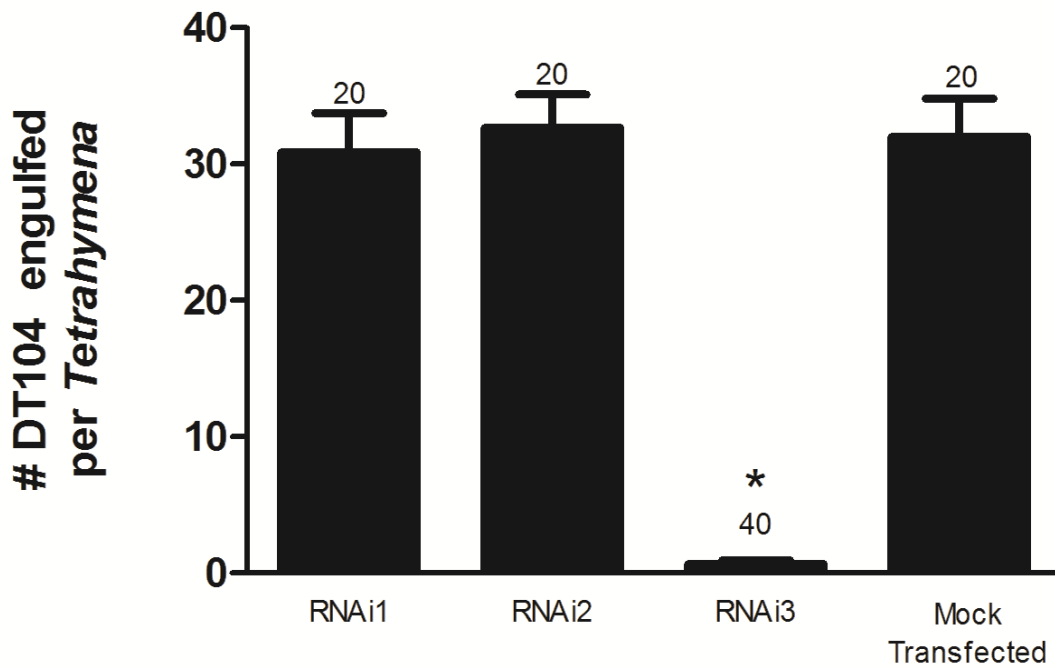
Figure 3.3. Engulfment of *Salmonella* DT104 by *Tetrahymena*. *Tetrahymena* were elecroporated with siRNA targeting G protein-coupled receptors (RNAi1 and RNAi2) or TetCNB-1(CNBP). Mock transfectants were electroporated without any siRNA. Data represented are the mean ± sem for three independent experiments performed in triplicate. Numbers above each bar represent the number of RT-PCR cycles needed to generate a visible amplicon for CNB-1. *p<0.05 versus mock transfected control.

CHAPTER 4. INVOLVEMENT OF A PUTATIVE INTERCELLULAR SIGNAL-RECOGNIZING G PROTEIN-COUPLED RECEPTOR IN THE ENGULFMENT OF *SALMONELLA* BY THE PROTOZOAN *TETRAHYMENA*

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ABSTRACT

In an effort to investigate the molecular basis of protozoa engulfment-mediated hypervirulence of *Salmonella* in cattle, we evaluated protozoan G protein-coupled receptors (GPCRs) as transducers of *Salmonella* engulfment by the model protozoan *Tetrahymena*. Our laboratory previously demonstrated that non-pathogenic protozoa (including *Tetrahymena*) engulf *Salmonella* and then exacerbate its virulence in cattle, but the mechanistic details of the phenomenon are not fully understood. GPCRs were investigated since these receptors facilitate phagocytosis of particulates by *Tetrahymena*, and a GPCR apparently modulates bacterial engulfment for the pathogenic protozoan *Entamoeba histolytica*. A database search identified three putative *Tetrahymena* GPCRs, based on sequence homologies and predicted transmembrane domains, that were the focus of this study. *Salmonella* engulfment by *Tetrahymena* was assessed in the presence of suramin, a non-specific GPCR inhibitor. *Salmonella* engulfment was also assessed in *Tetrahymena* in which expression of putative GPCRs was knocked-down using RNAi. A candidate GPCR was then expressed in a heterologous yeast expression system for further characterization. Our results revealed that *Tetrahymena* were less efficient at engulfing *Salmonella* in the presence of suramin. Engulfment was reduced concordantly with a reduction in the density of protozoa. RNAi-based studies revealed that

knock-down of one the *Tetrahymena* GPCRs caused diminished engulfment of *Salmonella*. *Tetrahymena* lysates activated this receptor in the heterologous expression system. These data demonstrate that the *Tetrahymena* receptor is a putative GPCR that facilitates bacterial engulfment by *Tetrahymena*. Activation of the putative GPCR seemed to be related to protozoan cell density, suggesting that its cognate ligand is an intercellular signaling molecule.

INTRODUCTION

Protozoa engulf *Salmonella* and, while the bacteria reside inside the protozoa, host cell invasion capabilities are hyperactivated and the bacteria are hypervirulent after exiting the protozoa. This protozoa-mediated hypervirulence occurs only for *Salmonella* strains bearing the multiresistance integron designated as SGI1, and its *in vivo* relevance has only been associated with protozoa that engulf *Salmonella* in the bovine rumen [1-5]. Free-living protozoa, like *Acanthamoeba* and *Tetrahymena*, are models for studying this phenomenon [1-5].

Protozoal determinants of engulfment are unclear but Renaud *et al*. determined that G protein-coupled receptors (GPCRs) are apparently involved in *Tetrahymena* phagocytosis of particulates [6]. Furthermore, Picazzari *et al.* demonstrated that bacterial engulfment is governed by a GPCR in the pathogenic protozoan *Entamoeba histolytica* [6]. Additionally, Lampert *et al.* presented evidence for GPCR-encoding genes in the *Tetrahymena* database [6].

The aim of this study was to assess the role of three putative GPCRs in the engulfment of *Salmonella* by *Tetrahymena*. These three putative GPCRs were identified in a GenBank database search. We used RNAi to preliminarily determine if any of these putative *Tetrahymena* GPCRs are involved in *Salmonella* engulfment. A heterologous yeast expression

system was used to partially characterize one of these candidate *Tetrahymena* GPCRs. Using these approaches we were able to identify a putative *Tetrahymena* GPCR involved in the engulfment of *Salmonella*.

MATERIALS AND METHODS

Tetrahymena culture

Tetrahymena thermophila was obtained from ATCC and axenically grown in the recommended ATCC medium (5.0 g/L proteose peptone, 5.0 g/L tryptone, and 0.2 g/L K_2HPO_4) at 25^oC. Media was replaced every four days and cells were diluted 1:14 in fresh media.

Tetrahymena engulfment of Salmonella in the presence of suramin

Approximately 10^5 to 10^9 CFUs/mL of bacteria were added to approximately 10^1 to 10^5 *Tetrahymena*/mL, maintaining a multiplicity of infection equal to 10^4. The *Salmonella-Tetrahymena* mixture was then gently rolled for 16 hrs at 37^oC in a sealed 5 mL glass tube, in the presence of various concentrations of suramin (0-200µM)- a non-specific inhibitor of GPCRs (Freissmuth *et al*., 1999). At the end of the 16-hr incubation period, extracellular *Salmonella* were killed using 300 µg/mL florfenicol [Schering-Plough; [4]]. Protozoa were then lysed for 60 sec at 4,800 rpm using 2.5 mm glass beads and a mini-beadbeater (Biospec Products). The lysate was centrifuged at 10,000 x g for 2 min, then resuspended in 350 µL Lennox L broth (Difco) that was plated on *Salmonella*-selective XLD agar plates (Fisher Scientific) and then incubated at 37^oC overnight. Characteristic black colonies were then counted the following

day. Percent engulfment was calculated as such: 100x (number of *Salmonella* recovered from protozoa/number of *Salmonella* added to *Tetrahymena*).

RNAi experiments

A preliminary GenBank database search identified three putative GPCR genes in *Tetrahymena* (XM_001009792.2, XM_001027519.2, and XM_001010055.2). siRNA was designed, using the Invitrogen webportal (http://rnaidesigner.invitrogen.com/rnaiexpress), to silence expression of these three genes in *Tetrahymena*. The sequences GAGATTACTACTAATAGCCTCTCTT, GCTGATTCATTTAATAGCCTTGCTT, and TGGCTCAGTGTAAGTGACTTAATAT were deemed to be appropriate siRNA targets for the three putative GPCRs, respectively. A random sequence (CTGACGACAGTTGCATAAAGC) was used as a control siRNA. Semi-quantitative RT-PCR (Carlson *et al*., 2007) was used to confirm the knock-down of the genes encoding the putative GPCRs (Table 1).

Tetrahymena thermophila were grown in 5 mL of media to reach confluency (3×10^4 cells/mL), and were then harvested by centrifugation at 3,000 x *g* for 10 min. The pellet was washed twice in 15 mL of deionized water and resuspended in 200 μL of deionized water. These cells were then electroporated (0.2cm electrode gap, 10μF, and 5 milliseconds) with 5nM siRNA.

At 24 hr after electroporation with siRNA, *Tetrahymena* cells were centrifuged at 4,000 x *g* for 5 min. *Tetrahymena* were then spectrophotometrically enumerated and resuspended in 1 mL of Luria-Bertani broth containing SGI1-bearing *Salmonella* at a multiplicity of infection equal to 10^4. After 1 hr of co-incubation, non-engulfed bacteria were then killed with florfenicol (300 μg/mL) and protozoa were lysed with bead-beating. Protozoal lysates were recovered and

plated on selective agar (XLD) for the enumeration of *Salmonella* engulfed by *Tetrahymena*, as described herein.

Manipulation of the gene encoding the Tetrahymena GPCR

RNAi studies identified one of the putative GPCRs [XM_001009792.2] as a potential determinant of *Salmonella* engulfment. In order to deorphanize this receptor in the yeast heterologous expression system, its gene was cloned into a yeast expression vector. Since *Tetrahymena* genes have read-through stop codons encoding glutamine residues [7], the gene sequence encoding this GPCR was synthesized (GeneScript) whereby the 13 read-through stops codons (TAA or TAG) were exchanged for glutamine codons (CAA or CAG). Codons were also optimized for expression in yeast.

Construction and cloning of the yeast expression vector containing the Tetrahymena *GPCR gene*

The synthetic *Tetrahymena* GPCR gene was PCR-amplified with forward (5'GCCATA*CCATGG*ACCAATCATTTGGAAATCAA3') and reverse (5'GCCATA-*GGATCC*TCAAGTTAGATTTATTTCACGTGAAT3') primers, which included filler sequences (underlined) and the restriction sites *NcoI* and *BamHI* (italicized) incorporated into the 5' and 3' ends of the amplicon, respectively. Purified amplicons and the linearized yeast expression vector Cp4258, which bears a leucine prototrophic marker [8,9], were co-digested with *NcoI* and *BamHI* restriction endonucleases (New England BioLabs). The digested vector and amplicons were ligated with T_4 DNA ligase (New England BioLabs) and the resulting plasmids were transformed into *Escherichia coli* K12 and individual clones were selected in ampicillin (resistance encoded by the Cp4258 vector) and grown aerobically in LB broth overnight at 37°C.

Plasmid DNA was purified using the HiSpeed Plasmid Mini Kit (Qiagen) and inserts were verified using PCR and then sequenced to confirm gene orientation and fidelity.

Transformation of yeast with the Cp4258/GPCR expression vector

Saccharomyces cerevisiae strain CY 19043 (J. Broach, Princeton University, USA) was used as the yeast recipient since these cells are leucine/histidine auxotrophs and exhibit a histidine prototrophic phenotype upon GPCR activation even if the receptor is exogenous [8,9]. Non-transformed CY 19043 yeast were grown in YPD media supplemented with all essential amino acids. Cells at mid-log phase (OD_{600} equal to 0.3 to 0.5) were co-incubated with 1 µg of Cp4258/GPCR construct, or Cp4258 bearing a non-*Tetrahymena* GPCR gene (an unpublished putative GPCR sequence from *Cryptosporidium parvum*), in the presence of 200 µg salmon sperm DNA (Invitrogen) and 0.1M LiAc (Sigma-Aldrich). Yeast cells were then incubated at $30^{o}C$ and then heat shocked at $42^{o}C$ for 15 min. Cells were plated on leucine-deficient media [1x YNB (Difco), 1x yeast synthetic dropout medium supplement without leucine (Sigma), 10 mM ammonium sulfate (Sigma), and 50% glucose] to select for transformation of Cp4258/GPCR. Transformants were verified by isolating plasmids (Promega Wizard Prep kit) and PCR-based detection of the *Tetrahymena* GPCR gene insert and its proper orientation.

Partial deorphanization of the putative Tetrahymena *GPCR using the yeast heterologous expression system*

A volume of 3 mL of leucine-deficient media was inoculated with yeast expressing the *Tetrahymena* GPCR, or the control, and grown at $30^{o}C$ to an OD_{600} equal to one. Cells were washed three times with leucine/histidine deficient medium [1x YNB (Difco), 1x yeast synthetic

drop out medium supplement lacking leucine and histidine (Sigma), 10 mM ammonium sulfate, 50% glucose, 50 mM 4-morpholinepropanesulfonic acid, pH 6.8], and then resuspended in 1 mL leucine/histidine-deficient media to a density of 15–20 cells/µL. Approximately 3,000 cells were added to each well of 96-well plates containing the same medium along with 50 µL of *Tetrahymena* culture media, *Tetrahymena* lysates (15–20 cells/µL lysed by bead-beating), or *Tetrahymena* lysates plus suramin (200 µM). Cells were grown at 30°C for approximately 24 hr after which growth was measured spectrophotometrically at OD_{600}. Yeast cells transformed with the *C. parvum* GPCR-encoding vector were used as a control for each treatment.

Statistical analysis

Statistical comparisons were made using ANOVA with Scheffe's F test for multiple comparisons. Comparisons were made across protozoal densities, suramin treatments, siRNA transformations, and yeast treatments. GraphPad Prism 5.0 was the software used, with $p<0.05$ indicating statistical significance.

Hydropathy analysis of the putative Tetrahymena *GPCR*

Since GPCRs are comprised of seven transmembrane domains, putative transmembrane domains were assessed using Kyte-Doolittle plots (Kyte and Doolittle, 1982) from a web-based platform (http://web.expasy.org/cgi-bin/protscale/protscale.pl). Transmembrane signatures were ascribed to peptide regions in which hydropathy scores rose from negative values to values greater than one. The codon-optimized deduced version of the protein, where

glutamine codons replace read-through amber (TAG) or ochre (TAA) stop codons, was used for the hydropathy analysis.

RESULTS

Suramin-mediated inhibition of the protozoa density-dependent engulfment of Salmonella *by* Tetrahymena

In order to assess the possible role of a GPCR in the engulfment of *Salmonella* by *Tetrahymena*, we incubated *Salmonella-Tetrahymena* co-cultures with the non-specific GPCR inhibitor suramin*.* As shown in Figure 4.1, bacterial engulfment increased with the density of *Tetrahymena*. As shown in Figure 4.2, this engulfment was significantly hampered by suramin in a concentration-dependent manner at all *Tetrahymena* densities.

RNAi-based screening of a GPCR involved in bacterial engulfment by Tetrahymena

Our preliminary database query identified three *Tetrahymena* genes encoding putative GPCRs (XM_001009792.2, XM_001027519.2, and XM_001010055.2). In order to assess the association of these GPCRs with bacterial engulfment, we knocked-down receptor expression with siRNA and then evaluated bacterial engulfment in *Tetrahymena*. As shown in Figure 4.3, bacterial engulfment was significantly hampered in *Tetrahymena* electroporated with one of the siRNAs corresponding to the gene with GenBank accession number XM001009792.2. Expression of this receptor was diminished as verified by semi-quantitative RT-PCR, and no off-target effects were noted in the transformants (Table 4.1).

Partial deorphanization of the putative Tetrahymena *GPCR using* Tetrahymena *lysates and suramin*

Results presented in Figure 4.1 identified a putative GPCR involved in *Salmonella* engulfment by *Tetrahymena*. In order to deorphanize this receptor, we expressed its codon-optimized cDNA in a yeast heterologous expression system that exploits a histidine prototrophic phenotype upon GPCR activation by a cognate ligand [8,9]. We hypothesized that a *Tetrahymena* surface molecule activates the putative GPCR on neighboring *Tetrahymena* cells, thus we used *Tetrahymena* lysates as "ligands". We also assessed the activation of the putative GPCR in the presence of suramin, a non-specific GPCR inhibitor. Treatment-control yeast were grown in leucine-deficient media in the absence of lysates or suramin. Transformation-control yeast were transformed with a vector encoding a non-*Tetrahymena* GPCR whose gene was cloned from *C. parvum*. As shown in Figure 4.4, *Tetrahymena* lysates elicited significant increases in yeast growth in the transformants expressing the *Tetrahymena* GPCR. This growth was blocked by the addition of suramin. *Tetrahymena* lysates had no effect on yeast expressing the non-*Tetrahymena* GPCR cloned from *C. parvum*. However, suramin was able to reduce the basal activity associated with this non-*Tetrahymena* GPCR.

Secondary structure analysis of the putative Tetrahymena *GPCR*

To assess the presence of motifs suggestive of a GPCR, a Kyte-Doolittle hydropathy plot (Kyte and Doolittle, 1982) was created for the putative *Tetrahymena* GPCR. Figure 4.5 reveals that the putative *Tetrahymena* GPCR has the characteristic seven transmembrane domains.

DISCUSSION

The objective of this study was to identify protozoal determinants involved in the engulfment of *Salmonella*, a phenomenon that hyperactivates the virulence of the bacteria [1-5]. Protozoa enhance the virulence of SGI1-bearing *Salmonella* by hyperactivating the expression of the pro-invasion gene designated as *hilA*, and this hyperexpression leads to enhanced virulence when the bacteria escape from the protozoa. The hyperactivation of *hilA* expression is dependent on the SGI1-specific gene designated as SO13 [2]. This phenomenon specifically occurs when SGI1-bearing *Salmonella* are engulfed by and then are liberated from free-living protozoa, such as *Acanthamoeba* and *Tetrahymena* [2], or from host-associated protozoa [1-5]. The protozoa/SGI1-bearing *Salmonella* hypervirulence relationship appears to be relevant to the ingestion of water contaminated with free-living protozoa harboring SGI1-bearing *Salmonella* [5], and cattle that naturally harbor large numbers of protozoa in their rumen [1-5].

GPCRs were the focus of the present study since these receptors are apparently involved in *Tetrahymena* phagocytosis of particulates [6], and another group presented evidence for GPCR-encoding genes in the *Tetrahymena* database [10]. Additionally, a GPCR was implicated in the engulfment of bacteria by the pathogenic protozoan *Entamoeba histolytica* [11]. In the present study we provide evidence that a putative GPCR governs *Salmonella* engulfment by *Tetrahymena*. It also appears that this engulfment process is dependent upon the density of protozoa, suggesting intercellular communication by *Tetrahymena*.

It appears that a specific seven transmembrane-spanning protein mediates the engulfment of *Salmonella*. This protein is putatively a GPCR given its ability to stimulate

histidine prototrophism in a yeast heterologous expression in which G proteins govern *de novo* synthesis of histidine following ligand occupancy of a GPCR. Histidine prototrophism was observed in the presence of *Tetrahymena* lysates while suramin was able to block the histidine prototrophism in the presence of *Tetrahymena* lysates, further indicating that the protein is a GPCR. Suramin has some unknown effects, but we found that this molecule inhibited the *Tetrahymena* GPCR in two disparate systems. Additionally, suramin reduced the basal activity of a non-*Tetrahymena* GPCR in the yeast system. Because of the unknown effects of suramin, however, we are unable to completely rule out GPCR-independent mechanisms contributing to its effects on *Tetrahymena* and the yeast.

Since *Tetrahymena* lysates activated the putative GPCR, an unknown *Tetrahymena* factor(s) appears to be an autocrine-like ligand for this receptor. This factor may be membrane-associated, whereby the receptor could be constitutively activated due to continual ligand occupancy when *Tetrahymena* are confluent. Alternatively, this may be a secreted factor. Although *Tetrahymena* has been established as an important model protozoan, there is little information on GPCRs in this organism except for a recent study [10]. Interestingly, there has been debate regarding the existence of *Tetrahymena* GPCRs [12].

In conclusion, we determined that a putative *Tetrahymena* GPCR is responsible for the engulfment of *Salmonella* by the protozoan. We also partially characterized this unique receptor that appears to be activated by intercellular ligands whose threshold appears to be dependent upon the density of protozoa.

REFERENCES

1. Brewer M, Xiong N, Dier J, Anderson K, Rasmussen M, et al. (2011) Comparisons of Salmonella conjugation and virulence gene hyperexpression mediated by rumen protozoa from domestic and exotic ruminants. Vet Microbiol 151: 301-306.

2. Carlson S, Sharma V, McCuddin Z, Rasmussen M, Franklin S (2007) Involvement of a Salmonella genomic island 1 gene in the rumen protozoan-mediated enhancement of invasion for multiple-antibiotic-resistant Salmonella enterica serovar Typhimurium. Infect Immun 75: 792-800.

3. McCuddin Z, Carlson S, Rasmussen M, Franklin S (2006) Klebsiella to Salmonella gene transfer within rumen protozoa: implications for antibiotic resistance and rumen defaunation. Vet Microbiol 114: 275-284.

4. Rasmussen M, Carlson SA, Franklin SK, Wu MT, McCuddin ZP, et al. (2005) Exposure to rumen protozoa leads to enhancement of invasion and pathogenicity for multiple antibiotic resistant *Salmonella* bearing SGI-1. Infect Immun 73: 4668-4675.

