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Molecular genetic analysis of bile salt hydrolase expression and transport in Lactobacillus Johnsonii strain 100-100

Christopher Anthony Elkins

To the Graduate Council:

I am submitting herewith a dissertation written by Christopher Anthony Elkins entitled "Molecular genetic analysis of bile salt hydrolase expression and transport in Lactobacillus Johnsonii strain 100-100." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Dwayne C. Savage, Major Professor

We have read this dissertation and recommend its acceptance:

Jeffrey M. Becker, Beth C. Mullin, Neil B. Quigley

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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(Microbiology)

Beth C. Mullin, Ph.D.
(Botany)

Neil B. Quigley, Ph.D.
(Microbiology)

Accepted for the Council:

Interim Vice Provost and
Dean of The Graduate School

MOLECULAR GENETIC ANALYSIS OF BILE SALT HYDROLASE EXPRESSION AND TRANSPORT IN *LACTOBACILLUS JOHNSONII* STRAIN 100-100

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Christopher Anthony Elkins
May 2001

DEDICATION

This dissertation is dedicated to my wife

Heather

who has given me much needed support

throughout this long and arduous process,

to my daughter Abigail,

AND

to my parents

Mr. Everette Elkins and Mrs. Bernadette Elkins

who have given me the opportunity to

attempt such a task

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Dwayne C. Savage, for his patience and guidance during my tenure as his student. He instilled confidence in my ideas and taught me how to think and write scientifically. I particularly appreciate the experimental independence he permitted in the laboratory. I understand why this is an important characteristic and hope to further this independence as a scientist. Finally, I, as his last student, am happy to have worked with and learned from him. I am convinced, from our candid scientific and personal conversations, that there is an important dynamic associated with one scientific generation to the next.

I am indebted to my committee members, Drs. Jeff Becker, Beth Mullin, and Neil Quigley, for their interest in and ideas on my project. I am especially thankful to Dr. Becker who made a positive impression on me of the University of Tennessee and convinced me to apply to the Department of Microbiology. I would like to thank Dr. Quigley for his helpful molecular discussions in the sequencing lab and Dr. Mullin who insisted that I commit to the preliminary examination twice, if necessary, before she would sit on my committee. I also recognize Drs. Tom Montie, Robert Moore, and Gary Stacey who sat on my examination committee and provided valuable ideas for my project in the process.

Special acknowledgment must go to Scott Moser, George Knauer III, Dr. Steve Wilhelm and lab, and Shilpa Deshpande who invaded my solitude but provided interesting and lively conversation. Finally, I thank the Department of Microbiology for accepting me as a graduate student.

ABSTRACT

Bile salt hydrolysis (BSH) activity of *Lactobacillus johnsonii* strain 100-100 is catalyzed by four cytosolic homo- and heterotrimeric isozymes composed of two antigenically distinct peptides, α and β. Two separate genomic libraries of strain 100-100 were prepared with *Sau*3AI and *Eco*RI and cloned into *Escherichia coli* cells. The libraries were screened on MRS *Lactobacillus* agar medium containing 0.5 % taurodeoxycholic acid for halos of deoxycholic acid precipitate in the medium surrounding the colonies (plate assay). Clones encoding the α and β peptides were identified from the *Sau*3AI and *Eco*RI libraries, respectively. The α peptide gene, *cbsHα*, was encoded at a locus separate from the β peptide gene and did not share tandem arrangement with other genes. The β peptide gene, *cbsHβ*, shared tandem arrangement with two other genes, *cbsT1* and *cbsT2*. These genes were duplicates with sequence similarity and hydropathy profiles consistent with transporters of the major facilitator superfamily (MFS). However, they represented a new subfamily of the MFS. All three genes, *cbsT1*, *cbsT2*, and *cbsHβ*, were coordinately regulated in stationary phase strain 100-100 cells and were present in *L. acidophilus* strain KS-13, a human isolate. *Lactobacillus* species from culture banks were screened for the BSH phenotype via plate assay and the *cbsHβ* genotype via the polymerase chain reaction. These properties were not always constant in isolates of the same species, which suggests a horizontal origin for the activity. *E. coli* cells expressing a construct containing a complete *cbsT2* was assayed for capacity to accumulate [24-^{14}C]taurocholic acid. When an extracellular factor (EF)

produced by strain 100-100 was present, uptake of [24-^{14}C]taurocholic acid was increased up to threefold over control levels. However, a statistically significant decrease in uptake of [24-^{14}C]cholic acid was also observed in these cells. Assays of net [24-^{14}C]taurocholic acid accumulation over time and concentration with CbsT2 and EF demonstrated that the increase is rapid (<15 sec), but saturable. Unlabeled cholic acid, if added to identical cells not exposed to EF, could produce an "EF-like" effect on uptake of [24-^{14}C]taurocholic acid. Studies with ionophores, 2,4dinitrophenol and CCCP did not decrease [24-^{14}C]taurocholic acid uptake in such cells and demonstrate that the uptake is independent of Δp. Moreover, uptake with [24-^{14}C]- and [^{3}H]taurocholic acid, labeled on the cholic acid and taurine respectively, demonstrated that [24-^{14}C]cholic acid leaves the cell, but [^{3}H]taurine remains intracellular in constructs expressing *cbsT2* and *cbsHβ*. The data support a hypothesis that CbsT2 (and CbsT1) is a facilitated taurocholic acid:cholic acid exchanger. I conclude that the known genetic determinants for BSH activity in strain 100-100 have been cloned and identified.

PREFACE

Approximately five years ago, I approached Dr. Dwayne C. Savage and inquired about his research and whether he would mentor a new Ph.D. student. I was interested in bacteriology. I wanted to study an area that would give me expertise in molecular biology and microbial genetics but permit some independence in experimentation. Dr. Savage, on the other hand, had just resigned as chairman of the Department of Microbiology. His laboratory suited my needs because it offered an independent environment. Dr. Savage was open to the idea of mentoring a new student and provided me with some reprints of his work. He advised me to come back if I found them interesting.

Evolution of the project. Dr. Savage was interested in gastrointestinal (GI) tract microbiota and factors that influence persistent microbial colonization within a host organism. A previous Ph.D. student, Scott Lundeen, worked on a rat stomach isolate that expressed the capacity to hydrolyze conjugated bile acids, endogenous compounds secreted by the host. I read the papers of Lundeen and Savage that described this bile salt hydrolase (BSH) system in *Lactobacillus* sp. strain 100-100. They had purified and characterized the cytosolic BSH enzymes at the protein level and determined regulation from a physiological perspective. However, the genetics of this system were not addressed. Although I was given a choice of projects and research avenues to pursue, I knew my choice was clear. I chose to characterize the system in strain 100-100 at the genetic level in accordance with my interests.

Upon gathering the literature in the field, I realized what little was known about BSH activity. At the time, the activity had been purified from only five organisms and the genetic determinant had been cloned from only two [with no detailed genetic analysis performed]. Furthermore, how this activity is important to the physiology of the bacterium or whether it is required for bacterium to colonize the GI tract was a matter of speculation. For instance, does the bacterium obtain an energy or carbon source from such activity or is it a mechanism to resist the toxicity of bile acids? I felt that a genetic approach could yield some answers to these basic questions and possibly lead to the discovery of genes, other than BSHs, that are involved in the process. Afterall, microbial bile acid dehydroxylation in *Eubacterium* is carried out by a coordinately regulated set of nine genes that play into a rather complicated model. These encoded properties would not necessarily have been identified from a biochemical approach at the protein level!

About the same time that the first genomic clone expressing BSH activity was identified from strain 100-100, I was enrolled in a seminar series entitled "Membrane Transport Processes: from Protons to Proteins." This course was conducted by Drs. Jeff Becker and Barry Bruce. Seminars, given by scientists that were experts in this field from around the world, covered a wide range of topics that included structural, mechanistic, and novel transport questions. I became increasingly interested in the field since it complemented my research. The genomic clone encoded what appeared to be two putative transport proteins [gene duplicates] positioned in tandem with the BSH gene. Could these two proteins be bile acid transporters? The literature contained no reports of conjugated bile acid importers in bacteria. The only known transporters of bile acids were multidrug (MDR) efflux pumps. MDR proteins have a broad specificity for

lipophilic molecules including bile acids but are a resistance mechanism from such molecules. Phylogenetic analysis of these two transporters by Saier and colleagues revealed that they represented a new subfamily of secondary active transporters in the major facilitator superfamily (MFS). My research focus expanded to include transport of conjugated bile acids.

It was hypothesized that the transporters import conjugated bile acids since strain 100-100 expresses the BSHs intracellularly. These molecules are almost completely ionized at physiological pH, however, unconjugated bile acids [the products from hydrolysis of conjugates] are not. It is logical to conclude that the cells would encode transporter(s) to import conjugated bile acids since these charged molecules, although lipophilic, have restricted passive diffusion across the bilayer. In the meantime, the genetic loci for the BSHs were identified and characterized, but revealed no additional genes of related function.

Kurdi et al. published a paper in November of 2000, in which they demonstrated that unconjugated bile acids in many lactobacilli are accumulated intracellularly, spontaneously, and independent of a protein carrier. I was left with a problem: if strain 100-100 encodes a method to import conjugated bile acids, why is there no exporter of the unconjugated products? Unconjugated bile acids are known to be more toxic to cells because they are largely neutral molecules at physiological pH that can disaggregate the cytoplasmic membrane. Sequence similarity of the transport proteins to oxalate:formate antiporters of the MFS may hold the answer. Perhaps these transporters catalyze import of conjugates concomitantly with export of unconjugated bile acids – an exchange

mechanism. This hypothesis could explain and support the designation of these genes into a new subfamily of the MFS.

Focus of the project. The focus of this thesis, therefore, is twofold. I address the genetics of BSH activity and bile acid transport in lactobacilli, particularly in strain 100-100. The work I present provides a basis to speculate that BSH activity is required for persistent colonization of the GI tract and that the activity may be encoded as a mobile genetic element that was acquired horizontally in lactobacilli. Furthermore, the bile acid transporters identified early in this work are characterized. The data suggest that the genes code for facilitated bile acid exchangers. It is speculated that they function in a manner analogous to flippases and capture substrates from within the membrane.

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PART I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Certain microbes have a persistent, lifelong association with our gastrointestinal (GI) tract and the GI tracts of other vertebrate lower life forms (Savage, 1977). The GI tract provides an anaerobic environment of limiting nutrients such that complex microbial ecologies develop. These microbes have evolved capacities selectively and competitively to occupy niches within this space and to survive and flourish in complex communities within the GI tract (Savage, 1981; 1984b; 1985b; 1989a). One capacity involves metabolism of endogenous compounds, such as bile acids, produced by the host organism (Savage et al., 1995; Savage, 2000). Since bile acids are not found in any other environment, it can be surmised that microbes, having the capability to "handle" these molecules, evolved this capability under a selective pressure for an advantageous gain. The larger question that underlies this activity is focused on the physiological relevance to the microbe. In essence, how is bile acid metabolism important to certain microbes in this environment?

This introduction will serve to acquaint one with the GI tract ecology, specifically that of the mouse model, the function of bile acids in the host organism, the interactions of microbes with such molecules, and the system of bile acid hydrolysis in *Lactobacillus* sp. strain 100-100. The work described was performed mainly with this organism, but also with certain other lactobacilli.

Principles of Microbial Colonization of the Gastrointestinal tract

The GI tract is an open and dynamic system with rapid turnover of contents. It can be likened to a stream flowing through a field with occasional stagnant eddies or more simplistically to a biological chemostat (Savage, 1985b). The idea that the GI tract of a host organism is associated with a defined complement of microbes was revolutionary and began a field of study known as gastrointestinal microecology (Dubos et al., 1965; Schaedler et al., 1965a; 1965b; Savage, 1977). The emergence of the concept of the "normal microflora" concomitantly established certain principles to determine whether a microbe was part of the GI microbiota and factors that regulate this association.

Allochthony and autochthony. Since the GI tract is open, transient (allochthonous) microbes are present along with indigenous (autochthonous) microbes (Savage, 1984b). Certain guidelines have been established to distinguish between these two designations and to experimentally satisfy the criteria for autochthony (Savage, 1984b; 1985b). Indigenous species grow anaerobically, but are sometimes aerotolerant. They colonize early in life and reach certain population levels and remain at those levels throughout the life of a healthy host. They colonize habitats in the tract geographically, both vertically and horizontally (Savage, 1985b). That is, autochthonous species colonize a particular horizontal segment of the GI tract (e.g., stomach, small intestine, or cecum and large intestine) and can associate vertically with the mucosa, epithelial cells, or crypts of

Lieberkühn (Savage, 1980; 1985b). Finally, authochthonous species are always found in normal adult hosts (Savage, 1984b; 1985b).

Allochthonous species can maintain a transient presence in the GI tract of disturbed adult hosts. This can occur if the adult is stressed, exposed to antimicrobial agents, subjected to a radical change in diet, or is otherwise in an unhealthy state that disrupts the microbiota (Savage, 1984b; 1985b). Therefore, an accurate appraisal of the health and the environment of the host organism is crucial for determining autochthony and, conversely, allochthony.

Allogenic and autogenic colonization factors. Factors that regulate which microbial species form climax communities have been classified into two groups. Allogenic factors are forces generated by the host organism that affect the ability of a microbe to colonize the habitat (Savage, 1984b; 1985b). Such factors include pH (Kunstyr et al., 1976), temperature (Savage, 1987), oxygen (Artwohl & Savage, 1979), oxidation-reduction potential (Celesk et al., 1976), stasis (peristaltic rate; Savage, 1984b), enzymes (Savage, 1977), and bile acids (Celesk et al., 1976). Peristalsis is particularly important in the stomach and small bowel. Microbes cannot colonize the lumen if peristalsis is faster than the rate at which microbes can multiply (Savage, 1984b). However, microbes can overcome this obstacle by forming a tight attachment to the lumenal surface (Savage, 1983). This is the case with segmented filamentous bacteria in the distal small bowel of the mouse (see below). Such attachment is representative of an autogenic colonization factor.

Autogenic factors are forces exerted by microbial populations that help establish climax communities (Savage, 1984b; 1985b). Such factors include competition for nutrients (Freter et al., 1983), nutritional synergy (Wolin, 1981), and microbial products such as volatile fatty acids, lactic acid, and hydrogen sulfide (Freter, 1983). A practical effect of these factors results in exclusion of allochthonous species from niches occupied by autochthonous ones (Savage, 1984b; 1985b). Allogenic and autogenic factors influence the development, or succession, of microbial communities in neonates into adult, or climax, communities. Obviously, the factors change dynamically in intensity and contribution during this process, but remain rather constant once a climax community is established (Savage, 1989a).

Murine Gastrointestinal Ecosystem

Much of what is known about indigenous microbes in the GI tract has been learned from laboratory rodents. Mice and rats can be housed in environments that can be tightly controlled. For instance, rodents can be rendered, maintained, and bred germ-free or associated with certain selective microbes such that a defined flora is established gnotobiotically (Heneghan, 1973). The effect of dietary intake, stress, and exposure to certain noxious compounds can also be studied in a controlled fashion (Kellogg, 1973). Moreover, rodents can be sacrificed for study of particular regions of the GI tract and compared to conventional germ-associated specimens (Gordon & Pesti, 1971; Heneghan, 1973).

Physiology. The GI canals of rats and mice are strikingly similar (Fig. 1; Hummel et al., 1966; Greene, 1968). The murine GI tract is monogastric and is separated into the foregut (esophagus and stomach), midgut (small intestine) and the hindgut (cecum and large intestine; Stevens, 1988). The cardiac antrum separates the nonsecreting, keratinized, epithelium of the upper stomach from the acid secreting, mucus lined, columnar epithelial cells of the lower stomach (Kaplan et al., 1983; Savage, 1985b; Stevens, 1988). The mucus of the lower stomach forms a gel containing a bicarbonate buffer and protects the underlying cells from the low pH (Bahari et al., 1982).

The small intestine is divided, in descending order, into the duodenum, jejunum, and ileum (Stevens, 1988); the epithelium is covered with a mucus gel (Kaplan et al., 1983; Savage, 1985b). The epithelium is convoluted, forming villi. The surface of the villi is convoluted further, forming microvilli (Stevens, 1988; Savage, 1985b). The base of the microvilli, called the crypts of Lieberkühn, is the site of epithelial cell mitosis (Kaplan et al., 1983; Savage, 1985b). The contents of the small intestine are subject to rapid peristalsis, and empty via the ileo-cecal valve, into the cecum and large intestine (Kaplan et al., 1983). The cecum is a blind pouch extending out from the ileo-cecal junction. The cecum and large intestine do not have villi and are covered in a mucus gel (Stevens, 1988). The lumenal contents move slowly through these areas permitting large microbial populations to establish (Savage, 1977).

Succession and climax communities. The fetus is sterile until birth, at which time, the pup is exposed to microorganisms in the birth canal and external genitalia (Gordon & Pesti, 1971; Savage, 1977; 1981; 1985b). Most of these organisms do not colonize the GI

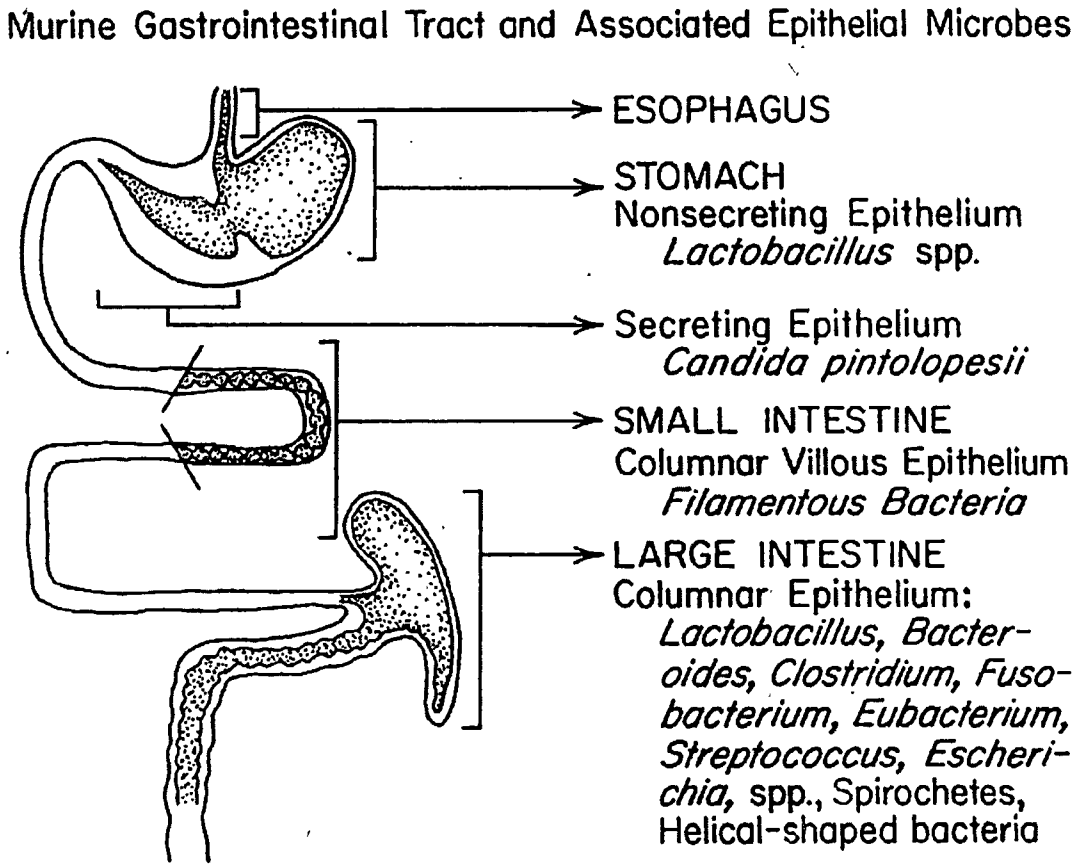


FIG. 1. Murine gastrointestinal tract and associated microbes (Savage, 1985b).

tract, but some such as lactic acid bacteria colonize the length of the tract. Facultative anaerobes, such as *Escherichia coli* and *Streptococcus faecalis*, soon appear and achieve high populations during the first and second week in suckling pups (Savage, 1981; 1985b). Once solid food is introduced, there is a decrease in the quantitative measure of facultative anaerobes and a concomitant increase in the populations of strict anaerobes. By the end of the fourth week, the pups are weaned and *Candida pintolopesii* establishes on the gastric epithelium (Savage, 1977; 1981). A filamentous bacterium colonizes the small intestine (Davis & Savage, 1974; Savage, 1977) and stable climax communities are established that persist, barring minor daily fluctuations, throughout the life of the animal (Savage, 1989a).

Lactobacillus communities are found in two distinct geographical locations in the climax microbiota of the mouse (Fig. 1). One community is located on the upper, nonsecreting portion of the stomach. It resists peristalsis by forming thick layers on the keratinized stratified squamous epithelium (Fig. 2; Savage et al., 1968). However, these organisms constantly slough and pass down the GI tract (Savage, 1977). In quantitative measure, this *Lactobacillus* population forms the predominate gastric microbial community in the mouse and has been estimated at 10^8 to 10^9 cells per gram of stomach tissue (Table 1). A second population is found in the cecum and large intestine (Fig. 1). This *Lactobacillus* community is substantial quantitatively but is not the predominate community in this area (Table 1).

Many types of microbes have been cultured from the contents of the stomachs of mice and rats (Savage, 1977; 1985b). Such organisms are regarded as allochthonous species shedding from habitats above the stomach or entering from ingested materials

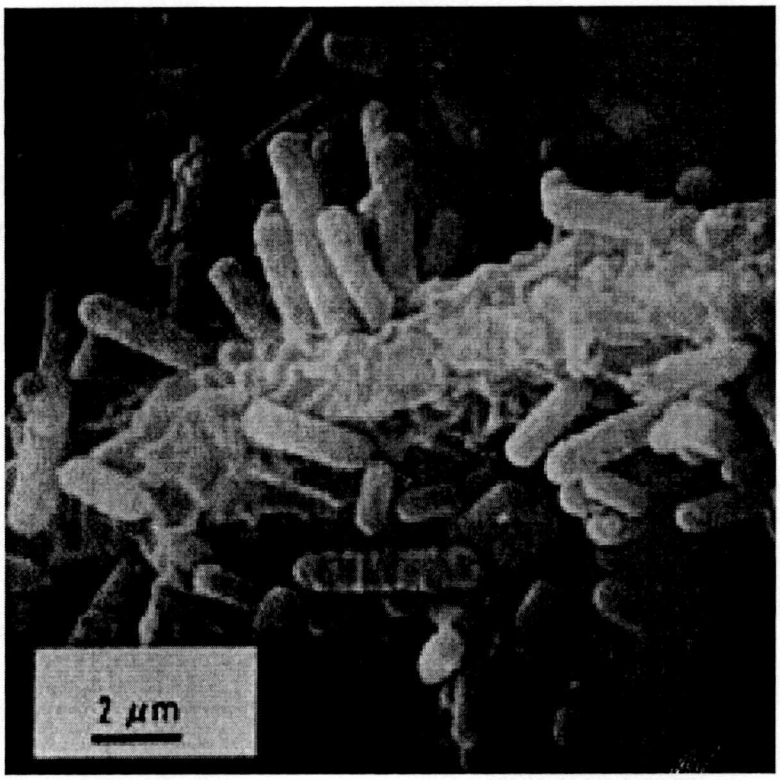


FIG. 2. *Lactobacillus* communities on the keratinized gastric epithelium of adult mice (Savage, 1983).

TABLE 1. Composite list of known autochthonous genera of microbes in the GI tract of the adult mouse[a]

Region of GI Tract	Microbial Genus[b]	Epithelium[c]	Population Level[d]
Stomach	*Lactobacillus*	Nonsecreting keratinized squamous	8 to 9
	Streptococcus	Nonsecreting keratinized squamous	6 to 7
	Candida	Secreting columnar	6 to 8
Small intestine	Segmented filamentous	Columnar villous	High[f]
(terminal ileum)	Helical bacteria	Columnar villous[e]	High[f]
Cecum and Colon	*Bacteroides*	Columnar	10 to 11
	Fusobacterium	Columnar	10 to 11
	Eubacterium	Columnar	10 to 11
	Helical bacteria	Columnar	9 to 10
	Bifidobacterium	Columnar	9 to 10
	Peptostreptococcus	Columnar	9 to 10
	Clostridium	Columnar	7 to 11
	Lactobacillus	Columnar	8 to 9
	Escherichia	Columnar	3 to 4
	Streptococcus	Columnar	3 to 4
	Spirochetes	Columnar	High[f]

[a] List is compiled from Savage (1981; 1984b; 1985b).
[b] Morphology if genus is unknown.
[c] Epithelium with which the microbes associate.
[d] Log_{10} per g of whole wet GI tissue with contents intact. Estimated from colonies grown on agar media inoculated with dilutions of homogenized tissue.
[e] Population associated especially in crypts of Lieberkühn.
[f] These prokaryotes have not been cultured *in vitro* so population cannot be estimated; judged from microscopy.

especially with coprophagous animals (Savage, 1977). Autochthonous organisms that colonize this area must be aciduric and capable of withstanding peristalsis of the gastric contents that can leave it empty for long periods (Savage, 1977). The lower secreting gastric epithelium is colonized by *C. pintolopesii* (Fig. 1). Although the yeast is capable of surviving at low pH, it associates intimately with epithelial cells underlying the mucus gel (Artwohl & Savage, 1979; Savage & Dubos, 1967), where it is protected from the acid-secreting parietal cells (Bahari et al., 1982). Therefore, it colonizes a habitat with a pH that is near its optimum for growth (Savage et al., 1995).

As mentioned previously, the small intestine is subject to rapid peristalsis that can restrict microbial colonization (Savage, 1984b). In mice and rats, two morphologically distinct filamentous bacteria, one segmented and the other unsegmented, form dense populations on the epithelium of the jejunum and ileum (Fig. 1; Table 1). These organisms have not been cultured *in vitro*. Microscopic evidence indicates that they resist peristalsis, and hence clearance, with a specialized "hold-fast" at one end of the filament that inserts into a "socket" in the membrane of the epithelial cell (Savage, 1983). Large numbers of helical bacteria are also found within the crypts of Lieberkühn in the terminal ileum (Table 1; Phillips & Lee, 1983).

Movement of the lumenal contents through the cecum and large intestine is decreased significantly, allowing large and diverse microbial populations to colonize (Fig. 1; Table 1). Approximately 10^{11} bacterial cells per gram dry weight of intestinal contents have been estimated in this area (Savage, 1977). More than 400 different microbial species of over 40 genera have been isolated from this region but represent only those organisms cultured in a laboratory environment (Savage, 1989b). In general,

these populations are composed of gram-positive and gram-negative bacteria that are unable to multiply in atmospheres containing oxygen (Fig. 1; Savage, 1981; 1985b). Such organisms are found associated with all regions of the cecum and large intestine such as the lumen (especially associated with particulate ingesta; Savage, 1977), mucus gel (Gustafsson and Maunsback, 1971; Savage, 1984a), and epithelial surfaces (Savage, 1972; 1985a).

The Bile acids

Bile acids are produced *de novo* in liver hepatocytes from cholesterol and have a steroid nucleus of cyclopentenephenantrene (Danielsson, 1973; Vlahcevic et al., 1996). The synthesis of bile acids from cholesterol was established some time ago using deuterium labeled cholesterol that was converted into cholic acid in the dog (Danielsson, 1973). Two bile acids that are typically produced in mammals are cholic acid and chenodeoxycholic acid although other bile acids such as α- and β-muricholic, hyocholic, and lithocholic acids are produced in certain species. Formation of these molecules occurs by hydroxylation of cholesterol at positions C-7 to produce chenodeoxycholic acid, C-7 and C-12 to produce cholic acid, or C-6 and C-7 to produce α- and β-muricholic acids, and hyocholic acid (Danielsson, 1973). In addition, some less polar bile acids are sulfated at the C-3 or C-7 position to produce lithocholic acid (Palmer, 1972). Collectively these molecules are defined as primary bile acids and are amphipathic. Therefore, such molecules can form spontaneous micelles only at critical micellar concentrations (Small, 1973; Wilson, 1981). Modification of the steroid moiety

such as hydroxylation or sulfation increases the solubility of these molecules (Small, 1973).

Bile acids in bile are conjugates of glycine and taurine. Primary bile acids are attached with an amide bond to one of these two amino acids at the carboxyl C-24 terminus and are secreted into the bile caniculi (Danielsson, 1973; Steinberg et al., 2000). Conjugation occurs in the liver through a two-step process (Vessey & Kelly, 1995; Solaas et al., 2000). Primary bile acids are activated by coupling to coenzyme A (CoA) to form a thioester, a process which is catalyzed by bile acid:CoA ligase (Danielsson, 1973; Steinberg et al., 2000). In the second step, the amino acid is attached to the primary bile acid via bile acid-CoA:glycine/taurine N-acyltransferase (Falany et al., 1994). The ratio between glyco- and tauroconjugates varies with species but is influenced by diet (Danielsson, 1973).

Enterohepatic circulation of bile acids. Conjugated bile acids function to trap and emulsify dietary lipids, fats, cholesterol, and fat-soluble vitamins via micellar formation (Kellogg, 1973; Hylemon & Harder, 1999). Bile is gathered and stored in the gall bladder and is released following feeding in humans and mice (Hummel et al., 1966). Certain other organisms, such as rats, do not have a gallbladder and continuously secrete bile (Greene, 1968). In either case, bile is secreted into the proximal end, or duodenum, of the small intestine via the common bile duct in the form of conjugated bile acids (Stevens, 1988). Insoluble lipid products of digestion effectively partition into the interior of bile acid micelles such that most lipid molecules are present in a mixed micelle (Small, 1973) rather than in an aqueous phase (Mazer et al., 1977). These aggregates

flow through the length of the small intestine and are actively and passively absorbed through the epithelium of the terminal ileum (Lack & Weiner, 1973; Wilson, 1981; Hylemon & Harder, 1999) although some evidence suggests that a major fraction of bile acids are absorbed by the proximal jejunum (McClintock and Shiau, 1983). Bile acids enter the portal blood circulation (Small et al., 1972; Hylemon & Harder, 1999) and are activity transported into the liver (Lack & Weiner, 1973), recycled, and secreted back into the small intestine (Baron & Hylemon, 1997; Hylemon & Harder, 1999). The enterohepatic circulation is defined as the cycling of bile acids between the liver and the small intestine. In humans, this process occurs approximately 6 to 10 times each day (Wilson, 1981; Vlahcevic et al., 1996), and in rats, approximately 10-13 (Danielsson, 1973).

The circulation of bile acids effectively and quantitatively forms a "total bile acid pool" (Kellogg, 1973). Early studies of the effect of feeding bile acids on biosynthesis and catabolism of cholesterol in rats suggested that formation of bile acids is regulated by a double feedback mechanism. That is, conversion of cholesterol to bile acids was inhibited followed by a decrease in the biosynthesis of cholesterol due to accumulated cholesterol (Danielsson, 1973). The finding that bile acid formation was regulated homeostatically by bile acids was later challenged (Danielsson, 1973). Furthermore, the ratios of various primary bile acids that are produced vary from species to species but remain somewhat constant within organisms of the same species (Danielsson, 1973). Regulation of this pool appears to be complex and has been the subject of an experimental resurgence at the molecular level in the past decade. Oxysterols, signaling molecules converted from excess cholesterol, mediate an intricate transcriptional control

mechanism to keep cholesterol levels within a narrow range in concentration (Schoonjans et al., 2000). One pathway involves a feedforward mechanism for cholesterol to bile acid production by nuclear receptors, such as the liver X receptor. The farnesol X receptor is activated by bile acids and regulates biosynthesis and enterohepatic reabsorption of bile acids (Schoonjans et al., 2000). Moreover, 7α-hydroxylase mRNA, the rate-limiting enzyme for the conversion of cholesterol into bile acids, is negatively but differentially regulated in cultured rat hepatocytes by bile acids, particularly more hydrophobic ones (Twisk et al., 1993). The regulation of hepatic bile acid formation is incompletely understood at best.

Bile acid transport. Bile acids are absorbed by the intestinal epithelium via active and passive transport. The type of transport that occurs depends largely on the chemical form of the bile acid (i.e., whether the bile acid is conjugated or unconjugated; Lack & Weiner, 1973; Wilson, 1981). Unconjugated and transformed bile acids are produced by the action of microbial enzymes of the autochthonous microbiota (discussed below). The physio-chemical properties of conjugated versus unconjugated bile acids differ significantly. Tauroconjugates have pK_a values of approximately 1.8 to 2.0, whereas unconjugated bile acids range from approximately 5 to 6.3 (Small, 1973). Hence, conjugated bile acids are completely ionized at the physiological pH of the intestinal lumen (Wilson, 1981). Parameters that define passive transport of such molecules across the mucosa of jejunum have been determined. Reflection coefficients compare the osmotic flow of water with the sieving effect of the membrane to retard the movement of bile acids. A vast majority of conjugated bile acids are transported via an active process

or ionic diffusion of the bile salt anion driven by an electrochemical difference (Lack & Weiner, 1973). Passive transport of bile acids occurs in the cecum and large intestine because of the capacity of bacteria to deconjugate and modify bile acids into less polar undissociated derivatives. It has been shown that bile acids absorbed in this manner do indeed enter into the enterohepatic circulation and are again conjugated in the liver (Lack & Weiner, 1973).

Active transport of bile acids occurs at three sites: the liver, distal small intestine (jejunum and ileum), and proximal renal tubule (Lack & Weiner, 1973). Since the third site is not part of the enterohepatic circulation it will not be addressed. Eukaryotic conjugated bile acid symporters have been identified and characterized from hamster ileum (Wong et al., 1994), human (Hagenbuch & Meier, 1994), and rat liver (Hagenbaugh et al., 1991). These transporters function as part of the enterohepatic circulation of bile acids. Bile acids are transported through the brush border apical membrane of the ileum in a Na^+-dependent process (Wilson, 1981; Kramer et al., 1993). These molecules are then transported in a Na^+-independent organic anion exchange system across the basolateral membrane and secreted into the portal blood circulation (Weinberg et al., 1986). The hamster ileal bile acid transporter is a 348-amino-acid protein with seven predicted transmembrane domains (TMD) and three putative N-linked glycosylation sites (Wong et al., 1994). This protein catalyzes Na^+-dependent transport of the conjugate, taurocholate, through the membrane of the intestinal epithelium. It shares considerable amino acid sequence identity and predicted structural similarity to the human and rat liver Na^+/bile acid co-transporters (Wong et al., 1994).