5. Xiong N, Brewer M, Day T, Kimber M, Barnhill A, et al. (2010) Evaluation of the pathogenicity and virulence of three strains of Salmonella organisms in calves and pigs. Am J Vet Res 71: 1170-1177.

6. Renaud F, Colon I, Lebron J, Ortiz N, Rodriguez F, et al. (1995) A novel opioid mechanism seems to modulate phagocytosis in Tetrahymena. J Eukaryot Microbiol 42: 205-207.

7. Adachi M, Cavalcanti A (2009) Tandem stop codons in ciliates that reassign stop codons. J Mol Evol 68: 424-431.

8. Kimber MJ, Sayegh L, El-Shehabi F, Song C, Zamanian M, et al. (2009) Identification of an Ascaris G protein-coupled acetylcholine receptor with atypical muscarinic pharmacology. Int J Parasitol 39: 1215-1222.

9. Wang ZX, Broach JR, Peiper SC (2006) Functional expression of CXCR4 in Saccharomyces cerevisiae in the development of powerful tools for the pharmacological characterization of CXCR4. Methods Mol Biol 332: 115-127.

10. Lampert T, Coleman K, Hennessey T (2011) A knockout mutation of a constitutive GPCR in Tetrahymena decreases both G-protein activity and chemoattraction. PLoS One 6: e28022.

11. Picazarri K, Luna-Arias J, Carrillo E, Orozco E, Rodriguez M (2005) Entamoeba histolytica: identification of EhGPCR-1, a novel putative G protein-coupled receptor that binds to EhRabB. . Exp Parasitol 110: 253-258.

12. Renaud FL, Chiesa R, De Jesús JM, López A, Miranda J, et al. (1991) Hormones and signal transduction in protozoa. Comp Biochem Physiol 100A: 41-45.

TABLE 4.1. Semi-quantitative RT-PCR of GPCR targets in *Tetrahymena* transformed with siRNA. DNA-free RNA was isolated and subjected to an RT-PCR assay that semi-quantitates transcript levels based on the number of PCR cycles required for amplicon visualization using agarose gel electrophoresis.

GenBank accession numbers corresponding to the source of siRNA used in *Tetrahymena*	Number of PCR cycles required for visualization of amplicons specific to the following GenBank sequences in *Tetrahymena* transformed with siRNA		
	XM_00100979.2	XM_001027519.2	XM_001010055.2
XM_00100979.2	50**	10	15
XM_001027519.2	15	>50**	10
XM_001010055.2	10	15	50**
Random RNAi	15	10	15
Mock RNAi	10	10	10

** on target effects on transcription
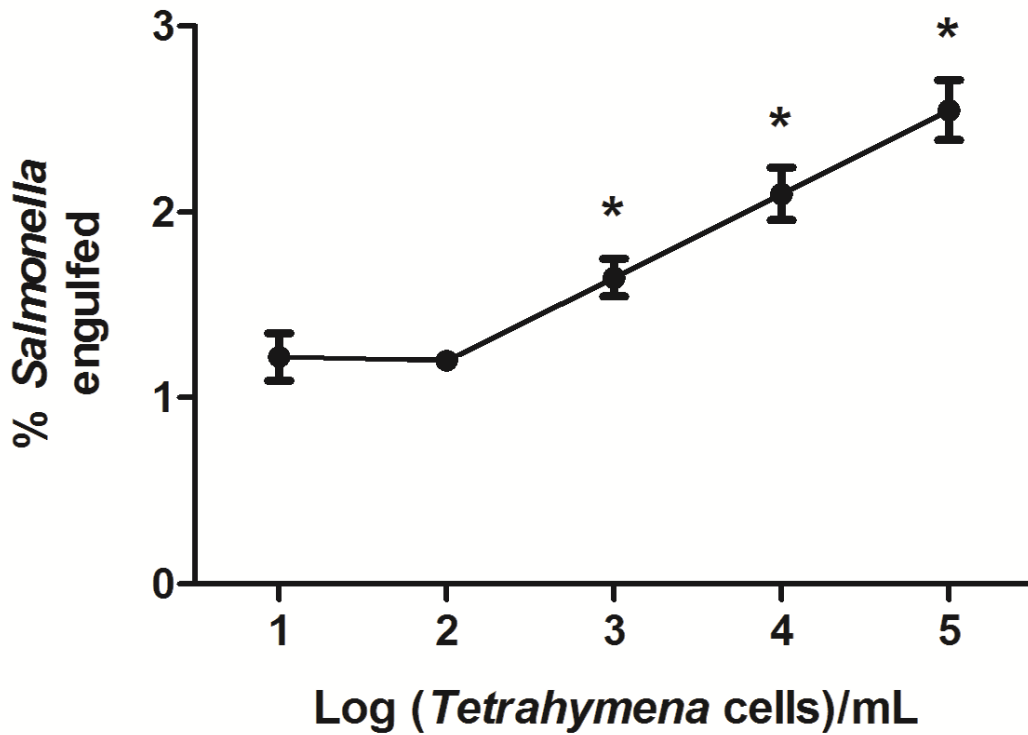
Figure 4.1. *Salmonella* engulfment at various densities of *Tetrahymena.* For each density of protozoa, *Salmonella* and *Tetrahymena* were co-cultured using a multiplicity of infection equal to 10,000. Bacterial engulfment (average number of bacteria per protozoa) was determined after lysing protozoa. Each data point represents the mean ± sem for three independent experiments performed in triplicate. *$p<0.05$ versus the rest of the data.
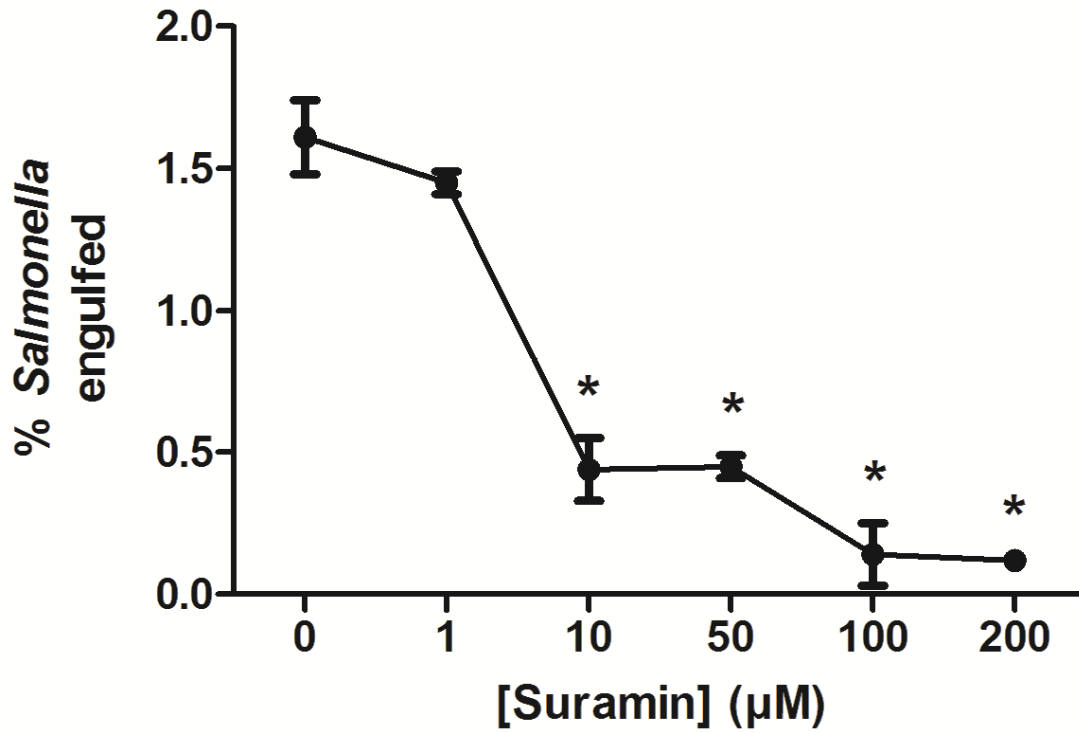
Figure 4.2. *Salmonella* engulfment by *Tetrahymena* exposed to suramin. For each concentation of suramin, *Salmonella* and *Tetrahymena* were co-cultured using an MOI equal to 10,000. Bacterial engulfment was determined after lysing protozoa. Each data point represents the mean ± SEM three independent experiments performed in triplicate. * $p< 0.05$ versus the results of suramin-free assays.
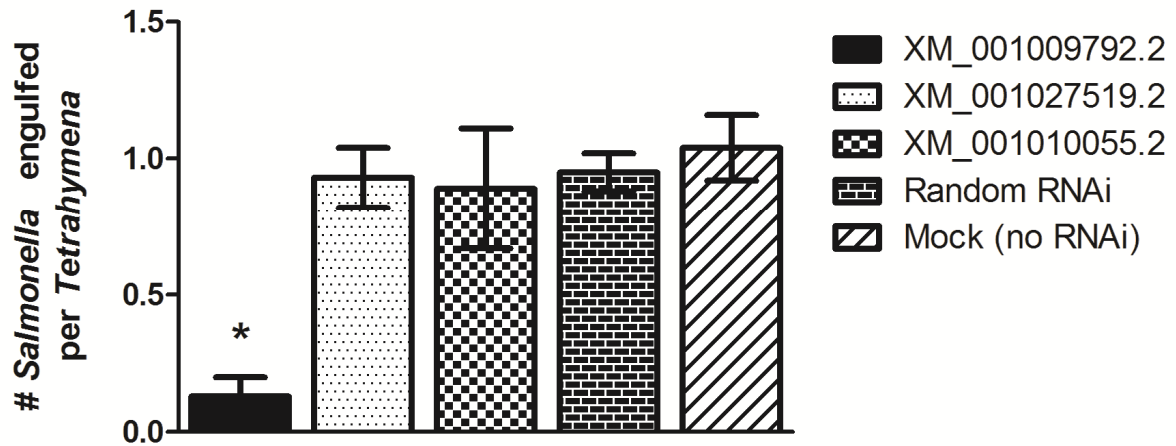
Figure 4.3. *Salmonella* engulfment by *Tetrahymena* electroporated with RNAi corresponding to putative GPCR genes. A preliminary database search identified three putative GPCR-encoding genes (GenBank accession numbers provided in the x-axis) in *Tetrahymena* and siRNA was designed based on these sequences. Bacterial engulfment (average number of bacteria engulfed per cell) was determined after electroporation with siRNAs. Random RNAi and mock transformants served as controls. Data presented are the mean $\pm$ sem for three independent experiments each performed in triplicate. *$p<0.05$ versus the rest of the data.
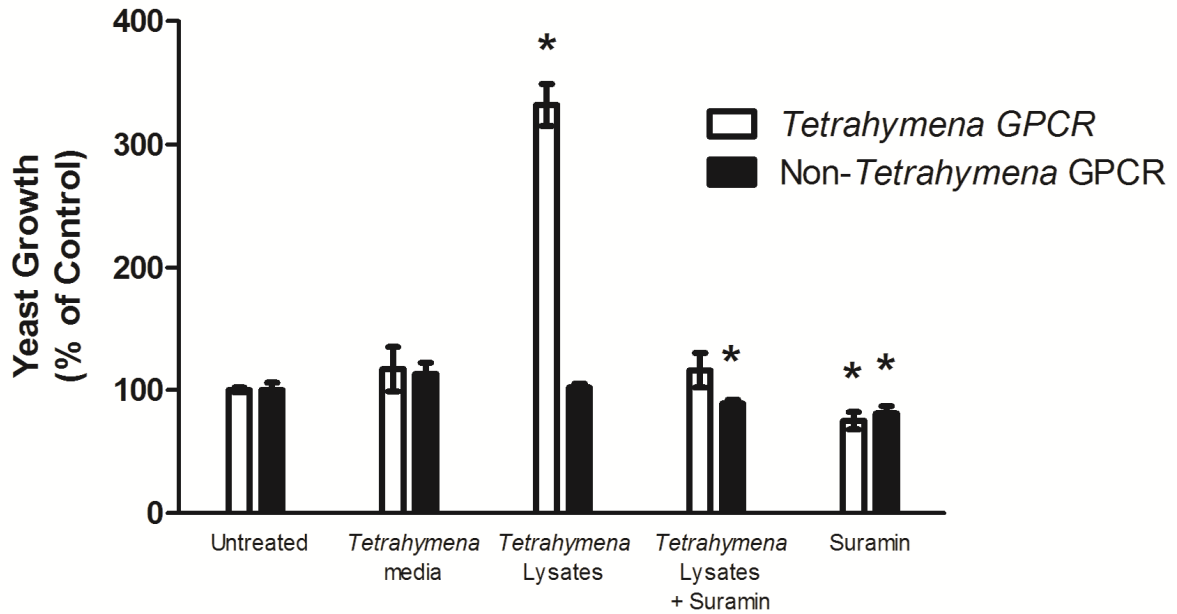
Figure 4.4. *Tetrahymena* lysate-mediated activation of histidine prototrophism in yeast expressing the *Tetrahymena* GPCR. Yeast transformants were exposed to the lysates and yeast growth was measured spectrophotometrically at OD_{600}. To determine background growth, yeast transformants were grown in media lacking leucine and histidine (untreated control). As an additional control, yeast cells were transformed with a vector encoding an unrelated GPCR (from *C. parvum*) and then grown in the same conditions. Growth is quantitated as compared to growth observed in untreated controls. Suramin, when used, was added at 200µM Data presented are the mean $\pm$ sem for three independent experiments each performed in triplicate. *$p < 0.05$ versus untreated controls.
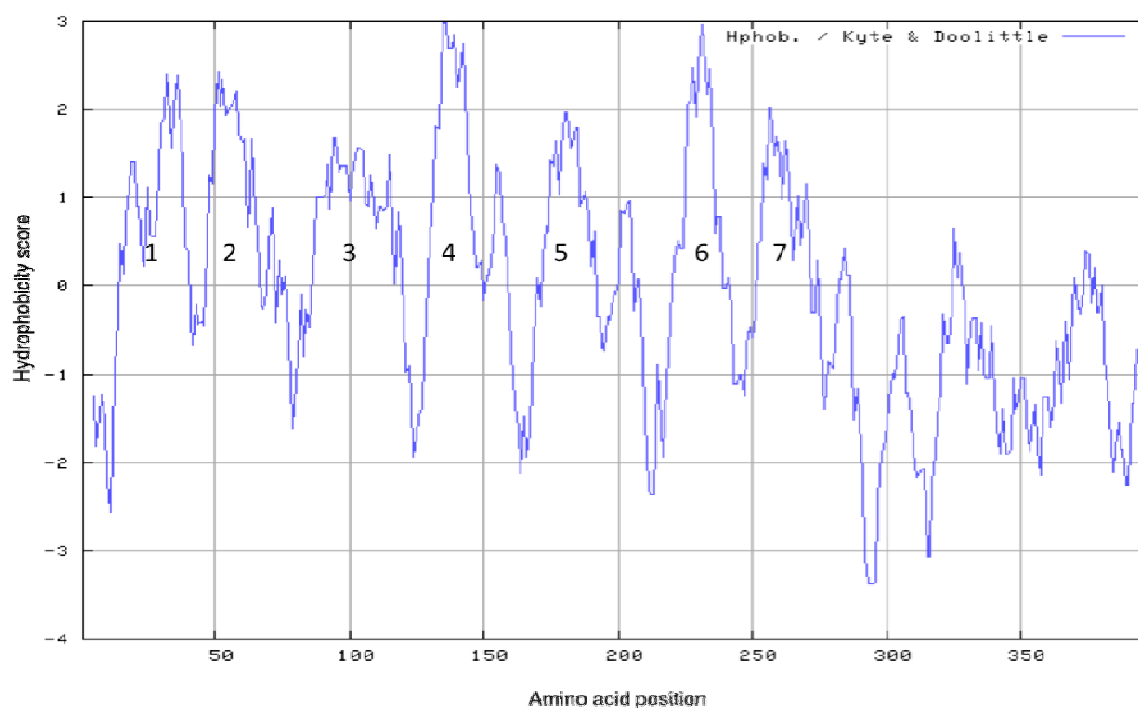
Figure 4.5. Kyte-Doolittle hydropathy plot of the putative *Tetrahymena* GPCR. Putative transmembrane domains were assessed using Kyte-Doolittle plots (Kyte and Doolittle, 1982) from a web-based platform (http://web.expasy.org/cgi-bin/protscale/protscale.pl). Transmembrane signatures were ascribed to peptide regions in which hydropathy scores rose from negative values to values greater than one. Putative transmembrane regions are indicated numerically from one to seven.

CHAPTER 5. EVIDENCE FOR A BACTERIAL LIPOPOLYSACCHARIDE-RECOGNIZING RECEPTOR IN THE BACTERIAL ENGULFMENT BY *ENTAMOEBA HISTOLYTICA*

Modification of a paper published in *Eukaryotic Cell*

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ABSTRACT

Entamoeba histolytica is the causative agent of amebic dysentery, a worldwide protozoal disease that results in approximately 100,000 deaths annually. The virulence of *E. histolytica* may be due to interactions with host bacterial flora whereby trophozoites engulf colonic bacteria as a nutrient source. The engulfment process depends on trophozoite recognition of bacterial epitopes that activate phagocytosis pathways. EhGPCR-1 was previously recognized as a putative G protein-coupled receptor (GPCR) used by *Entamoeba histolytica* during phagocytosis. In the present study, we attempted to characterize EhGPCR-1 using heterologous GPCR expression in yeast. We discovered that bacterial lipopolysaccharide (LPS) is an activator of EhGPCR-1, and that LPS stimulates EhGPCR-1 in a concentration-dependent manner. Additionally, we demonstrated that *Entamoeba histolytica* prefers to engulf bacteria with intact LPS and that this engulfment process is sensitive to suramin, which prevents interaction of GPCRs and G proteins. Thus, EhGPCR-1 is an LPS-recognizing GPCR that is a potentially druggable target for treating amebiasis, especially considering the well-established druggability of GPCRs.

INTRODUCTION

Entamoeba histolytica is an important cause of amebic dysentery [1]. *E. histolytica* infection often manifests as colitis but trophozoites can also gain access to the systemic

circulation resulting in liver or brain abscesses [2,3]. The majority of infections are asymptomatic [4] and many of the host factors determining the pathologic severity of infection have not been well characterized.

The virulence of *E. histolytica* may be due to interactions with host bacterial flora. Co-culture with bacteria can restore amoebic virulence in *E. histolytica* cell lines attenuated through serial passage [5,6], and this effect is related to an upregulation of genes associated with enhanced phagocytosis [7]. Specifically, *E. histolytica* exhibits enhanced adherence and cytotoxic abilities following engulfment of enteropathogenic bacteria [8]. *E. histolytica* is also dependent on colonic bacteria as a nutrient source for trophozoites. Prior to the advent of selective medium, trophozoites could only be grown in culture medium containing bacteria [9]. Although trophozoites are now routinely grown in bacteria-free culture medium, exposure to *E. coli* enhances their growth [8]. However, while it is clear that *E. histolytica* regularly engulf bacteria, the specific bacterial epitopes recognized by amoebic receptors are unknown.

Phagocytosis is a stepwise process that is initiated by activation of receptors that bind an extracellular target and ultimately activate cytoskeletal rearrangements. Phagocytic ability is essential for the pathogenesis of amebiasis and is strongly correlated with virulence; *E. dispar*, a non-pathogenic species, is less efficient at engulfing bacteria [10,11]. While many studies have established the importance of phagocytosis in amoebic pathogenesis, relatively few receptors mediating this process have been identified. Transmembrane kinase TMK96 is involved in erythrophagocytosis, while TMK39 is a cholesterol receptor that may also mediate bacterial engulfment [12,13]. In phagocytes of metazoa, G protein-coupled receptors (GPCRs) have been found to initiate the phagocytosis of bacteria [14]. GPCRs are transmembrane

receptors that act as guanine nucleotide exchange factors upon binding extracellular ligands. Recent studies by Bosch *et al.* described G protein signaling in *E. histolytica*, however, the receptors interacting with amoebic G proteins are not well established [15]. Picazarri *et al.* described EhGPCR-1, a putative GPCR associated with vesicular trafficking of proteins that localize to phagocytic cups [16]. EhGPCR-1 is highly expressed in pathogenic *E. histolytica* but not in *E. dispar* (www.amoebadb.org version 2.0), possibly accounting for the differential engulfment of bacteria by these divergent *Entamoeba* species [17]. The ligand for EhGPCR-1 had not been identified prior to the present study.

We hypothesized that since EhGPCR-1 has a putative role in the initiation of phagocytosis, it may recognize bacterial prey that are an essential nutrient source and potentiators of virulence for *E. histolytica*. In the present study, we used a heterologous yeast expression system to screen bacterial components for their ability to activate EhGPCR-1. In addition, we tested the ability of *E. histolytica* trophozoites to selectively engulf bacteria based on the presence of a bacterial component putatively identified as a ligand for EhGPCR-1.

MATERIALS AND METHODS

Creation of the yeast expression vector encoding EhGPCR-1

DNA encoding EhGPCR-1 (accession number AY880672) was synthesized by GeneScript using codon optimization for yeast expression. The gene was cloned into the pUC57 vector and the cDNA was amplified with forward and reverse primers adding the restriction sites *NcoI* and *BamHI* (5'GCCATACCATGGATCAATCATTCGGTAATCAA3') and (5'GCCATAGGATCCTTAA-GTCAAGTTAATTTCTCTTGAA3') to the 5' and 3' ends of the amplicon, respectively. Purified amplicons and the linearized yeast expression vector Cp4258, which contains a leucine

auxotrophic marker and encodes for ampicillin resistance [18,19], were co-digested with *NcoI* and *BamHI* restriction endonucleases. The EhGPCR-1 gene was then ligated into Cp4258 using T_4 DNA ligase (New England Biolabs). The resulting plasmid was transformed into *E. coli* and individual clones were selected and aerobically grown overnight at 37^oC in Luria-Bertani (LB) broth containing 32μg/mL ampicillin. Plasmid DNA was purified using HiSpeed Plasmid Mini Kit (Qiagen) and inserts were sequenced to confirm cDNA orientation and fidelity.

Transfection of yeast with the EhGPCR-1 expression vector

 Saccharomyces cerevisiae strain CY 18043 (J. Broach, Princeton University, USA) was used as the yeast recipient since this strain is a histidine auxotroph that exhibits histidine prototrophism upon GPCR activation even for exogenous receptors [18,19]. Non-transfected CY 18043 yeast were grown in YPD media supplemented with all essential amino acids. Cells at mid-log phase (OD_{600} equals 0.3 to 0.5) were transfected with 1μg cDNA construct or 1 μg empty vector (mock-transfectants) in the presence of 200μg salmon sperm DNA (Invitrogen) and lithium acetate (100mM, Sigma-Aldrich). Yeast cells were then incubated at 30^oC and heat shocked at 42^oC for 15 min. Cells were placed on leucine-deficient media [1x YNB (Difco), 1x yeast synthetic drop-out medium supplement without leucine (Sigma), 10 mM ammonium sulfate (Sigma), and 50% glucose] to select for transfection of Cp4258 with the EhGPCR-1-encoding plasmid. Transfectants were verified by isolating plasmids (Promega) and colonies expressing the EhGPCR-1 were verified by PCR prior to the functional assay.

Yeast Growth Assay

Since the Cp4258 vector encodes for leucine prototrophism, leucine-deficient media was inoculated with yeast expressing EhGPCR-1 or mock-transfected yeast that were grown at 30^oC to an OD_{600} equal to one. Cells were washed three times with leucine/histidine deficient medium [1x YNB (Difco), 1x yeast synthetic drop-out medium supplement lacking leucine and histidine (Sigma), 10 mM ammonium sulfate (Sigma), 50% glucose, 50 mM 4-morpholinepropanesulfonic acid, pH 6.8] and resuspended in 1mL leucine/histidine-deficient media, to a density of 15–20 cells/µL. Approximately 3,000 cells were added to each well of 96-well plates containing the same medium along with test agonists in a total volume of 200µL. Cells were grown at 30^oC for approximately 24 h. Initial and final OD_{600} values were measured with a spectrophotometer to determine growth of the yeast.

Agonist and Antibody Binding Studies

E. coli (K12 strain, Sigma-Aldrich) and LPS-free rough strain *E. coli* MG1655 (K12 derivative, N. Cornick, Iowa State University, USA) were grown aerobically overnight in LB broth at 37^oC. To produce bacterial lysates, cultures were incubated for 10 min at 100^oC. Purified lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Sigma-Aldrich) was also used as a test agonist.

Serial dilutions of whole bacteria, lysate, or LPS were added to leucine/histidine-deficient media for use in the yeast growth assay. Antibody-mediated inhibition of GPCR activation was performed by co-incubating test agonists in 200µL media with 25µL equine anti-LPS antiserum (MG Biologics, Ames, IA; 1:10 titer) in the absence or presence of proteinase K (Qiagen, 50µg/mL, 3 h, 37^oC).

Bacterial Engulfment Assay

Bacteria were fluorescently labeled by growing 8×10^8 CFUs in 1 mL of LB broth (Invitrogen) containing 10µg FITC (Sigma-Aldrich). Cells were washed three times with and were resuspended in M199 medium (Gibco) supplemented with 25mM HEPES and 5.7mM cysteine (M199s).

E. histolytica HM1 trophozoites (courtesy of Dr. William Petri, University of Virginia) were grown under anaerobic conditions at 37ºC in TYI Medium [9]. Trophozoites were harvested by centrifuging for 5 min at 1,000 RPM and 2.5×10^5 trophozoites were added to each well of a 24-well tissue culture dish in 500µL of TYI medium. Cells were allowed to adhere to the wells for 1 h at 37ºC under anaerobic conditions. For engulfment inhibition experiments, trophozoites were allowed to adhere and then were incubated with suramin (Sigma-Aldrich) for 1 hour. Each well was washed twice with pre-warmed M199s medium and inoculated with 8.75×10^6 CFUs bacteria in a final volume of 500µL of M199s medium. Bacteria and amoebae were co-incubated at 37ºC for 25 min in the presence or absence of suramin. The medium was aspirated and 500µL ice-cold 110 mM D-galactose was added to each well to detach trophozoites. Cells were pelleted by centrifuging for 5 min at 1,000rpm and washed with 500µL ice-cold 110 mM D-galactose. Cells were then fixed in 2% paraformaldehyde for 20 min at 37ºC. Paraformaldehyde was neutralized with 50mM ammonium chloride, and cells were pelleted and resuspended in phosphate-buffered saline. Aliquots (5µL) were fixed with Fluoromount-G mounting medium (Southern Biotech; Birmingham, AL) on a microscope slide. These specimens were examined by fluorescence microscopy on an Olympus BX51 microscope

with a UPlan F1 40x/0.75 objective equipped with an HBO lamp and dichroic FITC illumination filter for visualization of bacterial engulfment by trophozoites.

For flow cytometry experiments, at least 10,000 amoebic cells were analyzed for the presence of internalized bacteria on a Becton Dickinson FACScalibur or a Miltenyi MACSQuant (excitation wavelength of 488nm). Data was acquired using CellQuest Software (BD Biosciences) or MACSQuant software (Miltenyi Biotech) and analyzed by FlowJo Flow Cytometry Analysis software (Tree Star, Inc., Ashland, OR).

Search for Other GPCRs in E. histolytica

Using our GPCR search algorithm designated as transmembrane-focused support vector machine (TMf-SVM), we explored the *E. histolytica* genome for GPCR-like sequences as previously described for other eukaryotes (20). Expression of these sequences was then searched in the *Entamoeba* database (www.amoebadb.org version 2.0), using data from expressed-sequence tags and microarrays.

Statistical Analyses

Statistical analyses were performed using an analysis of variance with Scheffe's F test for multiple comparisons. StatView was used as the software.

RESULTS

EhGPCR-1 activation by bacterial lysate

To determine if EhGPCR-1 recognizes bacterial components, we monitored the response of the receptor to bacterial lysates using a histidine auxotrophic yeast heterologous expression

assay [18,19]. In this assay, the GPCR of interest is expressed in histidine auxotrophic *S. cerevisiae* engineered to lack their native GPCRs. When the expressed GPCR of interest is stimulated by its cognate ligand or agonist, a promiscuous GPCR-inducible pheromone pathway is activated which leads to the downstream expression of the *His3* reporter gene. *His3* encodes for *de novo* histidine synthesis enzymes, and thus *His3* expression provides the ability to grow on histidine-deficient media. As a result, heterologous receptor activation can by quantified by spectrophotometric analysis of yeast growth. Receptor activation is calculated as ligand-induced yeast growth compared to growth of yeast transfected with empty vector and exposed to the same ligand or agonist.

Application of *E. coli* K12 lysates to EhGPCR-1-expressing yeast produced significant increase in yeast growth; specifically, the EhGPCR-1-expressing yeast were stimulated more than 1400% compared to mock-transfected yeast (Figure 5.1). This growth was markedly attenuated by the addition of anti-LPS antibodies and the effect of the anti-LPS antibodies was abrogated by proteinase K. Addition of a rough strain of *E. coli* K12, which lacks the outer O-antigen of LPS [20], stimulated EhGPCR-1 to a significantly lesser extent. Histidine prototrophism was not observed when EhGPCR-1 expressing yeast were exposed to a panel of biogenic amines (histamine, serotonin, dopamine, epinephrine, and norepinephrine, 0-100mM) that are classic GPCR agonists or antagonists in mammals (data not shown). Specifically, these biogenic amines were used in early deorphanization attempts since they are important GPCR ligands in a variety of organisms.

Concentration-dependent activation of EhGPCR-1 by LPS

Since EhGPCR-1 was putatively activated by LPS, we examined the ability of LPS to stimulate EhGPCR-1 in a concentration-dependent manner. EhGPCR-1-expressing yeast were incubated with various concentrations of purified LPS isolated from *E. coli* 0111:B4 and, again, yeast growth in histidine-free medium was measured as an indicator of EhGPCR-1 activation. LPS activated EhGPCR-1 in a concentration-dependent manner with an EC_{50} of 15 nM (Figure 5.2).

Preferential engulfment of LPS-expressing E. coli by Entamoeba histolytica trophozoites

To determine if *E. histolytica* trophozoites selectively engulf bacteria based on the presence of LPS, we compared the engulfment of *E. coli* K12 and a rough isostrain of *E. coli* that lacks intact LPS*.* Bacteria were fluorescently labeled with FITC and co-incubated with *E. histolytica* HM1 trophozoites. Trophozoites were washed to remove bacteria that were not engulfed or attached and the number of *E. histolytica* trophozoites containing bacteria was quantitated by flow cytometry. Phagocytosis assays revealed that 23.2% of trophozoites contained *E. coli* K12 while only 3.8% of trophozoites engulfed the rough strain. This represents a >80% reduction in the bacterial engulfment capability of *E. histolytica* (Figure 5.3). Because no EhGPCR-1-specific drugs are known, we confirmed the role of G protein signaling in bacteria engulfment by conducting phagocytosis assays in the presence of suramin which uncouples GPCR and G proteins [21]. Suramin blocked phagocytosis in a concentration-dependent manner with an IC_{50} of approximately 80μM (Figure 5.4).

Identification of GPCR-like sequences in the E. histolytica genome

In order to assess the potential role of other GPCRs in the suramin-mediated effects on bacteria phagocytosis, we investigated the possible existence of other *E. histolytica* GPCRs. Using a search algorithm that mines occult GPCR-encoding sequences from non-mammalian genomes by targeting transmembrane signatures [20], we uncovered eight sequences encoding putative GPCRs. As summarized in Table 5.1, EST and microarray studies (www.amoebadb.org) revealed that none of these receptors appear to be expressed in *E. histolytica*. EhGPCR-1 is expressed in *E. histolytica,* but not in *E. dispar*.

DISCUSSION

Previous work indicated that EhGPCR-1 is linked to phagocytic pathways in *E. histolytica* [16]. The goal of the present study was to characterize EhGPCR-1 by determining its cognate ligand in a heterologous expression system, and to confirm the functional activity of the ligand in *E. histolytica* trophozoites.

GPCRs are cell surface receptors that sense the extracellular environment and are activated by a variety of ligands such as catecholamines, peptides, lipids, carbohydrates, *etc*. GPCRs are excellent drug targets, reflected by the fact that 30-50% of currently marketed drugs target these receptors [22,23]. While GPCRs have been well studied in vertebrates, the study of their role in *E. histolytica* physiology is only in its infancy. *E. histolytica* express heterotrimeric G proteins which have been shown to modulate pathogenic processes, yet few receptors interacting with these G proteins have been identified [15]. A review by Bosch and Siderovski indicated that *E. histolytica* G proteins lack homology to mammalian G proteins [24], suggesting

that GPCRs have not been identified in *E. histolytica* because of inadequacies in genomic search tools for divergent receptors. Classic GPCR agonists such as histamine and serotonin can modulate *E. histolytica* phagocytosis and virulence in a mouse model, but their receptors remain unidentified [25,26]. EhGPCR-1 did not respond to these ligands in our yeast assay (data not shown). Furthermore, our genomic search tool did not uncover any other GPCRs expressed by *E. histolytica* (Table 5.1)*.* In the absence of another GPCR expressed by *E. histolytica* (that could be targeted as a control that rules out collateral effects) and our inexperience with hairpin RNA*,* we were unable to perform the appropriate control experiments using RNAi-based knockdown of EhGPCR-1 expression.

The present study utilized a novel yeast auxotroph assay for screening GPCRs against potential ligands and agonists. This approach has recently been applied to GPCRs from parasitic helminths [18,19], and may represent a valuable tool for the study of protozoan receptors since culture of these microbes is often difficult. Utilizing this approach, we demonstrated that EhGPCR-1 is activated by a bacterial component of *E. coli*. This activation was abrogated in the presence of anti-LPS antibodies or a generic GPCR inhibitor. Purified LPS induced concentration-dependent EhGPCR-1 activation although this response was not as robust as the response to bacterial lysates. Structural variances between LPS from *E. coli* K12 and *E. coli* 0111:B4 might explain differences in receptor activation. Alternatively, additional bacterial components may be required for maximal occupancy of EhGPCR-1. Further research is needed to unveil the unique pharmacologic aspects of EhGPCR-1 in *E. histolytica* with respect to agonist affinity and binding cooperativity. EhGPCR-1 may represent a new GPCR subtype since an NCBI motif database search revealed that it is most related to the MIG-14-Wnt-bd superfamily of

proteins, and it lacks the E/DRY and NPXXY motifs found in class A types of GPCRs (29). Interestingly, one of the non-expressed *E. histolytica* GPCRs contains a DRY motif (Table 1).

Based on the results of our yeast expression assay, we hypothesized that *E. histolytica* initiates bacterial engulfment after recognizing bacterial LPS. Phagocytosis assays demonstrated a >80% reduction in the number of trophozoites containing bacteria when the bacterial prey lacked O-antigen, the outermost layer of the LPS. This result is supported by previous research indicating that *E. histolytica* primarily engulfs Gram-negative pathogens [27]. Other investigators have also demonstrated selective engulfment of bacteria by amoebae based upon bacterial O-antigen [28,29]. Therefore, other taxa of protozoa potentially express similar receptors for bacterial recognition. EhGPCR-1 mediated preferential bacterial feeding behavior may also lead to disruptions in intestinal microbiota which is observed during infection with *E. histolytica* [30]. Composition of intestinal flora is likely to be a factor allowing *E. histolytica* colonization of the gut, and the role of EhGPCR-1 in this process is a current line of research in our laboratory.

In summary, this study demonstrates the utility of a heterologous yeast expression system in the characterization of EhGPCR-1, a GPCR putatively used in phagocytosis by pathogenic *E. histolytica*. EhGPCR-1 is activated by bacterial LPS, suggesting that this GPCR may be used to initiate phagocytosis upon the recognition of bacterial prey. Functional studies supported the role of LPS in engulfment of *E. coli* by *E. histolytica*. EhGPCR-1 is the first GPCR to be characterized in *E. histolytica* and it may represent an important chemotherapeutic target in this pathogen.   While this may be the only GPCR expressed in *E. histolytica* despite the unexplained presence of non-expressed GPCR-encoding genes, other protozoan GPCRs may

represent innovative drug targets, and their roles in regulating protozoan physiology merits

further investigation.

REFERENCES

1. WHO (1997) World Health Organization/Pan American Health Organization/UNESCO report of a consultation of experts on amebiasis. Weekly Epidemiological Record 72: 97-99.

2. Petri WA, Singh U (1999) Diagnosis and Management of Amebiasis. Clin Infect Dis 29: 1117-1125.

3. Fotedar R, Stark D, Beebe N, Marriott D, Ellis J, et al. (2007) Laboratory diagnostic techniques for Entamoeba species. Clin Microbiol Rev 20: 511-532.

4. Huston C (2004) Parasite and host contributions to the pathogenesis of amebic colitis. . Trends Parasitol 20: 23-26.

5. Wittner M, Rosenbaum RM (1970) Role of bacteria in modifying virulence of Entamoeba histolytica: studies of amoebae from axenic cultures. . Am J Trop Med Hyg 19: 755-761.

6. Bos HJ, Van de Grind RJ (1977) Virulence and toxicity of axenic Entamoeba histolytica. . Nature 265: 341-343.

7. Debnath A, Tashker JS, Sajid M, McKerrow JH (2007) Transcriptional and secretory responses of Entamoeba histolytica to mucins, epithelial cells and bacteria. Int J Parasitol 37: 897-906.

8. Galvan-Moroyoqui JM, Dominguez-Robles MC, Franco E, Meza I (2008) The interplay between Entamoeba and enteropathogenic bacteria modulates epithelial cell damage. PLoS Neglected Tropical Diseases 2: e266.

9. Diamond LS, Harlow DR, Cunnick CC (1978) A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans R Soc Trop Med Hyg 72: 431-432.

10. Rodriguez MA, Orozco E (1986) Isolation and characterization of phagocytosis and virulence deficient mutants of Entamoeba histolytica. J Infect Dis 154: 27-32.

11. Hirata KK, Que X, Melendez-Lopez SG, Debnath A, Myers S, et al. (2007) A phagocytosis mutant of Entamoeba histolytica is less virulent due to deficient proteinase expression and release. Exp Parasitol 115: 192-199.

12. Boettner D, Huston C, Linford A, Buss S, Houpt E, et al. (2008) Entamoeba histolytica phagocytosis of human erythrocytes involves PATMK, a member of the transmembrane kinase family. PLoS Pathog 4: e8.

13. Christy N, Buss S, Petri WJ (2012) Common pathways for receptor-mediated ingestion of Escherichia coli and LDL cholesterol by Entamoeba histolytica regulated in part by transmembrane kinase 39. Int J Parasitol 42: 393-400.

14. Soumita D, Owen KA, Ly KT, Park D, Black SG, et al. (2011) Brain angiogenesis inhibitor 1 (BAI1) is a pattern recognition receptor that mediates macrophage binding and engulfment of Gram-negative bacteria. PNAS 108: 2136-2141.

15. Bosch D, Kimple A, Muller R, Giguère P, Machius M, et al. (2012) Heterotrimeric G-protein signaling is critical to pathogenic processes in Entamoeba histolytica. PLoS Pathog 8: e1003040.

16. Picazarri K, Luna-Arias J, Carrillo E, Orozco E, Rodriguez M (2005) Entamoeba histolytica: identification of EhGPCR-1, a novel putative G protein-coupled receptor that binds to EhRabB. . Exp Parasitol 110: 253-258.

17. Pimenta PF, Diamond LS, Mirelman D (2002) Entamoeba histolytica Schaudinn, 1903 and Entamoeba dispar Brumpt, 1925: differences in their cell surfaces and in the bacteria containing vacuoles. J Eukaryot Microbiol 49: 209-219.

18. Wang ZX, Broach JR, Peiper SC (2006) Functional expression of CXCR4 in Saccharomyces cerevisiae in the development of powerful tools for the pharmacological characterization of CXCR4. Methods Mol Biol 332: 115-127.

19. Kimber MJ, Sayegh L, El-Shehabi F, Song C, Zamanian M, et al. (2009) Identification of an Ascaris G protein-coupled acetylcholine receptor with atypical muscarinic pharmacology. Int J Parasitol 39: 1215-1222.

20. Johns M, Skehill A, McCabe W (1983) Immunization with rough mutants of Salmonella minnesota. IV. Protection by antisera to O and rough antigens against endotoxin. J Infect Dis 147: 57-67.

21. Chung W, Kermode J (2005) Suramin disrupts receptor-G protein coupling by blocking association of G protein alpha and betagamma subunits. J Pharmacol Exp Ther 313: 191-198.

22. Flower D (1999) Modeling G protein-coupled receptors for drug design. . Biochim Biophys Acta 1422: 207-234.

23. Wise A, Gearing K, Rees S (2002) Target validation of G protein-coupled receptors. Drug Discovery Today 7: 235-246.

24. Bosch D, Siderovski D (2013) G protein signaling in the parasite Entamoeba histolytica. Exp Mol Med 45: e15.

25. Khan M, Sen P (1988) Effect of histamine and serotonin on phagocytic activity of Entamoeba histolytica. Indian J Exp Biol 9.

26. Khan M, Sen P, Mishra B (1990) Enhancement of virulence of Entamoeba histolytica by histamine in vitro. Indian J Exp Biol 28: 376-377.

27. Mirelman D, Feingold C, Wexler A, Bracha R (1983) Interactions between Entamoeba histolytica, bacteria, and intestinal cells. J Exp Med 158: 1511-1521.

28. Wildschutte H, Wolfe DM, Tamewitz A, Lawrence JG (2004) Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella. PNAS 101: 10644-10649.

29. Wildschutte H, Lawrence JG (2007) Differential Salmonella survival against communities of intestinal amoebae. Microbiology 153: 1781-1789.

30. Verma A, Verma R, Ahuja V, Paul J (2012) Real-time analysis of gut flora in Entamoeba histolytica infected patients of Northern India. BMC Microbiol 12: 183.

Table 5.1. Identity of eight putative and novel *E. histolytica* GPCRs uncovered using TMf-SVM (20). Expression of each receptor is also listed, as determined from EST and microarray results at www.amoebadb.org. EhGPCR-1 is presented in the last row. The DRY motif (29) is bolded and underlined in the sequence for XP_657521.1/EDI_148210.