Bile formation is mediated by vectoral secretion of bile salts and other solutes from the hepatocyte across the canalicular, or apical, membrane (Nathanson & Boyer, 1991). The canalicular transport proteins are capable of exporting metabolites against steep concentration gradients. Investigation of hepatic bile acid secretion has demonstrated that it is an ATP-dependent process (Müller & Jansen, 1997). Molecular studies have identified several eukaryotic proteins all of which are integral membrane proteins of the ATP-binding cassette (ABC) superfamily that are capable of exporting bile acids. For example, a bile salt export pump from the mammalian liver/sister of P-glycoprotein (BSEP/spgp), that has homology to the multidrug resistance (MDR) P-glycoprotein gene family was cloned from the rat (Gerloff et al., 1998) and recently from the mouse (Green et al., 2000). This transporter is thought to be the major canalicular bile salt exporter of monovalent bile salts (Gerloff et al., 1998). Divalent bile salts, such as taurochenodeoxycholate-3-sulfate and 6-α-glucuronosyl-hyodeoxycholate, are excreted via the canalicular multidrug resistance-associated protein (MRP) 2 (Müller et al., 1994; Paulusma et al., 1996). MRP3, is active in transport of glycocholate, confers resistance to several anticancer agents (Zeng et al., 2000), and is a phospholipid flippase (Nies et al., 1996). Interestingly, the secretion of phospholipids across the canalicular membrane of hepatocytes occurs via MDR2 and is regulated by bile salts in rats (Gupta et al., 2000). An ABC transporter, Bat1p, shown to transport bile acids has been cloned from an unlikely source, *Saccharomyces cerevisiae.* This transporter has homology to the MRP gene family (Ortiz et al., 1997). Furthermore, ATP-dependent transport of bile salts has also been demonstrated in the vacuoles of plants (Hortensteiner et al., 1993) and the fission yeast *Schizosaccharomyces pombe* (St. Pierre et al., 1994). In the case of the

enterohepatic circulation, it is clear that several proteins are expressed in the apical hepatic membrane in order to export bile acids and that their regulation is likely to be complex.

Members of the autochthonous microbiota are also known to encode transporters of bile acids. Most bacterial transporters mediate resistance to bile acids since such molecules are toxic to living cells. Bile acids are present in high concentrations, approximately 20 mM in the duodenum and with their amphipathic nature, act as detergents that disrupt the ordered structure of biological membranes (Thanassi et al., 1997). With the exception of one case, BaiG from *Eubacterium* (Mallonee & Hylemon, 1996), all known bile acid transporters in bacteria are involved in efflux of, and hence resistance to, such molecules. Many bacterial transporters of bile acids are multidrug resistance (MDR) pumps that have a broad specificity for export of dyes, detergents, bile acids, and lipophilic antimicrobial agents (Nikaido, 1996). However, MDR proteins are present in organisms other than enteric microbes, suggesting that the broad specificity is an ancient property (Saier et al., 1998; Tseng et al., 1999).

E. coli is known to express at least two MDR efflux pumps that are responsible for export of a variety of lipophilic and amphiphilic compounds. These two pumps, AcrAB and EmrAB, export substrates with overlapping specificities (Ma et al., 1995; Nikaido, 1996). AcrB is a member of the resistance nodulation division (RND; Tseng et al., 1999) family of transporters. EmrB is a member of the major facilitator superfamily (MFS; Saier et al., 1999) of secondary active transporters. AcrA and EmrA are both periplasmic linkers that belong to the membrane fusion protein (MFP; Dinh et al., 1994) family. MFP proteins function as linkers to connect the corresponding cytoplasmic

efflux pump (AcrB or EmrB) to an outer membrane porin, in both cases TolC, and allow extrusion of the lipophilic substrate across both membranes of gram-negative bacteria (Nikaido, 1996). An *E. coli acrA* mutant was confirmed to be hypersensitive to bile acids, whereas an *acrA emrB* double mutant expressed even higher sensitivity to such molecules (Thanassi et al., 1997). Homologs of the AcrAB system are widespread in gram-negative bacteria and are found in *Salmonella typhimurium* (Nikaido et al., 1998), *Pseudomonas aeruginosa* (Li et al, 1995), and *Haemophilus influenzae* (Nikaido, 1996).

Apart from bile acid efflux activity described above, *E. coli* exports bile acids by a another mechanism, involving proton antiport (Thanassi et al, 1997). Using everted membrane vesicles prepared from *acrA emrB* mutants of *E. coli* strain K-12 cells, Thanassi et al. (1997) monitored accumulation of the bile salt, taurocholate, with a corresponding decrease in ΔpH. This efflux may be a defense mechanism for *E. coli* to resist the toxicity of bile acids. However, cloning and sequencing of a 7α-hydroxysteroid dehydrogenase gene from strain HB101 (Yoshimoto et al., 1991) indicates that *E. coli* may possess the ability to transform the steroid moiety of bile acids. Attempts to identify and clone the protein responsible for bile acid efflux in *E. coli* have not been successful. Random insertion mutagenesis was performed and yielded only deep rough lipopolysaccharide or regulatory mutants (Thanassi et al., 1997).

Cholate resistance has been shown in *Lactococcus lactis*. A cholate-resistant strain of *L. lactis* MG1363 was obtained through sequential transfer of the bacterium on media containing increasing concentrations of cholate (Yokota et al., 2000). The resulting strain, C41-2, was not cross-resistant to deoxycholate. The cholate efflux activity was not sensitive to conditions that dissipated the proton motive force but was

inhibited by *ortho*-vanadate, an inhibitor of P-type ATPases and ABC transporters. The cholate extrusion system in strain C41-2 represents the first known ATP-dependent bile acid transporter in prokaryotes (Yokota et al., 2000). In contrast, all known bile acid efflux systems in eukaryotic organisms are mediated in an ATP-dependent fashion (Müller & Jansen, 1997). This trend is interesting from an evolutionary perspective since the ABC superfamily is a large, diverse, and ancient family that is represented in all kingdoms of living organisms (Saier et al., 1998). The bile acid efflux described in *E. coli* and *L. lactis* represents the only reports in prokaryotes of such activity.

Cholic acid is accumulated by many lactobacilli in a ΔpH dependent fashion (Kurdi et al., 2000). This activity was shown to occur independently of a protein carrier and was the result of passive equilibration of cholic acid through the membrane. Cholic acid has a pK_a of 6.4 and acts as a hydrophobic weak acid. Intracellular accumulation factors of cholic acid could be predicted by the Henderson-Hasselbalch equation for equilibration of a weak acid. Since pH homeostasis is maintained intracellularly, lowering of the extracellular pH can lead to accumulation of cholic acid (Kurdi et al., 2000). Under conditions that lead to formation of a ΔpH, protonated cholic acid molecules can pass through the membrane, become deprotonated in a higher pH environment, and be trapped in the cytoplasm because of their polarity. Equilibration, and hence accumulation, occurs once concentrations of protonated cholic acid become equal on both sides of the membrane (Kurdi et al., 2000).

One case of a prokaryotic bile acid importer has been reported. The gene, *baiG*, is part of an operon of nine genes in *Eubacterium* sp. strain VPI 12708 (Mallonee & Hylemon, 1996). The genes in this operon encode collectively for 7α-dehydroxylation

activity, one of several bacterial bile acid transformation reactions that occur with primary unconjugated bile acids (see below). The BaiG gene of this pathway is the first reported bile acid transporter cloned and sequenced in a prokaryote. BaiG is a 477-amino-acid polypeptide that has been heterologously expressed and characterized in *E. coli* DH5α cells. This gene confers a four- to sevenfold increase in uptake of deconjugated bile acids on *E. coli* DH5α cells and uses the proton motive force for transport activity (Mallonee & Hylemon, 1996). The predicted amino acid sequence of this transporter shows significant homology to several sugar and antibiotic resistance transporters of the MFS and is proposed to have 14 transmembrane domains (Mallonee & Hylemon, 1996). The proteins described for import or export of bile acids in bacteria maintain no categorical similarities to eukaryotic transporters that catalyze the same function.

Modification of bile acids by the autochthonous microbiota. Bile acid absorption is an incomplete process. In humans, approximately 130 to 650 mg of bile acids/day escape reabsorption by the ileal epithelium and are lost to the colon (Baron & Hylemon, 1997). Members of the autochthonous microbiota have evolved the capacity to modify the structure of these endogenous compounds (Baron & Hylemon, 1997; Savage et al., 1995). Microbial bile acid reactions can be divided into two categories, deconjugation and transformation. The primary reaction is bile acid deconjugation and results in the removal of the amino acid from the conjugated bile acid, releasing the primary bile acid. There are several transformation reactions that can be subcategorized into three groups: dehydroxylation, dehydrogenation, and desulfation (Hylemon & Glass, 1983; Baron &

Hylemon, 1997). Transformation reactions require unconjugated primary bile acids (Batta et al., 1990) and produce unconjugated secondary bile acids (Bortolini et al., 1997). In addition, transformation reactions modify only the side groups of the steroid moiety of the bile acid (Baron & Hylemon, 1997; Bortolini et al., 1997).

The discovery that bile acid modification is a function of the intestinal microbiota was made from studies with germfree and conventional animals (Kellogg, 1973). The bile acid profiles in the feces of germfree animals revealed that these molecules were conjugated with the steroid nucleus unaltered. In contrast, conventional animals had fecal bile acid profiles that contained virtually all deconjugated bile acids (Gustafsson et al., 1957; Kellogg & Wostman, 1969). Most of these bile acids contained alterations of the side chains of the steroid moiety. Approximately 15 to 20 different bile acid metabolites are generated from cholic and chenodeoxycholic acid by the action of microbial enzymes (Hayakawa, 1973; Hylemon & Harder, 1999). In general, these reactions make the bile acids less water soluble and less active in forming micelles (Hylemon & Glass, 1983). Bile acid deconjugation and transformation reactions will be discussed in the following sections.

Bile Acid Deconjugation

Bile acid deconjugation results in the cleavage of the amide bond at the C-24 position of conjugated bile acids, between the steroid nucleus and the amino acid (Fig. 3). This activity is catalyzed by a class of microbial enzymes collectively named bile salt hydrolases (BSH; EC 3.5.1.24). The capacity for bacteria to hydrolyze conjugated bile

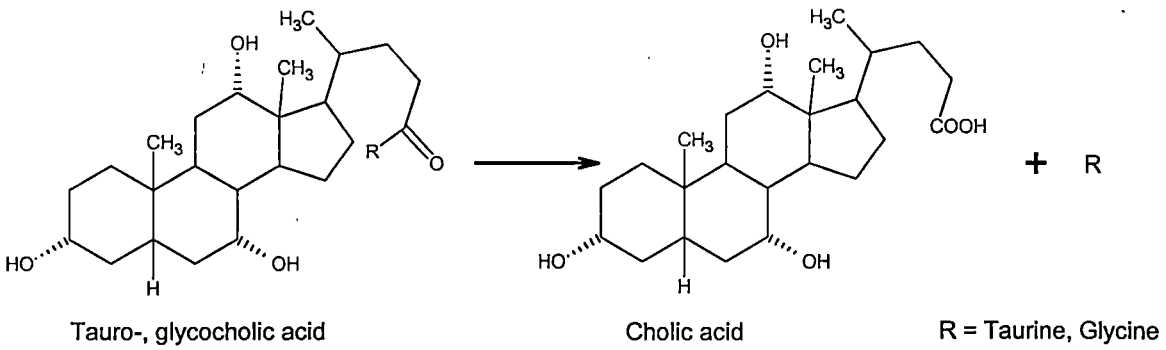


FIG 3. Bile acid deconjugation reaction is catalyzed by bacterial enzymes known as conjugated bile salt hydrolases (BSHs).

acids has been known for some time and represents the first bile acid modification reaction associated with bacteria (Frankel, 1936). The activity has been identified in several genera of the autochthonous microbiota, including species of genera *Lactobacillus* (Gilliland & Speck, 1977), *Bacteroides* (Midvedt & Norman, 1967; Masuda, 1981), *Clostridium* (Midvedt & Norman, 1967; Masuda, 1981), *Bifidobacterium* (Shimada et al., 1969; Ferrari et al., 1980), *Enterococcus* (*Streptococcus*; Kobashi et al., 1978), *Peptostreptococcus* (Hayakawa, 1973; Hylemon & Glass, 1983), *Fusobacterium* (Shimada et al., 1969), and *Eubacterium* (Hayakawa, 1973; Hylemon & Glass, 1983). High concentrations of conjugated bile acids can inhibit bacterial growth, *in vitro*, of both autochthonous and allochthonous microbes (Floch et al., 1971; Binder et al., 1976; Burke et al., 1977). For at least two autochthonous species, conjugated bile acids can increase BSH activity (Lundeen & Savage, 1990; Kishinaka et al., 1994). Therefore, BSH activity may be an advantageous phenotype for autochthonous microbes and prevent allochthonous species from colonizing the lower GI tract. Furthermore, this activity is pivotal in two respects. First, these enzymes perform a "gatekeeping" function since bile

acids must be deconjugated before transformation reactions can occur (Batta et al., 1990). Second, the level of BSH-producing microbes that colonize the GI tract and the relative activity of such enzymes can have substantial effects on the total bile acid pool (Kellogg, 1973), and hence, regulation of cholesterol in the host organism (Eyssen, 1973).

BSH enzymes have been purified and characterized from *Bacteroides vulgatus* VI-31 (Kawamoto et al., 1989), *Bacteroides fragilis* subsp. *fragilis* ATCC 25285 (Stellwag & Hylemon, 1976), *Bifidobacterium longum* BB536 (Grill et al., 1995) and SBT2928 (Tanaka et al., 2000), *Clostridium perfringens* MCV 815 (Gopal-Srivastava & Hylemon, 1988), and *Lactobacillus* sp. strain 100-100 (Lundeen & Savage, 1990; 1992a). The genetic determinants responsible for BSH activity have been cloned from only three organisms, *L. plantarum* 80 (Christiaens et al., 1992), *C. perfringens* 13 (Coleman & Hudson, 1995), and *B. longum* SBT2928 (Tanaka et al., 2000). Only one enzyme was purified or cloned in all but two cases. In *L.* sp. strain 100-100, two peptides with BSH activity were purified; they combine to form four native homo- and heterotrimers (Savage & Lundeen, 1992a). For *C. perfringens* 13, a gene encoding BSH activity was cloned, but crude extracts from the bacterium revealed a second BSH-active fraction that was antigenically distinct from the first BSH (Coleman & Hudson, 1995). BSH activity was maintained in strains deficient in the first BSH. The BSH from the second BSH-active fraction has not been purified or cloned from strain 13. Furthermore, this system appears to be different from that of strain 100-100 since the second BSH-active fraction does not cross react with antibody to the first BSH (Coleman & Hudson, 1995). Therefore, the system in strain 13 does not appear to form heteromultimers as in strain 100-100.

BSH enzymes are oxygen sensitive (Aries & Hill, 1970) but demonstrate remarkable heterogeneity in biochemical properties. The pH optima for BSH enzymes vary depending on the organism of origin. They range from lower pHs of approximately 4.5 in *L.* sp. strain 100-100 (3.8 to 4.5; Lundeen & Savage, 1992a), *B. fragilis* subsp. *fragilis* (Stellwag & Hylemon, 1976), *C. perfringens* 13 (Coleman & Hudson, 1995), and *L. plantarum* (4.7 to 5.5; Christiaens et al., 1992) to pHs that approach neutral conditions as in *B. longum* BB536 (5.5 to 6.5; Grill et al., 1995), *B. vulgatus* (5.6 to 6.4; Kawamoto et al., 1989), *C. perfringens* MCV 815 (5.8 to 6.4; Gopal-Srivastava & Hylemon, 1988), and *B. longum* SBT2928 (5 to 7; Tanaka et al., 2000). In general, BSH activity decreases rapidly once outside the respective range in pH optimum, although the BSH in strain SBT2928 is stable from pHs of 4 to 8 (Tanaka et al., 2000).

Some BSHs demonstrate a preference for particular conjugated bile acid substrates. For example, the BSHs from *B. longum* SBT2928 (Tanaka et al., 2000), *L. plantarum* 80 (Christiaens et al., 1992), and *C. perfringens* MCV 815 (Gopal-Srivastava & Hylemon, 1988) preferentially hydrolyze glycoconjugates, whereas the BSH from *B. vulgatus* (Kawamoto et al., 1989) will preferentially hydrolyze tauroconjugates. Furthermore, the BSH from *B. vulgatus* demonstrates a preference for particular taurochenodeoxycholic acid and suggests that this enzyme has specificity for the amino acid moiety and the steroidal moiety (Kawamoto et al., 1989). Some BSHs such as those from *Lactobacillus* sp. strain 100-100 (Lundeen & Savage, 1990) and *C. perfringens* 13 (Coleman & Hudson, 1995) demonstrate no obvious specificity for conjugated substrate. The BSH enzyme(s) from strain 13 differs, however, in this substrate specificity. The purified and characterized enzyme from strain 13 demonstrates no specificity for a

particular conjugate. A second potential BSH enzyme from this strain was studied in a genetic background deficient for the first enzyme and displays a small preference for glycoconjugates (Coleman & Hudson, 1995). The specificity for conjugated bile acid substrates can be rationalized since chemical properties of these differ. Tauroconjugates and glycoconjugates have pK_as of approximately 1.8 to 2 and 3.7 to 5, respectively (Kuksis, 1973). Thus, tauroconjugates are virtually ionized at physiological pHs, whereas a significant proportion of glycoconjugates are protonated.

Subunit molecular weights and composition of BSHs vary significantly. The BSH from *B. fragilis* subsp. *fragilis* (Stellwag & Hylemon, 1976) has the smallest subunit molecular weight, 32.5 kDa, of all characterized BSHs. In contrast, the BSH from *C. perfringens* MCV 815 (Gopal-Srivastava & Hylemon, 1988) has the highest molecular weight, 56 kDa. Except for the BSH peptides from *Lactobacillus* sp. strain 100-100, all BSH subunits form native homomultimers that range from tetramers (*C. perfringens* 13 and MVC 815, *B. vulgatus*, and *B. longum* SBT2928; Coleman & Hudson, 1995; Kawamoto et al., 1989; Tanaka et al., 2000) to hexamers (*B. longum* BB536; Grill et al., 1995) to octamers (*B. fragilis* subsp. *fragilis*; Stellwag & Hylemon, 1976). As mentioned earlier, strain 100-100 forms trimers from two different BSH peptides (Lundeen & Savage, 1992a).

Finally, BSHs differ in kinetic properties, regulation, and cellular location. V_{max} values range from 107 nmol of deconjugate formed per min per mg protein (*C. perfringens* 13; Coleman & Hudson, 1995) to 80 μmol formed per min per mg protein (*B. fragilis* subsp. *fragilis*; Stellwag & Hylemon, 1976). K_m values also differ and obviously depend on the preference for conjugated substrate. Growth phase of the bacterium can, in

some cases, affect BSH expression. In strain SBT2928, *B. longum* expresses BSH activity constitutively (Tanaka et al., 2000), but in strain BB536, BSH activity is regulated by and increases with growth phase (Grill et al., 1995). In *Lactobacillus* sp. strain 100-100, BSH activity is expressed constitutively upon entry into stationary phase but not in other phases of growth (Lundeen & Savage, 1990). In general, BSHs are expressed intracellularly, but there are exceptions. A *C. perfringens* isolate from human feces produces an extracellular BSH that is induced by conjugated bile acids (Kishinaka et al., 1994). BSH activity has also been identified in the periplasmic space of gram-negative bacteria (Hylemon & Stellwag, 1976).

There is little information available on BSH activity at the genetic level. The genetic determinants for the activity have been cloned from only three bacteria. However, the genetic architectures at these loci are different. The BSH gene from *L. plantarum* 80 is monocistronic and is followed by a putative stem-loop structure (Christiaens et al., 1992). The BSH gene from *B. longum* SBT2928 is coordinately regulated with at least one other gene (Tanaka et al., 2000). This gene shares similarity to glutamine synthetase adenylyltransferase, *glnE*, that is part of the nitrogen regulation cascade. The BSH gene is preceded by direct repeats and is flanked by inverted repeats. Analysis of mRNA indicates that the BSH gene is transcribed by its own promoter and to a lesser degree from an upstream region. The extent of the BSH operon, however, is not known (Tanaka et al., 2000). A BSH gene from *C. perfringens* 13 was cloned but no transcript analysis was performed. The DNA sequences flanking the gene in strain 13 have not been characterized (Coleman & Hudson, 1995).

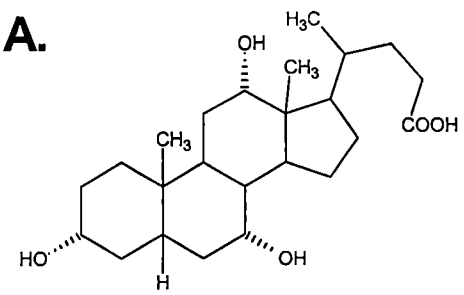
In contrast to 7α-dehydroxylation in *Eubacterium* sp. strain VPI 12708 (see below), there are no reports in the literature that identify genes or proteins functionally associated with BSH activity. The biochemical heterogeneity in BSH enzymes suggests that organisms may use the activity as a physiological and selective advantage in the ecology of the lower GI tract (i.e., bile acids may define and fulfill a niche). The range in regulation, substrate utilization, and catalytic activity of the BSH enzymes demonstrates this point. However, the physiological importance of this activity to the bacteria remains speculative. It can be hypothesized that bacteria gain an additional amino acid source from deconjugation (De Smet et al., 1995). The sulfonic acid group of taurine could serve as an electron acceptor for anaerobic respiration (Eyssen & Robben, 1989). Some strains of *Clostridium* have been shown to require taurine for growth or simply have increased growth rates in medium containing taurine (Huijghebaert et al., 1982). Alternatively, deconjugation may be a method to reduce the toxicity of bile acids (De Smet et al., 1995).

Bile Acid Transformation Reactions

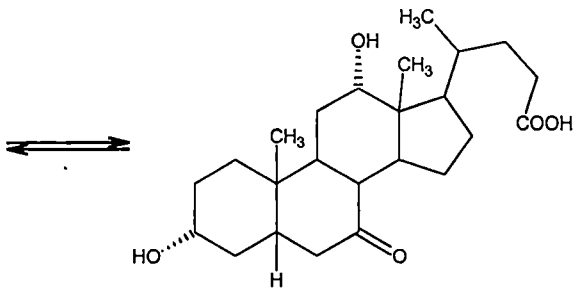
As mentioned before, the intestinal microbiota can generate a variety of bile acid metabolites from primary unconjugated bile acids. The products of such microbial actions are secondary bile acids. These molecules have modifications of the steroid side chains and are produced collectively by transformation reactions (Fig. 4; Bortolini et al., 1997). Similar reactions have been described in host mammalian systems and the autochthonous microbiota that affect a variety of other steroid molecules such as sterols

FIG. 4. Examples of bile acid transformation reactions catalyzed by enzymes produced by the intestinal microbiota. A, dehydrogenation by 7α-hydroxysteroid dehydrogenase; B, epimerization by 7β-hydroxysteroid dehydrogenase; C, dehydroxylation by 7-dehydroxylation pathways; D, desulfation by 3-alkylsulfatases. * Bonds can be in α or β configuration.

A.

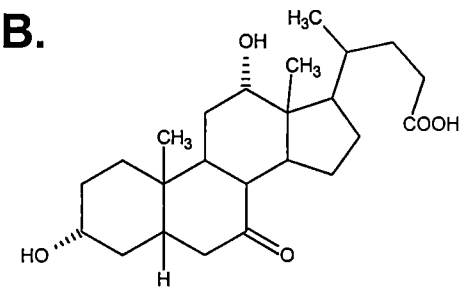


Cholic acid

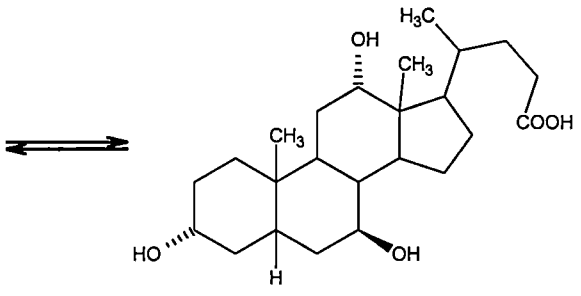


7-oxo-cholic acid

B.

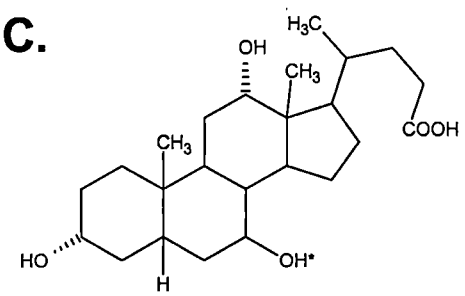


7-oxo-cholic acid

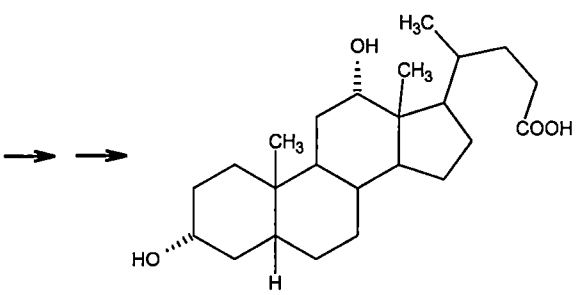


Ursocholic acid

C.

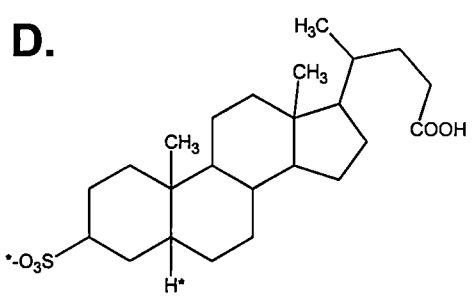


Cholic acid

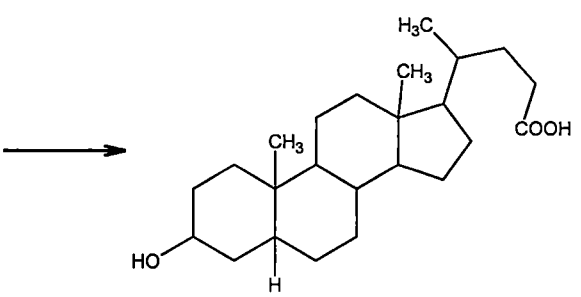


Deoxycholic acid

D.



Lithocholic acid-3α-sulfate



Lithocholic acid

and hormones (Groh et al., 1993; Labrie et al., 2000). Such molecules can flow with the enterohepatic circulation such that the intestine may be considered an endocrine active site (Groh et al., 1993). The host mammalian/autochthonous sterol and hormone reactions, however, are numerous, are not the focus of this thesis, and, therefore, will not be addressed. Three classes of microbial transformation reactions of bile acids will be described below.

Dehydrogenation. This class of transformation reactions is catalyzed by hydroxysteroid dehydrogenases (HSDHs). HSDH activity has been identified in several predominate members of the GI microflora, including *Bacteroides*, *Eubacterium*, *Clostridium*, *Bifidobacterium*, *Lactobacillus*, *Peptostreptococcus*, and *Fusobacterium* (Hylemon & Glass, 1983). These enzymes are stereospecific and can catalyze oxidation or epimerization of hydroxyl groups on the steroid moiety (Hylemon & Glass, 1983). Oxidation of α-hydroxyl groups is catalyzed by α-HSDHs (EC 1.1.1.159) and produces an oxo (keto) bile acid (Fig. 4A). This occurs at the C-3, C-7, and C-12 positions of cholic acid and the C-3 and C-7 positions of chenodeoxycholic acid (Hylemon & Harder, 1999). Oxo bile acids are reduced to β-hydroxyl groups by β-HSDHs in a reaction resulting in epimerization (Fig. 4B). Oxidation and reduction of the side chains are reversible reactions. Epimerization of hydroxyl groups can occur only through the action of both α- and β-HSDHs in an intraspecies or interspecies pathway. Bacteria that carry both α- and β-HSDHs are involved in intraspecies epimerization of hydroxyl groups. Alternatively, protocooperation between two species of bacteria, one expressing an α-

HSDH and a second expressing a β-HSDH, results in interspecies epimerization (Hylemon & Harder, 1999).

Two types of α-HSDHs that require NAD^+ or $NADP^+$, respectively, have been reported. The NAD^+-dependent HSDHs have been identified in *E. coli* strains B and 080 (Macdonald et al., 1973; Prabha et al., 1990), *B. fragilis* (Hylemon & Sherrod, 1975), and *Brevibacterium fuscum* (Kinoshita et al., 1988), whereas an $NADP^+$-dependent HSDH has been identified in *B. fragilis* (Macdonald et al., 1975). A NAD^+-dependent 7α-HSDH has also been cloned, and characterized in *E. coli* HB101 (Yoshimoto et al., 1991). The 3α-HSDH, named BaiA, from *Eubacterium* sp. strain VPI 12708 catalyzes a novel oxidation of cholyl-CoA to produce 3-oxo-cholyl-CoA and can use either NAD^+ or $NADP^+$ as elcctron acceptors (Baron et al., 1991; Mallonee et al., 1995; Hylemon & Harder, 1999). This, however, is part of a multistep pathway for 7α-dehydroxylation of cholic acid (see below).

Intraspecies epimerization of the 3α to 3β position has been demonstrated in strains of *C. perfringens*. Some strains in the same study oxidized the 3α position to an oxo group, but failed to epimerize these groups to the β position (Hirano et al., 1981). Such strains may participate in interspecies epimerization. An analogous system has been demonstrated in *Clostridium* strain S_2 that apparently reverse-epimerizes the C-6 position of β-muricholic acid via 6α and 6β-HSDHs to produce ω-muricholic acid (Robben et al., 1986). In a different study, a strain of *Clostridium*, called S_1, was isolated from rat feces and possesses 12α-HSDH and BSH activity (Huijghebaert et al., 1982). The HSDHs from *P. productus* represent another example of interspecies epimerization

(Edenharder et al., 1989). This organism is a human fecal isolate that expresses NAD^+-dependent 3α- and 3β-HSDH activity. In addition, it expresses $NADP^+$-dependent 7β-HSDH activity. Bile acid substrate specificities, pH optima, and estimated molecular weights of these proteins have been determined (Edenharder et al., 1989).

A novel intraspecies bile acid transformation was identified in a gram-positive rod from the rat intestinal microbiota (Eyssen et al., 1999). This organism is a member of an undescribed genomic species that is able to transform the 3α,6β,7β-trihydroxy bile acid, β-muricholic acid, into 3α,6α-dihydroxy acid, hyodeoxycholic acid. This occurs by dehydroxylation of the 7β-hydroxyl group and epimerization of the 6β-hydroxyl group to 6α-hydroxyl group (Eyssen et al., 1999). Apparently, novel combinations of enzymes capable of transforming bile acids can occur in an intraspecies fashion. Alternatively, new interspecies transformations may lead to unidentified bile acid intermediates that can account for the variety in fecal bile acid profiles of conventional laboratory rodents.

Dehydroxylation. Certain members of the genera *Eubacterium* and *Clostridium* are capable of removing the hydroxyl group at the C-7 position of primary unconjugated bile acids (Fig 4C; Hylemon & Glass, 1983; Doerner et al., 1997). This activity, termed 7-dehydroxylation, is quantitatively the most important bile acid transformation by the intestinal microbiota (Hylemon & Harder, 1999). Apart from the other transformation reactions described in this section, 7-dehydroxylation is not performed by a single enzyme, but is the result of a biochemical pathway involving the action of several proteins (White et al., 1988; Mallonee et al., 1990; Hylemon & Harder, 1999; Wells &

Hylemon, 2000). Dehydroxylation can occur in either orientation, α or β, of the hydroxyl group on the steroid ring. For example, *Eubacterium* sp. strain VPI 12708, a human intestinal isolate possesses bile acid 7α- and 7β-dehydroxylation activity (White et al., 1980; Hylemon, 1985).

At a genetic level, 7α-dehydroxylation is the best characterized example of a bile acid modification by the intestinal microbiota. An operon of nine genes is expressed when induced with primary bile acids in strain VPI 12708. These genes, called bile acid-inducible or *bai* genes, are assumed to encode a majority of the proteins involved in this pathway (Mallonee et al., 1990; Baron & Hylemon, 1997). Strain VPI 12708 encodes three *baiA* genes, named *baiA1*, *baiA2*, and *baiA3*, at three different loci on the genome that encode for 3α-HSDH activity (Gopal-Srivastava et al., 1990). *BaiA1* and *baiA3* are identical in sequence and transcribed monocistronically, whereas *baiA2* is part of the nine gene *bai* operon. It shares 92% amino acid sequence identity with the other *baiA* gene products. The significance of three copies of *baiA* is unknown (Hylemon & Harder, 1999). Moreover, the physiological role of 7-dehydroxylation in the bacterial cell is unclear, but is thought to provide the bacterium with an ancillary electron acceptor (Hylemon, 1985; Eyssen & Robben, 1989). In support of this theory, a *Clostridium* sp., isolated from rat feces and capable of 7β-dehydroxylation, has a strict growth requirement of 200 to 800 μg/mL of unconjugated trihydroxylated bile acids (Eyssen et al., 1985; Eyssen et al., 1987). The genes that comprise the *bai* operon in strain VPI 12708 have been purified and studied functionally in *E. coli* (Hylemon & Harder, 1999).

The 7α-dehydroxylation mechanism is complex and involves several intermediate oxidation and reduction steps (Fig. 5). Cholic acid (or chenodeoxycholic acid) is transported into the cell through a novel H^+-deconjugated bile acid importer, BaiG (Mallonee & Hylemon, 1996), and ligated to CoA via bile acid-CoA ligase, BaiB (Mallonee et al., 1992), in an ATP and Mg^{2+} dependent fashion. Oxidation of the bile acid-CoA conjugate by 3α-HSDH, BaiA (Baron et al., 1991), produces an oxo bile acid that is further oxidized by an oxidoreductase, supposedly BaiH or BaiC. BaiH has been functionally identified in *E. coli* and shown to encode NADH:flavin oxidoreductase activity (Baron & Hylemon, 1995). BaiC shares sequence similarity with BaiH but the function of this protein remains unclear (Franklund et al., 1993). The bile acid intermediate is deconjugated from CoA via a bile acid-CoA hydrolase, BaiF (Ye et al., 1999), and is dehydrated by the action of a 7α-dehydratase, BaiE (Dawson et al., 1996), to produce a 3-oxo-$\Delta^{4,6}$-steroid intermediate. BaiE is similar in sequence to another gene product in the operon, BaiI, which has not been identified functionally (Hylemon & Harder, 1999). Finally, the oxo bile acid intermediate is subject to several reductive steps that collectively produce deoxycholic acid (or lithocholic acid) that is exported from the cell by an unknown mechanism (Hylemon & Harder, 1999).

A similar set of genes has been described recently in a human feces isolate, *Clostridium* sp. strain TO-931 (Wells & Hylemon, 2000). The six gene operon in this strain is nearly identical in genomic architecture and exhibits approximately 75% DNA sequence identity to genes in the *bai* operon in strain VPI 12708. Similar to VPI 12708, transcription of the operon in strain TO-931 is inducible with deconjugated bile acids.

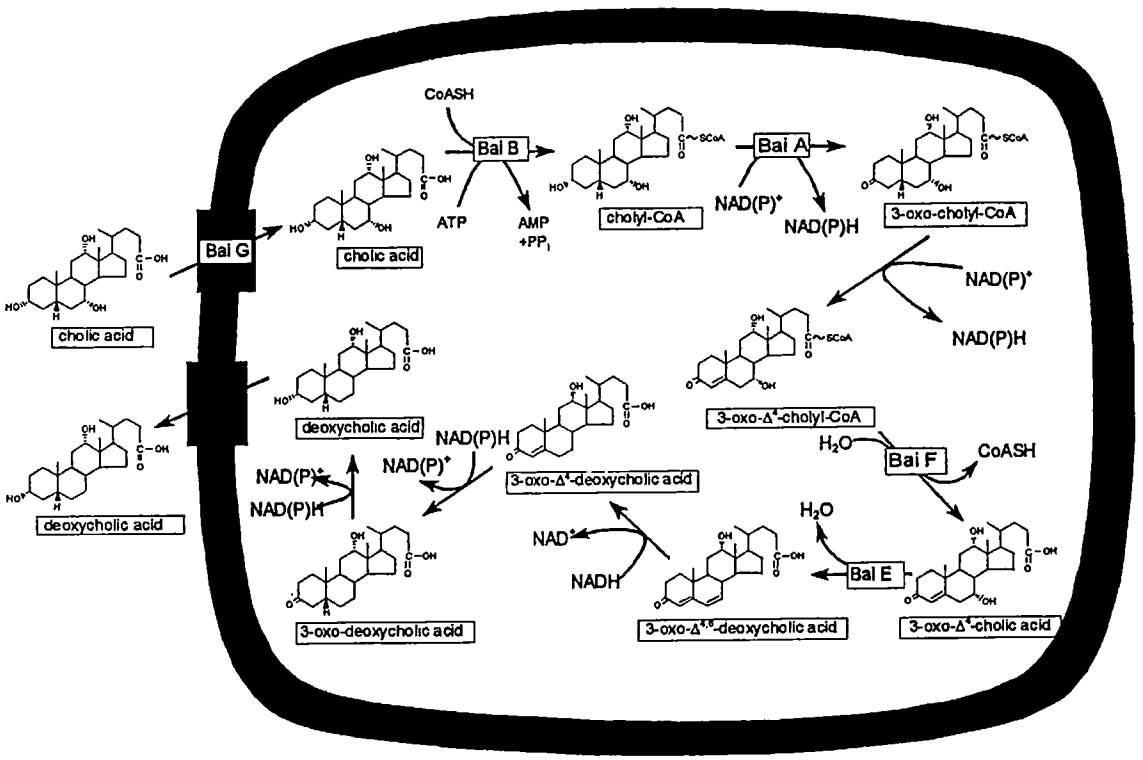


FIG. 5. Proposed pathway for 7α-dehydroxylation in intestinal bacteria involves several bile acid-inducible (*bai*) gene products. CoASH, coenzyme A-SH (Wells & Hylemon, 2000).

The promoter regions of the operons displayed significant sequence identity and a conserved element upstream of the putative –10 site (Wells & Hylemon, 2000). Some differences did exist between the *bai* loci of the two organisms. The operon in strain TO-931 was missing two genes, *baiH* and *baiI*, located at the 3' end of the operon in strain VPI 12708. *BaiC* and *baiD* were fused into one continuous gene in strain TO-931. Furthermore, comparison of intergenic DNA sequences showed little homology and were larger in strain TO-931 (Wells & Hylemon, 2000). A survey of *bai* genes in bacterial strains revealed that *bai* genes from strain VPI 12708 cross-hybridized with other *Eubacterium* strains but not with *Clostridium* strains (Doerner et al., 1997). The genetic work in strain TO-931 demonstrates that the *bai* pathway is highly conserved (Wells & Hylemon, 2000) and may be the major, if not only, bile acid 7α-dehydroxylation pathway in the human autochthonous microbiota (Björkhem et al., 1989; Hylemon et al., 1991; Hylemon & Harder, 1999).

Desulfation. Some bile acids, such as lithocholic acid, contain one hydroxyl group on the steroid ring and, hence, are less polar than di- or trihydroxylated bile acids. Such bile acids are made water soluble, but less capable of forming micelles, by sulfation (Low-Beer et al., 1969; Kellogg, 1973; DeWitt and Lack, 1980). These bile acids are produced in the liver (Palmer, 1972; Chen et al., 1977) by esterification of the hydroxyl group to sulfate at the C-3 position in rats and humans (Midvedt, 1987) and at the C-7 position in mice (Eyssen et al., 1976). Bile salt sulfates are not easily reabsorbed in the intestine and do not participate in enterohepatic circulation. Rather, these molecules are eliminated from host organism by fecal excretion (Eyssen et al., 1985). Some members of the

autochthonous microbiota are capable of desulfating these molecules (Fig. 4D; Eyssen & Robben, 1989). The desulfated bile acids can be reabsorbed through the intestinal epithelium and return to the enterohepatic circulation (Eyssen et al., 1985).

GI tract bacteria produce sulfatase enzymes that catalyze desulfation of endogenous steroid molecules, including bile acids and hormones (Eyssen & Robben, 1989). These enzymes can be divided into two major groups. Alkylsulfatase enzymes desulfate steroids, such as androstanes, pregnanes, and bile acids, containing a saturated A ring. Arylsulfatase enzymes desulfate steroids, such as estrogen sulfates, containing an aromatic A ring (Eldere et al., 1988). A survey of substrate specificity of steroid-desulfating strains revealed a variety of different bacterial sulfatase enzymes. The substrate specificity of sulfatases is stereospecific, relating to the equatorial orientation of the sulfate group (Huijghebaert & Eyssen, 1982). For example, alkylsulfatases with different stereospecificities have been identified in *Clostridium* strains S_1 and S_2 and *Peptococcus niger* strains H_4 and DSM 20475. *Clostridium* strain S_1 was capable of desulfating the 3β-sulfate of 5α- or 5β-bile acids (Van Eldere et al., 1988). However, this strain would desulfate the 3α-sulfate of 5β- and not 5α-bile acids (Huijghebaert & Eyssen, 1982; Van Eldere et al., 1988). Strain S_2 was capable of desulfating only 3α-sulfates of either 5α- or 5β-bile acids (Robben et al., 1986; Van Eldere et al., 1988). *P. niger* strain H_4 was capable of desulfating any stereoisomer of 3-sulfated bile acids, whereas strain DSM 20475 could desulfate only 3β-sulfates of 5α- or 5β-bile acids (Van Eldere et al., 1988). This type of discriminating specificity was shown with arylsulfates using these same strains and other genera of bacteria, including *Lactobacillus*,

Eubacterium, and *Bacteroides* (Van Eldere et al., 1988). Alkylsulfatase activity towards lithocholic acid-3-sulfate has also been identified in *P. aeruginosa* (Imperato et al., 1977).

The physiological importance of desulfation to the bacterium is speculative. Growth of *P. niger* strains H_4 and DSM 20475 is stimulated by sulfite, taurine, and sulfate esters but not sulfate or cysteine. The growth stimulation is accompanied by H_2S production (Van Eldere et al., 1987, Van Eldere et al., 1988). Clostridium strain S_2 demonstrates similar characteristics and expresses high levels of desulfation during exponential phase (Van Eldere et al., 1988). These observations indicate that sulfatase enzymes liberate reducible sulfur to act as an electron acceptor in anaerobic bacteria (Van Eldere et al., 1987; Van Eldere et al., 1988). Some aryldesulfating organisms, such as *E. cylindroides* strains H_1 and H_2, lack H_2S production and growth stimulation in the presence of taurine. They show no effect of exogenous cysteine on sulfatase activity and demonstrate slow desulfation during stationary phase. In these strains, sulfatase enzymes may provide sulfur for assimilatory or metabolic pathways (Van Eldere et al., 1988).

Significance of Bile Acid Modification to Human and Animal Health

Epidemiological evidence has demonstrated a connection between bile acid metabolism by the intestinal microbiota and colon carcinogenesis. Studies of individuals with colon cancer indicate that bile acid concentrations in the feces are elevated. In addition, fecal concentrations are elevated in individuals at high risk for the disease due to a high fat diet (Kay, 1981; Cheah, 1990). Animal studies have shown that secondary

bile acids more than their primary counterparts can promote colon cancer and act as co-carcinogens inducing colonic nuclear DNA damage in mice and mouse fibroblasts (Suzuki & Bruce 1986; Kawasumi et al., 1988). Secondary bile acids have also been shown to induce a DNA repair response in human foreskin fibroblasts and the bacterium *E. coli* (Kandell & Bernstein, 1991). The repair response in *E. coli* is not triggered directly by bile salts but is significantly enhanced by these molecules in the presence of fecal mutagens such as 4-nitroquinoline oxide or fecapentaene-12, -14. Therefore, bile salts have co-carcinogenic activity (Nair et al., 2000) in *E. coli.*

The majority of bile acids excreted in the feces are unconjugated secondary bile acids (Kandell & Bernstein, 1991). Such bile acids are produced by transformation of side chains on the steroid ring structure (e.g., bile acid dehydroxylation) by the autochthonous intestinal bacteria (Baron & Hylemon, 1997). Such transformations decrease the solubility of these molecules. Production of secondary bile acids by intestinal bacteria does not occur until primary bile acids are deconjugated by BSHs (Batta et al., 1990). Therefore, BSH enzymes are pivotal in the pathway to secondary bile acid production in the gut and have implications in human health.

Recent *in vitro* studies with *L. plantarum* have revealed a relationship between enhanced BSH activity and lowering of cholesterol levels (De Smet et al., 1994). Elevated serum cholesterol levels in humans have been associated with increased risk of atherosclerosis and coronary heart disease (De Smet et al., 1995). Bacterial deconjugation of primary bile acids produces free deconjugated bile acids that are easily precipitated at low pH and less efficiently reabsorbed through the intestinal epithelium as part of the enterohepatic circulation. Loss of these bile acids by precipitation and

excretion in the feces must be matched by *de novo* synthesis from cholesterol in the liver. It is suggested that elevated BSH activity leads to an increased demand on bile acid pools and a decrease in serum cholesterol levels. The *in vitro* study with *L. plantarum* concludes that a daily intake of highly BSH-active bacteria might lead to a significant reduction of cholesterol and a valid microbiological alternative treatment for hypercholesterolaemia (De Smet et al., 1994). A similar finding was obtained with BSH-active *L. reuteri* cells that were fed to pigs (De Smet et al., 1998). The data suggest that feeding of these cells caused a temporary shift within the indigenous *Lactobacillus* population of treated pigs. A significant lowering of total cholesterol concentrations was observed with treated pigs in comparison to control pigs, hence producing a probiotic effect (De Smet et al., 1998).

Research aimed at decreasing intestinal bile acid re-absorption has uncovered an experimental hypocholesterolemic compound, 2164U90, that selectively and competitively inhibits ileal sodium-dependent bile acid import in animal and human model systems (Root et al., 1995). This compound is a small, uncharged, lipophilic benzothiazepine derivative with a molecular weight similar to a bile acid and is effective at low concentrations. This compound, if given orally, will restrict bile acid flow at the level of ileal transport, another target to control levels of cholesterol (Root et al., 1995). Presumably, higher concentrations of bile acids in the intestinal lumen will result in increased microbial activity towards these molecules. Therefore, bile acids will become less soluble and excreted.

An elevated level of deoxycholic acid in bile is associated with increased risk for cholesterol gallstone disease (Marcus & Wheaton, 1988; Shoda et al., 1995). Studies of

patients with the disease have shown that fecal levels of 7α-dehydroxylating bacteria are 1,000-fold higher in individuals with elevated deoxycholic acid in the bile. In another study, over 42-fold higher levels of 7α-dehydroxylating bacteria, all of which belonged to the genus *Clostridium*, were isolated from feces of gallstone patients versus patients that had not developed gallstones (Wells et al., 2000). Moreover, antibiotic treatment of gallstone patients resulted in a decrease of deoxycholic acid, the biliary cholesterol saturation index, and fecal levels of 7α-dehydroxylating bacteria (Berr et al., 1996; Hylemon & Harder, 1999). As noted earlier, microbial desulfatases have a range of specificities not only for sulfated bile acids but also for hormones circulating enterohepatically (Van Eldere et al., 1987; Van Eldere et al., 1988). Antibiotic treatment can affect the steroid composition in feces and urine and affect levels of steroid hormones in plasma. Such treatment can reduce excretion of estrogenic hormones in urine and result in failure in contraceptive treatment in women (Van Eldere et al., 1987; Van Eldere et al., 1988).

Bile acid modification by the intestinal bacteria has health implications in animals grown for food. It has been known for some time that antibiotics promote improved growth responses in chickens. This phenomenon has been shown in studies in which growth responses have been compared in conventional and germfree chickens (Forbes & Pank, 1959; Coates et al., 1963). Subtherapeutic levels of antibiotics that increased the growth performance of chickens were shown to decrease concomitantly the BSH activity in ileal homogenates. Conversely, antibiotics that have no effect on growth rate demonstrated minimal effect on BSH activity in ilea homogenates (Feighner & Dashkevicz, 1987). Moreover, chickens fed dietary carbohydrates that cause growth

depression have a concomitant increase in BSH activity (Feighner & Dashkevicz, 1988). Studies in pigs also suggest an inverse relationship of BSH activity and growth performance (Tracy & Jensen, 1987). The use of subtherapeutic levels of antibiotics in animal feeds is controversial because of the potential spread of antibiotic resistance (Baldwin, 1970; Marsik & Parisi, 1975; Holmberg et al., 1984; Witte et al., 1999). These findings may be significant since they purport to identify a specific biochemical activity that is associated with the antibiotic effect on growth performance.

BSH system in *Lactobacillus* sp. strain 100-100

Gastric lactobacilli are responsible for a majority (86% and 75%) of the BSH pools respectively in the ileum and cecum of mice (Tannock et al., 1989). A study of BSH activity at the biochemical level was initiated in *Lactobacillus* sp. strain 100-100, an isolate from the keratinized, non-secreting, squamous epithelium in the stomach of a laboratory rat. Strain 100-100 expresses BSH activity that was compared, quantitatively, to that of certain other *Lactobacillus* strains of human, mouse, rat, and pig origin. Strain 100-100 expressed the highest levels of BSH activity relative to the other strains tested (Lundeen & Savage, 1990). The study revealed a BSH system that was unlike other microbes for which the activity had been purified and characterized (Lundeen & Savage, 1990; 1992a). Furthermore, strain 100-100 was shown to secrete an extracellular factor of unknown composition (see below; Lundeen & Savage, 1990; 1992b).

Identification of BSHs. BSH activity was purified from cytosolic fractions of strain 100-100. The activity was not detected or detected only at negligible levels in membrane fractions or culture supernatants, respectively. When compared to growth phase of strain 100-100, BSH activity was expressed constitutively upon entry into stationary phase. Initial purification of the protein(s) responsible for the activity revealed two anion-exchange HPLC fractions containing BSH activity. Each fraction contained a protein migrating as a single band on nondenaturing polyacrylamide gel electrophoresis (PAGE) with approximate molecular weights of 115,000 and 105,000 Da. Denaturing PAGE revealed that one subunit of approximately 42,000 Da was common to both hydrolases. These two hydrolase enzymes did not differ in substrate specificity for tauro- or glyco-conjugates. These proteins were assayed for activity over a pH range of 3.8 to 7.0. They were active optimally at pH < 4.5 with activity sharply decreasing at higher pHs until no activity could be detected at pH 6.5 (Lundeen & Savage, 1990).

Although purification of BSHs from other intestinal bacteria had been performed by this time, these studies had not demonstrated the existence of more than one BSH in a bacterium (Lundeen & Savage, 1992a). Therefore, the system in strain 100-100 was subjected to a more detailed biochemical analysis for the activity. The salt gradient used in anion-exchange HPLC was extended and revealed two additional BSHs. A total of four proteins, designated A, B, C, D, with BSH activity were purified from the cytosol of strain 100-100 cells with approximate molecular weights of 115,000, 105,000, 95,000, and 80,000 Da, respectively. Each of the four hydrolase-active cytosolic fractions contained at least one of two peptides, designated α and β, with molecular weights of 42,000 and 38,000 Da. Western blot analysis with specific polyclonal antibodies to α

and β was performed on purified hydrolases separated by denaturing PAGE. Hydrolases A and D contained only the α or β peptides, respectively, whereas hydrolases B and C contained both. The approximate molecular weights of the peptide subunits and the native hydrolases indicated that the four BSH isozymes exist as homo- and heterotrimers of the possible combinations of the α and β (α_3, $\alpha_2\beta$, $\alpha\beta_2$, β_3; Lundeen & Savage, 1992a).

The pH optima of the four isozymes were approximately the same, between 4.2 and 4.5. However, the isozymes were catalytically different with V_{max}s of 17, 53, 24, and 2.4 μmol of cholic acid formed per min per mg of protein for BSH A, B, C, and D, respectively. Although BSH A (composed of the α trimer) is catalytically more active than BSH D (composed of the β trimer), only a combination of the two peptides results in a more active protein. This finding suggests that α is the main catalytic subunit but that α and β interact to produce conformational changes that have a positive effect on enzyme activity. Amino acid compositions of the two peptides of the BSH isozymes demonstrate significant differences in the two proteins. Amino acid sequencing was performed on purified α peptide to reveal the amino terminal 25 amino acids of Gly-Thr-Ser-Ile-Val-Tyr-Ser-Ser-Asn-Asn-His-His-Tyr-Phe-Gly-Arg-Asn-Leu-Asp-Leu-Gln-Ile-Ser-Phe-Gly. The data suggested that strain 100-100 has two separate genetic determinants encoding BSHs that are structurally, and hence, antigenically distinct (Lundeen & Savage, 1992a).

Preliminary characterization of EF. The BSH activity of strain 100-100 differs from that of other reported genera not only by involving two peptides but also by involving an

extracellular factor that enhances activity. When conjugated bile acids are added to suspensions of stationary phase cells, the activity increases by as much as three- to five-fold within 20 minutes (Lundeen & Savage, 1990). Strain 100-100 cultures not exposed to conjugated bile acids but suspended in supernatants from cells that were exposed to such molecules demonstrate an immediate increase in BSH activity. Therefore, this increase is not due to induction of either enzymatic proteins or transport proteins. Rather, it is due to synthesis of a soluble extracellular molecule (Lundeen & Savage, 1990).

Preliminary characterization of the molecule (called BSH enhancing factor or EF) was undertaken. The molecule is air-, protease- and heat-resistant, partially partitions into organic solvents and is inhibited in function by the sulfhydryl group inhibitor, N-ethylmaleimide. It is resistant to pHs as low as 2.0 and as high as 12.0. Dialysis experiments with ^{14}C-taurocholic acid show that EF does not bind the bile acids. Attempts to determine molecular weight of EF vary depending on the method used. Ultrafiltration through membranes with molecular mass exclusion limits of 10, 30, and 100 kDa did not allow passage of the molecule. However, dialysis through membranes with molecular mass exclusion limits up to 12 to 14 kDa retained EF, whereas dialysis tubing with an exclusion limit of 25 kDa only partially retained the molecule. The effect of EF activity on other lactobacilli was also investigated. EF stimulated BSH activity in only two of twenty-six strains tested, *Lactobacillus* sp. strain 100-20 and *L. acidophilus* strain 100-37 (Lundeen & Savage, 1992b). It is clear from the preliminary characterization of EF that it is a stable, amphipathic molecule that can form large aggregates.

Aims of the Study

Autochthonous microbes have evolved the capacity to form intimate and persistent associations with the GI tracts of vertebrates. From a microbial perspective, factors influencing such associations are complex, multigenic traits (Savage, 2000). Such traits allow survival through harsh environments, adhesion, immune evasion, and metabolism of and resistance to endogenous compounds. Conversely, the microbiota can exert positive and negative influences on the health of the host organism (Savage, 2000). The role that BSH activity plays in the multifaceted environment of the lower GI tract is not understood. It is significant from a human and animal health perspective, but is it required for colonization of certain microbes? Why, then, do the same microbes from the same environment not express the capacity? Thus, the general focus of my work is ecological, and basic questions concerning particular microbial traits, I propose, can be approached from the perspective of the microbe.

BSH activity has been purified and characterized biochemically from strain 100-100 (Lundeen & Savage, 1990; 1992a). Genes responsible for BSH activity in three other organisms have been cloned but reveal very little about the role BSHs play in microbial physiology (Christiaens et al., 1992; Coleman & Hudson, 1995; Tanaka et al., 2000). The system in strain 100-100, however, is different from other BSH systems. There are two antigenically distinct BSH peptides (Lundeen & Savage, 1992a) and EF of unknown composition that is functionally related to but apart from hydrolysis of bile acids (Lundeen & Savage, 1990; 1992b). Therefore, the genetics of the system in strain 100-100 have the potential to reveal novel components that may define the activity

physiologically. The study had two objectives I) to characterize the BSH activity in strain 100-100 at the molecular genetic level and II) to identify and study proteins other than BSHs that may participate in the activity. These objectives will be addressed in the following chapters.

PART II

IDENTIFICATION OF GENES ENCODING CONJUGATED BILE SALT HYDROLASE AND TRANSPORT IN *LACTOBACILLUS JOHNSONII* STRAIN 100-100

(Elkins, C.A., and D. C. Savage. 1998. J. Bacteriol. 180:4344-4349)

Abstract

Cytosolic extracts of *Lactobacillus johnsonii* strain 100-100 (previously reported as *Lactobacillus* sp. strain 100-100) contain four heterotrimeric isozymes composed of two peptides, α and β, with conjugated bile salt hydrolase (BSH) activity. I now report cloning, from the genome of strain 100-100, a 2,977 bp DNA segment that expresses BSH activity in *Escherichia coli*. The sequencing of this segment showed that it contained one complete and two partial open reading frames (ORFs). The 3' partial ORF (927 nucleotides) was predicted by BLAST and confirmed with 5' and 3' deletions to be a BSH gene. Thermal asymmetric interlaced PCR was used to extend and complete the 948 nucleotide sequence of the BSH gene 3' of the cloned segment. The predicted amino acid sequence of the 5' partial ORF (651 nucleotides) was about 80% similar to the C-terminal half of the largest, complete ORF (1;353 nucleotides), and these two putative proteins were similar to several amine, multidrug resistance, and sugar transport proteins of the major facilitator superfamily. *E. coli* DH5α cells transformed with a construct containing these ORFs, in concert with an extracellular factor produced by strain 100-100, demonstrated levels of uptake of [^{14}C]taurocholic acid that were increased as much as threefold over control levels. [^{14}C]Cholic acid was taken up in similar amounts by strain DH5α pSportI (control) and DH5α p2000 (transport clones). These findings support a hypothesis that the ORFs are conjugated bile salt transport genes, which may be arranged in an operon with BSH genes.

Introduction

Bile acids are produced *de novo* in the liver from cholesterol. The steroid nucleus is conjugated with an amide bond at the carboxyl C-24 position to one of two amino acids, glycine or taurine (Savage et al., 1995; Baron & Hylemon, 1997). These conjugates are secreted via the common bile duct into the duodenum (Stevens & Hume, 1995). Because of their amphipathic nature, the conjugates form spontaneous micelles that trap dietary cholesterol and fats and facilitate their absorption by the intestinal epithelium. The bile acids are then actively transported by a sodium-dependent transporter (Wong et al., 1994) through the epithelium and into the bloodstream. Their return to the liver completes the enterohepatic cycle (Baron & Hylemon, 1997).

In humans, 130 to 650 mg of bile acids per day elude absorption through the intestinal epithelium (Baron & Hylemon, 1997). With bile acids flowing in such large amounts through the digestive tract, bacterial members of the autochthonous gastrointestinal microbiota have evolved the ability to alter these compounds (Savage et al., 1995; Baron & Hylemon, 1997). Isolates of indigenous bacteria of numerous genera and species produce enzymes that alter the side chains on the steroid ring system of bile acids. Such enzymes are involved in transformation reactions such as 7α- and 7β-dehydroxylation, 6β-dehydrogenation, and desulfation (Baron & Hylemon, 1997). Some of the best described enzymes, however, are bile salt hydrolases (BSH) which catalyze hydrolysis of the amino acid taurine or glycine from the C-24 position of conjugated bile salts (for example, choloylglycine hydrolase, EC 3.5.1.24). This reaction, deconjugation, may be required before side chain transformations can occur (Batta et al., 1990).

BSH activity has been detected in several bacterial genera of the autochthonous gastrointestinal microbiota of animals including mice, rats, humans, chickens, and swine (Savage, 1977; Baron & Hylemon, 1997). Enzymes with the activity have been purified and characterized from *Bacteroides vulgatus* VI-31 (Kawamoto et al., 1989), *Bacteroides fragilis* subsp. *fragilis* (Stellwag & Hylemon, 1976), *Bifidobacterium longum* BB536 (Grill et al., 1995), *Clostridium perfringens* MCV 815 (Gopal-Srivastava & Hylemon, 1988), and *Lactobacillus* sp. strain 100-100 (hereafter referred to as *Lactobacillus johnsonii* strain 100-100) (Lundeen & Savage, 1990; Savage et al., 1995). Genes encoding the enzymes in *Lactobacillus plantarum* 80 and in *C. perfringens* 13 have been cloned and sequenced (Christiaens et al., 1992; Coleman & Hudson, 1995). Except for *L. johnsonii* strain 100-100, only one protein has been purified in each case. For *L. johnsonii*, four proteins with the activity (A, B, C, and D) were purified from intracellular fractions of stationary phase anaerobic cultures (Lundeen & Savage, 1992a; Savage et al., 1995). These BSH enzymes exist as heterotrimers composed of two antigenically distinct subunits, designated α and β. The four possible subunit combinations, α_3, $\alpha_2\beta$, $\alpha\beta_3$,and β_3, coincide with the four observed isozymes expressed by the bacterium. The α trimer was determined to be catalytically more active than the β trimer with V_{max} values of 17 and 2.4 μmol of cholic acid formed per min per mg of protein, respectively (Lundeen & Savage, 1992a).

The BSH activity of *L. johnsonii* strain 100-100 differs in another way from that of other reported genera. When conjugated bile salts are added to suspensions of stationary-phase cells, the activity increases as much as three- to fivefold within 20

minutes (Lundeen & Savage, 1990; Savage et al., 1995). This increase is not due to induction of either the enzymatic proteins or the transport proteins. Rather, it is due to synthesis of a soluble extracellular molecule of a relatively small molecular size (12 to 25 kDa) that enhances BSH activity not only in *L. johnsonii* but also in certain other *Lactobacillus* isolates. The molecule (called BSH enhancing factor, or EF) is protease and heat resistant, partially partitions into organic solvents, and has not been shown to bind bile acids. Only the sulfhydryl group inhibitor N-ethylmaleimide inhibits its function (Lundeen & Savage, 1992b).

Because of the unique features of the BSH system of *L. johnsonii*, I have a long-term goal of learning how the enzymes in this complex system are regulated. To achieve this end, I have cloned from strain 100-100 and sequenced an approximately 3,000-bp genomic DNA sequence that heterologously expresses BSH activity in *Escherichia coli* cells. Along with a BSH gene, I am reporting two other predicted open reading frames (ORFs) on the fragment, which I implicate in cellular uptake of conjugated bile salts as part of a possible BSH operon.

Materials and Methods

Bacterial strains, plasmid vectors, and growth conditions. *L. johnsonii* strain 100-100 was maintained frozen at –80°C (Lundeen & Savage, 1990; Savage et al., 1995). It was grown anaerobically in MRS broth or agar medium (Becton Dickinson) as previously reported (Lundeen & Savage, 1990; Savage et al., 1995). *E. coli* HB101 (Promega) and *E. coli* DH5α (Gibco BRL) were used as host cell strains for cloning and sequencing and

for conjugated bile salt uptake assays, respectively. A plasmid secretion vector, pINIIIA3 (Lunn et al., 1986), was provided by B. Lampson (Energy Biosystems, The Woodlands, Tex.). The pINIIIA3 replicon was constructed from pBR322 and contains an ampicillin resistance gene and an *Eco*RI cloning site. The *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract [Difco Laboratories], 0.5% NaCl [Fisher], pH ~7 with 1M NaOH). Plasmid vectors pUC18 and pUC19 (Gibco BRL) were used for manual and automated fluorescent DNA sequencing. The pSportI (Gibco BRL) cloning vector was used to express the putative transport gene(s) in assays of uptake of bile salts.

BSH assays. BSH activity was detected in *L. johnsonii* strain 100-100 by two methods. In the first, the cells were plated on MRS-TDCA (MRS agar medium containing 0.5% taurodeoxycholic acid [Sigma]), and the activity was detected when deoxycholic acid precipitated around the colonies in the medium (Dashkevicz & Feighner, 1989). In the second, the bacterium was grown in MRS medium overnight to stationary phase. [24-^{14}C]taurocholic acid (New England Nuclear) was added to a 1:10 dilution of the culture in 5 mM sodium acetate buffer (pH 5.0) and incubated for 10 minutes at 37°C. The reaction was stopped by adding 1 mL of both ethyl acetate and 6 M HCl. [24-^{14}C]cholic acid released from the conjugate by BSH activity partitioned into the ethyl acetate. The ethyl acetate solution was placed in scintillation vials to which Ecoscint A scintillation cocktail (National Diagnostics) was added. The amount of [^{14}C]cholic acid produced was estimated with a Beckman LS 7000 liquid scintillation counter (Lundeen & Savage, 1990). The latter assay is referred to below as the isotopic BSH assay. The same assays

were used to detect the activity in *E. coli* strains containing chimeric plasmids. In this case, however, the MRS-TDCA medium contained 100 μg of ampicillin/mL (referred to as MRS-TDCA-AMP) to select for the plasmid chimeras. As previously reported, *L. johnsonii* expressed BSH activity when it was growing at 37°C on agar medium or in liquid medium incubated anaerobically in an atmosphere enriched with CO_2 (Lundeen & Savage, 1990; Savage et al., 1995). *E. coli* strains containing chimeric plasmids also expressed the activity when they were growing on agar media or in liquid media incubated anaerobically (BBL GasPak system with anaerobic system envelope containing palladium catalyst) at 37°C.

Recombinant DNA methods. Total chromosomal DNA was extracted from cells of *L. johnsonii* (Wizard Genomic DNA Purification Kit; Promega) and digested with *Eco*RI (New England Biolabs) according to the manufacturer's instructions. The *Eco*RI-digested pINIIIA3 vector was treated with calf intestinal alkaline phosphatase (New England Biolabs). The digested genomic fragments were ligated with phage T4 DNA ligase (New England Biolabs) into pINIIIA3 according to standard protocols (Protocols and Applications Guide; Promega). The vector chimeras were transformed into *E. coli* HB101 by a standard protocol (Promega). The transformants were plated onto LB agar medium containing 100 μg of ampicillin/mL. Colonies growing on the medium were either replicated or picked with sterile pipette tips to MRS-TDCA-AMP plates, which were then incubated anaerobically. Colonies growing on that medium and containing cells expressing BSH activity were detected within 72 hours by a white precipitate of deoxycholic acid in the surrounding medium (Dashkevicz & Feighner, 1989). Such

colonies were picked, plasmid DNA was extracted (Wizard Plus Midipreps DNA Purification Kit; Promega), and the DNA was electrophoresed in 1% agarose gels with 0.5 μg of ethidium bromide/mL by standard methods (Voytas, 1989).

A construct was engineered from the BSH^+ clone to test for uptake of bile salts. The 2,977-bp insert of pIN-BSH2 was excised with *Eco*RI. The BSH gene was eliminated from that fragment at a unique *Xba*I site 872 nucleotides from the 3' end. The vector, pSportI, was prepared by double digestion with *Eco*RI and *Xba*I by standard methods. Following dephosphorylation with calf intestinal alkaline phosphatase, the 2,109-bp product containing the predicted 5' partial ORF (ORF1) and the largest complete ORF (ORF2) was cloned into the digested pSportI vector under the control of the *lac* promoter. Chimeras were transformed into DH5α cells. Cells harboring the clone, designated p2000, were identified by blue/white colony screening on LB plates containing 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/mL, and 100 μg of ampicillin/mL.