Accession number and amoeba database number, respectively	Deduced amino acid sequence	Expression in *E. histolytica*
XP_648517.1 EDI_139040	MQSLAQTKTVLLTSLSPKGFSSIFIGFLMAVSFVMIMTMSLPTQYGTKTLNNTCAFSDSSACLSHLVQNGS NFTVKVDDIAATNQKVYIYLTYELQNAPRGRIKMSVNTALYKPKEKNKSITKYDEVKLIKSFTKKHRSFCN SNNSCEVLLASIESSKQYEDIYAVFSVNSVSLSESLGSPSLSIVYITPEFTSFEVFWRITLLTSMCCCTLF FMYVLRGIPFKQWSLEQKCTVLFMIALTISNNGFYSYEFMSANELFPSINAICDSIMLCSLLLYVLVVMDA LRKPLSQRTFKFFYFPRFLIVLLIGSFLVNLYLYNAENSDPVIVMMKDPINATMAILTGLLSLIYLFWLMF AIIRSFSEARKLGEKGQRIKMYGTFTIIAIFIFFVLLLSDYFIGYRSNAAVYLTLIAYVNIYAVVLMIFYL PSTKNQNEWNNRRREIVLDDVKVTQYDDDDDNELVVEENNEKNEAEILIAATNEEPTGDIDSKKIEL	No
XP_654063.1 EDI_137920	MEITQPEKKKLGILMNTISLKGILAIAMIFILITGAITIIAIISPKPYIQTKVGLWECSQSTQTFNPNICK GWDSYSTLTSPSTDKNISTSIVVGPFATLNKDVTFGLLLKNVFTSSNMPSFSFKLNLEYSFQPFNDIKLVS TVNYVREVAQNINVECTNGNEFCNVDFSVKRFGTLTYERFDIFSLSSSSLEKMDQAEFYGTIQTSNSSST VFEIYWRICLAFGNCVLLCLFFWFLRLYSVFNWALEQHLTALLIYGLILFNNPFCLLEHITGASFFLMVDS FFESFFTGYLMFYILAVFNSVRRPERAKGFIPLIVRAIMAIIISCSAFGSILSIRMTIQSNSKFHSNTLTI FFSVMFLLSIVIYLFWLLFEVIRSFSERRKMSGSNKNRVVIFGIVTFGCIFLLMAVYVGVYFSGYMVSNMF IGLLAYVNLYCFFVAILNLPSLQKQGSNTNGVNIAKLDTEFKVETYDDDDELVVENKNEEGTLEDSVALN	No
XP_652949.1 EDI_025380	MNVSNKDIYLKSKLVKDQDNSFAGFVLLPSEYSFNDICILTKNGQLSDISSKGYVFFQLKTQSTYINVKKG TYRLVYFSCGNKKIKWVNAKIIQGYTKDGIYHYLSSGEYPMIMITEIFGFILLVVILYDLYLLYKRETHTV QTLITGFSIIIFLHLCFDVLTLKATDSKGISNIRYFDYTFRIIETLSFYIVTLLVASGWSRKSKFSFEQKK TLLFFILLQVLSNVLYLVAEENELVNTRVTSVLRLIDIICSICILFPLIKTLNLLKKNTGTEAKTLKQYQR FSRCYSILLTIAYVKILLAYINDIASTREFDFIYLSVDKSLYIIYSIYLMIAFRPKKIISSSDEPKELMTE NTFDETKQEISKNTKNYQPEVSLNSFPTETSQNEEIKSVVLLDNNEHQEDTLSTTHNKNDICVELE	No
XP_651967.2 EDI_039820	MGIFDNKQTLLSSLDRKGLIIIGVIFSVLAVLLSILATVFTSTVIENSMDLWRCNNTEKFDSKQCMGYNAF NKEKEAIQYDKVKVHFGYFSRFNYEARISLSAEIKSEELTTQKTFIVPIHYFLGGYSDEEAMKNVDPELTE TMNINFECSQKSCKSDFLLKFKADYHFYYFIFQTDNSYKDEIIMKPTLTLMTSNSFFTVTELFWRIAFVCV GSIQLMTFMYCMRTIPIKEWIFEQILTFTLMYILLLFNNPLALIEYQAHHWFFSLISSVVESFAISFFLFY ILIIFDSLRKPINKMSTTTLWFKFIGVRVIFVGIIFITLCCFIFTQKVFDIKYLIAGTSNVVLIILGVIVL LIVLTYAFWLIFSLIRTFTERHKLGEKSRRVIGFSVFNIVIVFIFIIVLSILLFDGYDASSIYLASIVYFN LYCFAMALLMVKNDKQELSRRSKKFEISHPTEVSMEVNEYNDDDEIIIKEQPEEGILVDEQISL	No
XP_657521.1 EDI_148210	MESEIQLAHFRKIHYIVMTIFLILFFLFTIILGIFGPSEYSLKTQSGFKSVEGDGFQFTLPVLNKNELELK VMYQQKININNNYNMTIYEKKSQAIEVNFIDKDDKINETKIFNFTCTYRKDIICGRVEIIEQKVYRKGKGI ISLKLIDNEPQDGIKVLGNLNVFTST**<u>DRY</u>**TQINILYSLIVFSISVIIFIVYVIIELRYLQKKGRFNGLQLM NGLLVFAVVGHTFPWNAVAYSIQWIGVFALSKASYSVFNVVFICYLFFMLDSFMKQNGTSSKKVVWITRVL LLSIFVVCRLTNTTLHAVCSVQNVLIPKNTLHSTSIFFDVIEGLLYFFLYSWALYHLAMTYNKVTIPIIQK QLNYFAACTALFLLVLIFLLFFSGIISEETTFATYFENGSMVVYLISMLCAFLPSLSPVKNEWSIFYNEMD DDQPLYDEED	No
XP_656583.1 EDI_050770	MRNQLIVDTMNRWHFIILTVITILISLAVAGVSIYYHRVVLTTEITPLVVDVSSYSNGTYALMQSLDVQKY DKWLEVYLKLEPKNEINEEISFYAETQLHIVKDFHDNVNSFNRTYTTRCFIDKCDDIIFVTDDAVQEAKYI ILVAIKDIENKFNSTGLTISLRVSKAFKSHTIFSLSLTIILVFISTITFIILFVIAIKKKEFNIYVNFNLL LSFGMILFNDPFVALGFYANYISIEYVDNVLMELYMFILFVWIFFHVDHLKLLAIEEPYPILQWILRSCVL IAYFFCFILYFFMNILHHSSSITGSPNPIMDDVKAIRDLLQVVLVFWILMSEFFSFTQVMNPINRKRLFFL TPSTLLVAVACVIQLFFQNNSHNNIPIPVCMVKLMNTYFVIVMCFCFYQIKNDLATISLLHTTETNQTILY DEYDMFDSEDDKPIL	No
XP_001913666.1 EDI_135050	MPNGVIIDELGSIWKITLCVVWIICLIIGIFFSFGQILEIGIISNSTTFNVGSSFNGDDTVAFVSFVGNEKKIEGIVDSFNGM NIVNKIKEDKTDEIENELSSIGLKSSLISLEEDETSSSLSEGDTYYFFDVELLIKTYNQFQATTCKPYVKTDLNHQSVDKITI SGNATFWWLDHPELNSFDNINKPVKFETQCSFTGGECNRFSFIYLRNVFNSSYTFIFFFKVGFYNTLVMDSKRGIPGYSLFKI SFVTFLVILSVIATILYSILYFFMKLYKNDEDHFYINSFIMIILLNFYNNPFNILRFFFNADWISCIDTCIYTIYQIVLISFI FLHIDHSRNQALGKKNSVLIWILRATGLLAYFVFFLLYFILYYLPSNKSPFSVVDDTMSIGLMFGALSTFICFLLYGWTLIYM LFTYSEIRSPLYKNRIFWFSIFTSVVLIVTLLHLIFGQDMNYLPPISMICRQLLCNLYVYTLTFIFFPKEISNDDEYGITAYT QF	No
XP_650991.1 EDI_179320	MNYNNTATTNKEPKLLVSKLSKCGFLLIFAMFVVIVTFCISISMTIPNPWFSKTLSTWDCDEKVSNFDKKICHGIDLNEQKTK LTVAFGPFTVLNQETRISLIPQKRFGREGISAKGITEISYKLYGSALPIDKNSKVDTNSLELLEEKTITRKVKCPASRGLDCK AIPLASQIYLTHPNYLLEFTFTPTDISLGDIIVENSYISTSYSVFEMFWRMAFILFSSFCCMLYLSSIRGIPISQWCHEQKWT FILNIVLILFNNPFFFYEYIVDSVTFLLNFNTGIETFFICFLMFYVLSMFEALRKPLNIRKSFRFILPRALLLLTLFMLVFTEL MYNRTTPNYSITLKGIDDPANVVLSILILGIIVIYIFWLVFGIIRTYSESRKLGPVSLRLKYYGGFTICVIILYLCLLIAANF MGYHNNASITLTTLAYVNLYVMTLTILYLPVEMNNVPGNRVNLQNNVIHLDEEIKSGDDDLIVVDDEKGNDLIVIDE	No
XP_648234.1 EDI_110620	MNNSGVLNTNKEQRLLLTTISKIGFFLLFIVFAVLVVIALFVSLNAPKPWSTNEISSWKCAGDDAQFNQNC GGVNLLDKSKKITLQLGAYEIFNQESRLEYYFNKKSSKKDIAFDGFVKFRYTLYGTNVLNQPNQKVEVSSL KLIEEKTIESKVHCPARSTKCDIGYISSELYLDYPIYIIEISIINENVKDIVSDLIFKASFVSTFYTIFEL FWRLIFILFSCLCTCIYLWANRSIPQEKWSHEQKWTVVLLTFLIWENNPLYPYEFLMDNAFYLFVNSVIDT IFICFLMFYVLIMFDALRKPIRQRTSIRFYLPRGLLCGLLFSLILASFMYNKTRKIYSPTMTGSHDVFNVI ISLGIMGLLIIYLFWLIFSIIRSFSEVRKLGSAGYRVQVYGIFTIFILLFYVSLLLSVFFMGYRNNAAVSL TTIAFVNFYCMVLTILYLPSNVVDQKEERAKIVKLDDDEALELKVEDYDNEESHGDEVVIADDEN	Yes (EhGPCR-1)
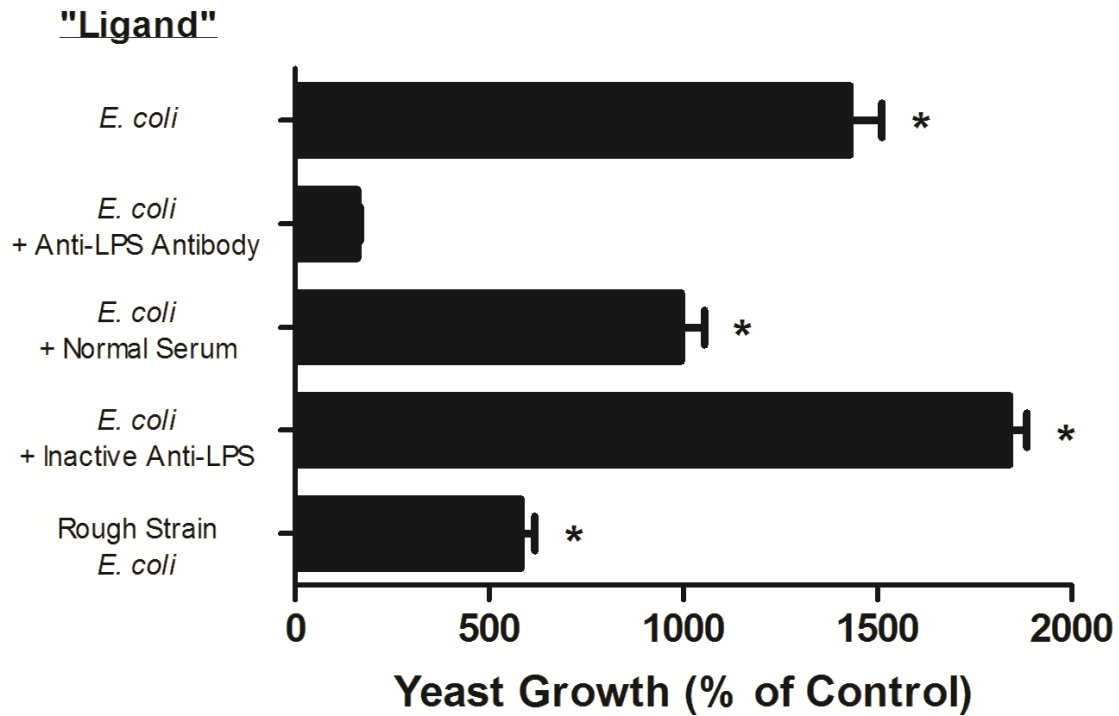
Figure 5.1. *E. coli* K12 lysate stimulation of histidine prototrophism in histidine auxotrophic yeast expressing EhGPCR-1. Yeast growth was measured in histidine-deficient in the presence of *E. coli* K12 lysates. Growth was also determined in the presence of these lysates plus intact anti-LPS antibodies or anti-LPS antibodies digested with proteinase K. EhGPCR-1 activation was also determined in the presence of a rough strain of *E. coli* K12. Receptor activation is expressed as percent growth over mock-transfected yeast. Data are expressed as mean $\pm$ sem. For bars with bacteria only, n=6; n=3 for antibody experiments n=3. *p<0.05 versus vehicle.
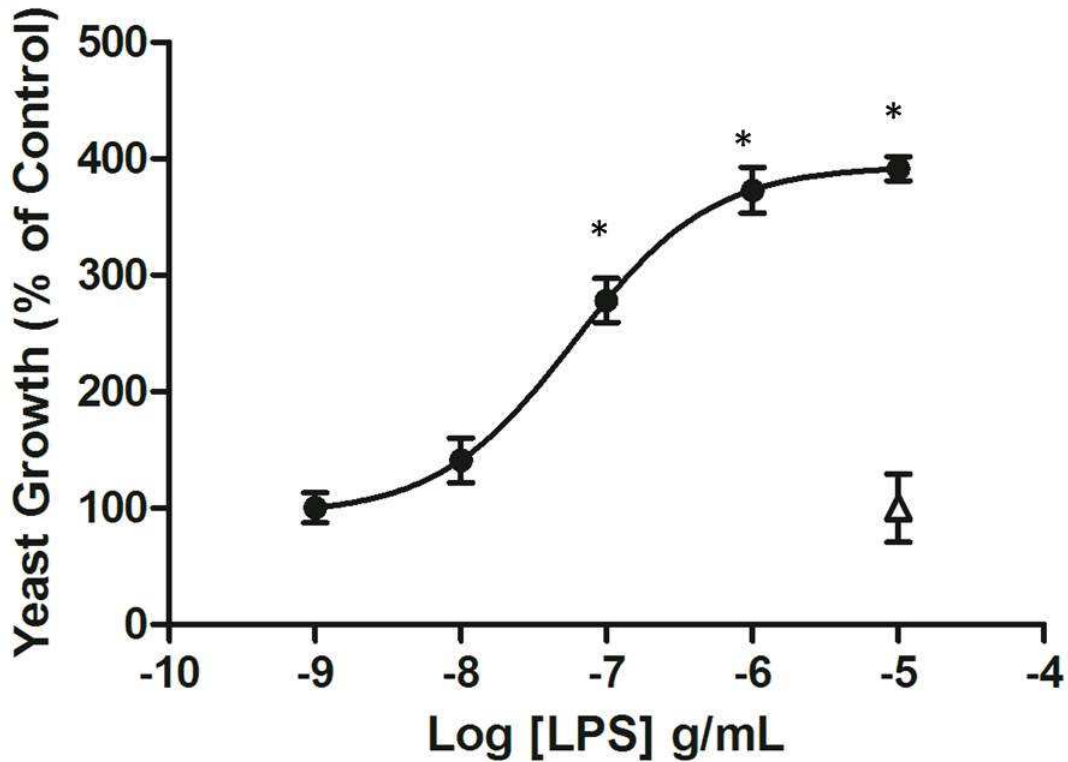
Figure 5.2. Activation of EhGPCR-1 by bacterial LPS is concentration dependent with an EC_{50} of 15 nM, based on an estimated molecular weight of 2,000 gm/mole for LPS. The open triangle represents the response of mock-transfected yeast to the highest concentration of LPS used. Each data point represents the mean $\pm$ sem for three independent experiments. *p<0.05 versus mock-transfected.
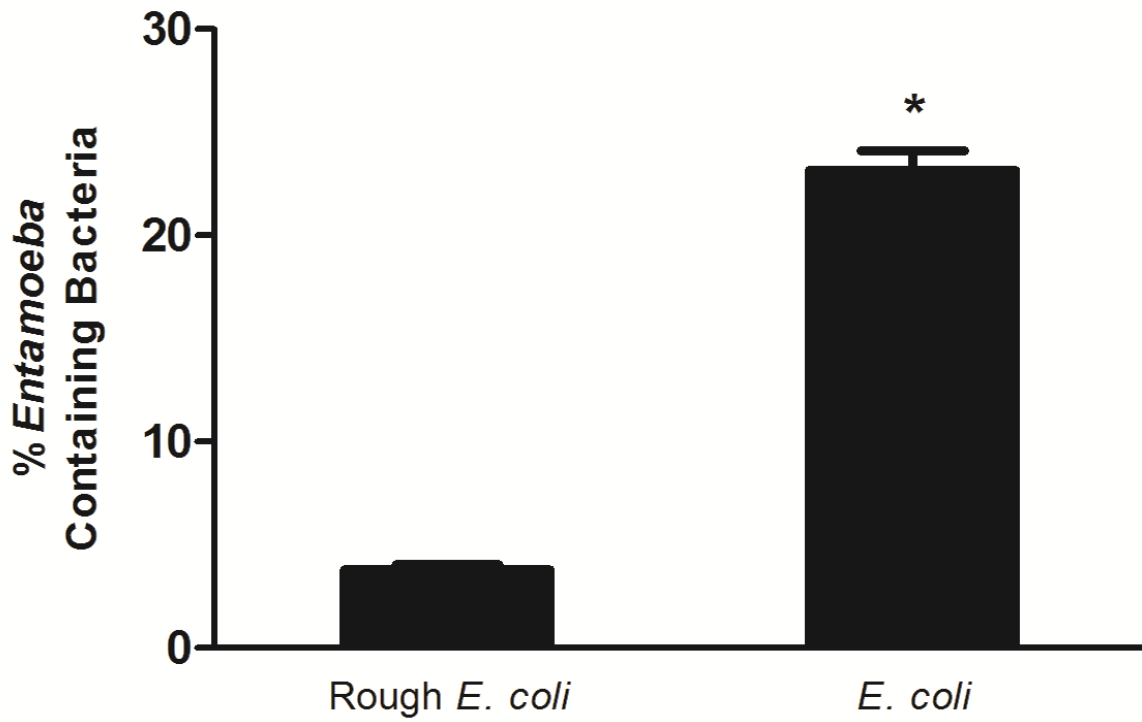
Figure 5.3. *E. histolytica* preferentially engulfs *E. coli* expressing LPS. Each bar represents the mean percentage± sem of trophozoites that contained bacteria after 25 min. of co-incubation (10,000 trophozoites counted per run; each bar represents 3 independent experiments with 3 replicates of each condition). *p<0.05 versus rough *E. coli*.
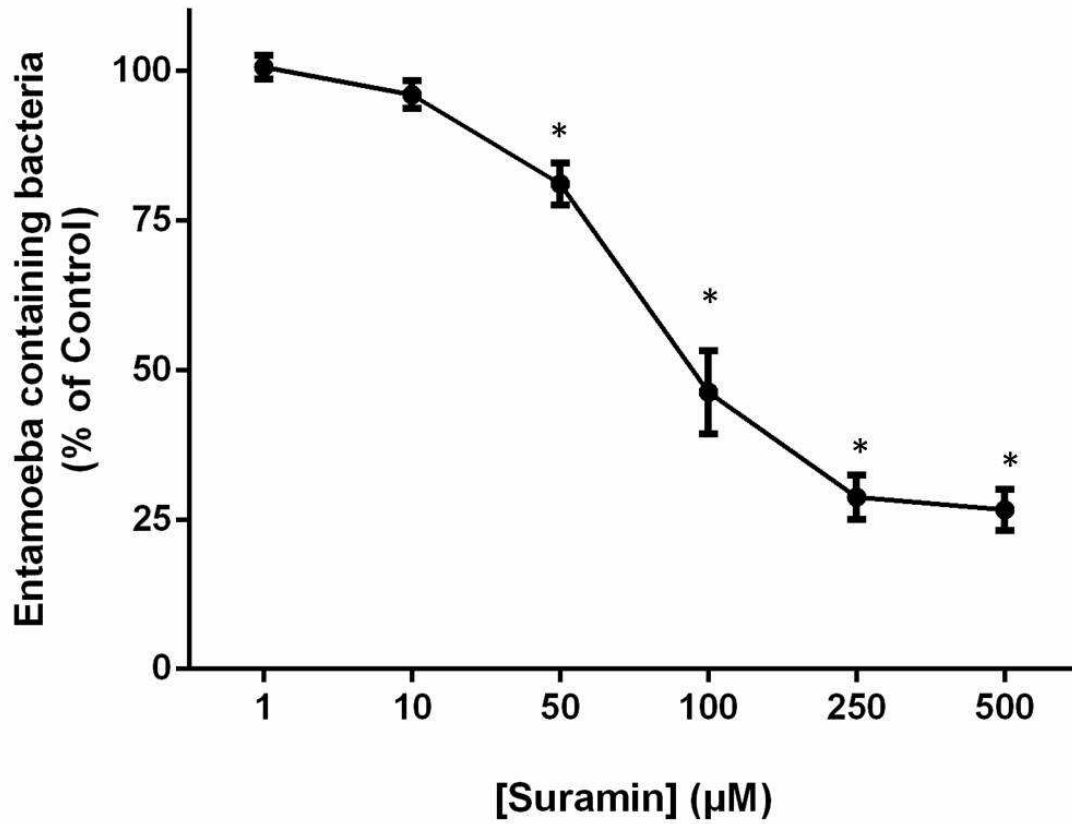
Figure 5.4. Suramin inhibits bacterial engulfment by *E. histolytica*. Circles represents the mean percentage ± sem of trophozoites that contained bacteria after 1 hr. of co-incubation (Each dot represents 3 independent experiments repeated in triplicate). *p<0.05 versus suramin-free control.