DNA sequencing methods. Nested deletions of cloned *L. johnsonii* DNA were prepared with exonuclease III (New England Biolabs) by standard procedures (Protocols and Applications Guide; Promega). The entire sequence and the truncated fragments were ligated with T4 DNA ligase into predigested and dephosphorylated pUC18 and pUC19 vectors. The chimeric plasmids were transformed into *E. coli* DH5α. Single-stranded DNA prepared from the chimeras was sequenced in both directions manually by the dideoxy chain termination method, as facilitated by the Sequenase Kit (Promega) and

reconfirmed with ABI Prism automated fluorescent sequencing (Molecular Biology Resource Facility, University of Tennessee, Knoxville). α-^{35}S-dATP (New England Nuclear), commercial universal forward and reverse primers (Promega), and other synthesized oligonucleotide walking primers (Gibco BRL) were used.

Extension of genomic DNA sequence. Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu & Whittier, 1995) was used to amplify and extend the genomic DNA sequence 3' of the cloned fragment (see Fig. 1). A nested set of three oligonucleotide primers, 5'-GCTACTCTTCTGGAAGCAAGACTTACTAC-3' (DN1-1), 5'-CTACTGTA ATTTTGAAGATGATTTTGAA-3' (DN1-2), and 5'-AAAGACTTATAAACTAGACGA TCACAC-3' (DN1-3; GIBCO BRL), to the 3' end of the clone were prepared. A degenerate primer, 5'-(G/C)TTG(A/T/G/C)TA(G/C)T(A/T/G/C)CT(A/T/G/C)TGC-3' (AD2), provided by Gary Stacey (University of Tennessee, Knoxville), was also used. Three successive high- and low-stringency PCR amplifications were performed with a genomic template from strain 100-100. Each successive PCR used a different nested sequence-specific primer, the same degenerate primer, and the template from the previous reaction according to guidelines established by Liu and Whittier (Liu & Whittier, 1995). The DNA product from the third TAIL-PCR was purified (Wizard PCR Preps DNA Purification System; Promega) and sequenced by automated fluorescent sequencing using DN1-3 as the primer. The sequence was reconfirmed by directly amplifying genomic DNA with the DN1-1 primer and another sequence-specific primer, 5'-CTTTACTAAAGTAAATCAAATAGTTAGAGGCTGGA-3' (DN1-4; GIBCO BRL), engineered to the 3' end of the new sequence.

DNA and amino acid sequence analysis. DNA sequence was analyzed for predicted start and stop codons with MacVector. ORFs were translated with MacVector into predicted amino acid sequences. The basic local alignment search tool (BLAST; NIH Website) was used to compare amino acid sequences of putative ORFs with published sequences. Kyte Doolittle hydropathy plots were obtained from both Genepro, version 5.00, and the Wisconsin Package (Genetics Computer Group).

Preparation of BSH EF. Taurocholic acid solubilized in 1 mL of sodium acetate buffer (pH 5.0) (final culture concentration, 0.4 mM) was added to 20 mL of an MRS culture of *L. johnsonii* grown anaerobically at 37°C for 20 to 24 hours. The cultures were then incubated for 30 minutes to obtain EF^+ supernatant solution. For EF^- supernatant solution, an equal volume of the acetate buffer was incubated with 20 mL of culture. The cells were pelleted at 5,000 x *g* for 10 minutes; the supernatant solutions were harvested and stored at 4°C (Lundeen & Savage, 1992b).

Isotopic conjugated bile acid uptake and specificity assays. A bile acid uptake assay was adapted from the transport assay of Mallonee and Hylemon (Mallonee & Hylemon, 1996). *E. coli* DH5α p2000 and *E. coli* DH5α pSportI cells were grown overnight to stationary phase (Klett reading, ~220) with shaking at 37°C in LB medium containing 100 μg of ampicillin per mL. The cultures were diluted 50% with LB medium and were incubated for 45 minutes in order to allow the cells to recover in the fresh medium. The cells were induced with 0.5 mM IPTG (final culture concentration) and incubated for an additional 90 minutes. Cell densities were normalized with LB medium to a Klett

reading of approximately 150. For both control (pSportI) and test (p2000) cultures, 8 mL of cells were harvested by centrifugation at 3,000 x *g* for 10 minutes at room temperature. Cell pellets were resuspended and washed in 5 mL of cold 50 mM Tris-HCl (pH 7.5) and centrifuged again at 3,000 x *g* for 10 min. Washed cell pellets were then resuspended in 600 μL of appropriate cold EF^+ or EF^- solutions and placed on ice. Three 200-μL portions of each culture were transferred into 1.5-mL Eppendorf tubes and pre-incubated in a 37°C water bath for 7 to 8 minutes. Fifty nanocuries of either [24-^{14}C]taurocholic acid (0.020 mCi/mL; specific activity, 51 mCi/mmol) (New England Nuclear) or [24-^{14}C]cholic acid (0.1 mCi/mL; specific activity, 54.5 mCi/mmol) (American Radiolabeled Chemicals, Inc.) was then added to each Eppendorf tube. The tubes were incubated at 37°C for 4 minutes, after which 1 mL of ice-cold 100 mM LiCl-100mM potassium phosphate (pH 7.0) was added to each. The samples were immediately centrifuged at 3,000 x *g* for 5 minutes. The supernatant solution was quickly removed from the cell pellet, and the 100 mM LiCl-100 mM potassium phosphate wash and pelleting was repeated once again. Each cell pellet was then digested with 1 mL of formamide (Fisher Biotech) in a 65°C water bath for 1 to 2 hours. The digested cell solution was placed into a scintillation vial containing 10 mL of scintillation cocktail (Ecoscint A; National Diagnostics) and 5 mL of ethanol. The samples were mixed by inverting the vial several times until it was clear. Radioactivity was quantitated in a Beckman LS 7000 liquid scintillation counter. Cholic acid uptake by DH5α cells harboring either pSportI or p2000 was also measured by an alternative procedure adapted from the work of Thanassi et al. (Thanassi et al., 1995). The procedure described above was followed up to and including incubation with [24-^{14}C]cholic acid. Thereafter, however, each of the 200-μL

aliquots was layered on top of 150 μL of silicon oil (105 μL of fluid no. 550 and 45 μL of fluid no. 510) (50 centistokes [cst]; Dow Corning Corp.) in 1.5-mL Eppendorf tubes. The samples were centrifuged at 12,000 x *g* for 2 minutes in order to pellet the cells and then frozen in a dry-ice-ethanol bath. The tips of the Eppendorf tubes containing the cell pellets were cut into scintillation vials. The pellets were resuspended in 0.3 mL of water to which 10 mL of scintillation cocktail was added and mixed. The radioactivity was quantitated as described above. The nonparametric Wilcoxon rank sum test was used as a statistical measure to compare the isotopic data.

Nucleotide sequence accession numbers. The sequences of ORF1, ORF2, and ORF3 have been registered with GenBank under accession no. AF054971 (Appendix A).

Results

Identification of *Lactobacillus* sp. strain 100-100 as *L. johnsonii* strain 100-100. *Lactobacilli* have been found to colonize the keratinized, nonsecreting, squamous epithelium in the stomachs of rodents (Savage, 1977). A rat stomach isolate was chosen for high levels of BSH expression. This isolate had been previously identified as a *Lactobacillus* sp. and given the strain designation 100-100 (Lundeen & Savage, 1990; Savage et al., 1995). It was identified to the species level by Martin Kullen and Todd Klaenhammer (North Carolina State University, Raleigh). Genomic DNA sequencing of the V1 region of its 16S rRNA gene identified it as *L. johnsonii*. The sequence, approximately 75 bp from the 5' end of the rRNA gene, identically matched the same

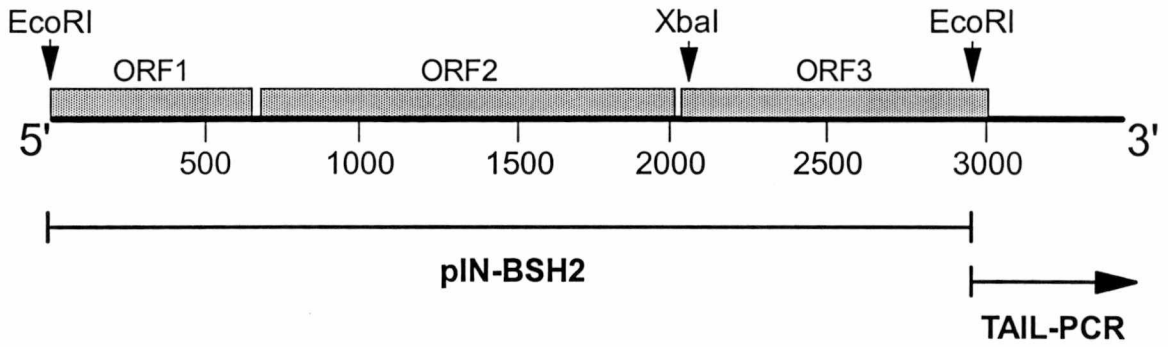


FIG. 1. BSH-positive genomic clone pIN-BSH2 with predicted ORFs.

sequence of GAGCGAGCTTGCCTAGATGATTTTAGTGCTTGCACTAAATGAAA CT in the type strain of *L. johnsonii,* strain VPI 7960.

Cloning of the BSH gene. Genomic DNA from *L. johnsonii* cut with *Eco*RI, ligated into pINIIIA3, and transformed into *E. coli* HB101 yielded two ampicillin-resistant colonies in which the cells expressed BSH activity on MRS-TDCA-AMP agar medium. The activity expressed by the cells in both of the colonies was stable upon transfer to fresh medium. Cells from each of the colonies yielded a plasmid chimera containing an approximately 3,000-bp insert (Fig. 1). The chimeras were designated pIN-BSH1 and pIN-BSH2. The insert in pIN-BSH2 proved to be more stable than that in pIN-BSH1 upon repeated transfer on MRS-TDCA-AMP. Therefore, pIN-BSH2 was selected for further study.

Sequencing and extension of the 2,977-bp insert in pIN-BSH2. Nested deletions in pUC18 and pUC19 of the 2,977 bp insert (Fig. 2) of lactobacillus DNA in pIN-BSH2 were sequenced manually and with an ABI Prism automated fluorescent sequencer. The

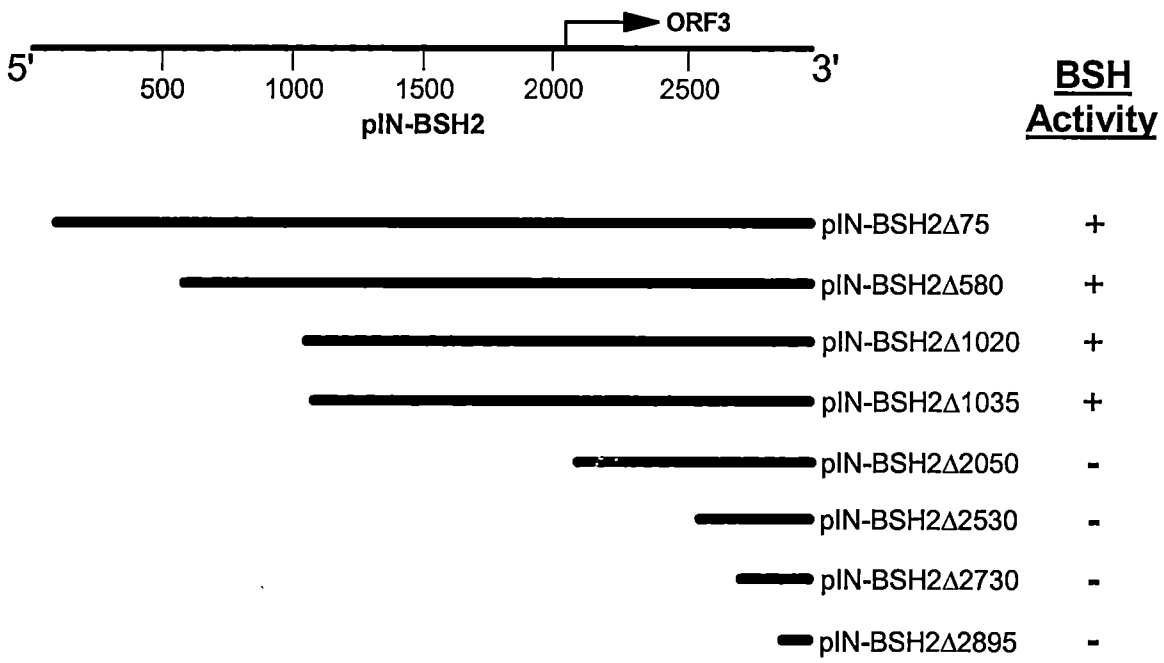


FIG. 2. Exonuclease III 5' deletion mutants of the 2,977-bp clone indicate that BSH activity is lost when the deletions disrupt the predicted start site of ORF3. BSH activity was assayed with MRS-TDCA-AMP. BSH^+ colonies were identified by precipitates of deoxycholate in the medium (Dashkevicz & Feighner, 1989).

sequence was predicted by MacVector to contain one complete ORF (designated ORF2) and two partial ORFs (ORF1 and ORF3) of 1,353, 651, and 927 nucleotides, respectively. The predicted ORFs were arranged in a unidirectional, tandem manner with minimal intergenic sequences of 27 and 19 nucleotides (Fig. 1). DNA sequences centered 8 to 13 nucleotides 5' of ORF2 and ORF3 (AAAGAAGGTAA and TAAGGAGGTTT, respectively) closely matched the Shine-Dalgarno 16S rRNA sequence of TAAGGAGGT GA. The partial ORF3 DNA sequence was extended and completed. A DNA product of approximately 550 bp was amplified by TAIL-PCR (Fig. 1). Once it was sequenced, by using DN1-1 as the primer, the amplified fragment extended the known genomic sequence 458 nucleotides 3' of the *Eco*RI-cloned DNA. A stop codon for ORF3 was predicted by MacVector, resulting in a complete coding sequence of 948 bp. MacVector did not predict any ORFs 3' of the stop codon for ORF3.

Analysis of ORFs. BLAST analysis of the predicted amino acid sequence of ORF3 revealed numerous proteins with high levels of similarity. The highest levels (approximately 70% similarity to the published consensus sequence [Coleman & Hudson, 1995]) were found for the products of the two BSH genes: the BSH from *C. perfringens 13* (Coleman & Hudson, 1995) and the choloylglycine hydrolase gene from *L. plantarum 80* (Christiaens et al., 1992). In addition, three conserved amino acid motifs, DXXNEXGL, GVXTNXP, and GXGXXGLPGD at residues 72, 174, and 217, respectively, were conserved within the two cloned hydrolases as well as within ORF3. BLAST analysis of ORF2 yielded many sequences with similarity. Those with highest levels of similarity were several monoamine, acetylcholine, and multidrug resistance

transporters of the major facilitator superfamily (MFS). BLAST analysis of the incomplete ORF1 yielded only one similar nucleotide sequence: that of ORF2. The nucleotide sequence of the entire partial ORF1 is 72% identical to the carboxyl half of ORF2 (Fig. 3). A Kyte-Doolittle hydropathy plot of ORF2 predicted 12 transmembrane domains arranged in a 6-plus-6 pattern (Fig. 4). This arrangement is characteristic of the MFS and the ATP-binding cassette (ABC) superfamily of transporters (Nikaido & Saier, 1992; Marger & Saier, 1993). The hydropathy plot for the carboxyl half of ORF1 revealed six transmembrane domains (Fig. 4).

Expression of BSH activity in *E. coli* HB101/pIN-BSH2 and in *E. coli* DH5α/pUC18 and *E. coli* DH5α/pUC19 nested deletion constructs. *E. coli* HB101 cells containing pIN-BSH2 but not pINIIIA3 expressed BSH activity not only while growing on MRS-TDCA-AMP plates but also in a radioisotopic assay after being grown in LB-AMP broth (Fig. 5). Cells containing nested deletion constructs, which were made with exonuclease III, ligated into pUC18 and pUC19 for sequencing, and transformed into *E. coli* DH5α, were plated on MRS-TDCA-AMP plates to monitor functional BSH activity. Once the exonuclease deletions compromised the predicted start site of ORF3, as with constructs pIN-BSH2Δ2050 through pIN-BSH2Δ2895 (Fig. 2), no deoxycholic acid precipitate, as an indicator of BSH activity, was produced in the medium. Therefore, ORF1 and ORF2 were not required for functional BSH activity in *E. coli* HB101. It should be noted that ORF3 in pIN-BSH2 lacks the 7 carboxyl-terminal amino acid residues of BSH. Thus,

FIG. 3. Predicted amino acid sequences of ORF1 and ORF2 (see Fig. 1). Identical residues are indicated by black highlighting. Grey shading indicates conservative substitutions.

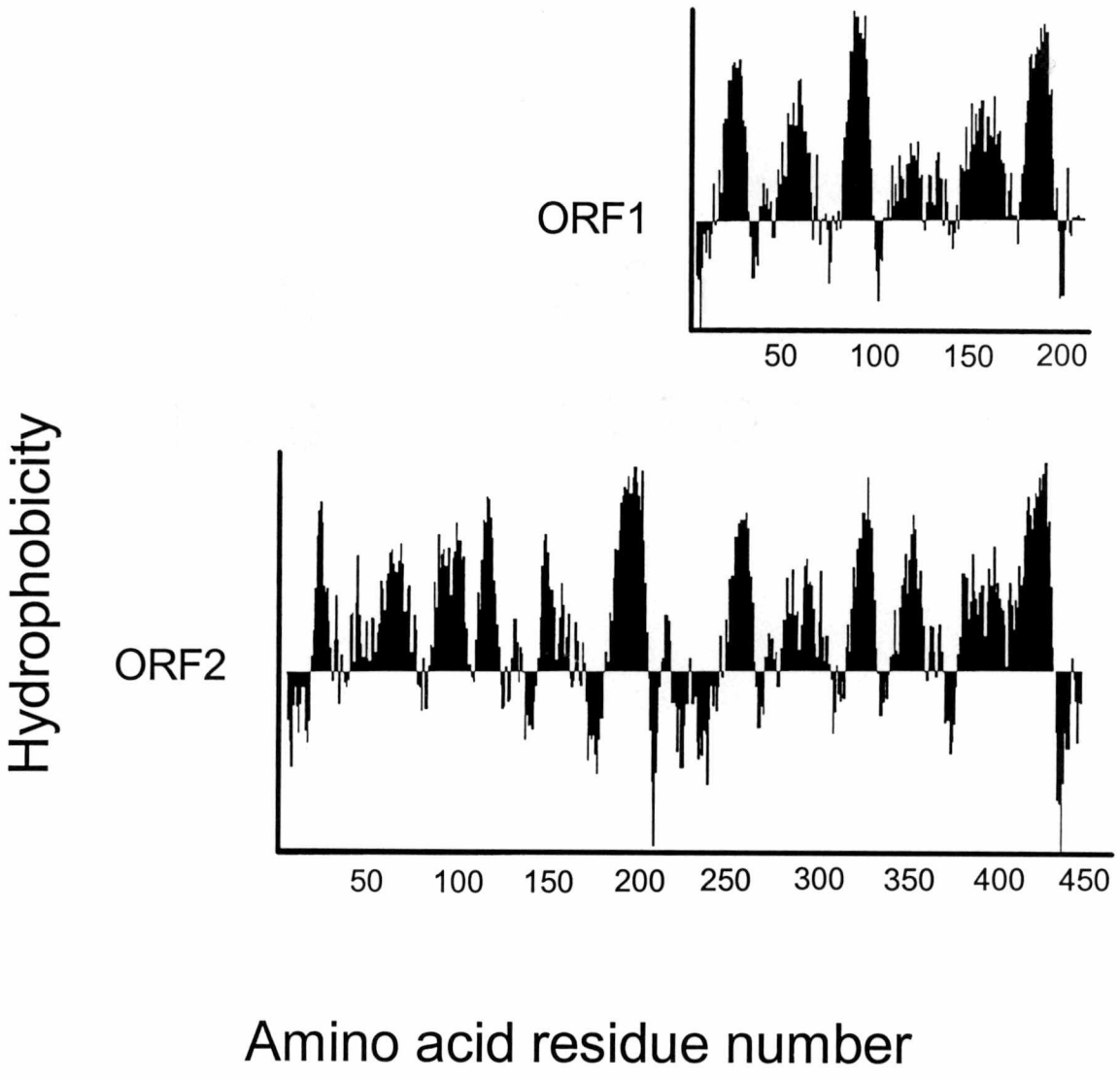


FIG. 4. Hydrophobicity plots of ORF1 and ORF2 (see Fig. 1). The hydrophobicity model of ORF2 predicts 12 transmembrane domains arranged in a 6-plus-6 motif characteristic of transport proteins in the MFS. The carboxyl end of the partial ORF1 indicates a similar structure. The plots were prepared with Genepro, version 5.00.

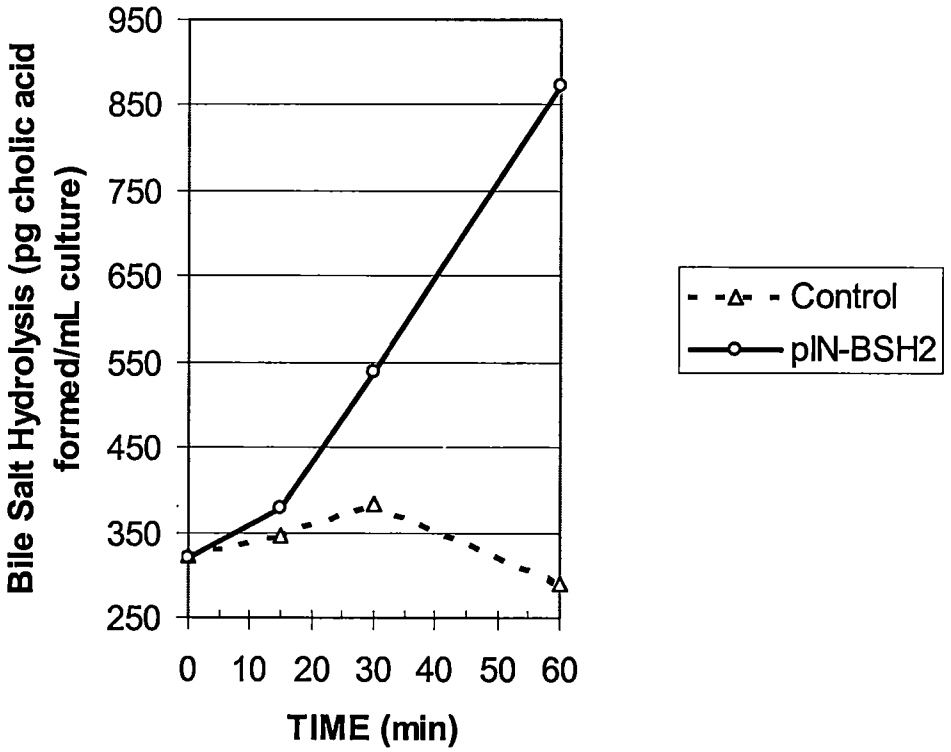


FIG. 5. Bile salt hydrolysis of *E. coli* HB101 cells harboring pIN-BSH2 as assayed by liquid scintillation counting of [24-^{14}C]cholic acid released from [24-^{14}C]taurocholic acid. *E. coli* HB101 cells containing pIN-BSH2 that has been heated to 85°C for 5 minutes were used as a control.

these residues are not required for BSH activity. However, all further deletion mutants from the 3' end of the clone resulted in loss of BSH activity.

Identification of ORF1 and ORF2. A hypothesis that ORF2 is involved in the uptake of conjugated bile salts was tested with a construct from which the predicted BSH had been deleted. Cells harboring the clone, p2000, were assayed for the ability to take up [24-^{14}C]taurocholic acid (Table 1). The uptake assays were performed on stationary-phase cultures grown aerobically at 37°C to a Klett reading of approximately 150. Cultures harboring either pSportI or p2000 and not treated with IPTG or EF took up [24-^{14}C]taurocholic acid in equivalent amounts. By contrast, the cultures harboring p2000 and induced with IPTG took up significantly more of the bile salt than cultures harboring pSportI similarly induced. Likewise, when not induced with IPTG, cells containing p2000 exposed to EF demonstrated more uptake of the bile salt than cells containing pSportI. The highest levels of uptake were detected when cells containing p2000 were both induced with IPTG and suspended in EF^{+} supernatant solution (Table 1). I conclude from these findings that ORF2, and possibly also ORF1, encode proteins involved in the transport of bile salts.

Role of EF. EF appeared to function as an enhancer of the transport of bile salts facilitated by ORF2. However, cells containing pSportI, which lacked the putative transporters, exhibited higher levels of uptake of [24-^{14}C]taurocholic acid when exposed to EF (Table 1). Therefore, EF appears to facilitate the uptake of bile salts, nonspecifically, at least in *E. coli.*

TABLE 1. Uptake of [24-^{14}C]taurocholic acid by *E. coli* DH5α cultures[a] containing cloned genomic DNA from *L. johnsonii* strain 100-100

Vector[b]	Treatment with:		Mean uptake ± σ (cpm)[e]	T value[f]
	IPTG[c]	EF[d]		
pSportI	-	-	1688 ± 120	
p2000	-	-	1782 ± 132	71
pSportI	+	-	1398 ± 168	
p2000	+	-	2037 ± 214	45
pSportI	-	+	2424 ± 172	
p2000	-	+	3066 ± 328	48
pSportI	+	+	2175 ± 229	
p2000	+	+	3507 ± 315	45

[a]Cultures were normalized to Klett readings of 144-158.
[b]pSportI vector has no genomic insert. p2000 is pSportI vector containing a 2,109-bp fragment of genomic DNA from *L. johnsonii* strain 100-100, including partial ORF1 and entire ORF2.
[c]Cultures were induced with 0.5 mM IPTG.
[d]Assays were performed with cell cultures suspended with or without EF produced by *L. johnsonii* strain 100-100.
[e]Arithmetical means were calculated from nine data points representing three separate experiments of three replicates each. σ, standard deviation.
[f]Statistical values were obtained from a nonparametric Wilcoxon rank sum test using a one-tailed *P* value of 0.025. T values <63 indicate that the two data sets tested are statistically different with 97.5% confidence.

Specificity of uptake of bile salts. EF is produced and BSH activity is increased three- to fivefold within 20 minutes after 0.4 mM (final culture concentration) conjugated bile salts such as taurocholic acid (but not deconjugated acids) are added to overnight cultures of *L. johnsonii* strain 100-100 (Lundeen & Savage, 1990; 1992b). This finding was confirmed and extended in assays of BSH activity in cells of strain 100-100 exposed to either conjugated or unconjugated bile salts or free amino acids. Only the conjugated bile salts enhanced BSH activity (Fig. 6). Both conjugates tested, taurocholic acid and taurochenodeoxycholic acid, increased the BSH activity of strain 100-100 more than threefold (5,234.0 ± 234.2 and 5,145.1 ± 850.6 pg of cholic acid formed/min/mL of culture, respectively) over control values (1,428.0 ± 424.6 pg of cholic acid formed/min/mL of culture) obtained with cultures incubated in medium free of bile salts. None of the unconjugated bile salts and free amino acids tested enhanced the BSH activity of strain 100-100. To test whether this specificity extended to the uptake of bile salts in *E. coli* DH5α cells containing p2000, I induced such cells with IPTG and exposed them to EF. These cells exhibited two- to threefold-higher levels of uptake of [24-^{14}C]taurocholic acid than control cells containing pSportI that had been induced with IPTG but not exposed to EF. By contrast, induced cells from the same culture containing p2000 and exposed to EF exhibited lower levels of uptake of the deconjugated [24-^{14}C]cholic acid than the control cells when uptake was assayed with wash buffer (Table 2). However, when the cells were centrifuged in silicon oil, levels of uptake by cells containing p2000 were similar to those of cells containing pSportI (Table 2).

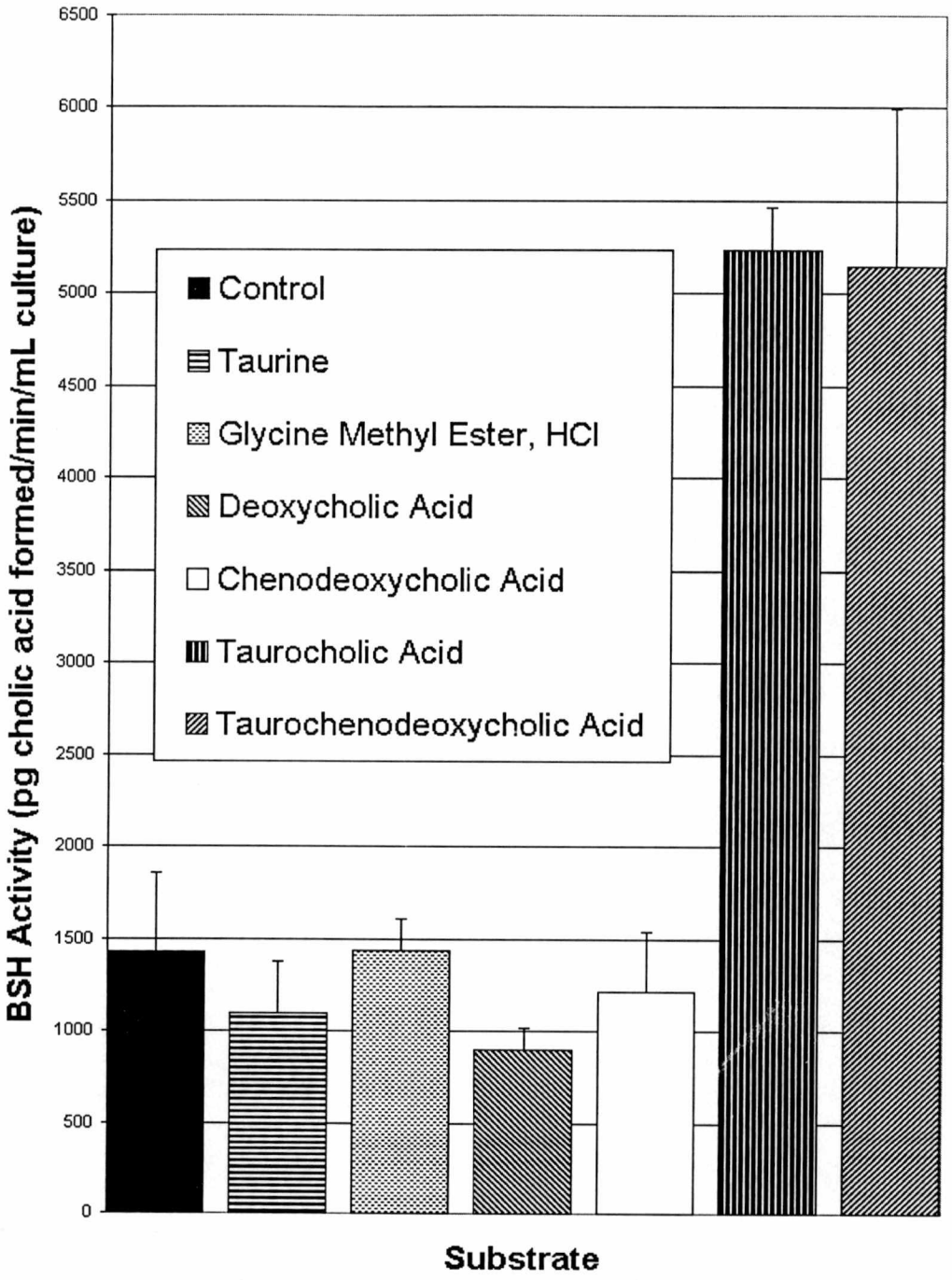


FIG. 6. BSH activity of *L. johnsonii* strain 100-100 when incubated with various bile acids and amino acids in the culture medium. Control values were obtained with cultures incubated in medium free of bile salts. Error bars represent standard deviation.

TABLE 2. Specificity of bile salt uptake by *E. coli* DH5α cultures[a] containing cloned genomic DNA from *L. johnsonii* strain 100-100

Vector[b]	Treatment with EF[c]	Bile acid[d]	Mean uptake ± σ (cpm)[e]	T value[f]
pSportI	-	TCA	1539 ± 151	
p2000	+	TCA	3050 ± 180	21
pSportI	-	CA	5396 ± 1121	
p2000	+	CA	4459 ± 176	21
pSportI	-	CA	10464 ± 644[g]	
p2000	+	CA	10900 ± 837[g]	33

[a]Cultures were normalized to a Klett readings of 153-155. All cultures were induced with 0.5 mM IPTG.
[b]See Table 1, footnote b.
[c]Assays were performed with cell cultures suspended with or without enhancing EF produced by *L. johnsonii* strain 100-100.
[d]TCA, taurocholic acid; CA, cholic acid.
[e]Uptake was measured by an assay involving washing of cells with 100 mM LiCl-100 mM potassium phosphate (pH 7.0). Arithmetical means were calculated from six data points representing two separate experiments of three replicates each. σ, standard deviation.
[f]Statistical values were obtained from a nonparametric Wilcoxon rank sum test using a one-tailed *P* value of 0.025. T values of <26 indicate that the two data sets tested are statistically different with 97.5% confidence.
[g]Uptake was measured by the method of Thanassi et al. (Thanassi et al., 1995), involving centrifugation of unwashed cells through silicon oil.

Discussion

My evidence supports a hypothesis that the 2,977-bp genomic clone from *L. johnsonii* strain 100-100 contains genes encoding two bile acid-related functions that are heterologously expressed in *E. coli* cells: BSH activity and transport of conjugated bile acids. Nucleotide sequence analysis of the cloned DNA predicted three clustered ORFs. 5' Deletion mutants of the genomic clone lost BSH activity when the predicted start site of ORF3 was compromised. In addition, BLAST analysis revealed that the predicted amino acid sequence of ORF3 had the greatest similarity to the predicted sequences of the two BSH genes previously reported (Christiaens et al., 1992; Coleman & Hudson, 1995). The molecular weight of 34,767 calculated for the protein product from ORF3 more closely approximates the estimated molecular weight of BSH β (38,000) than that of BSHα (42,000) (Lundeen & Savage, 1992a). Furthermore, the N-terminal 25-amino-acid sequence of BSHα does not match the predicted N-terminal amino acid sequence of BSH β (Lundeen & Savage, 1992a). I tentatively conclude, therefore, that ORF3 encodes peptide β. The gene encoding peptide α (Lundeen & Savage, 1992a) has yet to be cloned but may lie within close proximity to this group of functionally related genes.