CHAPTER 6. CONCLUSIONS

FACILITATION OF *SALMONELLA* DT104 BY PROTOZOA

Many protozoa engulf bacteria as a source of nutrition and bacterial mechanisms related to evasion of protozoan-induced death also enable bacterial pathogenesis in vertebrates. Past studies indicate that bovine rumen protozoa facilitate *Salmonella* DT104, an intracellular pathogen. First, DT104 is able to survive within the protozoan during transport through the rumen. Second, DT104 is capable of conjugal plasmid transfer while living intracellularly. Third, engulfment of DT104 by rumen protozoa induces a hyperinvasive phenotype that is measurable *in vitro* and leads to increased clinical severity of disease in a calf model.

Prior to the studies presented in this dissertation, it was unknown if other protozoa are capable of serving as a venue for DT104 plasmid transfer or inducing DT104 hypervirulence. First*, in vitro* assays were used to evaluate rumen protozoa from sheep, goats, and African ruminants. These assays demonstrated that conjugal plasmid transfer was achieved in all of these protozoa. Assessment of DT104 cell invasion following protozoan engulfment revealed that ovine protozoa increased invasion approximately 3 fold while the other types of protozoa did not induce a hyperinvasive phenotype. *In vivo* studies confirmed these observations; sheep and calves infected with DT104 had a more rapid onset of pyrexia and clinical signs. Altogether, these studies provide evidence that conjugal plasmid transfer is a more common phenomenon while induction of the DT104 hyperinvasive phenotype occurs only in certain bovine and ovine rumen protozoa. In subsequent studies, *Tetrahymena* was used as a model protozoan to study

engulfment of DT104. *In vitro* cell invasion assays indicated that engulfment of DT104 by *Tetrahymena* induced the hyperinvasive phenotype and this was confirmed in a *in vivo* (calf) model of disease.

PROTOZOAN GPCRS

G protein-coupled receptors are one of the most important group of transmembrane-signaling receptors. The investigation of GPCRs from protozoa is only in its infancy and to this point has been relegated to studies in model organisms. No studies have utilized a heterologous expression assay for deorphanization of putative protozoan GPCRs. A goal of this dissertation was to identify GPCRs from protozoa with the hypothesis that GPCRs could bind bacterial cells and initiate bacterial engulfment.

Initial studies entailed constructing a cDNA library from bovine rumen protozoa. Homologues of genes cloned from this library were identified in *Tetrahymena* and expression of these genes were targeted using an RNAi approach to assess their role in bacterial engulfment. Serendipitously, these RNAi studies revealed that knock-down of a cyclic nucleotide binding protein severely hampered Tetrahymena engulfment of DT104 and the ability of DT104 to induce the hyperinvasive phenotype. Additional studies conducted utilizing RNAi implicated a putative *Tetrahymena* GPCR in bacterial engulfment.

A yeast heterologous expression system was utilized for the deorphanization of protozoan GPCRs. In this system, the *Tetrahymena* GPCR was agonized by *Tetrahymena* cell lysates, suggesting that this receptor detects intercellular signaling molecules. In the context of bacterial engulfment, *Tetrahymena* GPCRs are probably most relevant in terms of chemotaxis since these organisms sweep food items indiscriminately into an oral feeding apparatus. In

contrast, amoebae such as *Entamoeba histolytica* may rely on receptor based interactions that bind bacterial epitopes and lead to engulfment of the bacteria. EhGPCR-1, a previously described GPCR from *E. histolytica*, was activated by bacterial lipopolysaccharide in the yeast expression assay. Subsequent experiments demonstrated that *E. histolytica* trophozoites engulf bacteria selectively based on the presence of lipopolysaccharide and this feeding could be inhibited by the G protein inhibitor suramin.

The work presented herein revealed the physiologic relevance to GPCR signaling in two protozoan organisms, *Tetrahymena* and *E. histolytica*. In addition, a novel yeast heterologous expression assay was used to deorphanize GPCRs from these organisms. These studies reinforce the need for further investigation of GPCRs from protozoa especially given the significance of these receptors as drug targets.

APPENDIX A. EFFECTS OF SUBTHERAPEUTIC CONCENTRATIONS OF ANTIMICROBIALS ON GENE ACQUISITION EVENTS IN *YERSINIA, PROTEUS, SHIGELLA,* AND *SALMONELLA* RECIPIENT ORGANISMS IN ISOLATED LIGATED INTESTINAL LOOPS OF SWINE

Modification of a paper published in the *American Journal of Veterinary Research*

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ABSTRACT

To assess antimicrobial resistance and transfer of virulence genes facilitated by subtherapeutic concentrations of antibiotics, we evaluated the conjugation frequency of antimicrobial resistance genes in 4 recipient pathogenic bacteria (*Salmonella enterica* serotype Typhimurium, *Yersinia enterocolitica*, *Shigella flexneri*, or *Proteus mirabilis*) in the presence of subinhibitory concentrations of 16 antimicrobials in isolated ligated intestinal loops in swine. Anesthetized pigs were experimentally inoculated with donor and recipient bacteria. After coincubations, intestinal contents were removed and assessed for pathogens that acquired new antimicrobial resistance or virulence genes following exposure to the subtherapeutic concentrations of antimicrobials. Three antimicrobials (apramycin, lincomycin, and neomycin) enhanced transfer of an antimicrobial resistance plasmid from commensal *E. coli* organisms to *Yersinia* and *Proteus* organisms, whereas seven antimicrobials (florfenicol, hygromycin, penicillin G, roxarsone, sulfamethazine, tetracycline, and tylosin) exacerbated transfer of an integron (*Salmonella* genomic island 1) from *Salmonella* organisms to *Yersinia* organisms. Sulfamethazine induced the transfer of *Salmonella* pathogenicity island 1 from pathogenic to nonpathogenic *Salmonella* organisms. Six antimicrobials (bacitracin, carbadox, erythromycin, sulfathiazole, tiamulin, and virginiamycin) did not mediate any transfer events. Sulfamethazine

was the only antimicrobial implicated in two types of transfer events. Use of subtherapeutic antimicrobials in animal feed may be associated with unwanted collateral effects.

INTRODUCTION

Subtherapeutic concentrations of antimicrobials have been used for decades as growth promotants and prophylactic agents. This practice has been scrutinized as a contributor to the dissemination of antimicrobial resistance. Specifically, subtherapeutic concentrations of chlortetracycline have been associated with an increase in resistance to multiple antimicrobials for swine intestinal microbes.[1] Another study[2] revealed that subtherapeutic concentrations of antimicrobials can lead to the propagation of antimicrobial-resistant *Enterococcus* organisms in swine. A recent study[3] indicated that intestinal bacteriophages are activated by subtherapeutic concentrations of antimicrobials, and this activation can lead to transfer of antimicrobial resistance genes. However, these studies have not addressed specific gene transfer events that precipitate resistance and virulence. Thus, little is known about the effects of these antimicrobials on specific gene transfer events that promote the dissemination of antimicrobial resistance and virulence genes in enteric pathogens.

The objective of the study reported here was to identify antimicrobials that, at subtherapeutic concentrations, augment the transfer of certain antimicrobial resistance and virulence genes. The main goals involved assessing three types of transfer events (plasmid transfer, integron transfer, and horizontal transfer of a pathogenicity island) mediated by subtherapeutic concentrations of antimicrobials *in vivo*. Specifically, the study was conducted to determine the relative rates of antimicrobial-mediated transfer events involving three representative antimicrobial resistance plasmids, one model integron, and one pathogenicity island in the presence of antimicrobials that are or were approved for use at subtherapeutic concentrations as feed additives for swine.

MATERIALS AND METHODS

Animals—Twenty juvenile swine were used to assess *in vivo* transfer events. Pigs were of mixed breeds and both sexes and weighed between 5 and 10 kg. Animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee.

Pigs were anesthetized with pentobarbital[a] (40 mg/kg, intraperitoneal). Isolated ligated loops of intestine (9 loops/pig; each ligated loop was 10 cm in length) were created[4] with 2-0 silk sutures.[b] At the end of the experiments, anesthetized pigs were euthanized by intracardiac administration of an overdose of pentobarbital (100 mg/kg).

Plasmid transfer—To determine plasmid transfer from donor bacteria to recipient pathogenic bacteria, each loop was injected with approximately 10^9 to 10^{11} CFUs of donor bacteria and 10^9 CFUs of recipient bacteria in 1 mL of saline (0.9% NaCl) solution that contained one of 16 feed additive antimicrobials[c] (apramycin, bacitracin, carbadox, erythromycin, florfenicol, hygromycin, lincomycin, neomycin, penicillin G, roxarsone, sulfamethazine, sulfathiazole, tetracycline, tiamulin, tylosin, and virginiamycin); antimicrobial-free saline solution was used as a negative control treatment. The concentration of each antimicrobial was 1 µg/mL. This concentration was chosen because it was less than the established breakpoints for all of the antimicrobial-bacteria combinations[5] and preliminary experiments conducted by our laboratory group on the minimum inhibitory concentration of these antimicrobials revealed that this was the lowest common concentration that permitted growth of all donor and recipient bacteria in each of the 16 antimicrobials (data not shown).

Plasmid transfer from a donor commensal intestinal *Escherichia coli*[6] (antimicrobial susceptible and lacking virulence genes) to each of four recipient pathogenic bacteria (*Salmonella enterica* serotype Typhimurium strain SL1344,[7] *Yersinia enterocolitica*,[d] *Shigella flexneri*,[e] or *Proteus mirabilis*[f]) was

measured. All of the recipient bacteria were susceptible to most antimicrobials (except tetracycline and streptomycin), as determined by use of microdilution broth assays performed in accordance with standards established by the Clinical and Laboratory Standards Institute.[5] The *E. coli* donor strain was experimentally transformed with one of three conjugative plasmids encoding an ESBL,[8] amikacin resistance (*aacC4*),[9] or fluoroquinolone resistance (via the *qnr* gene).[10]

Bacteria were allowed to incubate in the isolated ligated intestinal loops for 1 hour, after which loops were excised. Total volume of intestinal content in each isolated loop was 1.1 to 1.2 mL; approximately 10% of the total content of each loop was removed for plating on media selective for each of the four pathogens (XLD agar[g] for *Salmonella* and *Shigella* organisms, *Yersinia* selective agar base[h] for *Yersinia* organisms, and phenylalanine agar[g] for *Proteus* organisms) and that contained one of three antimicrobials (ceftiofur[i] at 32 μg/mL for the ESBL plasmid, amikacin[c] at 64 μg/mL, or enrofloxacin[j] at 8 μg/mL) at their respective breakpoint concentrations.[5] Each loop was cultured in triplicate (three agar plates/loop). Control experiments revealed that the donor *E. coli* did not grow on the selective media, except for the XLD agar; however, the *E. coli* colonies that grew on XLD agar were biochemically distinct from the *Salmonella* and *Shigella* colonies.

Plates were incubated at 37°C for 16 hours. Bacteria then were enumerated and the number of translocants/10^9 recipients was calculated by multiplying the number of recovered colonies by 10 to account for the fact that only 10% of the intestinal contents were plated. The $\log_{10}$ of the quotient was derived and used for final data analysis and statistical evaluation.

Transfer of the ESBL plasmid was assessed with a PCR assay with primers (5'-ATGATGAAAAAATCGTTATGCT-3' and 5'-TTATTGCAGCTTTTCAAGAAT-3') specific to the *bla$_{CMY-2}$* gene present on the ESBL plasmid.[8] Each transfer event was determined for 100 representative colonies.

SGI1 transfer—The possibility that subinhibitory concentrations of antimicrobials can modulate the transfer of SGI1 from *Salmonella* organisms to *Yersinia* recipients, *Shigella* recipients, or *Proteus* recipients was evaluated. The SGI1 is a multiresistance integron that encodes resistance to five antimicrobials in *Salmonella* organisms.[11] Integrons are mobile genomic elements putatively transferred by bacteriophages,[12] and studies[13–15] have indicated that *Yersinia* spp, *Shigella* spp, and *Proteus* spp are capable of receiving integrons.

To evaluate SGI1 transfer, approximately 10^9 CFUs of SGI1-free recipient *Yersinia enterocolitica*, *Shigella flexneri*, or *Proteus mirabilis* (all of which were susceptible to most antimicrobials except tetracycline and streptomycin), as determined by use of microdilution broth assays performed in accordance with standards established by the Clinical and Laboratory Standards Institute[5]) were each co-inoculated with 10^9 CFUs of SGI1-bearing donor *S enterica* serotype Typhimurium phagetype DT104 strain LNWI[4]) into ligated intestinal loops. Co-incubations included one of the 16 aforementioned antimicrobials (concentration, 1 μg/mL) and the negative control treatment (saline solution without an antimicrobial).

Bacteria were allowed to incubate in the isolated ligated intestinal loops for 1 hour, after which loops were excised. Total volume of the intestinal content in each isolated loop was 1.1 to 1.2 mL; approximately 10% of the total content of each loop was removed for plating on media selective for each of the 3 pathogens (XLD agar for *Salmonella* and *Shigella* organisms, *Yersinia* selective agar base for *Yersinia* organisms, and phenylalanine agar for *Proteus* organisms) and that contained one of two antimicrobials relevant for SGI1 (ampicillin[c] at 32 μg/mL and chloramphenicol[c] at 32 μg/mL) at their respective breakpoint concentrations.[5] Each loop was cultured in triplicate (three agar plates/loop).

Plates were incubated at 37°C for 16 hours. Bacteria then were enumerated and the number of translocants/10^9 recipients was calculated by multiplying the number of recovered colonies by 10 to

account for the fact that only 10% of the intestinal contents were plated. The $\log_{10}$ of the quotient was derived and used for final data analysis and statistical evaluation.

A PCR assay specific to the *floR-tetR* sequence in SGI1[16] was used to assess the presence of the SGI1 integron in 100 representative recipient bacteria that grew on selective media containing ampicillin and chloramphenicol. Additionally, transferrants were assessed for the absence of a *Salmonella* virulence gene segment (*sipB/C*).[16] Control experiments revealed that the donor *Salmonella* organisms were not able to grow on the media selective for *Yersinia* spp and *Proteus* spp, whereas *Salmonella* organisms were distinguishable from *Shigella* organisms on XLD agar.

SPI1 transfer—Transfer of SPI1 from pathogenic to nonpathogenic *Salmonella* organisms was evaluated. The SPI1 is a major determinant of virulence in *Salmonella* organisms, and avirulent *Salmonella* organisms lack SPI1.[17] The genomic structure of SPI1 suggests that this island is transferrable.[18] *In vivo* coincubations with the 16 antimicrobials and the negative control treatment (saline solution without an antimicrobial) were performed as described previously for the plasmid and integron transfer experiments. The SPI1-bearing *S enterica* serotype Typhimurium (antimicrobial-susceptible strain SL1344[7]) was incubated with one of four SPI1-free strains (*S enterica* serotypes Litchfield,[17] Senftenberg,[17] Seminole, or Betiocky), all four of which were transformed with a nonconjugative plasmid encoding green fluorescent protein.[19] For this experiment, 10^{11} CFUs of donor bacteria and 10^{11} CFUs of recipient bacteria were used for the incubations.

Bacteria were allowed to incubate in the ligated loops for 1 hour. Total volume of each loop was 1.1 to 1.2 mL; approximately 10% of the total content of each loop was removed and used in a large-volume tissue culture invasion assay[20] in which recovered bacteria were plated on XLD agar that contained 50 µg of zeocin[k]/mL (zeocin is the selective marker for the fluorescence plasmid[19]). Each loop was cultured in triplicate (three agar plates/loop). Fluorescent colonies that were invasive (*i.e.*,

recovered from inside tissue culture cells) were individually expanded in fresh nutrient broth and then subjected to a second invasion assay. Amount of invasion was compared with that of strain SL1344.[7] Serotype analysis was conducted at another laboratory,[l] and a PCR assay that detected the *sipB-sipC* sequence in SPI1[16] was performed on each clone for which invasion was indistinguishable from that of strain SL1344 (approximately 1% invasion).

Statistical analysis—Statistical differences were assessed via an ANOVA.[m] There were 51 combinations of antimicrobials and transfer events ([16 antimicrobials plus one antimicrobial-free treatment] times three transfer events), each assessed in three separate ligated loops (153 total loops) and three agar plates/loop (459 total loops). Frequency data for all transfer events were analyzed *en masse* to allow for inter-antimicrobial and intra-event comparisons and intra-antimicrobial and inter-event comparisons [*i.e.*, comparisons were made among the antimicrobials within the 3 transfer events (plasmid, SGI1, and SPI1) and among the three transfer events within a specific antimicrobial]. The Scheffe *F* test was chosen as the *ad hoc* test because our research group has empirically found it to be the most conservative for detecting differences, in contrast to the Bonferroni test. Values of $P < 0.05$ were considered significant.

RESULTS

Plasmid transfer—*In vivo* transfer events of three clinically relevant antimicrobial resistance plasmids were assessed by use of ligated intestinal loops of swine. Loops were co-inoculated with donor commensal *E coli* bearing one of three antimicrobial resistance plasmids, one of four recipient pathogenic *Enterobacteriaceae*, and an antimicrobial (1μg/mL) approved as a feed additive in swine. Commensal *E. coli* and recipient *Enterobacteriaceae* were chosen because these microbes are highly representative of enteric bacteria that transfer genetic information. Three antimicrobials mediated a significant increase in the frequency of a specific plasmid transfer event *in vivo* **(Figure 1)**. An increased

frequency of transfer of the ESBL plasmid from *E coli* to *Yersinia* recipients and from *E. coli* to *Proteus* recipients was evident in the presence of apramycin, lincomycin, or neomycin. None of the antimicrobials caused significant changes in the frequencies of ESBL plasmid transfer from *E. coli* to *Salmonella* recipients and from *E. coli* to *Shigella* recipients. Additionally, none of the antimicrobials caused significant changes in transfer of amikacin resistance plasmids or fluoroquinolone resistance plasmids.

The PCR assay of 100 representative ceftiofur-resistant colonies from each of the aforementioned transfer events revealed that 96% to 97% of the putative transconjugates contained *bla*$_{CMY-2}$. Colonies that lacked this sequence were numerically discounted as transconjugates in the final calculations (Figure 1).

SGI1 transfer—*In vivo* transfer events of an integron were assessed via ligated intestinal loops co-inoculated with donor *Salmonella* organisms bearing SGI1,[11] one of four recipient pathogenic *Enterobacteriaceae*, and an antimicrobial (1 µg/mL) approved as a feed additive in swine. Seven antimicrobials mediated a significant increase in the frequency of *in vivo* transfer of a specific integron **(Figure 2)**. An increased frequency of transfer of the SGI1 integron from *Salmonella* organisms to *Yersinia* organisms was evident in the presence of florfenicol, hygromycin, penicillin G, roxarsone, sulfamethazine, tetracycline, and tylosin. None of the antimicrobials caused significant changes in the frequencies of SGI1 transfer events from *Salmonella* organisms to either of the other two pathogens examined. The PCR assay of 100 representative colonies from each of the 16 aforementioned transfer events revealed that 98% to 99% of the putative SGI1 recipients contained the *floR-tetR* sequence indicative of SGI1.[16] Colonies that lacked this sequence were numerically discounted as translocants in the final calculations.

SPI1 transfer—*In vivo* transfer events of a pathogenicity island were assessed via ligated intestinal loops

co-inoculated with donor *Salmonella* organisms, one of four recipient nonpathogenic *Salmonella*

organisms that lacked SPI1, and an antimicrobial (1 µg/mL) approved as a feed additive in swine.

Sulfamethazine mediated the horizontal transfer of SPI1 *in vivo* **(Figure 3)**. Three of four SPI1-free

Salmonella serovars (Betiocky, Seminole, and Litchfield) had transfer events in the presence of

sulfamethazine. Transfer of SPI1 was detected in the absence of an antimicrobial for one clone of

Salmonella serovar Litchfield. *Salmonella enterica* serovar Senftenberg did not acquire SPI1 in this

experiment. Results of the PCR assays confirmed the presence of the *sipB-sipC* genomic segment in all

clones that had invasion indistinguishable from that of strain SL1344.

DISCUSSION

The use of subtherapeutic concentrations of antimicrobials in livestock feed is a controversial practice

that is being scrutinized. Of concern are the unknown collateral effects on bacterial gene transcription,

plasmid transconjugation from commensals to pathogens, and viral-mediated transduction of genes

from commensals to pathogens and from one pathogen to another. Specifically, these collateral effects

can activate molecular processes culminating in gene transfer events that yield pathogens with multiple

antimicrobial resistance or pathogens with new virulence capabilities.

The experiments in the present study involved the use of low concentrations (1 µg/mL) of

antimicrobials to mimic field conditions without directly harming the donor or recipient bacteria.

Although this concentration may be lower than concentrations in the intestinal tract of swine fed feed

that contains antimicrobials, this concentration was considered relevant for swine in which the

antimicrobial is removed from the diet prior to slaughter.

The present study revealed that there were transfer events in the absence of an antimicrobial and that certain antimicrobials mediated gene transfer events at a higher frequency than did other antimicrobials **(Table 1)**. Sulfamethazine mediated two separate transfer events (SGI1 transfer and SPI1 transfer), whereas a related drug, sulfathiazole, did not mediate any transfer events. Bacitracin, carbadox, erythromycin, tiamulin, and virginiamycin were also not implicated in transfer events. Transfer events were confirmed with a PCR assay, although a few (1% to 4%) transconjugates and translocants did not harbor the transferrable element, which suggested that efflux systems may have been activated in these few clones.