The similarities of the predicted amino acid sequences of the incomplete ORF1 and the complete ORF2 to each other and to transporter proteins, as well as the proximity of these genes to the BSH gene, indicated that they may function as bile acid transporters. The p2000 construct containing the partial ORF1 and the complete ORF2 increased the uptake of conjugated bile salts in *E. coli* cells, especially when they were supplemented

with EF. I conclude from these findings that ORF2, and possibly ORF1, may encode a conjugated bile acid transporter.

In addition to enhancing the uptake of conjugated bile salts in *E. coli* transformed with p2000, EF also enhanced the uptake of the conjugates in cells transformed with the vector pSportI. EF may act as a general enhancing factor mimicking the binding proteins of ABC transport systems in gram negative bacteria (Nikaido & Saier, 1992). EF is produced when conjugated but not deconjugated bile salts are present in the growth medium. Since EF also enhances the uptake of conjugated bile salts, I tested whether the putative transporter would transport only such salts. Therefore, the p2000 construct was tested for the ability to take up either taurocholic or cholic acid.

As consistently observed (Table 1), *E. coli* cells transformed with p2000 and treated with EF took up more conjugated bile salts than cells containing the vector. However, such cells took up no more [24-^{14}C]cholic acid than cells transformed with pSportI (Table 2). When silicon oil centrifugation was used, the amount of deconjugate taken up was over sixfold higher than the amount of conjugate. The physical properties of deconjugated and conjugated bile salts differ significantly. The deconjugate, cholic acid, has a pK_a value close to 7 and consequently may diffuse readily across a bacterial membrane. When I assayed the uptake of cholic acid without using silicon oil centrifugation and washed the cells twice with 100 mM LiCl-100 mM potassium phosphate, much of the deconjugate may have diffused out from the cells. Recent work by Thanassi et al. indicates that the *E. coli* genome encodes a deconjugated bile acid exporter (Thanassi et al., 1997). The capacity of *E. coli* to export deconjugated bile acids suggests that these molecules enter the cells passively. In contrast, conjugated bile acids,

with their low pK_a values, cannot enter bacterial cells passively and consequently require specific transporters.

My data from BSH expression and bile acid uptake experiments indicate that the three clustered ORFs of the 2,977 bp clone are functionally related. I suggest that this clone is part of a coordinately regulated operon. The genes encoding two phenotypes have yet to be cloned: the peptide of BSH α and the compound with EF activity. In addition, there may be a gene encoding a deconjugated bile acid exporter. The 7α-dehydroxylation (*bai*) pathway of a *Eubacterium* sp. (White et al, 1988; Mallonee et al., 1990) consists of several genes arranged in an operon. That pathway is involved, however, in one of several known bile acid side chain transformations (Baron & Hylemon, 1997), while the putative BSH operon in *L. johnsonii* is involved in intracellular deconjugation of conjugated bile salts.

The BaiG gene of the *bai* pathway was the first bile acid transporter reported in a prokaryote (Mallonee & Hylemon, 1996). Its sequence similarity to other transport genes and proposed membrane topology suggest that it may be part of the MFS of transport proteins (Mallonee & Hylemon, 1996). However, the BaiG gene is a deconjugated bile acid transporter, while ORF2 is a transporter of conjugated bile acids. In addition, ORF2 is enhanced in function by EF. ORF2, like BaiG, exhibits homology to multidrug resistance MFS transporters; unlike BaiG, ORF2 also exhibits homology to vesicular monoamine transporters.

Bacterial members of the autochthonous microbiota may have evolved BSH-related functions in order to obtain energy sources under anaerobic conditions (Baron & Hylemon, 1997) and therefore to be able to outcompete allochthonous species in habitats

in the digestive tract. Alternatively or in addition, the functions may operate to protect the bacteria from bile acid toxicity. Since high concentrations of bile acids are toxic to bacteria (Floch et al., 1971), tight regulation of conjugated bile acid transport by the bacterium may exist to protect it from toxic levels of the substrate. Identifying the genes involved and genetic regulation of the BSH capacity in *L. johnsonii* strain 100-100 may help explain this function in the ecology of the gut.

Acknowledgments

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PART III

GENETIC CHARACTERIZATION OF BILE SALT HYDROLASE ACTIVITY IN CERTAIN *LACTOBACILLUS* SPECIES

Abstract

Lactobacillus johnsonii strain 100-100 expresses two antigenically distinct conjugated bile salt hydrolases (BSH), α and β, that combine to form native homo- and heterotrimers. I now report characterization of loci within the genome that encode this capacity. Genomic DNA sequence from strain 100-100 was extended 5' and 3' of a clone of a locus that encodes BSHβ (*cbsHβ*) and a partial (*cbsT1*) and a complete conjugated bile salt transporter (*cbsT2*). DNA sequence analysis revealed a complete open reading frame (ORF) for *cbsT1* and no other ORFs in tandem. Stationary-phase mRNA from strain 100-100 was analyzed with long primer extension and amplification PCR. With this method, the three genes, *cbsT1*, *cbsT2*, and *cbsHβ*, were shown to encode an operon and a putative promoter was identified. A second locus that expresses BSH activity in strain 100-100 was identified functionally from a genomic library of partially digested *Sau*3AI fragments. Sequence analysis of the clone predicted a 978 nt ORF that did not share tandem organization with other ORFs, was similar in sequence to other BSH genes, and matched, in predicted protein sequence, the first 25 amino acids of BSHα. A phenotypic screen for BSH activity and a genetic screen for the *cbsHβ* locus were performed on fifty *Lactobacillus* isolates from humans or dairy products. Nearly all of the isolates that were positive for *cbsHβ* were from human sources. Variability in the BSH phenotype and *cbsHβ* genotype was identified in isolates of the same species. I obtained and analyzed DNA sequence from the *cbsHβ* locus of one human isolate, *L. acidophilus* strain KS-13. This organism encodes three genes that are 85% identical in

DNA sequence to those of strain 100-100. DNA sequence identity to strain 100-100 ends 5' and 3' of this locus. I have cloned the genetic determinants for BSH activity in strain 100-100 and have extended my analysis to lactobacilli of several species. My findings suggest that BSH genes have been acquired horizontally and that the activity is important at some level for lactobacilli to colonize the lower gastrointestinal tract.

Introduction

The autochthonous microbiota inhabiting the lower gastrointestinal (GI) tract of animals (Savage, 1977) have evolved the ability to alter bile acids biochemically (Baron & Hylemon, 1997). Prokaryotic organisms from several genera express proteins that catalyze hydrolysis of an amide bond between the C-24 position of the steroid moiety and an amino acid, either glycine or taurine (deconjugation; Savage et al., 1995; Baron & Hylemon, 1997; Savage, 2000). This activity may affect animal health including that of humans. For example, it is associated with growth depression in monogastric animals grown for food (Eyssen & De Somer, 1963; Cole & Fuller, 1974). In humans, it may contribute to colon carcinogenesis (Kay, 1981; Cheah, 1990; Kandell & Bernstein, 1991) but may lower blood cholesterol levels (De Smet et al., 1994). Some bacterial species express enzymes that catalyze side-chain (sterol) transformations such as 7α- and 7β-dehydroxylation, 6β-dehydrogenation, and desulfation (Baron & Hylemon, 1997; Hylemon & Harder, 1999). Bile salt hydrolysis, however, is the principle microbial reaction in that it performs a "gatekeeping" function; bile salts must be deconjugated before sterol transformations can occur (Batta et al., 1990).

The class of microbial enzymes that catalyze deconjugation have been collectively named conjugated bile salt hydrolases (BSH, EC 3.5.1.24). These enzymes can have demonstrable specificity for tauro- versus glyco-conjugates (Aries & Hill, 1970; Gilliland & Speck, 1977), and with the exception of one expressed by *Clostridium perfringens* (Kishinaka et al., 1994), are soluble cytosolic proteins (Stellwag & Hylemon, 1976; Gopal-Srivastava & Hylemon, 1988; Kawamoto et al., 1989; Lundeen & Savage, 1990; Grill et al., 1995; Tanaka et al., 2000). The molecular fate of the amino acid released by deconjugation in the bacterial cell and the benefit that the organisms may derive from such activity is unclear (De Smet et al. 1995). The deconjugated product, or primary bile acid, is a toxic hydrophobic molecule that can dissagregate the cytoplasmic membrane (Thanassi et al., 1997). These molecules are not metabolized and leave the cell passively or through some unidentified bile acid export mechanism. At the physiological pH of the intestinal lumen, deconjugates can be actively transported through the epithelium (Wong et al., 1994; Hylemon & Harder, 1999) and into the bloodstream of the host or are precipitated in the feces and excreted (Baron & Hylemon, 1997).

BSH enzymes have been purified and characterized from *Bacteroides vulgatus* VI-31 (Kawamoto et al., 1989), *B. fragilis* subsp. *fragilis* (Stellwag & Hylemon, 1976), *Bifidobacterium longum* BB536 (Grill et al., 1995) and SBT2928 (Tanaka et al., 2000), *C. perfringens* MCV 815 (Gopal-Srivastava & Hylemon, 1988), and *Lactobacillus johnsonii* strain 100-100 (Lundeen & Savage, 1990; 1992a). Genes encoding this activity have been cloned from *L. plantarum* 80 (Christiaens et al., 1992), *C. perfringens* 13 (Coleman & Hudson, 1995), *L. johnsonii* strain 100-100 (Elkins & Savage, 1998), and *B.*

longum SBT2928 (Tanaka et al., 2000). Genetic characterization of these loci reveal different architectures. The *L. plantarum* 80 BSH transcript is monocistronic (Christiaens et al., 1992); *L. johnsonii* strain 100-100 BSH is arranged in tandem with at least two other functionally-related genes (Elkins & Savage, 1998); and the *B. longum* SBT2928 BSH is coordinately regulated with at least one other gene that shares a high level of amino acid homology with glutamine synthetase adenylyltransferase (*glnE*; Tanaka et al., 2000). The extent of the operon in *B. longum* SBT2928 and the putative operon in *L. johnsonii* strain 100-100 is not known (Elkins & Savage, 1998; Tanaka et al., 2000); DNA sequence flanking the *C. perfringens* 13 BSH gene has not been characterized (Coleman & Hudson, 1995).

L. johnsonii strain 100-100 expresses high levels of BSH activity (Lundeen & Savage, 1990). In contrast to other bacteria studied, the activity in strain 100-100 involves four enzymes that exist as homo- and heterotrimers of two antigenically distinct proteins, designated α and β (Lundeen & Savage, 1992a). A 2,977-bp *Eco*RI genomic clone, pIN-BSH2, that expresses hydrolase activity in *Escherichia coli* cells has been identified and shown to encode the β peptide (ORF3, hereafter referred to as *cbsHβ*; Elkins & Savage, 1998). A 651 nucleotide (nt) partial ORF1 (hereafter referred to as *cbsT1*) and a 1,353 nt complete ORF2 (hereafter referred to as *cbsT2*) were encoded on the fragment 5' of *cbsHβ*. These ORFs share similarity with transport proteins of the major facilitator superfamily (MFS; for review, see Saier et al., 1999) and approximately 80% predicted amino acid sequence similarity to each other. They increase uptake of conjugated bile acids by as much as threefold over control levels when expressed in *E.*

coli cells that were exposed to an extracellular factor (EF; Lundeen & Savage, 1990; 1992b) produced by strain 100-100 (Elkins & Savage, 1998).

I have characterized this locus and report here that *cbsT1*, *cbsT2*, and *cbsHβ* comprise a BSH operon. In addition, I report a second locus that encodes BSH α. I have also assayed a collection of fifty *Lactobacillus* sp. for BSH activity and the *cbsHβ* locus. I speculate that BSH activity was acquired horizontally and implicate the activity as an important factor for colonization of the GI tract.

(A preliminary account of this study was presented at the 100th General Meeting of the American Society for Microbiology, Los Angeles, CA.)

Materials and Methods

Culture techniques. *L. johnsonii* strain 100-100 was maintained at –80°C in 15% glycerol for cryoprotection and grown anaerobically in MRS broth or agar medium (Becton Dickinson) as previously reported (Lundeen & Savage, 1990; Savage et al., 1995). Strains of numerous *Lactobacillus* sp. obtained from several culture collections (Table 1) were received as lyophiles, revived anaerobically in MRS media, and subcultured. Glycerol stocks of each strain were prepared from isolated colonies and handled identically to strain 100-100.

Oligonucleotide primers. Primers used in this study (Table 2) were synthesized by Gibco BRL custom primers division under standard conditions.

TABLE 1. BSH phenotype and *cbsHβ* genotype in *Lactobacillus* sp. strains

Lactobacillus sp.	Collection[a]/Strain	Source	BSH Activity[b]	*cbsHβ* locus[c]
acidophilus	ATCC/4356	Human	+	-
acidophilus	ATCC/4357	Human	+	-
acidophilus	ATCC/53544	Human infant, rectal swab	+	-
acidophilus	BCCM/8151	Acidophilus milk	0	-
acidophilus	JCM/1028	Human intestine	+	+
acidophilus	JCM/1034	Human intestine	+	+
acidophilus	JCM/1229	Human intestine	+[d]	-
acidophilus	NCSK/1070 (ATCC/700396)	Unknown	+	-
acidophilus	PI/KS-13	Human	+	+
Brevis	ATCC/14869	Human feces	-	-[e]
brevis	BCCM/7944	Human feces	+	-
brevis	BCCM/11998	Starter from dairy	+	-
brevis	BCCM/18022	Zabady (yogurt)	+	-
buchneri	JCM/1068	Human intestine	-	-
buchneri	JCM/1069	Human intestine	+	-
delbrueckii subsp. *bulgaricus*	ATCC/11842	Bulgarian yogurt	0	+
delbrueckii subsp. *bulgaricus*	BCCM/12168	Homemade yogurt	0	-
delbrueckii subsp. *bulgaricus*	DSMZ/20080	Yogurt	0	-[e]
fermentum	ATCC/11976	Intestine of infant	+[d]	-
fermentum	ATCC/23271	Human intestine	-	-
fermentum	BCCM/6902	Fermented Beets	-	-
fermentum	DSMZ/20050	Unknown	-	-
gasseri	ATCC/19992	Feces	-	+
gasseri	BCCM/9203	Human	+	+
gasseri	JCM/1025	Human intestine	+	+
gasseri	NCSK/99	Unknown	+[d]	+
johnsonii	JCM/1022	Human intestine	+	+
johnsonii	JCM/8791	Human feces	+	+
plantarum	BCCM/18021	Milk	+[d]	-
plantarum	BCCM/18027	Laban Rayeb	+[d]	-
plantarum	BCCM/18035	Milk	-	-

[a] American Type Culture Collection, ATCC; Japanese Collection of Micro-organisms, JCM; Belgian Co-oridinated Collections of Micro-organisms, BCCM; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), DSMZ; North Carolina State University - Todd Klaenhammer, NCSK; Principal Investigator - our lab, PI.

[b] From S. A. Moser and D. C. Savage, submitted for publication; *Lactobacillus* strains were streaked on MRS plates containing 0.5% taurodeoxycholic acid (MRS-TDCA). +, a halo of deoxycholic acid precipitate around bacterial growth (Dashkevicz & Feighner, 1989); -, no halo present; 0, no growth on MRS-TDCA plates.

[c] *cbsHβ* locus was screened with oligonucleotide primers Hβ675a and Hβ675b using standard PCR. Positive, a single 675 bp PCR product; negative, no amplification of genomic DNA.

[d] Weakly positive on MRS-TDCA plates.

[e] Results negative, a ladder of bands were obtained.

TABLE 2. Oligonucleotide primers used in this study

Primer	Nucleotide sequence listed 5' to 3'	Strand[a]	Use
AP	CGAGGAATTCGGGGGGGGGGGGG	NA*	LDGW[b], LPEA[c]
UP-1	CCCTAAACCAATAATTAAAAAGCCG	-	LDGW
UP-2	GCCGATACTAAAAATCCAGAACCATTTC	-	LDGW
UP-3	CCATTTCATGTGAAGAACTTGCTTGCCGCTCC	-	LDGW
UP-4	CCTTACAGAAAGTTAATTTTAAAATTTTGTATTTG	-	LDGW
DN-1	GCTACTCTTCTGGAAGCAAGACTTACTAC	+	LDGW
DN-2	CTACTGTAATTTTGAAGATGATTTTGAA	+	LDGW
DN-3	AAAGACTTATAAACTAGACGATCACAC	+	LDGW
DN-4	CTTTACTAAAGTAAATCAAATAGTTAGAGGCTGGA	+	LDGW
Hβ-1	AATCCTGTCCAACATCTAGATTACG	-	LPEA
T1-1	CCATTTCATGTGAAGAACTTGCTTGCCGCTCC	-	LPEA
T1-2	ACACCTTGACCAGAATAAAAGTTGAACCAACC	-	LPEA
T1strt	CGACAATATAAGTGGgTAccGTCAAAGCTC[d,e]	+	Cloning, pT1T2Hβ
T2strt	GGTTTTGCTATTGGTaCCCCACTATTTGGC[d,e]	+	Cloning, pT2Hβ
Hβstrt	GGCTATGCCATTGGTaCcCCACTATTTGG[d,e]	+	Cloning, pHβ
Hβend	CGACCAGGCTTGATgGATCCGC[d,f]	-	Cloning, pT1T2Hβ, pT2Hβ, and pHβ
Hβ675a	ATGTGTACTGGTTTAAGATTC	+	PCR screen for *cbsHβ*
Hβ675b	GGCTGGAATGCTGTCACCAGGCAGACC	-	PCR screen for *cbsHβ*
La-Hβa	GCTATTGGTaCcCCATTATTCGG[d,e,g]	+	Cloning, pLaHβ
La-Hβb	GTCATTTGGGAgGTAcCTGTTGAATCAGG[d,e,g]	-	Cloning, pLaHβ

[a] Primers were engineered to the coding strand (+) or the noncoding strand (-) of the *cbsT1, cbsT2,* and *cbsHβ* locus in *L. johnsonii* strain 100-100. * Not applicable, AP primer duplicated from procedure outlined by Min & Powell (1998).

[b] LDGW, long distance genome walking (Min & Powell, 1998).

[c] LPEA, long primer extension and amplification.

[d] Lower case nucleotides indicate base substitutions in the sequence used to create restriction enzyme sites (underlined).

[e] *Kpn*I site.

[f] *Bam*HI site.

[g] Primers were engineered to *cbsHβ* locus in *L. acidophilus* strain KS-13.

Extension of genomic DNA sequence. Long distance genome walking polymerase chain reaction (LDGW PCR; Min & Powell, 1998) was used to amplify and extend DNA sequence 5' and 3' of the strain 100-100 locus in pIN-BSH2 (Fig. 1). The hemianchored PCR method extends from genomic DNA of known sequence into adjacent DNA of unknown sequence using a long and accurate Advantage cDNA Polymerase Mix (ADP; Clontech), nested sequence specific primers (SSPs), oligo-dG adaptor primer (AP), and thermocycling parameters established by Min and Powell (Min & Powell, 1998). Genomic DNA was purified from strain 100-100 cells (Wizard Genomic DNA Purification Kit; Promega). LDGW was performed on genomic DNA with nested SSPs, UP-1 and UP-2, that were engineered to the 5' end of the 2,977-bp *Eco*RI fragment. The LDGW protocol was modified to include a second nested amplification with UP-3 that eliminated contaminating DNA species and enriched for the genomic DNA sequence of interest. DNA from the second reaction was purified (Wizard PCR Preps DNA Purification System; Promega) and sequenced with a fourth nested SSP, UP-4, and walking primers. The sequence was reconfirmed by directly amplifying genomic DNA with UP-1 and a primer engineered to the 3' end of the new sequence. The same strategy was applied to DNA sequence 3' of the cloned locus with primers, DN-1, DN-2, DN-3, and DN-4. DNA sequence 5' and 3' of a 675 bp PCR amplified locus in *L. acidophilus* strain KS-13 was also obtained with LDGW (primers not shown).

RNA transcript analysis. Strain 100-100 is known to express BSH activity upon entry into stationary phase (Lundeen & Savage, 1990). Whole cell RNA was harvested from stationary phase cells (TRI Reagent; Molecular Research Center, Inc.). Approximately 2

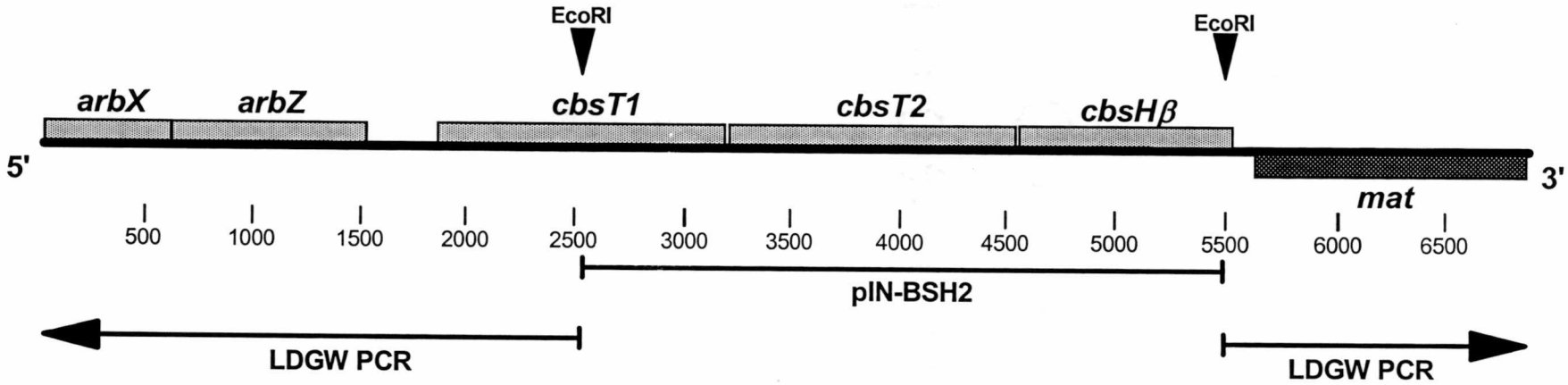

FIG. 1. LDGW PCR (Min & Powell, 1998) was used to amplify strain 100-100 genomic DNA 5' and 3' of an *Eco*RI cloned locus, pIN-BSH2 (Elkins & Savage, 1998), and revealed a complete ORF for *cbsT1* and three additional ORFs: *arbX*, *arbZ*, and *mat*. BLAST analysis identified high similarity of the three ORFs to glycosyltransferases, phospho-β-glycosidases, and group II intron encoding maturases, respectively.

mL of cells were incubated with a 120 μL solution of 10 mg/mL lysozyme (Sigma)-10 mg/mL lysostaphin (Sigma) for one hour at 37°C prior to RNA isolation to faciliate lysis of the gram positive bacteria. Analysis of the mRNA that encodes *cbsHβ* was performed with long primer extension and amplification (LPEA) PCR. LPEA was developed by my lab and is a hybrid protocol of two PCR-based methods: rapid amplification of cDNA ends (RACE; Edwards et al., 1995) and LDGW (Min & Powell, 1998). It involves the use of Moloney Murine Leukemia Virus-reverse transcriptase (M-MLV RT) and ADP to extend over long distances (>7-kb) on template nucleic acids. A microcentrifuge tube containing approximately 2 μg of strain 100-100 RNA and 50 pmol of primer Hβ-1, engineered complementary to the 5' end of *cbsHβ*, was adjusted to a final volume of 15 μl with dH_2O, heated to 70°C for 5 minutes, and immediately cooled on ice. Two hundred units of M-MLV RT (Promega), 5 μL of 5X M-MLV RT reaction buffer, 10 pmol of each dNTP (UltraPure; Clontech), and 25 units of rRNasin Ribonuclease inhibitor (Promega) were added to a final volume of 25 μL. The tube was incubated at a stringent temperature for the M-MLV RT of 42°C for 60 minutes. DNA was purified (Wizard PCR Preps DNA Purification System; Promega) from two such RT reactions and concentrated to approximately 15 μL. A dCTP nucleotide tail was added to the 3' end of the cDNA and two successive, nested PCR reactions were performed following the protocol and thermocycling parameters for LDGW (Min & Powell, 1998). Nested SSPs, T1-1 and T1-2, engineered complementary to nts 718-749 and 403-435 of *cbsT1* respectively, and AP were used. The first reaction contained T1-1, AP, and tailed cDNA. The second nested reaction contained template DNA produced in the first reaction, T1-2,

and AP. Total DNA from the second PCR reaction was purified and sequenced with T1-2. The DNA complement of the nucleotide adjacent to the oligo-dC tail was identified as the putative transcriptional start site.

Recombinant DNA methods. Plasmid vector pSportI (Gibco BRL) was used in all recombinant DNA procedures. It contains an ampicillin resistance gene for selection, a *lac*-inducible promoter, and is capable of blue/white colony screening when transformed into *lacZΔ* cells. Universal forward and reverse primer binding sites flank the multiple cloning site for sequencing purposes. Three constructs that harbor strain 100-100 genomic loci coding for *cbsT1-cbsT2-cbsHβ*, *cbsT2-cbsHβ*, or *cbsHβ* were cloned under control of the pSportI *lac*-inducible promoter. Primers containing engineered *Kpn*I sites, T1strt, T2strt, and Hβstrt, were constructed 5' of *cbsT1*, *cbsT2*, and *cbsHβ* respectively and used with a primer containing an engineered *Bam*HI site 3' of *cbsHβ*, Hβend. Genomic DNA was amplified by PCR, digested with *Kpn*I and *Bam*HI (Promega), and cloned into identically digested vector DNA with T4 DNA ligase (Promega). The chimeras were designated pT1T2Hβ, pT2Hβ, and pHβ, respectively, and transformed into *E. coli* DH5α cells. Constructs were identified by blue/white colony screening on Luria-Bertani (LB) medium containing 100 μg of ampicillin (AMP)/mL, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (XGAL)/mL (LB-AMP-IPTG-XGAL). Plasmid DNA for each construct was purified (Wizard Plus Minipreps DNA Purification Kit; Promega) and sequenced to verify genetic content and orientation within the vector. The vector was

also used to clone and express the predicted BSH gene from *L. acidophilus* strain KS-13. Chromosomal DNA was purified from strain KS-13 cells and PCR amplified under standard conditions with primers, La-Hβa and La-Hβb. The primers contain engineered *Kpn*I sites and flank the BSH gene. The DNA was purified, digested with *Kpn*I, and ligated into dephosphorylated pSportI DNA that was digested identically. Chimeras were transformed into DH5α cells, plated on LB-AMP-IPTG-XGAL media, and screened for BSH activity.

Creation of a *L. johnsonii* strain 100-100 genomic library. A genomic library of strain 100-100 was created with pSportI and screened for BSH activity. Chromosomal DNA was purified from strain 100-100 cells and partially digested with *Sau*3AI (Promega) over a 54-fold range in enzyme concentration (Bloch, 1992). The restriction enzyme recognizes a four bp DNA sequence and is insensitive to *dam* methylation. The fragments were purified and ligated into the *Bam*HI site of pSportI that was dephosphorylated with calf intestinal alkaline phosphatase (Promega). The chimeras were transformed into *E. coli* DH5α cells and plated on LB-AMP-IPTG-XGAL media. The library was screened for clones expressing BSH activity.

DNA sequencing and analysis. DNA generated from LDGW and LPEA PCR, direct amplified DNA, and cloned DNA was sequenced with the ABI Prism automated fluorescent method (Molecular Biology Resource Facility, University of Tennessee, Knoxville). Direct amplified DNA and cloned DNA were sequenced bidirectionally using universal forward and reverse primers and/or synthesized oligonucleotide walking

primers. DNA sequence was analyzed for start and stop codons and translated into predicted amino acid sequence with MacVector. Open reading frames (ORF) were putatively identified from similarity with published sequences using the advanced basic local alignment search tool (BLAST [NIH website]).

Assay for BSH activity. Transformed *E. coli* DH5α cells were screened for the ability to hydrolyze conjugated bile acids with an agar plate assay (Dashkevicz & Feighner, 1989). Cultures were streaked or replica plated onto MRS agar containing 0.5% taurodeoxycholic acid (Sigma), a conjugated bile acid, 100 μg of AMP/mL, and 0.5 mM IPTG (MRS-TDCA-AMP-IPTG). Cultures were incubated anaerobically for 24-48 hours. BSH activity was detected when a halo of deoxycholic acid precipitated around colonies in the medium.

Screen for *cbsHβ*. Coleman and Hudson identified conserved amino acid motifs in cloned BSHs by aligning predicted protein sequences (Coleman & Hudson, 1995). A motif at the amino terminus and a motif centered around residue 225 were selected and used to construct primers Hβ675a and Hβ675b, respectively. The primers were engineered to *cbsHβ* sequence in strain 100-100 and encompass a region of approximately 675 bp. The primers were used in standard PCR reactions to amplify genomic DNA from other lactobacilli.

Nucleotide sequence accession numbers. The sequence of the complete bile salt hydrolase operon in *L. johnsonii* strain 100-100 and *L. acidophilus* strain KS-13 are registered with GenBank under accession numbers AF054971 (Appendix A) and AF091248 (Appendix C), respectively. The sequence of *cbsHα* is registered under accession number AF297873 (Appendix B).

Results

Genomic analysis of the *cbsHβ* locus. Genomic DNA sequence 5' and 3' of the cloned locus in pIN-BSH2 was extended and analyzed. DNA fragments of approximately 2,000 to 2,500 bps were amplified in both directions by LDGW (Fig. 1). Sequence 5' of the locus yielded a complete ORF of 1,356 nts for *cbsT1* that is 75% identical and 86% similar in predicted amino acid sequence to *cbsT2* (Fig. 2). A putative Shine-Dalgarno 16S rRNA sequence was identified that is centered 8 to 13 nts 5' of the ATG start site for *cbsT1*. The sequence, AAAGAAGAGAG, closely matched the previously identified putative ribosomal binding site for *cbsT2*, AAAGAAGGTAA (Elkins & Savage, 1998). Advanced BLAST analysis of CbsT1 and CbsT2 revealed similarities to several families of the major facilitator superfamily (Saier et al., 1999). The highest scoring, with approximately 42% similarity to the first 209 and 223 amino acids of CbsT1 and CbsT2 (6 and 10% gaps), respectively, were oxalate:formate antiporters from *Archaeoglobus fulgidus* and *Oxalobacter formigenes* (Abe et al., 1996). MacVector also predicted two additional clustered ORFs 305 nts 5' of *cbsT1* on the coding strand. These ORFs are highly similar in spatial arrangement and predicted amino acid sequence, as identified by

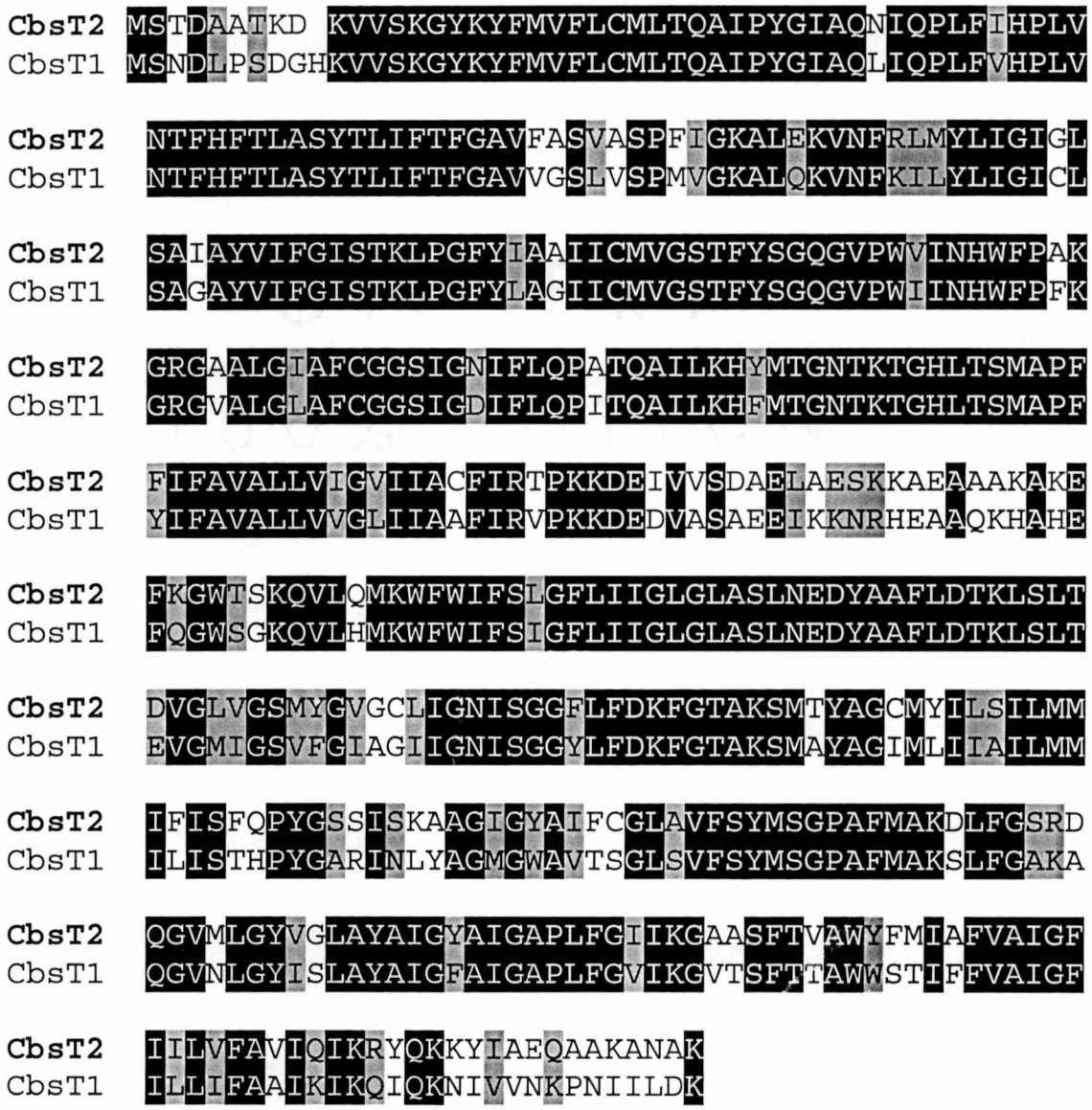

FIG. 2. The predicted amino acid sequence of CbsT1 is 75% identical and 86% similar to CbsT2. Identical residues are indicated by black highlighting. Grey shading indicates conservative substitutions.

advanced BLAST, to a set of two coordinately regulated genes, *arbX* and *arbZ*, in *L. delbrueckii* subsp. *lactis* that encode a putative glycosyl transferase and a phospho-β-glycosidase, respectively (Weber et al., 1998). DNA sequence 3' of the cloned locus on the coding strand yielded no ORFs of significant size within approximately 1,500 nts. However, an ORF of 1,245 nts located complementary to the coding strand for *cbsT1*, *cbsT2*, and *cbsHβ* was identified 119 nts 3' of *cbsHβ*. BLAST analysis putatively identified the ORF, designated *mat*, as a group II intron encoding maturase (Fig. 1; Edgell et al., 2000).

BSH operon in strain 100-100. LPEA produced long cDNA that extended to the 5' terminus of the mRNA species complementary to primer Hβ-1. This cDNA was amplified with AP and nested primers, T1-1 and T1-2, in two, successive, nested, LDGW PCR reactions. The first PCR reaction produced two visible bands of approximately 2 and 0.8 kbps when analyzed by 1% agarose gel electrophoresis (Fig. 3, lane 2). The second, nested reaction produced a single fragment of approximately 0.5 kbps (Fig. 3, lane 3). Considering the respective placement of T1-1 and T1-2, a decrease of approximately 0.3 kbps from 0.8 to 0.5 kbps is proportional to and consistent with genomic DNA sequence from strain 100-100. I believe the fragment of 2 kbps (Fig. 3, lane 2) is an artifact. LDGW PCR is known to produce artifacts of unrelated sequence that can be eliminated, in my experience, in subsequent, nested amplifications with SSPs. Alternatively, or in addition, the 2 Kbp fragment could have been amplified from a similar primer binding site for T1-1 that is found within *cbsT2*. Purified DNA from the second reaction was sequenced with T1-2 and matched identically the DNA sequence

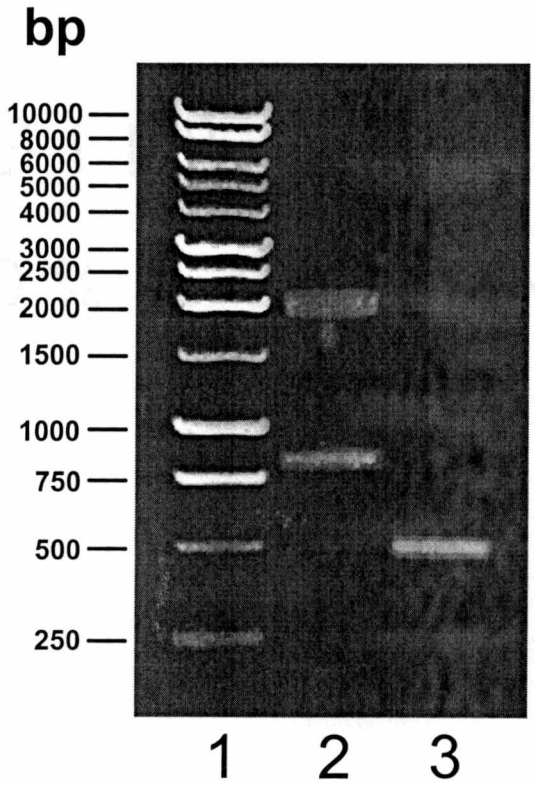

FIG. 3. Ethidium bromide-agarose gel electrophoresis of LPEA PCR products. Lane 1, 1 kb DNA ladder (Promega) with fragment sizes in bp; lane 2, DNA products from first, nested PCR reaction containing oligo-dC tailed cDNA, T1-1, and AP; lane 3, second, nested PCR reaction containing DNA template from lane 2, T1-2 and AP.

from the genome of strain 100-100 directly 3' of the T1-2 binding site (Fig. 4). The complement of the DNA sequence extended 78 nts upstream of *cbsT1* and was tailed with a string of guanocines. Sequences centered approximately -10 and -35 of the transcriptional start site, AATATAA and TTGATT respectively, were identified and compare with *E. coli* -10 and -35 consensus promoter sequences, TATAAT and TTGACA respectively, that are recognized by σ^{70} (Fig. 4). Expression analysis of *cbsHβ* was undertaken with three clones, pT1T2Hβ, pT2Hβ, and pHβ. All three clones expressed BSH activity when transformed into *E. coli* DH5α cells incubated anaerobically on MRS-TDCA-AMP-IPTG plates.

Identification of *cbsHα*. A genomic library of strain 100-100 was prepared and screened anaerobically on MRS-TDCA-AMP-IPTG plates. Two colonies, designated pBSH1 and pBSH2, produced a halo of deoxycholic acid. DNA sequence analysis of pBSH1 revealed a fragment encoding the *cbsHβ* locus. The second BSH-positive clone, pBSH2, contained a 1,610-bp DNA fragment from the genome of strain 100-100 encoding a complete ORF of 978 nts and two putative partial ORFs of 123 and 315 nts. The 5' and 3' partial ORFs were located 229 nts upstream and 56 nts downstream of the complete ORF, respectively. The 3' partial ORF, however, was located complementary to the coding strand for the other ORFs and does not share tandem arrangement (Fig. 5). Advanced BLAST analysis of the complete ORF displayed sequence similarities (DNA and predicted amino acid) to all cloned BSH genes (Christiaens et al., 1992; Coleman & Hudson, 1995; Elkins & Savage, 1998; Tanaka et al., 2000); the BSH gene from *L.*

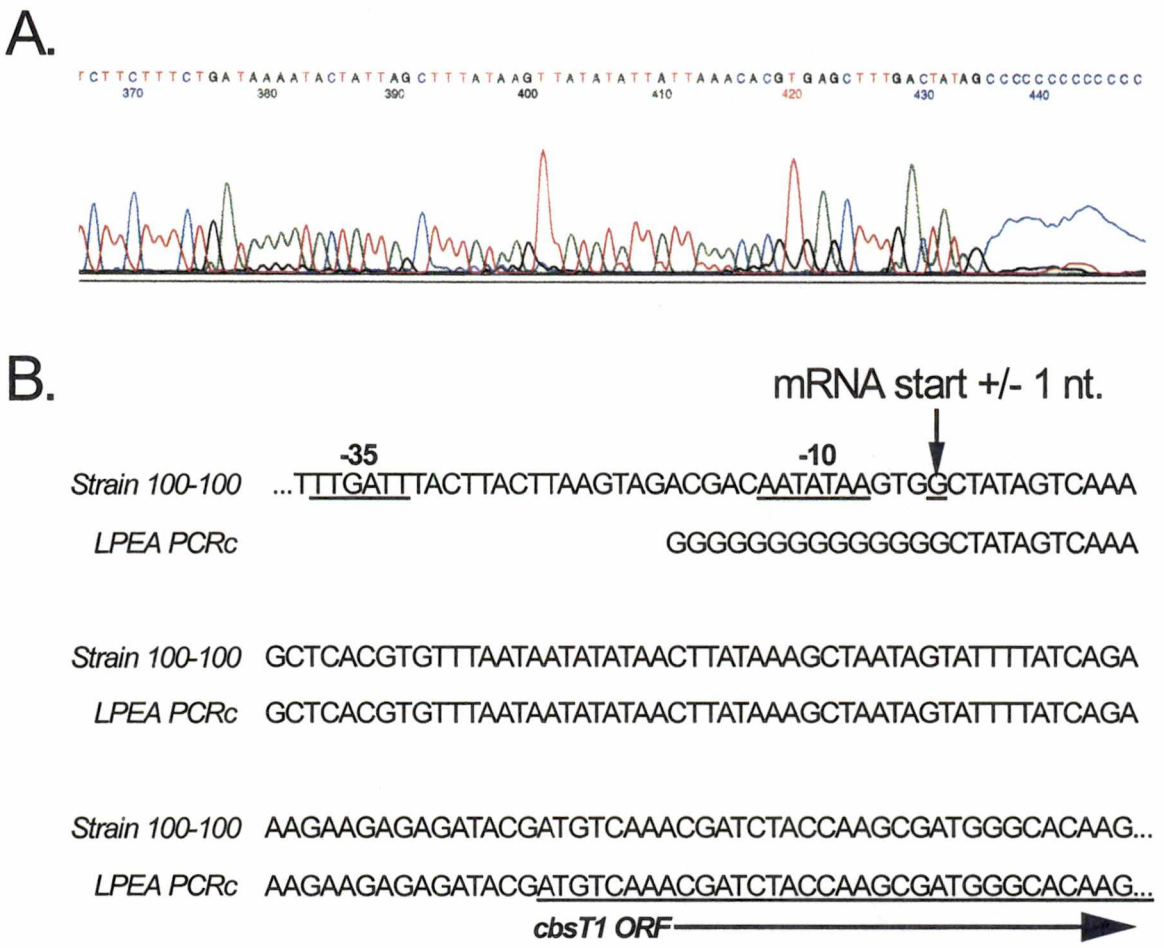

FIG. 4. LPEA PCR produced one DNA fragment of approximately 0.5 kbps (Fig. 3). The complement of this DNA sequence (LPEA PCRc) is identical to genomic sequence from strain 100-100. A transcriptional start site (arrow) and putative promoter (underlined) were identified.

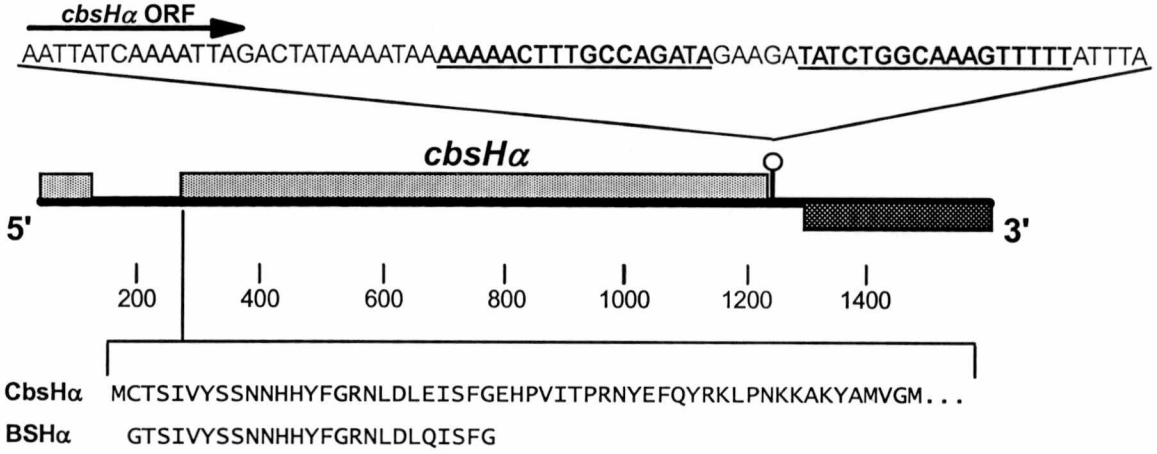

FIG. 5. A BSH-positive genomic clone from strain 100-100, pBSH2, with predicted open reading frames. The predicted amino acid sequence of *cbsHα* matched the protein sequence of purified BSHα (Lundeen & Savage, 1992a). An inverted repeat (underlined) was identified 3' of the *cbsHα* stop codon.

plantarum 80 (Christiaens et al., 1992) scored the highest. The partial ORFs did not display strong sequence similarity to any known genes. Microsequencing of the first 25 amino acids of the α hydrolase (BSHα) was determined previously from purified protein blotted onto polyvinylidene difluoride (PVDF) membranes (Lundeen & Savage, 1992a). The predicted amino acid sequence from the amino terminus of the complete ORF, designated *cbsHα,* was virtually identical (23/25 amino acids) to the BSHα sequence (Fig. 5). DNA sequence centered approximately 8 to 13 nts 5' of *cbsHα*, TAAGGAGGAAT, closely matched the Shine-Dalgarno 16S rRNA sequence of TAAGGAGGTGA. An inverted repeat capable of forming a stem-loop structure was identified 12 nts 3' of the stop codon of this gene (Fig. 5).

Survey of 50 *Lactobacillus* isolates for *cbsHβ* locus. Fifty strains of *Lactobacillus* species from humans or dairy products were obtained from culture banks and screened

for BSH activity and the *cbsHβ* locus. Genomic DNA from each was screened using standard PCR with primers Hβ675a and Hβ675b. A total of ten isolates screened positive for *cbsHβ.* Nine of these isolates were cultured from human sources and belong to species *acidophilus, gasseri,* and *johnsonii.* Only one diary isolate from Bulgarian yogurt, *L. delbrueckii* subsp. *bulgaricus,* screened positive for this locus. Some isolates from the same species, however, displayed a variable BSH phenotype (S. A. Moser and D. C. Savage, submitted for publication) and *cbsHβ* genotype (Table 1). Strains of species [number screened]: *amylovorous* [2], *crispatus* [3], *curvatus* subsp. *curatus* [1], *kefir* [1], *maltaromicus* [1], *paracasei* subsp. *paracasei* [3], *reuteri* [2], *rhamnosus* [3], *ruminis* [1], undetermined [2] were screened for *cbsHβ* but were negative.

***cbsHβ* locus in *L. acidophilus* strain KS-13.** Strain KS-13, a human isolate from our lab, screened positive for the *cbsHβ* locus (Table 1) and was chosen for an extended genetic study. Primers Hβ675a and Hβ675b amplified a single fragment of approximately 675 bps from the genome of strain KS-13 that was 85% similar in DNA sequence to that of *cbsHβ* of strain 100-100. DNA fragments 5' and 3' of this region in strain KS-13 were obtained by LDGW and sequenced. The composite DNA sequence was analyzed with MacVector and revealed an identical genomic architecture to the operon in strain 100-100 (Fig. 6). Three tandem ORFs of 1344, 1353, and 948 nts were predicted that displayed 84, 87, and 85% DNA sequence identity and 95, 95, and 97% predicted amino acid sequence similarity to *cbsT1*, *cbsT2*, and *cbsHβ* of strain 100-100, respectively. Intergenic regions of 28 nts (*cbsT1-cbsT2*) and 21 nts (*cbsT2-cbsHβ*) in

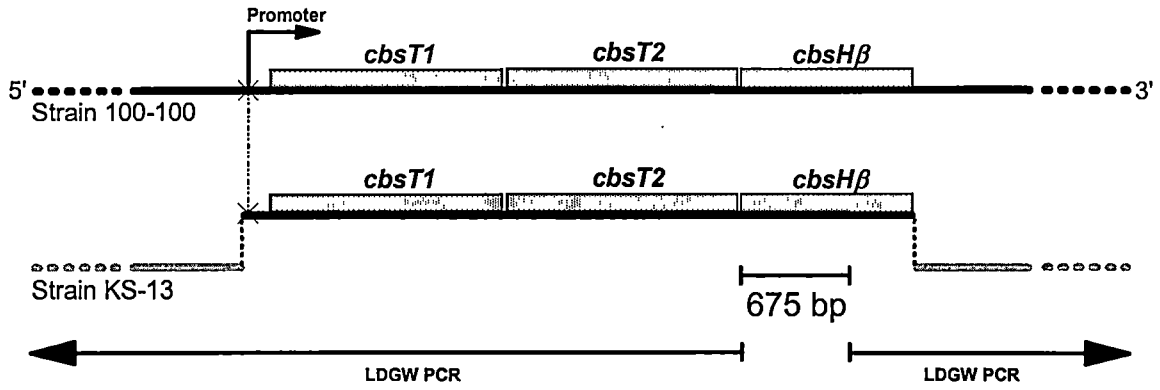

FIG. 6. *L. acidophilus* strain KS-13 genomic sequence of the *cbsHβ* locus was generated by standard (675 bp) and LDGW PCR (Min & Powell, 1998). The DNA sequence identity to strain 100-100 (85%) ends 5' of the putative promoter and 3' of the *cbsHβ* stop codon.

strain KS-13 compare with 27 nts and 19 nts in strain 100-100 (Elkins & Savage, 1998), respectively. A two sequence BLAST of strain KS-13 sequence and strain 100-100 sequence revealed no similarity in DNA sequence 26 nts 5' of the putative –35 promoter and directly 3' of the *cbsHβ* stop codon (Fig. 6). Primers La-Hβa and La-Hβb that contain engineered *Kpn*I sites were used to amplify and clone the predicted *cbsHβ* gene in strain KS-13. *E. coli* DH5α cells were transformed with a pSportI plasmid construct containing this ORF. Colonies from such cells precipitated deoxycholic acid in the surrounding medium when grown anaerobically on MRS-TDCA-AMP-IPTG plates.

Discussion

I have demonstrated that BSH activity is encoded by two separate loci on the genome of strain 100-100. One locus encodes *cbsHβ* and is part of an operon of three

genes related in function. To my knowledge, this is the first report of a complete BSH operon. Unlike the partial operon in *B. longum* SBT2928 (Tanaka et al., 2000), *cbsHβ* is encoded with functionally-related genes. The second locus encodes another BSH, *cbsHα*, which is not arranged in tandem with other ORFs. This discovery is novel since other enteric bacteria are known to encode only one BSH (Stellwag & Hylemon, 1976; Gopal-Srivastava & Hylemon, 1988; Kawamoto et al., 1989; Christiaens et al., 1992; Kishinaka et al., 1994; Coleman & Hudson, 1995; Grill et al., 1995; Tanaka et al., 2000).

The BSH operon of strain 100-100 contains two genes, *cbsT1* and *cbsT2*, that share a high level of similarity in their DNA and predicted amino acid sequence. This finding suggests that the genes are duplicates. Functional studies have shown that *cbsT2* and partial *cbsT1*, when expressed in *E. coli*, increase uptake of conjugated bile acids (Elkins & Savage, 1998). These genes, while demonstrating putative topologies characteristic of MFS transporters (Saier et al., 1999), do not display impressive sequence similarity (<40% of the amino halves), as identified by BLAST, to any known genes. Members of several subfamilies of the MFS provide the closest sequence similarity. In support of my observations, Saier and colleagues have phylogenetically classified *cbsT1* and *cbsT2* as members of a new subfamily of the MFS called the conjugated bile salt transporter family (BST; Saier et al., 1999; http://www-biology.ucsd.edu/~msaier/transport/titlepage.html).

The MFS subfamily that shares the highest level of similarity in amino acid sequence to CbsT1 and CbsT2 contains oxalate:formate antiporters. These transmembrane proteins catalyze the electrogenic exchange of $oxalate^{-2}$ for $formate^{-1}$ via intracellular decarboxylation of oxalate (Anantharam et al., 1989; Abe et al., 1996). I

hypothesize that CbsT1 and CbsT2 may function to exchange conjugated^{-1} for deconjugated bile acids via intracellular hydrolysis. This hypothesis is based upon the similarity to electrogenic antiporters, the absence of a putative bile acid efflux pump, and the physio-chemical properties of conjugated versus deconjugated bile acids (Thanassi et al., 1997). Since high concentrations of deconjugated bile acids are toxic to cells, I suggest that the transporters are sensitive to intracellular concentrations of deconjugates. Furthermore, I suggest that the duplication of the transporters provides a "gene dosage" effect to protect the cell and drive a membrane potential.

I was able to produce and amplify cDNA from strain 100-100 using primers engineered to *cbsHβ* and *cbsT1*. LPEA produced one fragment of 0.5 kbps that was identical in sequence to genomic DNA from the 5' end of *cbsT1*. This sequence extended 78 nucleotides 5' of the start site of *cbsT1*. I conclude that *cbsT1*, *cbsT2*, and *cbsHβ* are encoded polycistronically. The expression analysis with *cbsHβ* supported this conclusion. When expressed under *lac*-inducible promotion, clones containing *cbsT1-cbsT2-cbsHβ* or *cbsT2-cbsHβ* produced precipitates of deoxycholic acid in the medium. Therefore, *cbsT1* and *cbsT2* do not contain mRNA termination sequences, at least that are recognized by *E. coli.*

The genetic analysis of BSH activity supports protein characterization studies. Strain 100-100 produces two hydrolases, α and β, that combine to form homo- and heterotrimeric complexes. Purified α and β hydrolases have estimated molecular weights of 42,000 and 38,000 Da, respectively (Lundeen & Savage, 1992a). The calculated molecular weights of the BSH from the *Sau*3AI genomic clone (complete ORF) and

cbsHβ are 36,667 and 34,916 Da, respectively. In addition to having the larger molecular weight, the amino-terminal sequence of the *Sau*3AI BSH matches, almost identically, the first 25 amino acids of the α hydrolase (Lundeen & Savage, 1992a). I conclude that the complete 978 nt ORF from the *Sau*3AI genomic clone encodes BSHα and designate this ORF *cbsHα*. I also conclude that the known genetic elements responsible for BSH activity in strain 100-100 have been identified and cloned.

CbsHα shares the highest amino acid sequence similarity with the BSH from *L. plantarum* 80 (Christiaens et al., 1992). Similar to the BSH in strain 80, *cbsHα* is not arranged in tandem with other ORFs and contains an inverted repeat 3' of the stop codon. The BSH protein(s), however, has not been isolated or characterized from strain 80 (Christiaens et al., 1992). I suggest that the strain may also contain a second hydrolase locus encoding additional functionally-related genes if the activity was acquired horizontally. I also tentatively conclude that *cbsHα*, similar to the BSH gene from 80, is encoded monocistronically.

My phenotypic and genetic screens suggest that BSH activity was acquired horizontally in lactobacilli. Lactobacilli within species *acidophilus*, *brevis*, *buchneri*, *fermentum*, *gasseri*, and *plantarum* express a variable BSH phenotype. PCR screening within species of *acidophilus* and *delbrueckii* subsp. *bulgaricus* also produced variable results. Nearly all (9/10) of the *cbsHβ*-positive isolates were from human sources which suggests that this locus is important for persistent colonization of the GI tract. Taken in aggregate, these results suggest that the BSH phenotype and genotype is variable within a given species. It is not known whether BSHs and related genes are encoded on mobile

genetic elements. It is known, however, that the BSH gene from *B. longum* SBT2928 is flanked by inverted repeats (Tanaka et al., 2000). It is interesting to note, furthermore, that strain 100-100 encodes a maturase, *mat*, downstream of *cbsHβ*. Although primary literature on bacterial maturases is sparse, the enzymes have endonuclease and reverse transcriptase activity that faciliate movement and splicing of cDNA into the genome in a process known as retrohoming (Edgell et al., 2000). Group II maturases are encoded by self-contained ORFs with independent promoters. Most group II maturases are inserted in or associated with mobile genetic elements (Edgell et al., 2000). The putative maturase in strain 100-100 is located complementary to but in close proximity with DNA encoding the BSH operon.

A BSH operon identical in genomic architecture to strain 100-100 is conserved in *L. acidophilus* strain KS-13. DNA sequence identity to strain 100-100 ends 5' of the putative promoter and 3' of *cbsHβ*. I suggest that this locus forms a genomic mobile element and supports my hypothesis that BSH genes in lactobacilli were obtained horizontally. *L. acidophilus* is one of only two organisms that respond to an EF of unknown composition produced by strain 100-100 (Lundeen & Savage, 1992b). Unlike other BSH systems, hydrolase activity in strain 100-100 is increased by as much as three- to fivefold within 20 min after conjugated bile acids are added to suspensions of stationary phase cells. The increase is due to induction of a soluble extracellular molecule and not enzymatic or regulatory proteins (Lundeen & Savage, 1992b). EF also enhances uptake of conjugated bile acids in *E. coli* cells expressing *cbsT2* and partial *cbsT1* from strain 100-100 (Elkins & Savage, 1998). Therefore, the transporters in strain KS-13 are related to the bacterium's ability to respond to EF.

The genetics of BSH activity in strain 100-100 are not as complex as that of 7α-dehydroxylation in *Eubacterium* sp. strain VPI 12708 (Mallonee et al., 1990) and *Clostridium* sp. strain TO-931 (Wells & Hylemon, 2000). The genomes of these two organisms contain large bile acid inducible operons that contain nine and six genes, respectively (Mallonee et al., 1990; Wells & Hylemon, 2000). The operons exhibit approximately 75% DNA sequence similarity that translates into 90% predicted amino acid sequence similarity. Genetic architecture of the operon in *Clostridium* is nearly identical to that of *Eubacterium* (Wells & Hylemon, 2000). The proposed model for 7α-dehydroxylation contains a number of intermediate products that require coenzyme A-SH, hydrolysis of ATP, and repetitive reduction of $NAD(P)^+$ to NAD(P)H (White et al., 1988; Mallonee et al., 1990; Hylemon & Harder, 1999; Wells & Hylemon, 2000).

The BSH operon in strain 100-100 contains two bile acid-related functions: hydrolysis and transport. The BSHs in strain 100-100 have been purified and physiologically characterized. Bile acid transport by contrast could be studied with the focus on several questions. Is there a bile acid exporter or is export activity catalyzed concomitantly with import? What is the function of EF in bile acid transport? Experiments designed to identify the energy source and kinetics of transport may provide functional information that supports the phylogenetic assignment of *cbsT1* and *cbsT2* into a new family of the MFS. Optimistically, this information, once obtained, may lead to a working model for BSH activity in enteric bacteria.

Acknowledgements

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PART IV

CONJUGATED BILE SALT TRANSPORTERS IN *LACTOBACILLUS JOHNSONII* STRAIN 100-100: A NEW SUBFAMILY OF THE MAJOR FACILITATOR SUPERFAMILY

Abstract

Two conjugated bile salt transporters, CbsT1 and CbsT2, have been identified in *Lactobacillus johnsonii* strain 100-100 that are enhanced in function by an extracellular factor (EF) produced by the bacterium. They are duplicates that share amino acid sequence similarity to electrogenic oxalate:formate antiporters of the major facilitator superfamily and are coordinately regulated with *cbsHβ*, a bile salt hydrolase (BSH). This report contains evidence on bile salt transport activity obtained with these duplicates in *Escherichia coli* cells. *E. coli* DH5α cells containing p2000 (CbsT2 cloned into pSportI) suspended in EF^+ supernatant from strain 100-100 demonstrated a rapid initial uptake (<15 sec) of [24-^{14}C]taurocholic acid that was higher than pSportI cells suspended in EF^- supernatant (control). Uptake velocity of [24-^{14}C]taurocholic acid due to CbsT2 and EF^+ was measured over a 64-fold range in concentration and was saturable in *E. coli* HN971 cells (deficient in two bile acid efflux pumps). DH5α cells expressing transporter(s) and BSH (pT12BSH and pT2BSH) demonstrated less uptake of ^{14}C radiolabel than cells expressing p2000, suggesting that [24-^{14}C]cholic acid (produced by the BSH) leaves cells via the transporter(s). This finding was confirmed with cells exposed to [^{3}H]taurocholic acid that produces [^{3}H]taurine upon hydrolysis by the BSH. ^{3}H radiolabel remained associated with cells expressing pT2BSH and p2000. Furthermore, EF^- supernatant could be "converted" to EF^+ supernatant by adding 0.4 mM unlabeled cholic acid. Taurocholic acid uptake by DH5α p2000 cells was insensitive to the presence of ionophores, 2,4dinitrophenol and CCCP, but was dependent on temperature, suggesting that the

process is independent of the protonmotive force but is protein-mediated. Unlike oxalate:formate antiporters, CbsT2 and CbsT1 are not electrogenic since they do not confer enhanced growth rate on DH5α cells expressing pT12BSH or pT2BSH. Furthermore, ATP levels in strain 100-100 cells did not increase when exposed to conjugated bile acids. These findings support a hypothesis that CbsT2 and CbsT1 facilitate electroneutral exchange of taurocholic for cholic acid that may be analogous, in mechanism, to phospholipid flippases.

Introduction

Conjugated bile acids are secreted through the common bile duct into the duodenum of the small intestine of mammals and birds (Stevens & Hume, 1995). Their main function is to aid in the absorption of dietary lipids and fats (Hylemon & Harder, 1999). These molecules are synthesized from cholesterol in the liver as primary bile acids and attached at the carboxyl C-24 terminus, via an amide bond, to glycine or taurine (Savage et al., 1995; Baron & Hylemon, 1997). The amphipathic nature of bile acids permits spontaneous micellar formation for fat emulsification but also causes the acid to be toxic to living cells. The molecules can act as detergents that disaggregate biological membranes. This phenomenon can especially affect microorganisms that enter the intestine; bile acid concentrations can reach as high as 20 mM in the duodenum (Thanassi et al., 1997). Some of these microbes have intrinsic resistance to bile acids; some even have genes encoding proteins that chemically alter bile acids via bile acid deconjugation and transformation (Savage, 2000; Hylemon & Harder, 1999). However, the

physiological importance of those deconjugation and transformation activities remains unclear.

Resistance to bile acids in *Escherichia coli* is due in part to the multidrug efflux pumps AcrB, in the resistance nodulation division of membrane transporters (RND; Tseng et al., 1999), and EmrB, in the major facilitator superfamily (MFS; Saier et al., 1999). These pumps in concert with periplasmic linkers, AcrA and EmrA respectively, are responsible for efflux of a range of antimicrobial agents (Nikaido, 1996). They associate with an outer membrane porin, TolC, to extrude such molecules into the environment, bypassing the periplasm, by consuming ΔpH (Nikaido, 1996). An analogous system comprised of MexAB-OprM has also been shown in *Pseudomonas aeruginosa* (Li et al., 1995). *E. coli*, however, encodes another genetic determinant that is responsible for high level efflux of taurocholic acid, a conjugated bile acid, and chenodeoxycholic acid, a primary bile acid. This activity is catalyzed by a proton antiporter for which the genetic determinant has not been identified (Thanassi et al., 1997).

Flux of bile acids across bacterial membranes is not limited to gram-negative, intestinal bacteria, a particular energy coupling, or to simple efflux. Genomic analyses have revealed that many bacteria have genes encoding multispecific efflux pumps that are responsible for bile acid extrusion. Nonenterics such as *Bacillus subtilis* (Ito et al., 1999), *Lactococcus lactis* strain MG1363 (Yokota et al., 2000), and *Neisseria gonorrhoeae* (Hagman et al., 1995) have such pumps. Energy coupling for bile acid efflux is also not limited to the proton motive force. For example, strain MG1363 possesses an ATP-dependent mechanism for effluxing cholic acid that is the first to be shown in

prokaryotes. The activity in this organism was inhibited with *ortho*-vanadate suggesting that the transporter is a member of the ATP-binding cassette superfamily (Yokota et al., 2000). Many lactobacilli can intracellularly accumulate cholic acid independent of a protein carrier (Kurdi et al., 2000). This accumulation relies on ΔpH and the property of cholic acid, with a pK_a of 6.4, to act as a weak acid. Thus, lowering extracellular pH can cause cholic acid to accumulate in the pH-homeostatic environment of the cell to levels predicted by the Henderson-Hasselbalch equation (Kurdi et al., 2000). Finally, bile acid importers have been identified in *Eubacterium* sp. strain VPI 12708 (Mallonee & Hylemon, 1996) and *Lactobacillus johnsonii* strain 100-100 (Elkins & Savage, 1998). BaiG from strain VPI 12708 is a member of the 14 transmembrane domain (TMD) drug efflux subfamily of the MFS and catalyzes deconjugated bile acid import using ΔpH to drive translocation (Mallonee & Hylemon, 1996). Two transporter duplicates, *cbsT1* and *cbsT2*, have been identified in strain 100-100 that are involved in uptake of conjugated bile acids. Their encoded proteins have not been characterized (Elkins & Savage, 1998).

The genes encoding the two bile acid transporters strain 100-100 are coordinately regulated with *cbsHβ*, a bile salt hydrolase (BSH; Part III). The transporters are gene duplicates that share 75% identity and 86% amino acid sequence similarity (Part III). They share sequence similarity, albeit unimpressive (<40% of the amino halves), only to transporter proteins from several subfamilies of the MFS but interestingly not to BaiG (Elkins & Savage, 1998). The subfamily with the highest level of sequence similarity to CbsT1/T2 contains oxalate:formate electrogenic antiporters. Hydropathy plots of both predicted protein sequences display a 6-plus-6 TMD pattern that is characteristic of the MFS (Elkins & Savage, 1998; Part III). When the 3' half of *cbsT1* and a complete *cbsT2*

are expressed in *E. coli* DH5α cells, the cells accumulate up to a threefold more radiolabeled taurocholic acid than control cells. This threefold increase occurred only when the DH5α cells expressing the transporters were exposed to an extracellular factor (EF) produced by strain 100-100. These same cells accumulate less radiolabeled cholic acid than control cells but only by a small but statistically significant amount (Elkins & Savage, 1998).

The few reports of bile acid transport in prokaryotes, their lack of sequence similarity and putative TMD structure to BaiG, and their similarity to electrogenic antiporters in the MFS suggest that the transporters from strain 100-100 may catalyze a novel bile acid flux. This report contains evidence from a functional study of these transporters. A model for conjugated bile acid:deconjugated bile acid facilitated exchange is proposed from the data. The model accounts for the role of EF as cholic acid.

Materials and Methods

Culture techniques and strains. Bacterial strains were maintained in 15% glycerol for cryoprotection and stored at –80°C. *L. johnsonii* strain 100-100 was grown anaerobically in MRS (Becton Dickinson) broth or agar media as needed. *Escherichia coli* strains DH5α (Gibco BRL) and HN971 (provided by H. Nikaido, University of California-Berkeley) were grown aerobically at 37°C in Luria-Bertani (LB) media (Fisher Biotech). Strain HN971 is a derivative of *E. coli* strain K-12 and contains mutations in *acrA* and

emrB (Thanassi et al., 1997). Since *emrB* is interrupted with a *kan* cassette, this strain was cultured with 60 μg of kanamycin/mL in the growth medium for selection.