Three classes of antimicrobials were implicated in transfer to the ESBL plasmid. Lincomycin is a lincosamide, apramycin is an aminocyclitol, and neomycin is an aminoglycoside (although the latter 2 are sometimes grouped in the same class). Lincomycin is an inhibitor of the 50S ribosome in bacteria, yet 3 other 50S inhibitors (erythromycin, tiamulin, and virginiamycin) did not mediate transfer events. Apramycin and neomycin are inhibitors of the 30S ribosome, but another 30S inhibitor, tetracycline, did not exacerbate transfer of the ESBL plasmid. It is possible that apramycin, lincomycin, and neomycin can selectively alter protein synthesis that impacts sex pheromones and conjugation.

Seven antimicrobials (florfenicol, hygromycin, penicillin G, roxarsone, sulfamethazine, tetracycline, and tylosin) from 7 antimicrobial classes were implicated in transfer of SGI1. Tylosin and florfenicol are 50S inhibitors, but that is the extent of the similarities among these seven antimicrobials. Because movement of SGI1 may be a phage-mediated event,[12] it is possible that those seven antimicrobials activated phage recrudescence in SGI1-bearing *Salmonella* organisms, similar to that recently reported for sulfamethazine, chlortetracycline, and penicillin.[3] It is also possible that naked SGI1 DNA was

transferred from *Salmonella* organisms to *Yersinia* organisms, although to our knowledge this process has not been described in the literature.

Analysis of results of the present study indicated that certain antimicrobials at subtherapeutic concentrations are more likely to mediate unwanted gene transfer into pathogenic bacteria in ligated intestinal loops in swine. Sulfamethazine mediated 2 different types of transfer, whereas apramycin, lincomycin, and neomycin exerted the greatest quantitative effect on a single transfer event into two genera of *Enterobacteriaceae*. Bacitracin, carbadox, erythromycin, sulfathiazole, tiamulin, and virginiamycin did not significantly influence any of the three transfer events evaluated. No antimicrobial class–specific patterns were observed in the three transfer events. Transfer of the ESBL plasmid was detected at the highest frequency. Protein synthesis irregularities may underlie transfer of the ESBL plasmid, and activation of bacteriophages may be involved in SGI1 transfer or SPI1 transfer (or both). Regardless of the mechanisms involved, subtherapeutic concentrations of antimicrobials have potential ecologic impacts that involve the dissemination of antimicrobial resistance and virulence genes.

References

1.   Funk J, Lejeune J, Wittum T, et al. The effect of subtherapeutic chlortetracycline on antimicrobial resistance in the fecal flora of swine. *Microb Drug Resist* 2006;12:210–218.

2.   Poole T, Hume M, Genovese K, et al. Persistence of a vancomycin-resistant *Enterococcus faecium* in an anaerobic continuous-flow culture of porcine microflora in the presence of subtherapeutic concentrations of vancomycin. *Microb Drug Resist* 2001;7:343–348.

3.   Allen H, Looft T, Bayles D, et al. Antibiotics in feed induce prophages in swine fecal microbiomes. *mBio* 2011;2:e00260–e00211.

4.   Wu MT, Carlson SA, Meyerholz DK. Cytopathic effects observed upon expression of a repressed collagenase gene present in *Salmonella* and related pathogens: mimicry of a cytotoxin from multiple antibiotic-resistant *Salmonella enterica* serotype Typhimurium phagetype DT104. *Microb Pathogen* 2002;32:1–9.

5.   Clinical and Laboratory Standards Institute. MIC Interpretive Standards (µg/mL) for Enterobacteriaceae. In: *Performance standards for antimicrobial disk and dilution susceptibility tests.* 2nd ed. Wayne, Pa: Clinical and Laboratory Standards Institute, 2008;16-19.

6.   Lyczak JB. Commensal bacteria increase invasion of intestinal epithelium by *Salmonella enterica* Serovar Typhi. *Infect Immun* 2003;71:6610–6614.

7.   Wray C, Sojka WJ. Experimental *Salmonella typhimurium* in calves. *Res Vet Sci* 1978;25:139–143.

8.   Brewer M, Xiong N, Dier J, et al. Comparisons of *Salmonella* conjugation and virulence gene hyperexpression mediated by rumen protozoa from domestic and exotic ruminants. *Vet Microbiol* 2011;151:301–306.

9.   Tosini F, Visca P, Luzzi I, et al. Class 1 integron-borne multiple-antibiotic resistance carried by IncFI and IncL/M plasmids in *Salmonella enterica* serotype typhimurium. *Antimicrob Agents Chemother* 1998;42:3053–3058.

10.   Gay K, Robicsek A, Strahilevitz J, et al. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. *Clin Infect Dis* 2006;43:297–304.

11.   Briggs CE, Fratamico PM. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob Agents Chemother* 1999;43:846–849.

12.   Schmieger H, Schicklmaier P. Transduction of multiple drug resistance of *Salmonella enterica* serovar *typhimurium* DT104. *FEMS Microbiol Lett* 1999;170:251–256.

13.   Soto S, Lobato M, Mendoza M. Class 1 integron-borne gene cassettes in multidrug-resistant *Yersinia enterocolitica* strains of different phenotypic and genetic types. *Antimicrob Agents Chemother* 2003;47:421–426.

14.   McIver C, White P, Jones L, et al. Epidemic strains of *Shigella sonnei* biotype g carrying integrons. *J Clin Microbiol* 2002;40:1538–1540.

15.   Ahmed A, Hussein A, Shimamoto T. *Proteus mirabilis* clinical isolate harbouring a new variant of *Salmonella* genomic island 1 containing the multiple antibiotic resistance region. *J Antimicrob Chemother* 2007;59:184–190.

16.   Carlson SA, Bolton LF, Briggs CE, et al. Detection of *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. *Mol Cellular Probes* 1999;13:213–222.

17.   Ginocchio CC, Rahn K, Clarke RC, et al. Naturally occurring deletions in the centisome 63 pathogenicity island of environmental isolates of *Salmonella* spp. *Infect Immun* 1997;65:1267–1272.

18.   Hensel M, Shea J, Bäumler A, et al. Analysis of the boundaries of *Salmonella* pathogenicity island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. *J Bacteriol* 1997;179:1105–1111.

19.   Frana T, Carlson S. Development and use of a plasmid encoding green fluorescent protein in multiple antibiotic-resistant *Salmonella*. *BioTechniques* 2001;30:28–32.

20.   Gianella RA, Washington O, Gemski P, et al. Invasion of HeLa cells by *Salmonella typhimurium*: a model for study of invasiveness of *Salmonella*. *J Infect Dis* 1973;128:69–75.
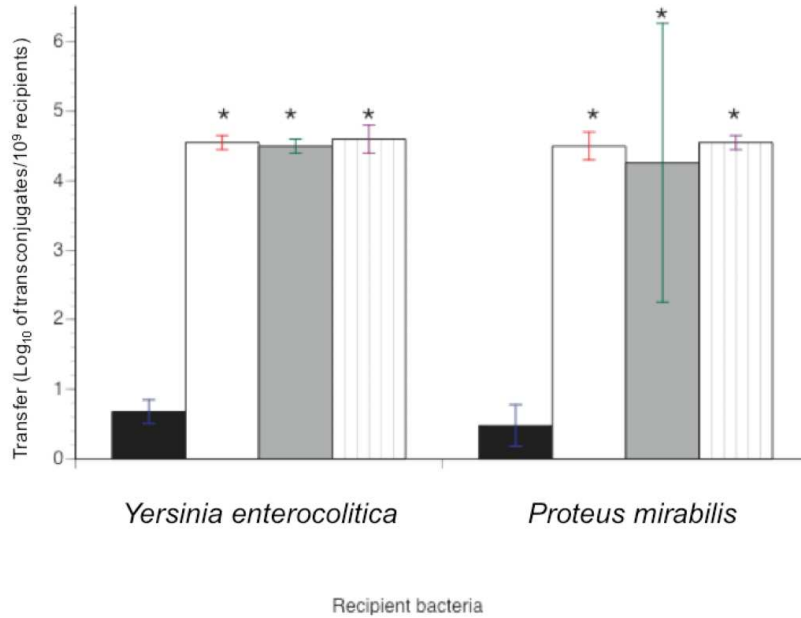
Figure 1—Plasmid transfer from commensal *Escherichia coli* to *Yersinia* organisms and *Proteus* organisms after co-incubation with subtherapeutic concentrations (1 ug/mL) of lincomycin (white bars), apramycin (gray bars), or neomycin (striped bars) or saline (0.9% NaCl) solution that did not contain an antimicrobial (negative control treatment [black bars]) for 1 hour in isolated ligated intestinal loops in swine. Results represent the mean ± sem for three experiments that each was performed in triplicate. Notice that there was enhancement of transfer of the ESBL plasmid[8] in the presence of subtherapeutic concentrations of antimicrobials in *Yersinia* recipients and *Proteus* recipients. *Within a bacterium, value differs significantly ($P < 0.05$) from the value for the co-incubation with the negative control treatment.
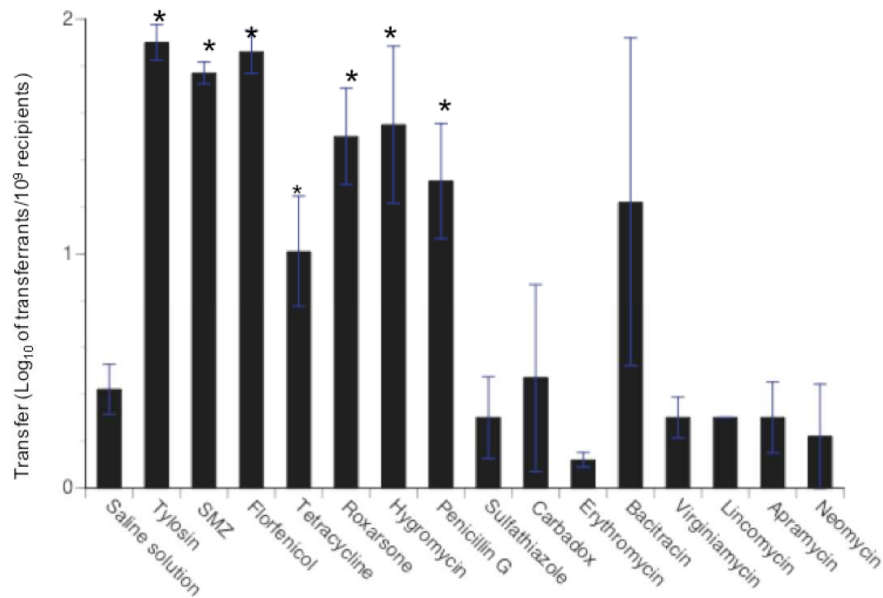
Figure 2—Transfer of SGI1 from *Salmonella enterica* serotype Typhimurium phagetype DT104 to *Yersinia enterocolitica* after co-incubation with subtherapeutic concentrations (1 ug/mL) of 16 antimicrobials or saline solution that did not contain an antimicrobial (negative control treatment) for 1 hour in isolated ligated intestinal loops in swine. Results represent the mean ± sem for three experiments that each was performed in triplicate. Notice that transfer of the integron was enhanced in the presence of seven antimicrobials. *Values differs significantly (*P* < 0.05) from the value for coincubation with the negative control treatment. SMZ = Sulfamethazine.

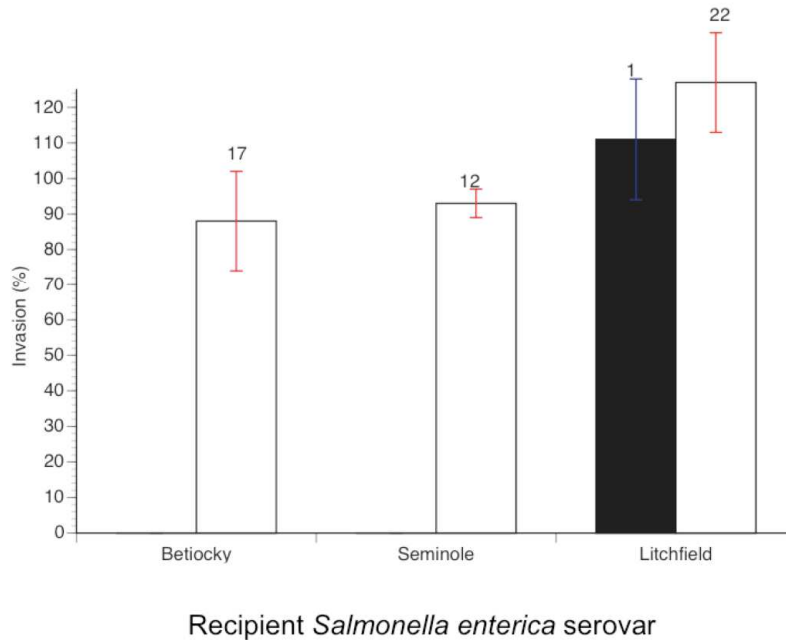Figure 3—Transfer of SPI1 from pathogenic *Salmonella* organisms to nonpathogenic *Salmonella* organisms after co-incubation with subtherapeutic concentrations (1 ug/mL) of sulfamethazine (white bars) or saline solution that did not contain an antimicrobial (negative control treatment [black bars]) for 1 hour in isolated ligated intestinal loops in swine. Fluorescent bacteria were subjected to two invasion assays, and colonies that were invasive (*i.e.*, recovered from inside tissue culture cells) were quantitated by enumerating the bacteria recovered from tissue culture cell lysates at the end of the second assay. Invasion represents the percentage as compared with that of the donor *Salmonella* organisms. Results represent the mean ± sem for three experiments that each was performed in triplicate. Notice that transfer of SPI1 was only detected in the presence of sulfamethazine and that one clone of *S. enterica* serotype Litchfield was invasive in the absence of an antimicrobial. The number above each column indicates the cumulative number of serovar-specific clones that were invasive across three separate experiments.

Table 1—Gene transfer events for donor bacteria to recipient bacteria after co-incubation with subtherapeutic concentrations (1 ug/mL) of 16 antimicrobials or saline (0.9% NaCl) solution that did not contain an antimicrobial (negative control treatment) for 1 hour in isolated ligated intestinal loops in swine.

Antimicrobial	ESBL plasmid transfer	SGI1 transfer	SPI1 transfer	Ratio of total transfer frequencies†
Saline solution	NA	NA	NA	1.0
Apramycin	*Yersinia* recipients and *Proteus* recipients*	—	—	3,471
Bacitracin	—	—	—	2.3
Carbadox	—	—	—	0.6
Erythromycin	—	—	—	0.7
Florfenicol	—	*Yersinia* recipients*		9.8
Hygromycin	—	*Yersinia* recipients*		5.1
Lincomycin	*Yersinia* recipients and *Proteus**	—	—	4,596
Neomycin	*Yersinia* and *Proteus**	—	—	4,641
Penicillin G	—	*Yersinia* recipients*		4.4
Roxarsone	—	*Yersinia* recipients*		4.7
Sulfamethazine	—	*Yersinia* recipients*	3 recipient nonpathogenic serovars of *Salmonella enterica*†	13.6
Sulfathiazole	—	—	—	1.1
Tetracycline	—	*Yersinia* recipients*	—	4.1
Tiamulin	—	—	—	1.2
Tylosin	—	*Yersinia* recipients*	—	10.3
Virginiamycin	—	—	—	0.9

Mean transfer frequency in the absence of an antimicrobial was approximately 1.4×10^{-8} transconjugates/recipient for the ESBL plasmid, 2.6×10^{-9} translocants/recipient for SGI1, and 3×10^{-11} invasive clones/noninvasive recipient for SPI1; these frequencies were calculated by dividing the total number of all recovered recipients by the total number of donor bacteria added to all of the co-incubations in the absence of an antimicrobial. For each antimicrobial, the ratio of total transfer frequency = (total number of all bacteria that received the new genetic element when co-incubated with an antimicrobial/total number of recipients co-incubated with an antimicrobial)/ (total number of all bacteria that received the new genetic element when co-incubated without an antimicrobial/total number of recipients co-incubated without an antimicrobial).

*Transfer frequency was significantly ($P < 0.05$) greater than the transfer frequency for the antimicrobial-free (negative control) treatment. †*Salmonella enterica* serotypes Betiocky, Litchfield, and Seminole.

NA = Not applicable. — = Transfer frequency was not significantly greater than the transfer frequency for the antimicrobial-free (negative control) treatment.

APPENDIX B. AMELIORATION OF SALMONELLOSIS IN PRE-WEANED DAIRY CALVES FED *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCTS IN FEED AND MILK REPLACER

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ABSTRACT

Salmonellosis is an insidious and potentially epidemic problem in pre-weaned dairy calves. Managing this disease, or any other diarrheal disease, is a financial burden to producers. Calf mortalities and medicinal treatments are overt costs of salmonellosis, while hidden costs include hampered weight gains and persistent intestinal colonization of the pathogen. In this study, we examined the anti-*Salmonella* effects of *Saccharomyces cerevisiae* fermentation products (SCFP) incorporated into both the milk replacer and the starter grain. In a blinded study, 2 to 8 day-old calves were fed SCFP (n = 20 calves) or an SCFP-free Control (n = 20 calves) for two weeks before and three weeks after experimental challenge with *Salmonella enterica* serotype Typhimurium. Following the challenge, calves were monitored for clinical signs and parameters associated with salmonellosis. Calves were then euthanized and examined for rumen development and intestinal *Salmonella* colonization. When compared to calves that received milk replacer and feed lacking SCFP, calves fed SCFP had fewer bouts of diarrhea and fever. Rumens from these calves were more developed, as measured by the length of papillae, which is consistent with the enhanced weight gain observed in this treatment group. Additionally, *Salmonella* intestinal colonization was reduced in SCFP-fed calves and *Salmonella* fecal shedding disappeared at an earlier stage in these calves. This study revealed that the

combination of two proprietary *Saccharomyces cerevisiae* fermentation products provide marked benefit for preventing the negative effects of salmonellosis in pre-weaned dairy calves, while also boosting productivity. The mechanism of action needs to be clarified, but it may be related to the observed decrease in colonization by the pathogen and increase in rumen development.

Key words: *Salmonella* infection, dairy calf, yeast culture

INTRODUCTION

Salmonellosis is one of the many diarrheal diseases affecting pre-weaned dairy calves. *Salmonella* organisms are commonly isolated from dairy farms and the fecal-oral transmission route can occur from dam to offspring. Calves can also acquire the organism from fecal-contaminated fomites or the environment. Calves manifest the disease as diarrhea, fever, anorexia, and dehydration all of which significantly compromise the development and maturation of the animal. Further costs include treatment with electrolytes or antibiotics or both, and some calves still perish because of the increasing prevalence of antibiotic resistance in *Salmonella* [1] and hypervirulence associated with multi-resistant strains (Rasmussen et al., 2004). Furthermore, calves that survive salmonellosis can be long-term carriers of the pathogen [2] and these adult animals can serve as a persistent source for new infections in the herd [3]. Environmental persistence also contributes to this problem [3].

Preventing *Salmonella* infections currently focuses on a vaccine technology [4]. Unfortunately, this vaccine is only for cattle that are six months or older thus pre-weaned calves are dependent upon colostral passive immunization from the vaccinated dam. Anti-

Salmonella bacterins have been tried but are frequently unsuccessful because of the immunodominance of the *Salmonella* O-antigen (Barat et al., 2012) and serovar specificity (House et al., 2001). Anti-lipopolysaccharide antiserum and lipopolysaccharide toxoids are available but the anti-*Salmonella* benefits have not been clearly established. Thus *Salmonella* prophylaxis is not optimal at this time in the pre-weaned calf (Lanzas et al., 2008), although vaccinating the dam will reduce the environmental exposure of the calf.

It has been shown that soluble components present in *Saccharomyces cerevisiae* fermentation products (**SCFP**) enhance gut health [5] and promote immune function (Jensen et al. 2007). When supplemented to the starter grain, SCFP improved rumen development, starter grain intake, and BW gain of non-challenged calves (Lesmeister et al., 2004). Additionally, SCFP was shown to improve the gastrointestinal health of calves in a *Salmonella* endemic herd [6]. Because of these benefits associated with SCFP, we examined its anti-*Salmonella* effects when fed to pre-weaned dairy calves experimentally infected with *Salmonella*. The specific aims of this study were to determine the effects of the combination of two proprietary SCFP (Diamond V SmartCare™ and Diamond V Original XPC™) on the growth and rumen development, clinical signs of salmonellosis, *Salmonella* shedding, and intestinal colonization of *Salmonella* in pre-weaned dairy calves experimentally infected with *Salmonella*.

MATERIALS AND METHODS

Calves and Pre-infection Treatments

Animal experiments were approved by the Animal Care and Use Committee at Iowa State University. Forty Holstein or Holstein-cross calves (32 females and eight males) were purchased from a local supplier in northwest Iowa. Calves were fed colostrum for the first two days after birth and then fed a standard milk replacer until shipment to Iowa State University at two to eight days of age. Upon arrival at an animal biosafety level-2 building at Iowa State University, calves were weighed (28 to 47 kg, with Holstein-Jersey crosses representing the lower weights) and randomly assigned (without redistribution) to one of two separate but adjacent rooms. Each room had constant ambient temperatures (about 22°C) and humidity (about 40%) and was ventilated by negative pressure through HEPA filters. Calves were housed in individual 18 m^2 pens on Tenderfoot-type flooring without bedding.

Two separate experiments were performed each using 20 calves (10 per group) of similar ages (2-4 days in one experiment and 6-8 days in the other experiment), and treatment groups were alternated in the two different rooms in each experiment in order to avoid a "room effect". Each room was fed either SCFP or the Control to avoid the potential for inappropriate administration of a treatment within a room. Rooms were alternated between the two experiments, i.e., in the first experiment calves in "Room A" received SCFP while calves in this same room received the Control in the second experiment.