Plasmid constructs. All plasmid constructs were generated with vector pSportI (Gibco BRL). The vector is a high copy number plasmid that contains an ampicillin resistance gene for selection, a *lac*-inducible promoter for expression of cloned inserts, and is capable of blue/white colony screening when transformed into *lacZΔ* cells (*E. coli* DH5α). The p2000 construct is a derivative of an *Eco*RI genomic clone from strain 100-100 from which the *cbsHβ* (BSH) gene has been eliminated with an endogenous *Xba*I site (Elkins & Savage, 1998). The construct contains the 3' end of *cbsT1* and a complete *cbsT2*. Constructs pT12BSH, pT2BSH, and pBSH were generated by PCR of strain 100-100 genomic DNA with a long and accurate Advantage cDNA polymerase (Clontech) as described previously (Part III). Plasmid pT12BSH contains genes *cbsT1*, *cbsT2*, and *cbsHβ*. Plasmid pT2BSH contains *cbsT2* and *cbsHβ*, and plasmid pBSH contains only *cbsHβ*. Transformed *E. coli* strains harboring the purified plasmid constructs were grown and subcultured on LB medium containing 100 μg of ampicillin (AMP)/mL (except for strain HN971 which was maintained under ampicillin and kanamycin selection), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (XGAL)/mL (LB-AMP-IPTG-XGAL).

Chemicals and radiolabeled bile acids. Taurocholic acid, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and 2,4dinitrophenol (DNP) were obtained from Sigma Chemical Co. [24-^{14}C]Taurocholic acid (0.020 mCi/mL; specific activity, 46.3

mCi/mmol) was obtained from New England Nuclear Corp. [24-^{14}C]Cholic acid (0.1 mCi/mL; specific activity 54.5 mCi/mmol) and [^{3}H]taurocholic acid (1.0 mCi/mL; specific activity 50 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc.

Preparation of EF. EF^+ and EF^- supernatant solutions were prepared from strain 100-100 as described previously (Lundeen & Savage, 1992b). Briefly, a 40 mL culture of strain 100-100 was grown to stationary phase (approximately 20-24 hrs.) and split into two 20 mL aliquots. Taurocholic acid (0.4 mM final culture concentration) solubilized in 1 mL of sodium acetate buffer (pH 5.0) was added to one aliquot (EF^+). One mL of buffer only was added to the second aliquot (EF^-). The cultures were incubated for 30 minutes at 37°C. The cells were pelleted at 5,000 x *g* for 10 minutes. The supernatant solutions were harvested and stored at 4°C (Lundeen & Savage, 1992b).

Assay for isotopic bile acid uptake. A bile acid uptake assay was performed as described previously (Elkins & Savage, 1998). The protocol was adopted from the transport assays with BaiG from *Eubacterium* (Mallonee & Hylemon, 1996) and from assays of nafcillin uptake, a strongly hydrophobic penicillin, in *Salmonella typhimurium* (Nikaido et al., 1998). Uptake of radiolabeled bile acids was compared between dense suspensions of *E. coli* cells harboring different plasmid constructs (described above). *E. coli* cultures harboring such plasmids were grown overnight, induced with isopropyl-B-D-thiogalactopyranoside (IPTG) normalized to the same Klett reading (approximately 150). In brief, the method involved pelleting and washing 8 mL of cells in 50 mM Tris-

HCl (pH 7.5) then suspending these cells in 600 μL of EF solution (EF^+ or EF^-) on ice. Three 200 μL aliquots were prepared from each suspension, preincubated at 37°C for 7 to 8 min., and exposed to radiolabeled bile acid for four min. The cells were repeatedly washed and pelleted in ice-cold 100 mM LiCl-100 mM potassium phosphate (pH 7.0) then digested in formamide (Fisher Biotech) for approximately 1 to 2 hours. The digested cells were placed in 10 mL of scintillation cocktail (Ecoscint A; National Diagnostics) containing 5 mL of ethanol. Radioactivity was quantitated in a Beckman LS 7000 scintillation counter (Elkins & Savage, 1998).

In certain experiments, EF^+ and EF^- supernatant solutions were replaced by sodium acetate buffer (pH 5.0), fresh MRS media (pH 6.5 and 4.2), or an MRS salts buffer (pH 4.2). Fresh MRS medium or MRS salts buffer were prepared and adjusted to pH 4.2 with lactic acid. MRS salts buffer contained the components of MRS medium without carbon and energy sources. The formula per liter includes 2 g dipotassium phosphate, 1 g polysorbate 80, 5 g sodium acetate, 2 g ammonium citrate, 0.2 g magnesium sulfate, and 0.05 g manganese sulfate.

Growth assays for *E. coli* transformants. *E. coli* DH5α cultures transformed with constructs pT12BSH, pT2BSH, and pBSH were assayed for growth in media containing conjugated bile acids. The method was derived from a plate assay used to detect bile salt hydrolase activity when halos of precipitated deoxycholic acid formed around colonies in the medium (Dashkevicz & Feighner, 1989). The method has been used successfully to detect such activity in lactobacilli and *E. coli* genomic libraries of strain 100-100 (Elkins & Savage, 1998; Part III). MRS agar medium containing 0.5% taurodeoxycholic acid,

100 μg of AMP/mL and 0.5 mM IPTG (MRS-TDCA-AMP-IPTG) was spot inoculated with 3 μL of each of the three *E. coli* cultures that had been normalized to the same Klett reading (~150). Relative growth of the cultures after 24 and 48 hours was monitored by measuring the diameter of the halos of deoxycholic acid.

ATP pools in strain 100-100. ATP pools in cultures of strain 100-100 were measured with a modified luciferase assay (Cole et al., 1967). A 40 mL culture of strain 100-100 was grown anaerobically in MRS broth to stationary phase (approximately 20-24 hours). The culture was split into two 20 mL aliquots. Taurocholic acid was added to one aliquot at 0.4 mM (final culture concentration). Both aliquots were incubated for 30 minutes in a 37°C water bath, then three, 2 mL samples of each culture were added immediately to 0.5 mL of ice-cold 30% (w/v) $HClO_4$. After 10 minutes, the samples were shaken and neutralized with 1.5 mL KOH. After the precipitate settled, 20 μL of supernatant solution was added to a room temperature mixture of 0.2 mL glycylglycine buffer (pH 7.4) and 0.7 mL dH_2O. Either 20 or 50 mL of filtered firefly lantern extract in arsenate-magnesium buffer (Sigma FLE-250) was added and luminescence was quantitated immediately in a Zylux FB14 luminometer.

Results

Sequence analysis of CbsT1 and CbsT2. CbsT1 and CbsT2 share predicted amino acid sequence similarity, hydropathy profiles, and putative topological models (Fig. 1). These properties are consistent with transporters of the MFS (Elkins & Savage, 1998). The two

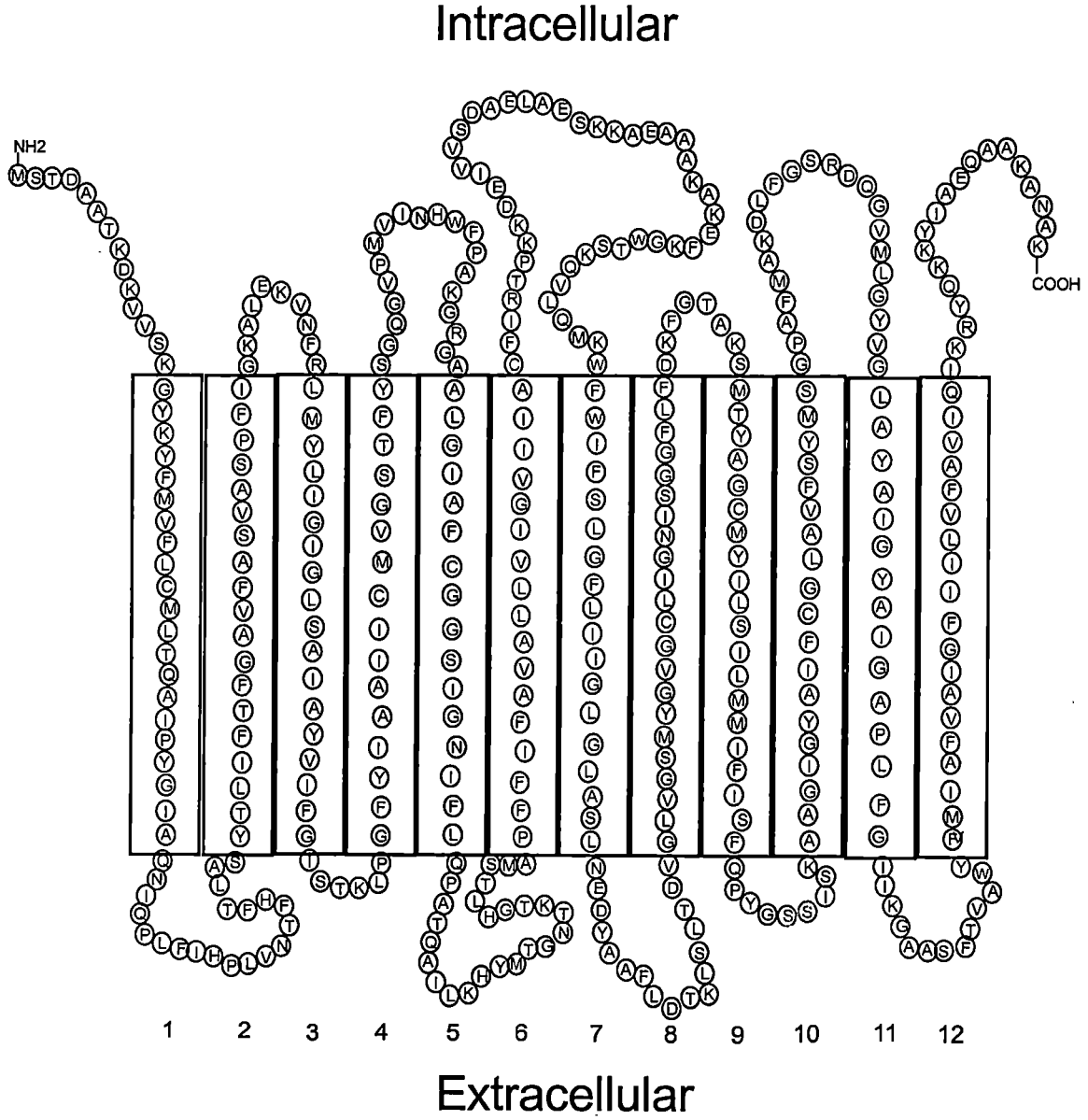


FIG. 1. Proposed model of transmembrane organization of CbsT2 (and CbsT1) prepared with Tmpred program (http://www.ulrec3.unil.ch/software/TMPRED_form.html).

transporters from strain 100-100 each contain an amino acid motif between predicted TMDs 2 and 3 that is generally conserved among all MFS transporters, even those members that exhibit extensive sequence divergence (Pao et al., 1998). The motifs identified in CbsT1 and CbsT2, GKALQKVNFKILY and GKALEKVNFRLMY respectively, compare well with the 13-residue MFS consensus motif of G-[RKPATY]-L-[GAS]-[DN]-[RK]-[FY]-G-R-[RK]-[RKP]-[LIVGST]-[LIM] (Jessen-Marshall et al., 1995; Pao et al., 1998). The broad category of MFS H^+/drug antiport proteins such as tetracycline transporters and multidrug resistance pumps contain a motif in TMD 5 of consensus gxxxGPxxGGxI (Lewis et al., 1997). Predicted TMDs 5 of CbsT1 and CbsT2 contain a similar motif of GxxxGIxxxGGxI and GxxxGLxxxGGxI, respectively. However, CbsT1 and CbsT2 have no similarity to the deconjugated bile acid importer, BaiG, from *Eubacterium* sp. strain 12708 (Mallonee & Hylemon, 1996). BaiG has similarity to the 14 TMD TetA from *B. subtilis* (Ogasawara et al., 1994). Phylogenetic assignment of transporters into subfamilies provides some putative information on structure, substrate class, and empirical mechanism or energy coupling (Saier, 1998; Saier, 1999). Conserved amino acid motifs within subfamilies of the MFS (Pao et al., 1998) have not been identified in CbsT1 or CbsT2 (Elkins & Savage, 1998; Part III). They exhibit highest amino acid sequence similarity to the oxalate:formate antiport (OFA) subfamily (Part III). OFA proteins catalyze an electrogenic exchange of $oxalate^{-2}$ for $formate^{-1}$ via an intracellular decarboxylase (Anantharam et al., 1989; Abe et al., 1996). OFA proteins from *Archaeoglobus fulgidus* and *Oxalobacter formigenes* (Abe et al., 1996) were approximately 42% similar in amino acid sequence to the N-terminal half of CbsT1 and CbsT2 (Fig. 2; Part III). Phylogenetic analyses by Saier and colleagues

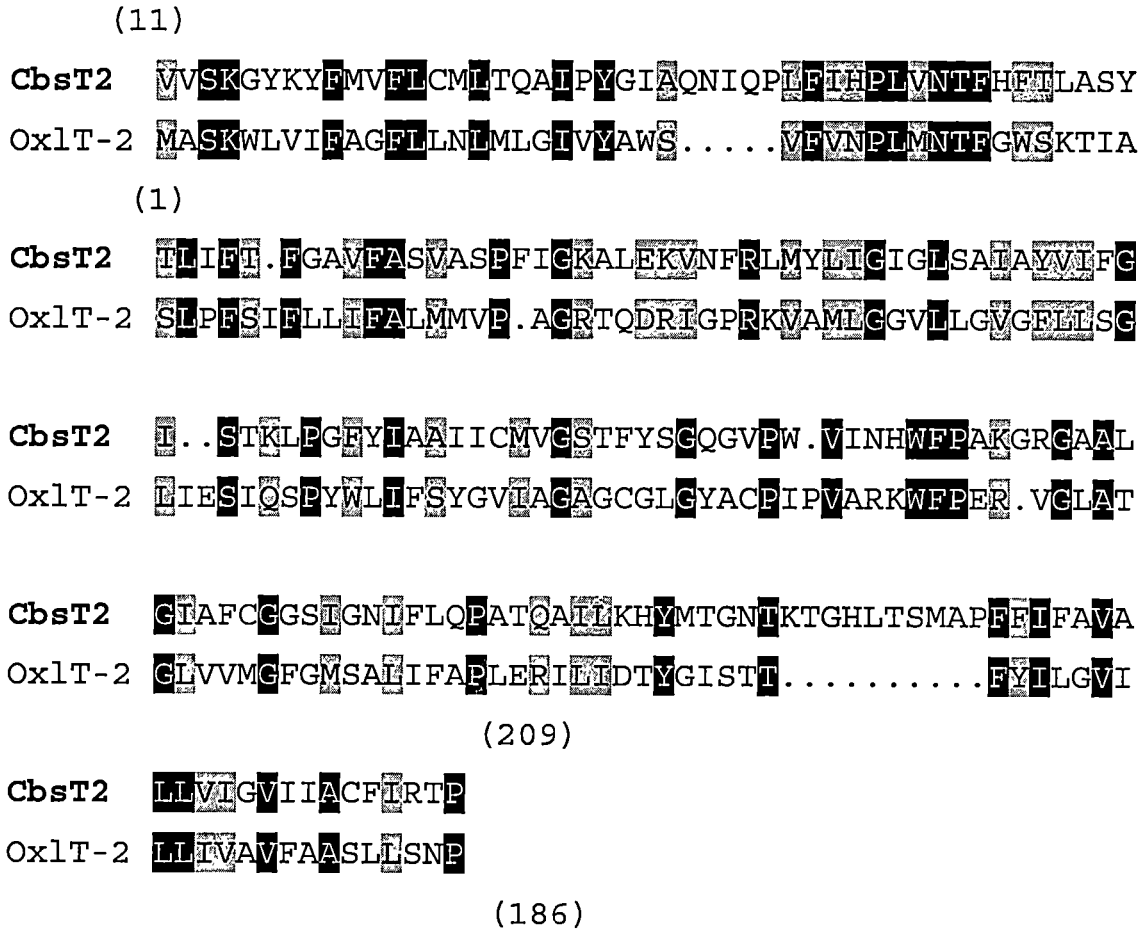


FIG. 2. Predicted amino acid sequence comparison of CbsT2 from strain 100-100 to oxalate:formate antiporter, OxlT-2, from *A. fulgidus*. The first half of CbsT1/T2 is approximately 42% similar to antiporters from *A. fulgidus* and *O. formigenes* (Abe et al., 1996). Identical residues are indicated by black highlighting. Grey shading indicates conservative substitutions.

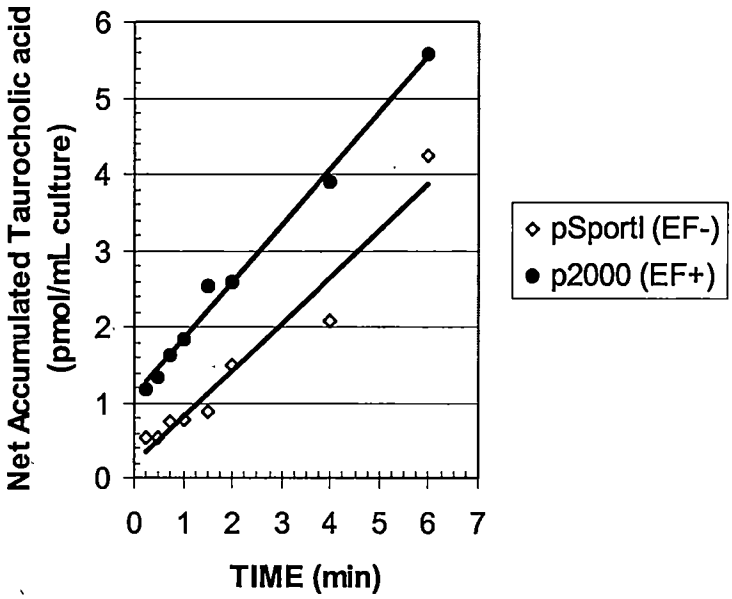
have assigned CbsT1 and CbsT2 to a new subfamily of steroid transporters within the MFS called the conjugated bile salt transporter family (BST; http://www.biology.ucsd.edu/~msaier/transport/titlepage.html). This assignment suggests that CbsT1 and CbsT2 may have substrate specificities, empirical function, or polarity of transport different from other MFS subfamilies.

Time and concentration dependent accumulation of taurocholic acid in *E. coli* DH5α and HN971 cells. *E. coli* DH5α cells expressing a construct encoding the 3'-half of *cbsT1* and a complete *cbsT2* (p2000) accumulate [24-^{14}C]taurocholic acid by as much as threefold over control levels when induced with 0.5 mM IPTG and exposed to EF^+ supernatant from strain 100-100. The control levels were obtained with induced DH5α cells harboring pSportI (vector alone), normalized to the same Klett reading but exposed to EF^- supernatant (Elkins & Savage, 1998). Aliquots of these two cell cultures were assayed over time with 100 nCi of [24-^{14}C]taurocholic acid (Fig 3A). Both cultures demonstrated a rapid increase (<15 sec) in initial uptake of taurocholic acid. The culture expressing the p2000 construct at the first time point (15 sec) accumulated more taurocholic acid than the culture containing pSportI. Neither the p2000 construct nor EF^+ affected taurocholic acid uptake after the first timepoint since uptake for both cultures from that point was linear over time with no obvious difference in rate.

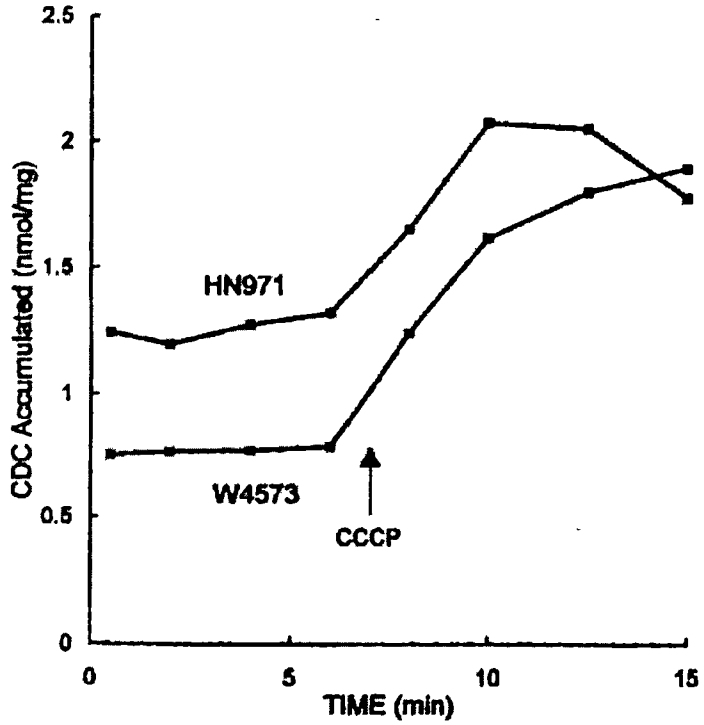
These data must be analyzed with reference to the findings in a study of bile acid flux in the *E. coli* background (Fig 3B; Thanassi et al., 1997). In that work, a similar but converse experiment with an *acrA emrB* mutant of *E. coli* (HN971) demonstrated a rapid initial increase (<30 sec) in chenodeoxycholate accumulation. That increase was higher

Fig. 3. Accumulation of [^{14}C]bile acids in *E. coli* cells over time. (A) *E. coli* DH5α cultures induced with 0.5 mM IPTG and normalized to a Klett reading of 108 were exposed to 100 nCi of [24-^{14}C]taurocholic acid. Cells were resuspended in EF solution (pH 4.2) from strain 100-100. (B) Accumulation of [24-^{14}C]chenodeoxycholate in wild-type (W4573) and HN971 *E. coli* cells centrifuged through silicone oil. Cells were incubated in phosphate buffer (pH 7.0) containing glucose and exposed to [24-^{14}C]chenodeoxycholate at a final concentration of 200 μM. CCCP was added to final concentration of 100 μM (Thanassi et al., 1997).

A.


Net Accumulated Taurocholic acid (pmol/mL culture)
0
1
2
3
4
5
6
0 1 2 3 4 5 6 7
TIME (min)
pSportI (EF-)
p2000 (EF+)

B.


CDC Accumulated (nmol/mg)
0
0.5
1
1.5
2
2.5
0
5
10
15
TIME (min)
HN971
W4573
CCCP

than that of wild-type cells (W4573). Both genes are part of separate bi-membrane pumps responsible for efflux of bile acids (Thanassi et al., 1997). After the rapid initial increase however, a steady-state net accumulation is reached that can be disrupted upon addition of CCCP, an ionophore that eliminates $\Delta\psi$ and ΔpH. CCCP results in a linear increase over time in both HN971 and wild-type cells with no obvious difference in rate (Fig. 3B; Thanassi et al., 1997). Measurements in my system (Fig. 3A) resemble the linear increase over time that is obtained in HN971 and W4573 cells treated with CCCP.

The rates at which [24-^{14}C]taurocholic acid is accumulated when present at differing concentrations were studied in HN971 cells harboring pSportI and p2000. The parameters of the initial rapid rate at which the bile acid was taken up (see above) were not defined for these cultures. Therefore, cells containing either construct were incubated with various concentrations of radiolabeled substrate for a period of four min, the standard time used in the uptake assay. HN971 cells were chosen because they contained mutations in two bile acid efflux pumps (Thanassi et al., 1997) that render them more sensitive than wild-type *E. coli* to bile acids in small concentrations. Rate of uptake was measured over a 64-fold range in concentration yielding a saturable curve for p2000 cells expressing CbsT2 and exposed to EF^+ (Fig. 4). The capacity for *E. coli* to mediate flux of bile acids independent of CbsT2 or EF^+ was eliminated by subtracting the background uptake of [24-^{14}C]taurocholic acid by HN971/pSportI cells. Kinetic values such as K_m and V_{max} were not calculated for CbsT2 because initial rates (<15 sec) were not measured in this experiment. Uptake was also measured by filtering and washing cells with a vacuum manifold. This method would permit measurements at short timepoints (<15

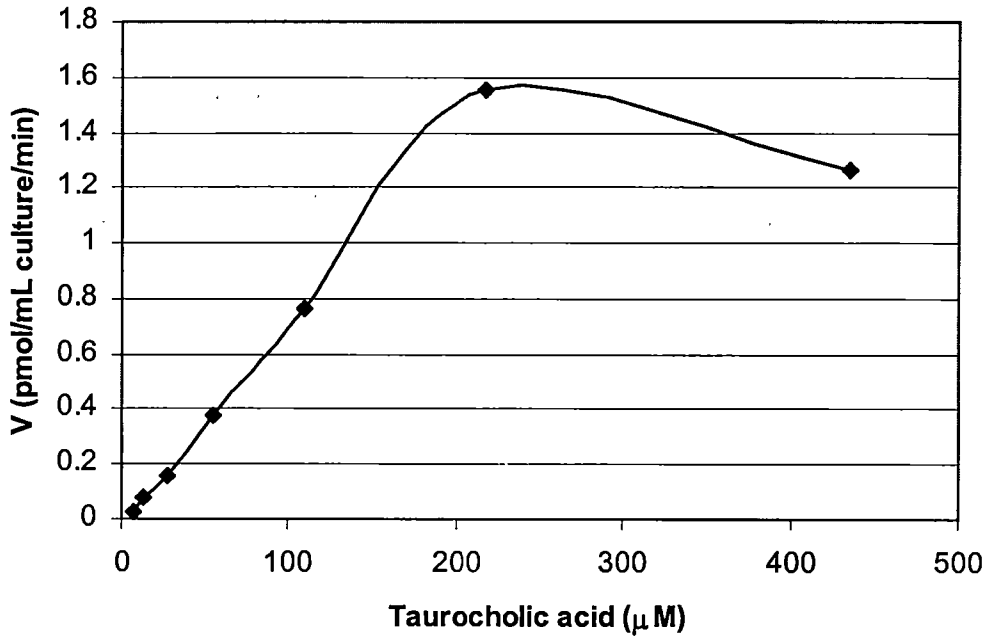


FIG. 4. Accumulation rates at different external concentrations of [24-^{14}C]taurocholic acid by *E. coli* HN971 cells harboring p2000 and exposed to EF^+. Mean accumulation rates of HN971 cells harboring pSportI and exposed to EF^- were subtracted from the mean rates of HN971 cells harboring p2000 and exposed to EF^+. Both cultures were normalized to a Klett reading of 120. Mean accumulation rates were obtained from three replicates from the same bacterial suspension.

sec) but yielded erratic data that could be due to binding of taurocholic acid to the filter (Thanassi et al., 1997).

Accumulation of taurocholic acid by DH5α cells containing constructs of the *cbsHβ* operon. [24-^{14}C]Taurocholic acid accumulated by DH5α cells containing pBSH, pT2BSH, and pT12BSH was compared to that of cells harboring p2000. [24-^{14}C]taurocholic acid is labeled on the bile steroid. For this reason, the primary bile acid could be traced in cells expressing *cbsHβ* (BSH) that hydrolyzes the peptide bond (C-24 position), releasing the amino acid. Fifty nCi of [24-^{14}C]taurocholic acid was added to cells harboring p2000 and exposed to EF^+. Such cells accumulated 3,322 ± 578 cpm of taurocholic acid. This value is comparable to values published previously (Elkins & Savage, 1998). Similar cells under identical conditions containing pBSH, pT2BSH, and pT12BSH accumulated 1,914 ± 456, 1,482 ± 91, and 2,005 ± 272 cpm of ^{14}C radiolabel, respectively. By comparison, cells exposed to EF^- harboring pSportI accumulated 1,675 ± 390 cpm, a value also comparable to previously published data (Elkins & Savage, 1998). Therefore, all cells containing the gene expressing BSH accumulated taurocholic acid in amounts that were similar to each other and to cells expressing pSportI. Most [24-^{14}C]taurocholic acid is converted into [24-^{14}C]cholic acid in cells expressing BSH (Elkins & Savage, 1998). Therefore, the taurocholic acid in cells containing transporters and BSH are undoubtedly converted to cholic acid which leaves the cell.

Identification of EF. Cholic acid is secreted directly into the culture supernatant solution by strain 100-100 cells. Such cells generate EF^+ solution when incubated with 0.4 mM cold taurocholic acid (final culture concentration) for 30 min (Lundeen & Savage, 1990; Lundeen & Savage, 1992b). Assuming 100% conversion, one would expect that EF^+ supernatant solution contains 0.4 mM cholic acid. DH5α cells suspended in EF^- solution supplemented with 0.4 mM unlabeled cholic acid (EF^-/UCA) were assessed for uptake of [24-^{14}C]taurocholic and [24-^{14}C]cholic acids. These values were compared to uptake by the same cells but suspended in EF^- and EF^+ supernatant solutions. DH5α cells (pSportI or p2000) suspended in EF^-/UCA solution and exposed to 50 nCi of [24-^{14}C]taurocholic acid accumulated radiolabel at levels similar to cells suspended in EF^+ solution. Likewise, these same cells suspended in EF^-/UCA but exposed to 50nCi of [24-^{14}C]cholic acid also accumulated radiolabel at levels similar to cells suspended in EF^+ solution (Table 1). EF solutions were preincubated at 37°C with the cells for 7 to 8 min. During this time, cholic acid can equilibrate across the membrane. It is evident that this process occurs from the data with cells exposed to [24-^{14}C]cholic acid. DH5α cells (pSportI or p2000) display a decrease in accumulated [24-^{14}C]cholic acid when suspended in EF^+ or EF^-/UCA versus identical cells suspended in EF^- supernatant (Table 1). The ability of cold cholic acid to produce an "EF^+-like" increase in taurocholic acid accumulation suggests that CbsT1 and CbsT2, may act as bile acid exchangers.

Effect of ionophores and temperature on accumulation of taurocholic acid. 2,4Dinitrophenol and CCCP are ionophores that collapse the components, ΔpH and ΔΨ,

TABLE 1. Effect of unlabeled cholic acid on [24-^{14}C]taurocholic and [24-^{14}C]cholic acid accumulation in *E. coli* DH5α cultures[a]

Vector[b]	Treatment with EF[c]	Bile acid[d]	Mean uptake ± σ (cpm)[e]
pSportI	-	TCA	1,513 ± 107
pSportI	+	TCA	1,732 ± 418
pSportI	UCA	TCA	2,011 ± 376
p2000	-	TCA	1,990 ± 385
p2000	+	TCA	3,086 ± 112
p2000	UCA	TCA	3,487 ± 313
pSportI	-	CA	6,180 ± 1,629
pSportI	+	CA	2,677 ± 505
pSportI	UCA	CA	3,682 ± 200
p2000	-	CA	4,182 ± 1,306
p2000	+	CA	3,141 ± 704
p2000	UCA	CA	2,874 ± 1,458

[a]Cultures were normalized to a Klett reading of 155. All cultures were induced with 0.5 mM IPTG.

[b]p2000 is derived from pSportI and encodes the 3'-half of *cbsT1* and a complete *cbsT2* (Elkins & Savage, 1998).

[c]Assays were performed with cell cultures suspended in *L. johnsonii* strain100-100 supernatant solutions: EF^+ (+), EF^- (-), EF^- but containing 0.4 mM unlabeled cholic acid (UCA; final concentration).

[d]TCA, [24-^{14}C]taurocholic acid; CA, [24-^{14}C]cholic acid.

[e]Arithmetical means were calculated from data representing three replicates from the same bacterial suspension. σ, standard deviation.

of the protonmotive force, Δp (White, 1995). These compounds inhibit the function of secondary active transporters such as AcrB or EmrB that are dependent on Δp (Nikaido, 1996). Uptake of taurocholic acid via CbsT2 was tested in cell suspensions containing the ionophores. 2,4Dinitrophenol and CCCP were added to DH5α cell suspensions (1 mM or 50 μM final culture concentrations, respectively; Mallonee & Hylemon, 1996; Thanassi et al., 1997) immediately before the 7 to 8 min preincubation of cells at 37°C. [24-^{14}C]Taurocholic acid was accumulated by DH5α cell suspensions (pSportI or p2000) containing the ionophores in slightly higher amounts than in comparable cell suspensions not exposed to ionophores (control; Fig. 5). If CbsT2 is dependent on the components of Δp (i.e., a proton symporter), then [24-^{14}C]taurocholic acid should accumulate in p2000 cells to a lesser extent than the same cells lacking ionophores. The data demonstrated no such decrease, but rather indicated that uptake occurs by an energy-independent manner such as facilitated diffusion.