Calves were randomly assigned to one of two treatments: Control (no additive in milk replacer or starter grain) or diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products delivered separately (SCFP; 1 g/head/d SmartCare™ [0.15% inclusion

rate in conventional milk replacer] and 3.5 g/head/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). SmartCare is a water dispersible product that can be added directly to milk or milk replacer as a supplement for pre-weaning liquid calf diets (starting at d 1). Original XPC is dry feed product commonly used in pre-weaning calf starter diets. The combination of these products is the basis for Diamond V's dairy calf program during the pre-weaning phase. A gelatin capsule containing 3.5 g/head/d grain matrix used to produce XPC was given to Control calves to equalize the nutrients, although minimal, contributed by XPC.

All calves were fed a non-medicated milk replacer (20% all-milk protein, 20% fat; Land O'Lakes Animal Milk Products, Shoreview, MN) at a volume equivalent rate of approximately 10% of arrival BW twice per day (*i.e.*, 5% of BW each feeding) for the duration of the trial. Milk replacer was mixed in single batches using warm water and a cordless drill-driven stirrer. Specifically, each calf received 6 oz of milk replacer in 1 qt water *bid*, in which the milk replacer was 18.8% w/v of the solution.

Calves were fed calf starter (Calf Startena™, 18% crude protein, 0.005% decoquinate, Purina Mills, LLC, St. Louis, MO) and water *ad libitum*, although it was not feasible to measure intake of either because of spillage and other uncontrollable factors. The Iowa State University investigators (M.T.B., K.L.A., and S.A.C.) were blinded as to which calves received the Control or SCFP treatments. Specifically, the Diamond V investigators (I.Y. and M.F.S.) notified a third party (scientists at the Office of Intellectual Property at Iowa State University) as to the identity of the treatment groups prior to the onset of the studies. Once the studies were completed,

the Iowa State University investigators revealed the data to the Office of Intellectual Property who then revealed the identity of the treatment groups.

In the two-week pre-infection phase, six to seven calves from each group were orally treated with one dose of sulfamethazine (356 mg/kg; Sustain™, Bimeda, Oakbrook Terrace, IL) for veterinarian diagnosed coccidial infections manifested by blood in the feces. This treatment alleviated the bloody feces within 3 d of treatment. Treatment with sulfamethazine was deemed to not have a negative effect on the outcome of the trial since the *Salmonella* strain used in this study is resistant to sulfonamides and equivalent numbers of calves from each experimental group were subjected to sulfamethazine treatment.

Salmonella Infection of Calves

Calves were confirmed to be *Salmonella*-free by fecal culture on arrival and on d 7 and 12 post-arrival. Specifically, 1 g of freshly voided feces was diluted in 20 mL of Lennox L broth (Invitrogen, Carlsbad, CA). After settling, an aliquot (100 μL) of this mixture was streaked onto and then incubated overnight at 37°C on XLD agar (Fisher Scientific, Pittsburgh, PA) selective for *Salmonella* that appear as red colonies with black centers. All pre-infection fecal samples were free of *Salmonella*. Sulfamethazine was not used in the XLD agar for pre-infection assessment. SL1344 was plated on XLD as a positive control during the experiments.

At d 14 post-arrival (d 0 post-infection), calves were orally inoculated with *Salmonella enterica* serotype Typhimurium strain SL1344 [7] at the dose of 2 x 10^6 CFU/kg BW [8-10]. The *Salmonella* inoculum was prepared and dosed as described previously by the Carlson laboratory [8-10]. The inoculum was placed in the gelatin capsule containing the Control or SCFP, which

was administered using a small balling gun. Our empirical studies revealed that the strain

SL1344 was viable after incubation with either the Original XPC^{TM} or the Control treatment.

That is, pre-inoculated experiments revealed that SL1344 was 100% viable and recoverable

after remaining in the Control- or SCFP-containing gelatin capsule for 24 hrs (data not shown).

Assessment of Clinical Parameters in Calves

Pyrexia and diarrhea are frequently observed components of salmonellosis in calves

[11]. On d 0, 1, 2, 3, 4, 5, 6, 7, 10, and 21 post-infection, rectal temperatures were measured

and diarrhea was assessed on an ordinal scale. Diarrhea scoring was as follows: 0, no diarrhea;

1, mild diarrhea; 2, profuse diarrhea; or 3, profuse diarrhea with blood. This determination was

performed by one investigator (S.A.C.), who was blinded to the treatment groups, with vast

experience with experimental salmonellosis.

Assessment of Salmonella Shedding in Calves

Fecal shedding of *Salmonella* is a highly variable and sporadic occurrence in calves

infected with *Salmonella* [12]. Nonetheless, this event is of importance for the spread of this

pathogen [13]. On d 0, 1, 2, 3, 4, 5, 6, 7, 10, and 21 post-infection, 1 g of freshly voided feces

was briefly vortexed in 20 mL of Lennox L broth (Invitrogen, Carlsbad, CA). An aliquot of this

mixture (100 µL) was incubated overnight at 37°C on XLD agar containing the Committee on

Laboratory Standards Institute-derived [14] breakpoint concentration (512 µg/mL) of

sulfamethazine (Sigma Aldrich, St. Louis, MO), i.e., a concentration that will enable the growth

of SL1344 but will inhibit the growth of many other enteric bacteria. The following day red

colonies with black centers were enumerated and CFU/g of feces was calculated based on a dilution factor equal to 200.

Assessment of Intestinal Colonization by Salmonella

Intestinal colonization by *Salmonella* contributes to the persistence of the pathogen [3] and the fecal shedding that transmits the microbe to other cattle. On d 21 post-infection, all calves were euthanized using xylazine (0.5 mg/kg, intramuscular, Lloyd Laboratories, Walnut, CA) and pentobarbital (100 mg/kg, intravenous, Fort Dodge Laboratories, Fort Dodge, IA). A 2 cm section (approximately 1 g) of distal ileum was aseptically removed from each calf and cut longitudinally. Each section was placed in 20 mL Lennox L broth (Invitrogen, Carlsbad, CA) and briefly vortexed to dislodge the *Salmonella*. An aliquot (100 μL) of this mixture was then dispersed onto XLD agar containing sulfamethazine (to prevent the growth of other bacteria) that were incubated overnight at 37°C. The following day red colonies with black centers were enumerated and CFU/g of ileum was calculated based on a dilution factor equal to 200.

Assessment of Rumen Development in Calves

Salmonella, and any other enteric pathogen, can have a negative impact upon performance in the pre-weaned calf. It was hypothesized that SCFP may abrogate salmonellosis by promoting overall gastrointestinal health and improving rumen development in pre-weaned calves. Therefore, both weight gain and the size of rumen papillae were assessed in *Salmonella*-infected calves fed SCFP. Following euthanasia, a 4 cm^2 section of a ventrolateral portion of the rumen was removed and placed in 10% buffered-neutral formalin.

Rumen tissues were submitted to the Histopathology Laboratory at the College of Veterinary Medicine at Iowa State University. Tissues were prepared using standard hematoxylin and eosin staining. Length and width of rumen papillae were measured using an intra-ocular ruler. Measurements were collected from 10 randomly selected papillae present on two different sections (*i.e.*, five papillae from each section). Widths were measured at mid-shaft.

Statistical Analyses

For data in which assessments were performed on multiple days (rectal temperatures, diarrhea scores, and fecal shedding), statistical comparisons were made using a repeated measures analysis of variance with Tukey's *ad hoc* test for multiple comparisons (GraphPad Prism, Version 6, La Jolla, CA). For data involving single measurements from each calf (intestinal colonization and rumen papillae length), statistical comparisons were performed using a student's t-test (GraphPad). Significant differences were defined at $P \leq 0.05$. Statistical trends were consistent when the two sets of experiments were examined independently (data not shown).

RESULTS

Assessment of Clinical Parameters in Calves

Rectal temperatures and diarrhea we monitored in the calves on d 0, 1 through 7, 10, and 21 post-infection. As shown in Figures 1 and 2, pyrexia (rectal temperature > 39.2^{o}C) and diarrhea were observed less frequently ($P < 0.05$) in calves fed SCFP. The largest differences between calves supplemented with SCFP and Control calves were observed on d 3 to 5 post-

infection for rectal temperatures (38.8 to 39.0°C versus 39.5 to 39.7°C, respectively), and d 3

through 7 post-infection for diarrhea scores (0.10 to 0.25 versus 0.61 to 0.86 arbitrary units,

respectively). None of the SCFP-fed calves exhibited pyrexia at anytime throughout the study,

while nearly all Control calves exhibited pyrexia on d 2 through 6 post-infection. The relative

incidence of diarrhea was less in SCFP-fed calves on all days except day 0 (Fig. 2b).

Assessment of Salmonella Shedding in the Calves

Salmonella fecal shedding was monitored on d 0, 1 through 7, 10, and 21 post-infection.

As shown in Figure 3a, there was a quantitative difference in fecal shedding between the two

groups of calves on d 6 post-infection in which shedding was less ($P < 0.05$) in calves fed SCFP

(undetectable versus 955 CFU/g of feces). There also was a qualitative difference in fecal

shedding of *Salmonella* on d 3 through 7 post-infection (Fig. 3b).

Assessment of Intestinal Colonization by Salmonella in the Calves

Upon euthanasia, ileal sections were excised and subjected to *Salmonella* culture and

enumeration that quantifies intestinal colonization of *Salmonella*. As shown in Figure 4, fewer

($P < 0.05$) *Salmonella* were present in the ilea of calves fed SCFP (1,620 versus 9,289 CFU/g of

ileum).

Assessment of Rumen Development and Weight Gain in the Calves

As shown in Figures 5 and 6, rumen papillae length was greater ($P < 0.05$) in calves fed

SCFP (236 versus 203 μm) while papillae width was indistinguishable (50 versus 40 μm). Table 1

shows the body weight at different stages of experiment and Figure 7 reveals the superior ($P <$

0.05) weight gain in *Salmonella*-infected calves fed SCFP (23.8 versus 17.2%).

DISCUSSION

Salmonella is an insidious problem for the dairy industry. This problem represents a

critical animal health issue since cattle of all ages are affected by the pathogen. Adult cattle will

exhibit diarrhea and anorexia, both of which compromise the performance of the animal. In

the pre-weaned calf, *Salmonella* can cause diarrhea and malaise that will also hamper

performance and expose caretakers to the pathogen. Furthermore, salmonellosis in the pre-

weaned calf can lead to persistent infection and the carrier state. These animals become an

asymptomatic source of *Salmonella* for the herd, while some animals may perish because of the

infection.

Identifying *Salmonella* mitigation strategies is of critical significance yet the progress is

very slow. In this study, the anti-*Salmonella* effects of SCFP were examined and three critical

indicators of salmonellosis (pyrexia, diarrhea, and intestinal colonization) were significantly

reduced by SCFP. The absence of pyrexia and the diminished diarrhea in the calves fed SCFP

are consistent with the reduced intestinal colonization by *Salmonella*. Both pyrexia and

diarrhea are dependent upon pathogen burden and it appears that *Salmonella* may be less

efficient at attaching to the intestinal tract in the presence of SCFP. This diminished intestinal

colonization was manifested by a cessation of *Salmonella* shedding in calves fed SCFP at 4 d

prior to the last day of fecal shedding in calves fed the Control. In Control-fed calves, a higher

number of *Salmonella* colonized the intestinal tract thus extending the overall shedding period, which ultimately increased the risk of disease transmission.

Our results are consistent with a previous study [6] in which feeding SCFP led to an improvement of gastrointestinal health in pre-weaned dairy calves naturally exposed to *Salmonella*. Although the mechanism of action is yet to be clarified, the effect of SCFP on intestinal colonization (Ibukic et al., 2012) and growth (Broomhead, et al., 2012; Nsereko et al., 2013) of *Salmonella* has also been reported in poultry.

Other significant findings in this study are the improved weight gain and rumen papillae maturation in calves fed SCFP, which is consistent with a non-infectious study whereby supplemental SCFP improved pre-weaning calf growth, feed intake, and corresponding rumen development parameters [15]. Although it is unclear how these parameters were improved by feeding the *Saccharomyces cerevisiae* fermentation products, these findings suggest an economic benefit for inclusion in milk replacer and starter grain as demonstrated previously (Magalhães et al., 2008). It is likely that these benefits extend to calves in herds even in which *Salmonella* is not endemic. Furthermore, it is possible that these benefits extend to protection from related enteric pathogens such as *E. coli* since previously reported studies suggested SCFP could inhibit the growth of *E. coli* (Jenson et al., 2008b). Future studies will assess this possibility.

CONCLUSIONS

In summary, *Salmonella*-infected dairy calves were significantly less likely to exhibit clinical signs associated with salmonellosis when fed SCFP. Specifically, these calves were less

likely to exhibit pyrexia and diarrhea, possibly as a direct result of diminished intestinal colonization by *Salmonella*. Ultimately, these protective effects augmented growth and improved rumen development in the calves infected with a serious enteric pathogen.

REFERENCES

1. Cummings K, Warnick L, Elton M, Gröhn Y, McDonough P, et al. (2010) The effect of clinical outbreaks of salmonellosis on the prevalence of fecal Salmonella shedding among dairy cattle in New York. Foodborne Pathog Dis 7: 815-823.
2. Van Kessel J, Karns J, Wolfgang D, Hovingh E, Schukken Y (2012) Dynamics of Salmonella serotype shifts in an endemically infected dairy herd. Foodborne Pathog Dis 9: 319-324.
3. Cobbold R, Rice D, Davis M, Besser T, Hancock D (2006) Long-term persistence of multi-drug-resistant *Salmonella enterica* serovar Newport in two dairy herds. J Am Vet Med Assoc 228: 585-591.
4. Hermesch D, Thomson D, Loneragan G, Renter D, White B (2008) Effects of a commercially available vaccine against Salmonella enterica serotype Newport on milk production, somatic cell count, and shedding of Salmonella organisms in female dairy cattle with no clinical signs of salmonellosis. Am J Vet Res 69: 1229-1234.
5. Jensen G, Patterson K, Yoon I (2008) Yeast culture has anti-inflammatory effects and specifically activates NK cells. Comp Immunol Microbiol Infect Dis 31: 487-500.
6. Magalhães V, Susca F, Lima F, Branco A, Yoon I, et al. (2008) Effect of feeding yeast culture on performance, health, and immunocompetence of dairy calves. J Dairy Sci 91: 1497-1509.
7. Wray C, Sojka WJ (1978) Experimental *Salmonella typhimurium* in calves. Res Vet Sci 25: 139-143.
8. Xiong N, Brewer M, Anderson K, Carlson S (2013) Non-typhoidal Salmonella encephalopathy involving lipopolysaccharide in cattle. Vet Microbiol 162: 285-287.
9. Xiong N, Brewer M, Anderson K, Watrous G, Weeks K, et al. (2012) Beta-lactam antibiotics prevent Salmonella-mediated bovine encephalopathy regardless of the beta-lactam resistance status of the bacteria. The Vet J 192: 535-537.
10. Xiong N, Brewer M, Anderson K, Weeks K, Carlson S (2011) Expression of a collagenase that enables blood-brain barrier penetration for Salmonella implicated in bovine encephalopathies. Microb Pathog: [Epub ahead of print].
11. Smith B, Habasha F, Reina-Guerra M, Hardy A (1979) Bovine salmonellosis: experimental production and characterization of the disease in calves, using oral challenge with Salmonella typhimurium. Am J Vet Res 40: 1510-1513.
12. Kirchner M, McLaren I, Clifton-Hadley F, Liebana E, Wales A, et al. (2012) A comparison between longitudinal shedding patterns of Salmonella Typhimurium and Salmonella Dublin on dairy farms. Vet Rec 171: 194.

13. Lanzas C, Warnick L, James K, Wright E, Wiedmann M, et al. (2010) Transmission dynamics of a multidrug-resistant Salmonella typhimurium outbreak in a dairy farm. Foodborne Pathog Dis 7: 467-474.

14. CLSI (2008) Performance standards for antimicrobial disk and dilution susceptibility tests. Wayne, PA.

15. Lesmeister K, Heinrichs A, Gabler M (2004) Effects of supplemental yeast (Saccharomyces cerevisiae) culture on rumen development, growth characteristics, and blood parameters in neonatal dairy calves. J Dairy Sci 87: 1832-1839.

Table 1. Effect of *Saccharomyces cerevisiae* fermentation products (SCFP) on body weight of calves before and after *Salmonella* challenge[1].

Treatment[2]	Body Weights (mean $\pm$ SEM) (kg)			% Growth (mean $\pm$ SEM)		
	d 0 (start)	d 14 (at challenge)	d 35 (end)	% growth from d 0 to d 14	% growth from d 14 to d 35	% growth from d 0 to d 35
Control	42.8 $\pm$ 0.7	45.1 $\pm$ 0.7	50 $\pm$ 0.8	5.3 $\pm$ 0.2	10.9 $\pm$ 0.2	16.7 $\pm$ 0.5
SCFP	40.3 $\pm$ 1	42.5 $\pm$ 1.1	50 $\pm$ 1.4	5.5 $\pm$ 0.5	17.4 $\pm$ 0.9*	23.8 $\pm$ 0.8*

[1]*Salmonella enterica* serotype Typhimurium strain SL1344 at the dose of 2 x 10^6 CFU/kg BW.

[2]Control – no additive in milk replacer or starter grain; SCFP - diet that contained two proprietary SCFP products (1 g/head/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/head/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa)

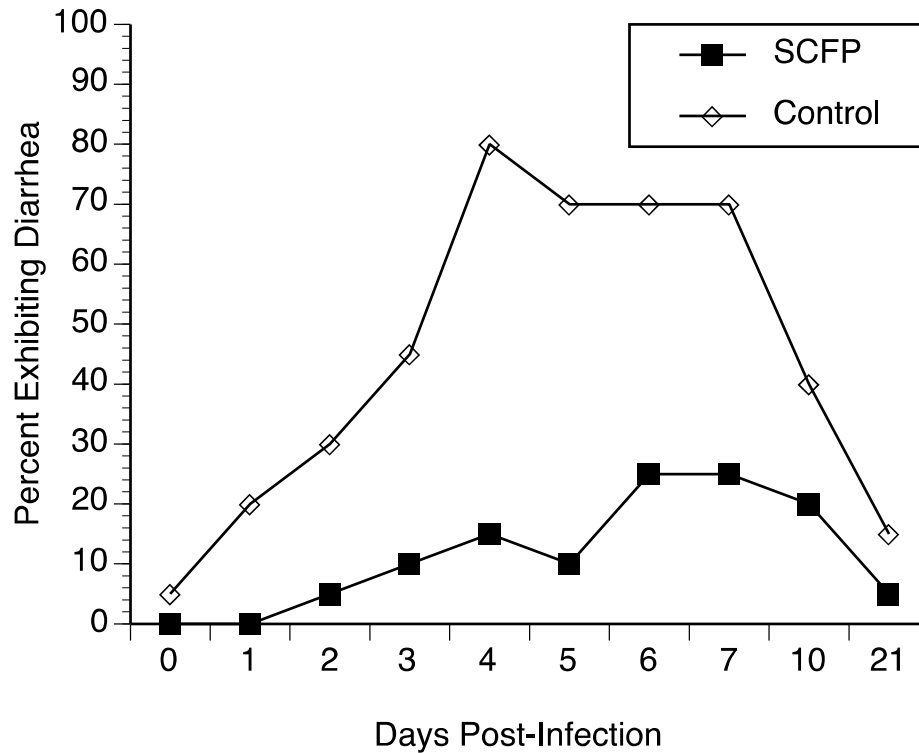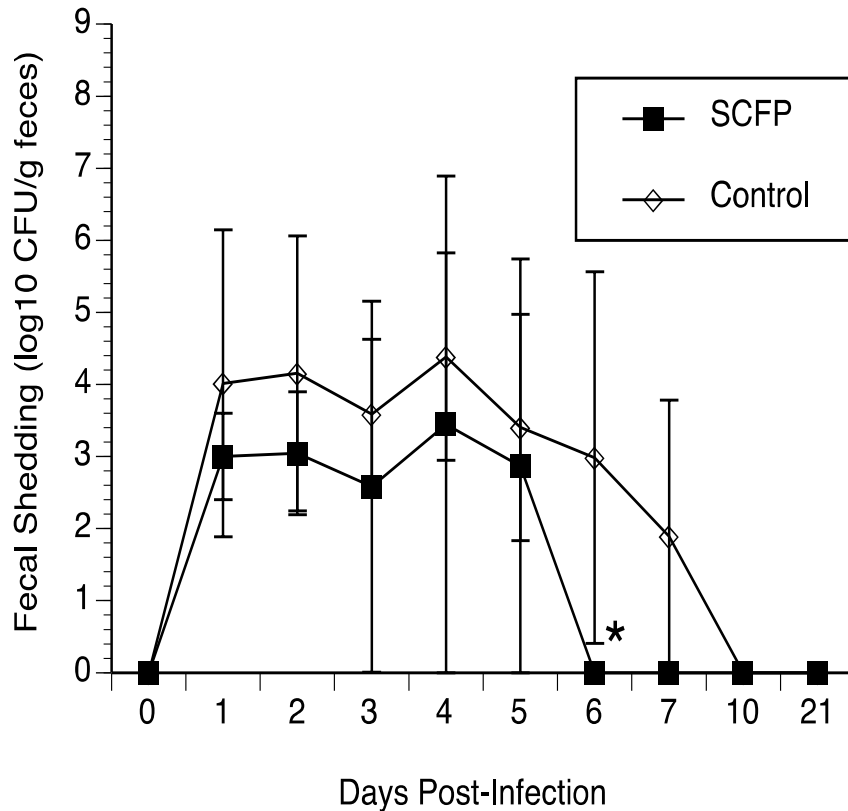*P <0.05 versus Control when comparing growth rates.
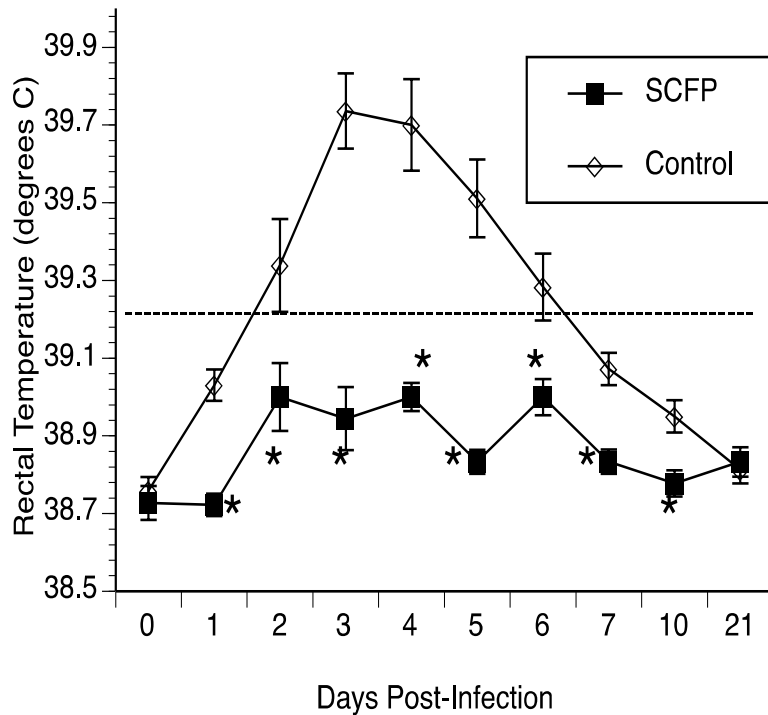
Figure 1. Time course of rectal temperatures in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Data represent the mean + SEM for single daily measurements on 20 calves in each group. The dashed line represents the upper limit of the normal rectal temperature for a calf. *P <0.05 versus Control.
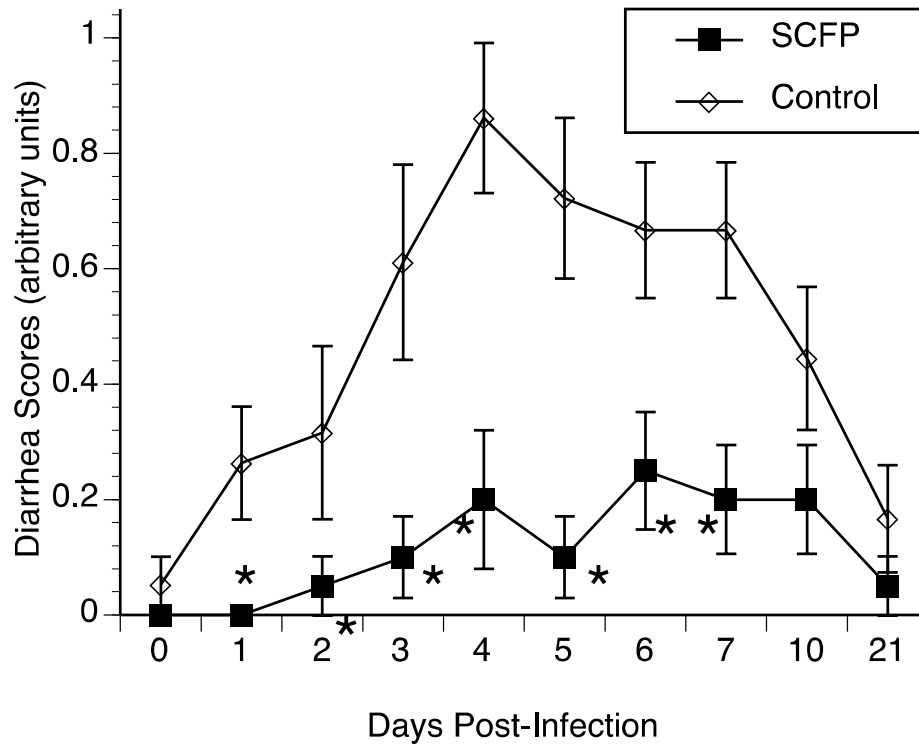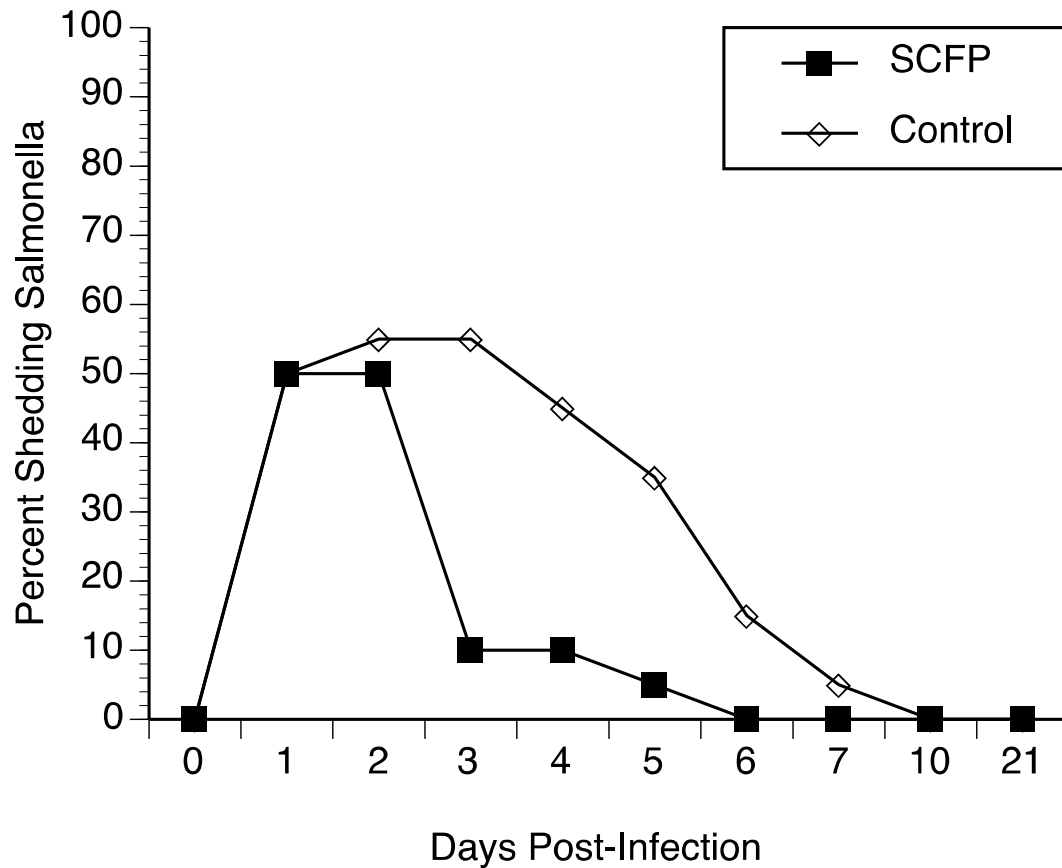
Figure 2a. Time course of diarrhea scores in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Diarrhea scoring was as follows: 0, no diarrhea; 1, mild to moderate diarrhea; 2, profuse diarrhea; or 3, profuse diarrhea with blood. Data represent the mean $\pm$ SEM for single daily measurements on 20 calves in each group. *P <0.05 versus the Control.
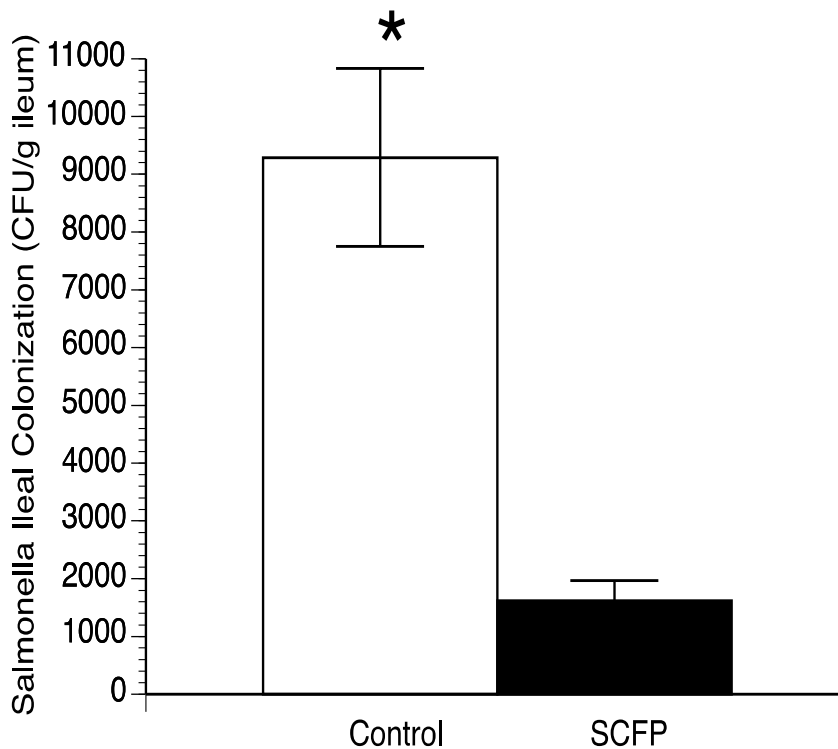
Figure 2b. Time course of diarrhea incidences in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Percentages are based on the number of calves exhibiting any type of diarrhea on a given day, with n=20 per group on all days.
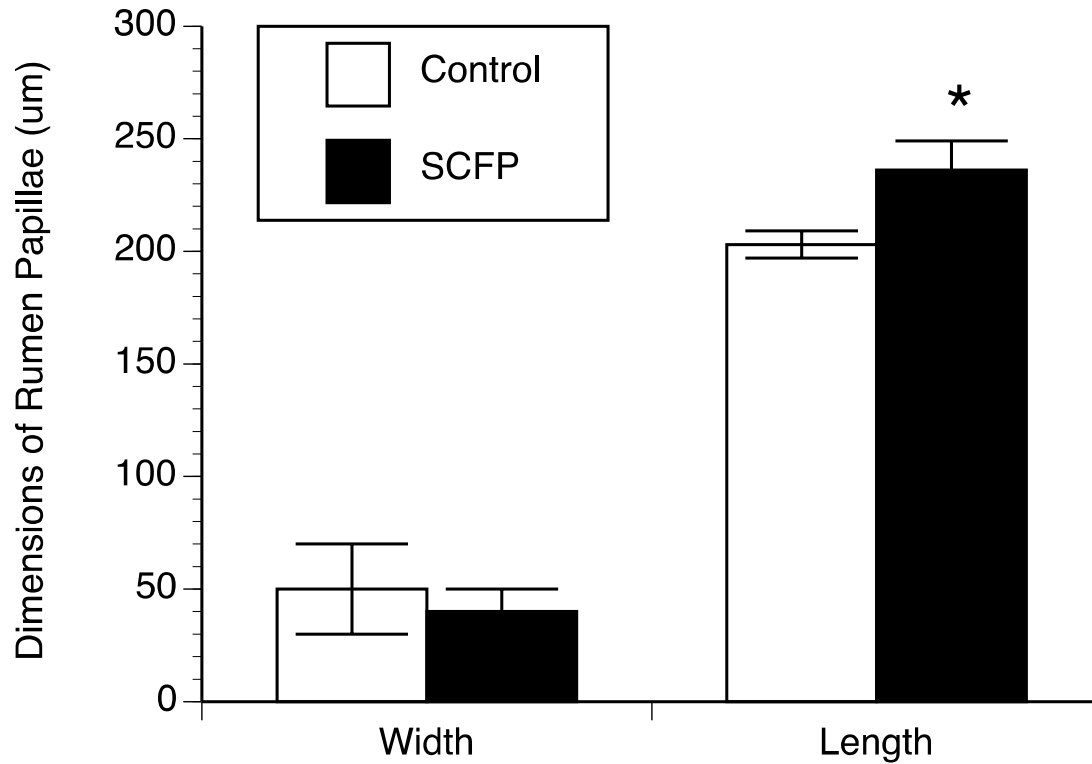
Figure 3a. Time course of *Salmonella* fecal shedding in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Data represent the mean ± SEM for single daily measurements on 20 calves in each group. *$P < 0.05$ versus SCFP.
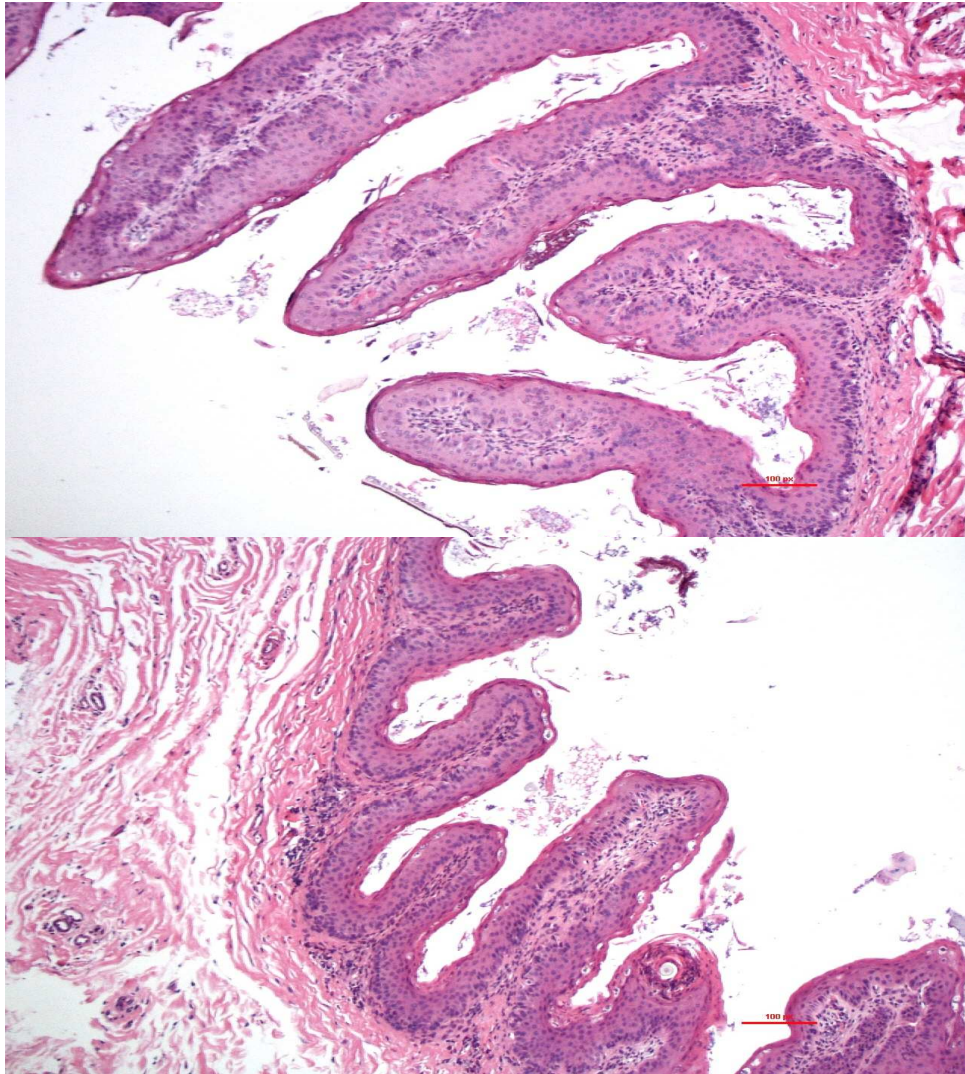
Figure 3b. Time course of fecal shedding incidences in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Percentages are based on the number of calves shedding any detectable amount of *Salmonella* on a given day, with n=20 per group on all days.

Figure 4. *Salmonella* ileal colonization in *Salmonella*-infected calves fed SCFP (filled column) or the Control (open column). Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Data represent the mean + SEM for single measurements on 20 calves in each group. *P <0.05 versus the Control.
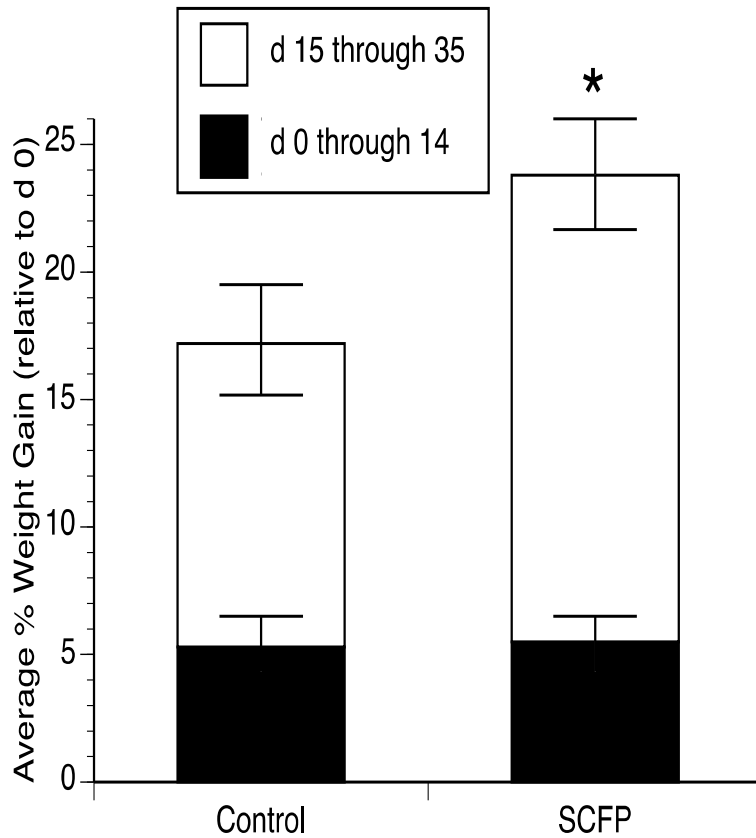
Figure 5. Rumen papillae dimensions in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Data represent the mean $\pm$ SEM for measurements on 20 calves in each group. Rumen papillae were measured ten times for each animal. Widths were measured at the mid-shaft of the papillae. *P <0.05 versus the Control.

Figure 6. Representative rumen papillae photomicrographs (40X) of hematoxylin-eosin stained sections from *Salmonella*-infected calves SCFP (top) or the Control (bottom). Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Papillae are longer in calves fed SCFP, whereas papillae widths are indistinct. A 100μm scale bar is present on the bottom right of each photo.

Figure 7. Percent body weight gains in *Salmonella*-infected calves fed SCFP or the Control. Treatments include Control (no additive in milk replacer or starter grain) or SCFP (diet that contained two proprietary *Saccharomyces cerevisiae* fermentation products (1 g/hd/d SmartCare™ [0.15% inclusion rate in conventional milk replacer] and 3.5 g/hd/d Original XPC™ administered orally via gelatin capsule; Diamond V, Cedar Rapids, Iowa). Data represent the mean $\pm$ SEM for measurements on 20 calves in each group. Body weights were measured on d 0, 14, and 35. *P <0.05 versus the Control.

APPENDIX C. MOLECULAR CLONING OF GPCRS FROM *TRYPANOSOMA BRUCEI*

ABSTRACT

Classical deorphanization of GPCRs often begins with cloning of a putative GPCR. For this study, a PCR strategy was used to clone GPCRs from the genomic DNA of the protozoan parasite *Trypanosoma brucei.* Approximately 25 pairs of degenerate primers were designed based on a multiple sequence alignment of vertebrate and arthropod GPCRs and PCR reactions were conducted using all combinations of these 25 pairs. Secondary PCR reactions were then performed on amplicons from the initial reaction by utilizing combinations of primers that provided a nested PCR strategy. Lastly, the amplicons from the nested reaction were utilized as the template in a multiplex PCR reaction containing all 25 pairs of primers. Amplicons of the correct size (~1500 BP) were cloned into the TA vector pBAD and sequenced at the ISU DNA facility. BLAST searches of these sequences were conducted in the NCBI database utilizing these sequences. With this approach, a putative GPCR was identified. These sequences contain 7 predicted transmembrane domains and were not present in mammalian databases, indicating that our PCR reactions were not contaminated with human DNA. The sequence for this putative receptor are provided.

TRYPANOSOMA BRUCEI PUTATIVE GPCR AMINO ACID SEQUENCE

maffyaifwi sltvlllqcc vsqwltvqas namtnpdlrk vslsllgnlh ydvlfgkpgg
ygvcnqgqps iatpgcdspl klirsvmrdv skwkesftli tggllrhgtd tiatteiesm
mkdvvveian ssresylkke gatytvalsf ggtdfipans ftpegkqphf trllnllely
ellnsqeskk lgncgyffrd lngtklrvis lntllwsnal rpgfgvgdvd pcgqfpflqg
aieqakqrgr sviilgdtpp vinvadalrk ssvqeaesyw redfteayfr iiatyrfsia
aqffghtnsf afvdspevgp plyivppisp vtgsnpsylr atldtntgrv vtlkqrylse
ngkwvegesl edaigaplre mgeslpkdll litesekkwe klaamrvggr fltergacsl
wcrrviacas lyyskmaier casidlpsqr lglilaivft cfgmlmitfs fgymishyki
ifhppvvvga gkgrhrlfle hteeefs