The uptake of taurocholic acid by cells expressing CbsT2 should be temperature dependent if the activity is associated with a protein. [24-^{14}C]Taurocholic acid uptake by control cells (pSportI and p2000) was compared to cells lacking ionophores but incubated on ice. Those suspensions incubated on ice accumulated far less taurocholic acid than the respective control cell suspensions (pSportI or p2000). In addition, p2000/EF^{+} cells incubated on ice accumulated taurocholic acid in amounts similar to p2000/EF^{-} cells incubated identically (Fig. 5). Uptake of taurocholic acid by p2000/EF^{+} cells is, therefore, a temperature dependent (protein-mediated) process. It is an interesting observation that DH5α pSportI cells demonstrated an increase in accumulated

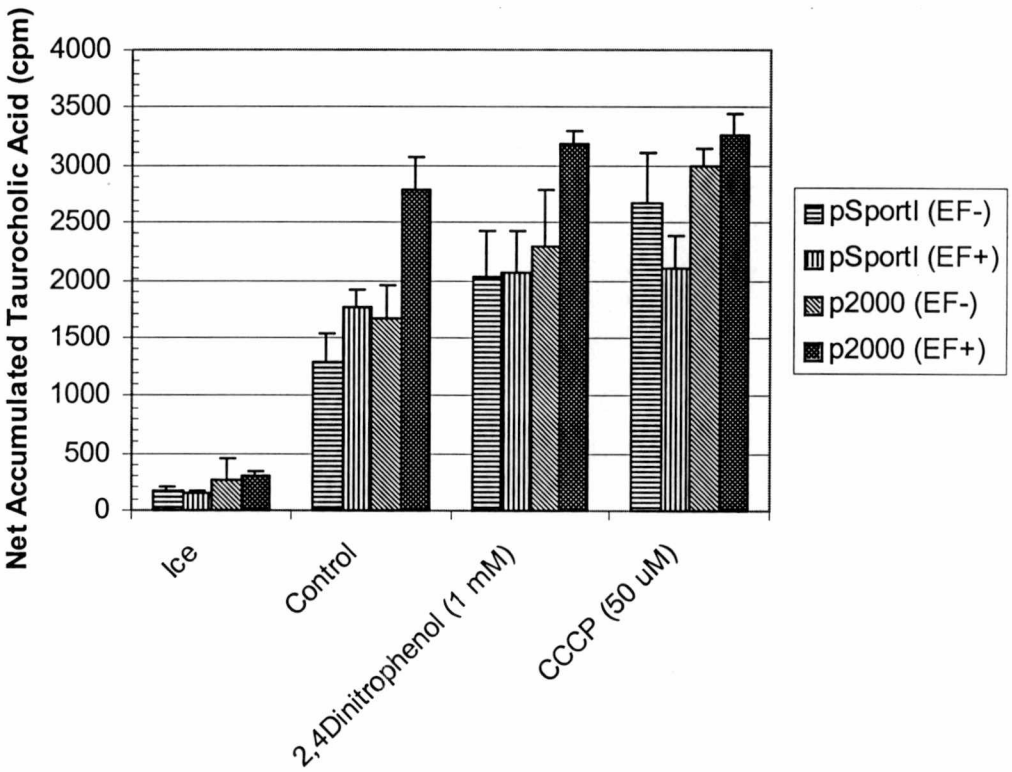


FIG. 5. Accumulation of [24-^{14}C]taurocholic acid by DH5α cultures on ice and in the presence of ionophores, 2,4dinitrophenol and CCCP. Taurocholic acid (50 nCi) was incubated with each cell suspension for four min. All cultures were normalized to Klett readings of 150 to 160. Error bars represent standard deviation of two separate experiments of three replicates each for 2,4dinitrophenol and ice treatment. Standard deviations with cells exposed to CCCP represent three replicates from the same bacterial suspension. Control cells suspensions were treated identically but contain no ionophore; accumulation was measured for 10 min for cells on ice. Standard deviation represents four separate experiments of three replicates each.

taurocholic acid versus identical cells on ice. This finding suggests that bile acids may bind to membrane proteins with specificity for these molecules (e.g., efflux pumps). These proteins, however, may not function efficiently in EF supernatant solution (see below). Alternatively or in addition, taurocholic acid would not diffuse into cells on ice at the same rate as cells incubated at a higher temperature since diffusion is temperature dependent.

Accumulation of taurocholic acid by DH5α cells in a defined transport medium. EF preparations are undefined since they are supernatant solutions from strain 100-100 cells (Lundeen & Savage, 1992b). EF^+ and EF^- solutions were found to be identical in pH (4.2). Furthermore, unlabeled cholic acid produced an "EF^+-like" effect when added to EF^- solution. A hypothesis was tested that DH5α cells (pSportI or p2000) could accumulate taurocholic acid in defined resuspension solutions to the same extent observed in EF supernatant solutions. Cells were suspended in MRS medium, MRS salts medium, and sodium acetate buffer at different pHs with or without 0.4 mM unlabeled cholic acid. DH5α cells (pSportI or p2000) resuspended at higher pHs (5.0 and 6.5) accumulated [24-^{14}C]taurocholic acid in much lower amounts than the comparable cells resuspended in solutions at pH 4.2. The highest levels of uptake (3,674 ± 425 and 3,301 ± 222 cpm) were detected in p2000 cells suspended in pH 4.2 MRS and MRS salts buffer, respectively, supplemented with 0.4 mM cholic acid (Fig. 6). These levels were similar to values previously published of p2000 suspended in EF^+ supernatant (3,507 ± 315 cpm; Elkins & Savage, 1998). However, pSportI cells suspended in MRS or MRS

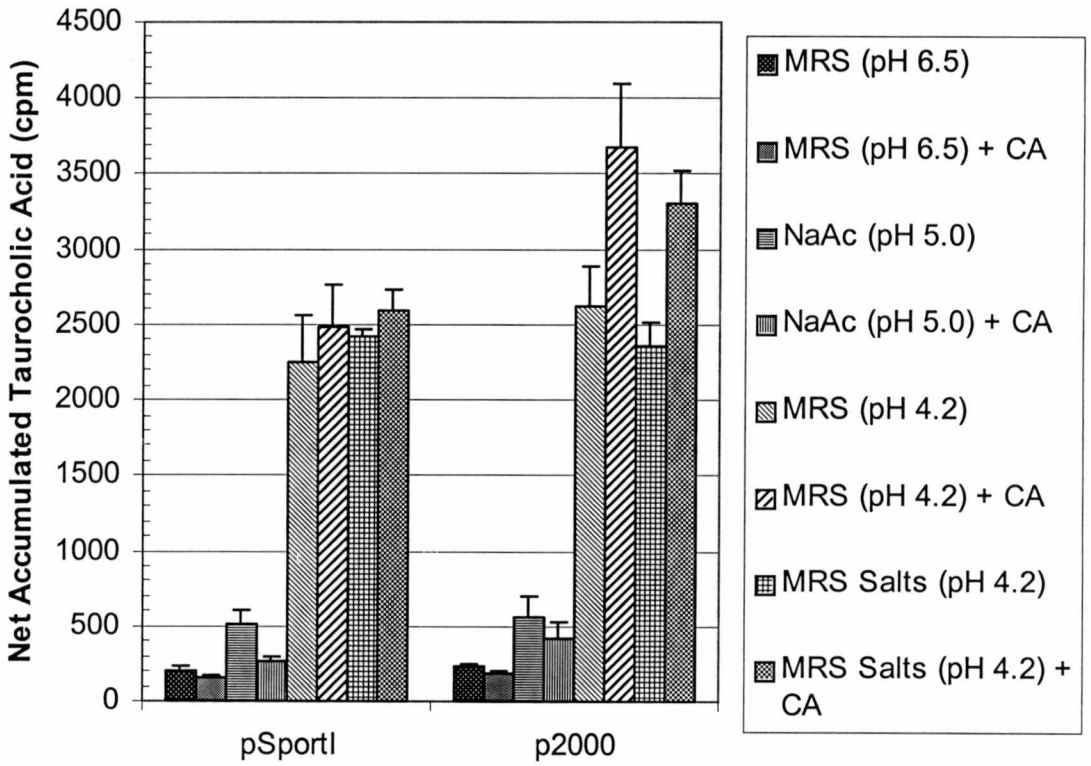


FIG. 6. Accumulation of [24-^{14}C]taurocholic acid by DH5α cells suspended in fresh MRS, MRS medium without carbon and energy sources (MRS salts), and sodium acetate buffer (NaAc) at different pHs. Cell cultures were grown and normalized to Klett readings of 150 to 155. Error bars represent standard deviation of three replicates from the same bacterial suspension. CA, 0.4 mM (final concentration) of cold cholic acid.

salts medium accumulated taurocholic acid in amounts higher than obtained for cells suspended in EF^- or EF^+ (1,398 ± 168 and 2,175 ± 229 cpm, respectively; Elkins & Savage, 1998). The data suggest that *E. coli* exports bile acids more efficiently at pHs that approach native *E. coli* environments (5.0 and 6.5) than at an aciduric pH (4.2). Membrane proteins responsible for this efflux may still retain the capacity to bind bile acids. Similar results were reported for uptake of [24-^{14}C]cholic acid in *E. coli* DH5α cells. Accumulation of [24-^{14}C]cholic acid in pSportI cells increased as pH decreased (Mallonee & Hylemon, 1996). Energy sources in fresh MRS medium (dextrose, beef extract, and yeast extract) had little effect on the amounts of taurocholic acid accumulated by cells as compared to cells suspended in MRS salts buffer not containing such sources. Energy sources would be expected to enhance levels of substrate uptake if that uptake were mediated by an active process requiring Δp. It is unknown why pSportI cells accumulated more taurocholic acid in defined medium than in EF solutions. It is speculated, however, that the salt concentrations in MRS or MRS salts medium may affect bile acid partitioning into the membrane.

The defined transport medium, MRS salts buffer, was used in an experiment with taurocholic acids that were radiolabeled with ^{14}C or ^{3}H. As mentioned before, [24-^{14}C]taurocholic acid is labeled on the bile acid steroid (cholic acid). [^{3}H]taurocholic acid is labeled on the taurine and was used to confirm and extend the studies with constructs p2000, pT2BSH, and pBSH (see Accumulation of taurocholic acid by DH5α cells containing constructs of the *cbsHβ* operon). If CbsT2 catalyzes an exchange of taurocholic and cholic acid, cells expressing pT2BSH should accumulate ^{3}H radiolabel in

amounts similar to p2000. It is assumed that free taurine, released from taurocholic acid, would remain inside the cell since it is hydrophilic. As consistently observed, p2000 cells with or without 0.4 mM cholic acid accumulated more ^{14}C radiolabel than did pT2BSH cells treated similarly (Table 2). However, these same cells, when treated identically, accumulated similar amounts of ^{3}H radiolabel. Cells expressing p2000 or pT2BSH and exposed to 0.4 mM cholic acid accumulated decreased amounts of ^{3}H radiolabel as compared to the same cells without cholic acid. Cells expressing pBSH accumulated less ^{3}H radiolabel than cells expressing p2000 or pT2BSH. Moreover, these cells did not demonstrate decreased levels of ^{3}H radiolabel in the presence of unlabeled cholic acid (Table 2). The concentration of [^{3}H]taurocholic acid added to cell suspensions was 1,000-fold lower than the concentration of [24-^{14}C]taurocholic acid. Facilitated diffusion of taurocholic acid by CbsT2 would be dependent on concentration of the substrate. If taurocholic acid is exchanged for cholic acid via a facilitated mechanism, taurocholic acid uptake would not occur readily if cholic acid was added in excess.

Electrogenic studies. The similarity of the amino acid sequences of CbsT1 and CbsT2 to those of electrogenic OFA proteins suggests that CbsT1 and CbsT2 may function as electrogenic antiporters of conjugated and unconjugated bile acids. As mentioned previously, unconjugated bile acids can act as weak acids since they have a pK_as of 5 to 6.5 (Small, 1973; Thanassi et al, 1997; Kurdi et al., 2000). In pools of deconjugated bile acids that are produced by BSHs, a large fraction exists in deprotonated form. CbsT1 and CbsT2 could create a membrane potential if exchange of conjugated^{-1} for deconjugated

TABLE 2. Accumulation of ^{14}C and ^{3}H labeled taurocholic acids in *E. coli* DH5α cultures[a]

Vector[b]	Cholic acid[c]	Radiolabeled taurocholic acid[d]	Mean uptake ± σ (cpm)[e]
p2000	-	^{14}C	2,505 ± 170
p2000	+	^{14}C	2,796 ± 112
pT2BSH	-	^{14}C	1,512 ± 152
pT2BSH	+	^{14}C	1,267 ± 135
p2000	-	^{3}H	3,140 ± 423
p2000	+	^{3}H	1,771 ± 115
pT2BSH	-	^{3}H	3,109 ± 251
pT2BSH	+	^{3}H	1,984 ± 125
pBSH	-	^{3}H	653 ± 278
pBSH	+	^{3}H	613 ± 190

[a]Cultures were normalized to a Klett reading of 150 to 155. All cultures were induced with 0.5 mM IPTG.

[b]Constructs were derived from pSportI. p2000, see Table 1, footnote b; pT2BSH encodes *cbsT2* and *cbsHβ* (Part III).

[c]Cells were assayed in MRS salts buffer (-) or MRS salts buffer containing 0.4 mM (final concentration) cholic acid (+).

[d]Either 50 nCi of [24-^{14}C]taurocholic acid (^{14}C) or 0.4 μCi of [^{3}H]taurocholic acid (^{3}H) was incubated with cell suspensions.

[e]Arithmetical means were calculated from data representing three replicates from the same bacterial suspension. σ, standard deviation.

bile acids occurs. This membrane potential should have positive effects on bacterial growth. Suspensions of DH5α cells expressing pT12BSH, pT2BSH, and pBSH were grown, normalized to same Klett reading, and spot inoculated onto MRS-TDCA-AMP-IPTG plates. Halos of precipitated deoxycholic acid surrounding colonies from each culture were the same size after 24 and 48 hours of anaerobic growth. In a related experiment, ATP levels in strain 100-100 cultures were measured using a modified luciferase assay (Cole, 1967). Strain 100-100 culture exposed to 0.4 mM taurocholic acid (final culture concentration) demonstrated no difference in luminescence when compared to the same culture not exposed to the bile acid (data not shown).

Discussion

These data support a hypothesis that CbsT2 (and CbsT1) facilitates uptake of taurocholic acid. *E. coli* cells expressing CbsT2 (p2000) demonstrated a rapid initial increase in uptake of taurocholic acid. After this initial increase, such cells demonstrated no contribution of CbsT2 or EF^+ to rate of uptake versus pSportI/EF^- control cells. Therefore, CbsT2 in the presence of EF^+ did not act as a pump of taurocholic acid in DH5α cells. However, cells expressing p2000 did demonstrate a saturable kinetic uptake of taurocholic acid that was contributed exclusively by CbsT2 in the presence of EF^+. Such characteristics resemble a facilitated mechanism for uptake of taurocholic acid. The data generated in presence of ionophores also supports this conclusion. Uptake of taurocholic acid by DH5α cells expressing p2000 remained constant when incubated with

2,4dinitrophenol or CCCP. Many secondary active transporters of the MFS use Δp as energy source for uptake of substrates and would be inhibited by either ionophore.

Any measurement of bile acid uptake in *E. coli* harboring a bile acid importer is a measurement of net accumulation of the substrate. The results obtained in the time dependent uptake of taurocholic acid (Fig. 3A) suggest that the EF supernatant solution ($^+$ or $^-$), used to resuspend the *E. coli* DH5α cells, has an effect on bile acid uptake. My studies and the studies of Thanassi et al. (1997) demonstrate a rapid initial increase in bile acid uptake for all *E. coli* cultures. Since bile acids are amphipathic molecules that form micelles, they can diffuse through the membrane down a concentration gradient. The initial rapid increase suggests that the bile acids, tauro- and chenodeoxycholic acid, partition directly into the membrane. However, my studies with p2000 and pSportI do not demonstrate a steady-state accumulation of substrate, but rather a linear increase over time. Since the pH of EF^+ and EF^- solutions are approximately 4.2, the contributions of $\Delta\Psi$ and ΔpH to the Δp are different from those in the native environment for *E. coli*. For example, the Δp of a neutrophile like *E. coli* is comprised of 70 to 80% $\Delta\Psi$ and 20 to 30% ΔpH. The Δp of an aciduric organism is comprised entirely of ΔpH (White, 1995). *E. coli* efflux pumps dependent on the Δp may not function efficiently or cease functioning in supernatant solutions of an aciduric organism (see Accumulation in a defined transport medium) and therefore may not reach a steady-state accumulation of taurocholic acid.

The lack of steady-state accumulation in DH5α cells suspended in EF is not the result of an increased concentration of taurocholic acid. The amount of [24-

^{14}C]taurocholic acid added to each sample, 5.4 μM, was 37-fold lower than that used by Thanassi et al. (1997). An alternative explanation of these data involves the physio-chemical differences in tauro- versus chenodeoxycholic acid. Taurocholic acid has a pK_a of approximately 1.5 to 2.0 whereas chenodeoxycholic acid has a pK_a of approximately 6 to 6.5 (Thanassi et al., 1997). Chenodeoxycholic acid can act as a weak acid that can accumulate in the cell. Since the intracellular pH is higher in respiring cells than the extracellular pH, protonated species of the weak acid remain equal on both sides of the membrane but deprotonated, charged species spontaneously accumulate in the cell (Kurdi et al., 2000). Taurocholic acid is completely charged at physiological pHs and therefore cannot act as a weak acid.

The function of EF^+ supernatant solution on uptake of taurocholic acid by CbsT2 was investigated in this study. Taurocholic acid uptake in EF^+ supernatant by DH5α cells expressing pT12BSH, pT2BSH, and pBSH, was lower than uptake in p2000 cells. Rather, the levels of uptake of pT12BSH, pT2BSH, and pBSH cells were similar to uptake in pSportI cells suspended in EF^- supernatant. It is suggested that [24-^{14}C]cholic acid, liberated by the BSH, leaves the cell via CbsT2 (and CbsT1). Therefore, levels of uptake of taurocholic acid in cells expressing transporter(s) and BSH can approximate levels of cells expressing BSH alone or control vector (pSportI). Taurocholic acid uptake with cells expressing CbsT2 (p2000) in EF^+ supernatant was similar to uptake in EF^- supernatant containing unlabeled cholic acid. Likewise, in cells expressing control vector (pSportI), uptake in EF^+ solution was similar to uptake in EF^- solution supplemented with cold cholic acid (EF^-/cholic acid). The "EF^+-like" effect of EF^-/cholic acid supernatant suggests that cold cholic acid has an effect on [24-^{14}C]taurocholic acid uptake. At pHs

below 6.4, cholic acid exists as primarily as a neutral hydrophobic molecule that readily equilibrates on either side of the membrane (Kurdi et al., 2000). These data suggest that CbsT2 may exchange taurocholic acid for cholic acid and can account for the positive effect on uptake with EF^+ solution. Moreover, EF^+ solution enhances levels of BSH activity in *L. acidophilus* strain 100-37 (Lundeen & Savage, 1992b). A *cbsHβ* locus encoding CbsT1 and CbsT2 homologs was identified in *L. acidophilus* strain KS-13 (Part III). Therefore, the presence of bile acid transporters can account for EF^+-effect on BSH activity in this species.

Preliminary characterization of EF^+ supernatant indicates that the molecule responsible for enhanced BSH activity has properties similar to a bile acid (Lundeen & Savage, 1992b). EF^+ supernatant is air-, protease-, and heat-resistant. It does not bind taurocholic acid but can partially partition into organic solvents. It does not elute through ultrafiltration membranes with high molecular weight exclusion limits (100 kDa), but dialyzes through tubing with molecular mass exclusion limit of 25 kDa (Lundeen & Savage, 1992b). Therefore, the molecular weight of the molecule is speculative. These results can be explained and expected if EF is a bile acid. Bile acids are amphipathic molecules that can associate with organic solvents and form large aggregates (micelles) in the proper environment (Small, 1973; Wilson, 1981).

EF^+ solution is inhibited by N-ethylmaleimide (NEM), a sulfhydryl group inhibitor. Furthermore, cholic acid when added to EF^- supernatant does not produce an EF^+ effect on BSH activity (Lundeen & Savage, 1992b). Three explanations can be given for these observations. (i) NEM, added at concentrations of 1 and 10 mM, were dialyzed out of EF^+ solution but may have remained in residual amounts that would have affected

strain 100-100 cells negatively. (ii) The pH of the dialysis buffer (7.0) could have raised the pH of EF solution and have a negative effect on transport via CbsT2 (and CbsT1) in strain 100-100 cells. The data on uptake of taurocholic cholic acid obtained with DH5α cells suspended in solutions of various pHs (see Accumulation of taurocholic acid by DH5α cells in a defined transport medium) supports this explanation. *E. coli* is less capable of retarding influx via export of taurocholic acid at lower (aciduric) than at higher pHs. The converse may hold true for strain 100-100. Transporters CbsT2 and CbsT1 may not function efficiently at pHs significantly higher than aciduric conditions. (iii) The inability of cholic acid to enhance BSH activity in strain 100-100 cells may be due to precipitation of cholic acid since EF solution has a pH of 4.2. I observed no such enhanced BSH activity with deoxycholic acid, a less soluble primary bile acid than cholic acid. In my assays, EF solutions were mixed thoroughly such that any cholic acid is resuspended. A thick suspension of *E. coli* cells was then created in EF solutions similar to assays with nafcillin, an extremely hydrophobic penicillin (Nikaido et al., 1998). Therefore, cholic acid could partition directly into a soluble membrane environment.

That cholic acid leaves cells expressing CbsT2 (and CbsT1) is supported by experiments with [24-^{14}C]- and [^{3}H]taurocholic acid and by recent studies on spontaneous accumulation of cholic acid in lactobacilli (Kurdi et al., 2000). DH5α cells expressing transporter and BSH (pT2BSH) accumulated less ^{14}C radiolabel (by approximately half) than cells expressing transporter alone (p2000; Table 2). In contrast, these same cells (pT2BSH and p2000) accumulated similar amounts of ^{3}H radiolabel when comparing cells suspended identically in transport medium with or without 0.4 mM cold cholic acid. Furthermore, cells expressing pBSH accumulated less ^{3}H radiolabel

than either pT2BSH or p2000 cells. I conclude from these data that [24-^{14}C]cholic acid leaves cells expressing transporter and BSH. The levels [^{3}H]taurine in these same cells demonstrate that taurocholic acid has access to the intracellular BSH. Therefore, taurine does not leave the cell and is accumulated to levels similar to cells expressing transporter alone (p2000).

Recent studies in several strains of lactobacilli reveal that cholic acid is accumulated spontaneously, driven by ΔpH (Kurdi et al., 2000). Deprotonated, charged cholic acid accumulates within the cell and can be described by the Henderson-Hasselbalch equation for weak acids. This system assumes that protonated, uncharged cholic acid remains equal on either side of the membrane. Deprotonated, charged cholic acid accumulates intracellularly for two reasons. (i) The internal pH that is higher than the extracellular pH. (ii) The charged species of cholic acid cannot diffuse readily across the membrane because of polarity. Glucose energized *L. salivarius* JCM 1044 cells accumulated increased levels of cholic acid upon addition of valinomycin (an ionophore that dissipates ΔΨ) versus identical cells without valinomycin. However, the same experiment was performed with *L. acidophilus* JCM 1028 resulted in a slight decrease of accumulated cholic acid versus identical glucose energized control cells (Kurdi et al., 2000). Strain JCM 1028 encodes the *cbsHβ* locus containing CbsT1 and CbsT2 homologs (Part III). I suggest that the decrease in accumulated cholic acid in strain JCM1028 is facilitated by these transporters in the absence of taurocholic acid (for exchange) and ΔΨ. The authors conclude that accumulated cholic acid in the presence of

valinomycin by strain JCM 1044 was due to an increase of ΔpH as a result of the dissipation of $\Delta\Psi$ and was verified by measuring internal pH (Kurdi et al., 2000).

The data contained in this report can be culminated into models for the empirical mechanism of CbsT2 (and CbsT1) function (Fig. 7). Taurocholic acid is exchanged for cholic acid without need for energy input and in a facilitated fashion such that Michaelis-Menten kinetics are obeyed. The question of which species of cholic acid is exchanged would determine if the process is electrogenic. Exchange of taurocholic $acid^{-1}$ for cholic acid would produce a polarizing effect across the membrane and increase both $\Delta\Psi$ and ΔpH (i.e., a negative charge moves into the cell while a cytosolic proton is consumed and leaves the cell via cholic acid; Fig. 7A). Such an effect was tested with bacterial growth experiments with *E. coli* and with studies of strain 100-100 ATP levels. *E. coli* transformed with pT12BSH, pT2BSH, and pBSH demonstrated no differences in growth, monitored by halo size of precipitated deoxycholic acid. Furthermore, strain 100-100 cells exposed to taurocholic acid demonstrated no increase in ATP levels within 30 min versus cells not exposed to such molecules. If exchange of conjugated bile acids is electrogenic, increases in bacterial growth or ATP levels should occur with constructs pT12BSH and pT2BSH or strain 100-100 cells, respectively.

The rapid initial uptake of taurocholic acid by DH5α cells (<15 sec), the energy independent facilitated exchange of taurocholic and cholic acids (Fig. 7B), and the amphipathic nature of bile acids suggest that these transporters may function as flippases. Flippases are associated with transbilayer movement of phospholipids (Hrafnsdóttir et al., 1997; Hrafnsdóttir & Menon, 2000). The process is important because phospholipids are

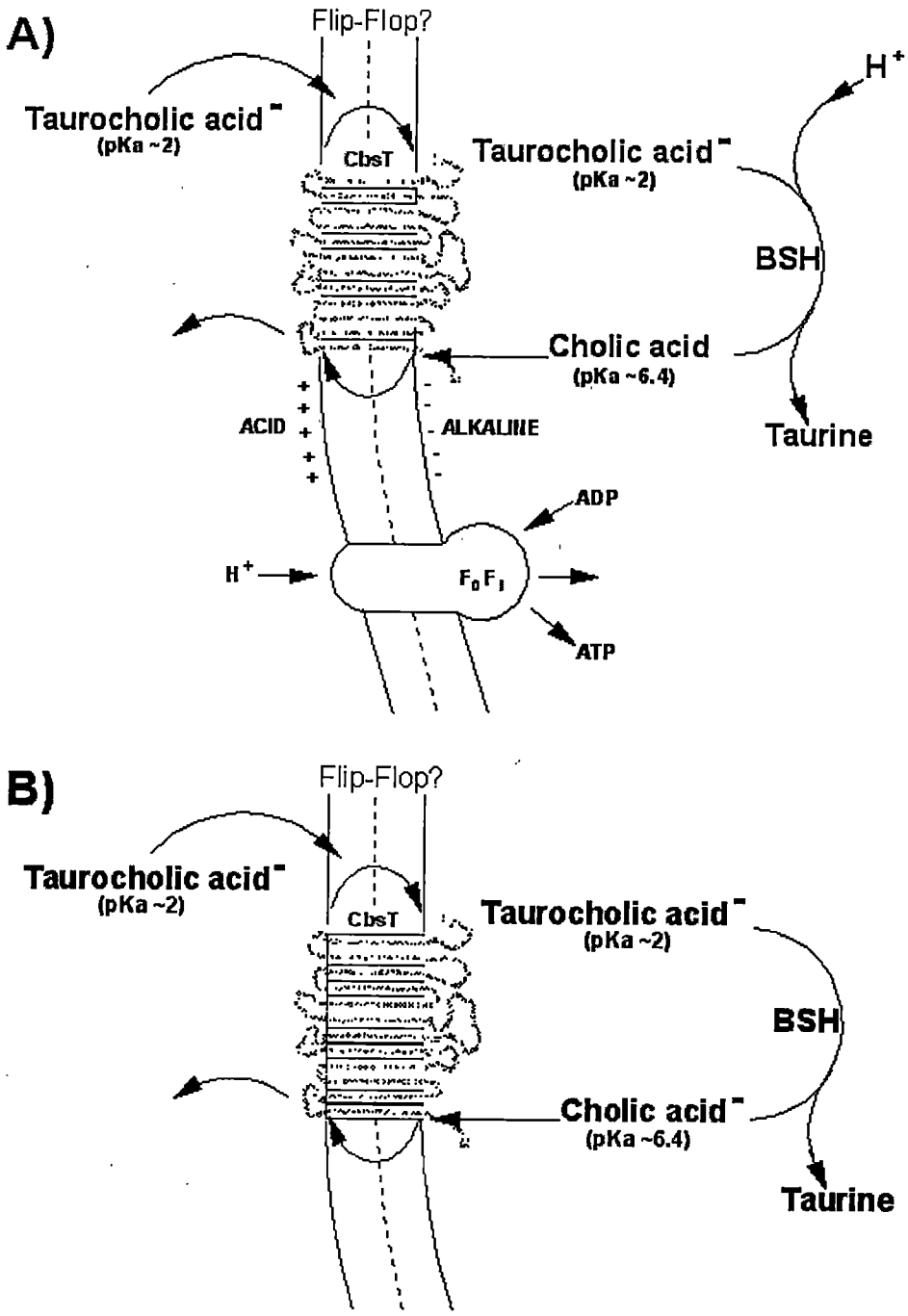


Fig. 7. Proposed models for exchange of bile acids by CbsT2 (or CbsT1). The transporters demonstrate characteristics of flippases than may permit flip-flop of taurocholic acid and cholic acid. (A) Model for electrogenic exchange of taurocholic for cholic acid. (B) Model for electroneutral exchange of the bile acids.

synthesized on the inner leaflet of the cytoplasmic membrane but must translocate to the outer leaflet to propagate the bilayer. The process is associated with transmembrane proteins and occurs very fast (15 to 30 sec; Hrafnsdóttir et al., 1997; Hrafnsdóttir & Menon, 2000). Similar to phospholipids, bile acids are amphipathic molecules that could flip-flop from one leaflet to the other, and therefore, be captured from within the membrane. A flipping mechanism has been proposed for the multidrug efflux pump AcrAB of *Salmonella typhimurium* (Nikaido et al., 1998) and a mouse protein, MDR2, capable of translocating phosphatidylcholine (Ruetz & Gros, 1994). In light of the observations in this report, it is interesting that MDR2-mediated phosphatidylcholine translocation is enhanced by taurocholic acid (Ruetz & Gros, 1995). Whether CbsT1 and CbsT2 do indeed flip bile acids within the membrane warrants further investigation that may require fluorescent conjugates of such molecules. In addition, it would be interesting to determine whether the transporters have a broad specificity, similar to multidrug efflux pumps (e.g., AcrAB and EmrAB; Nikaido, 1996), that would include phospholipids.

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PART V

CONCLUSIONS

Summary

This study was focused on the molecular genetics of BSH activity in strain 100-100. The work of Lundeen and Savage demonstrated that strain 100-100 encoded two peptides, α and β, that combine to form four, native homo- and heterotrimeric BSH isozymes (Lundeen & Savage, 1990; Lundeen & Savage, 1992a). In addition, strain 100-100 was shown to produce and respond to a soluble and secreted EF of unknown composition (Lundeen & Savage, 1990; Lundeen & Savage, 1992b). The data presented in this work support a conclusion that the genetic determinants responsible for BSH activity have been identified and cloned (Elkins & Savage, 1998; Part III). As hypothesized earlier, this molecular genetic approach has yielded additional genes, related to BSH capacity, that encode transporters of the conjugated bile acid substrates (Elkins & Savage, 1998; Part IV). Furthermore, EF has been identified and a functional role at the level of transport has been postulated (Part IV).

BSH Genetics

Strain 100-100 contains two separate loci that encode the two BSH peptides α and β. At one locus, the gene encoding the β peptide, *cbsHβ*, is in tandem with two other genes, *cbsT1* and *cbsT2* (Elkins & Savage, 1998). The three genes are coordinately regulated and transcribed polycistronically during stationary phase from a putative promoter 78 nts 5' of *cbsT1* (Part III). Physiological studies demonstrate that the BSH activity is expressed constitutively upon entry into stationary phase (Lundeen & Savage,

1990). Identification of *cbsHβ* transcripts from stationary phase strain 100-100 culture complements this earlier finding. The gene encoding the α peptide, *cbsHα*, is at a locus separate from that of *cbsHβ* with no other predicted genes in tandem. CbsHα shares the highest level of sequence similarity to the BSH from *L. plantarum* 80. Moreover, the architecture of this locus is identical to that in strain 80: a single gene flanked 3' by an inverted repeat capable of forming a strong stem loop structure (Part III).

The protein analysis of BSH activity in strain 100-100 by Lundeen and Savage is supported by my genetic studies. SDS-PAGE demonstrated that the α peptide is larger than the β peptide with predicted molecular weights of 42 and 38 kDa, respectively (Lundeen & Savage, 1992a). Molecular weights based on the predicted amino acid sequence of the two genes confirm this observation. *CbsHα* encodes a larger gene than *cbsHβ* with predicted molecular weights of 36.7 and 34.9 kDa, respectively (Elkins & Savage, 1998; Part III). Convincing evidence that supports my designation of the genes encoding α versus β peptides is given from the predicted protein sequence of *cbsHα*. This sequence matches the first 25 amino acids obtained from microsequencing of a purified preparation of the α peptide by Lundeen and Savage (1992a).

BSH activity: a colonization factor. The *cbsHβ* locus was the subject of a detailed genetic study in other lactobacilli. This locus was chosen because it represented the first identified and characterized BSH operon in the literature (Elkins & Savage, 1998). The operon encoded, in addition to BSH activity, a novel function of transport of conjugated bile acids in genes *cbsT1* and *cbsT2* (see below). Questions, interesting from an

ecological perspective, could be addressed since the genetic determinants for BSH activity were identified. Is this activity required for microbial colonization of the GI tract? In other words, is this property important to the physiology of the organism in its enteric environment? How extensive is the *cbsHβ* locus in the genus *Lactobacillus*? These questions served as the focus for the genetic analysis at the ecological level.

Fifty *Lactobacillus* strains were selected from worldwide culture banks based on the following criteria. The strain must be identified to species level and the strain must originate from either human GI tracts or dairy products. Primers engineered to the *cbsHβ* gene from strain 100-100 were used to detect this locus in each of the fifty strains. Almost all of the strains that were positive for *cbsHβ* (9/10) were from human sources. As a corollary and although not explicitly discussed in the body of this work, a majority (71%) of strains of human origin were positive for the BSH phenotype whereas only 25% of strains of dairy origin were positive for the activity (Part III). These two lines of evidence suggest that BSH activity and specifically the *cbsHβ* locus are important factors for lactobacilli to colonize the GI tract.

That some strains from human sources were not positive for BSH activity can be explained. Other factors may contribute to microbe's capacity to persistently colonize a niche in the GI tract. One of these factors could include the capacity to transform bile acids. One transformation reaction, 7α-dehydroxylation, has been studied at a genetic level in *Clostridium* sp. strain TO-931 (Wells & Hylemon, 2000). Genus *Clostridium* has been shown to deconjugate bile acids (Midvedt & Norman, 1967; Masuda, 1981; Gopal-Srivastava & Hylemon, 1988; Coleman & Hudson, 1995). Both activities, however, have

not been shown in *Clostridium*. It has been speculated, as with BSH activity, that 7α-dehydroxylation provides the bacterium with an ancillary electron acceptor for anaerobic respiration (Hylemon, 1985; Eyssen & Robben, 1989). However, some BSH-positive *Lactobacillus* strains do express the capacity to transform bile acids via 7α-HSDH (Stellwag & Hylemon, 1976). Alternatively, BSH-negative lactobacilli of human origin may encode other factors apart from deconjugation or transformation of bile acids that facilitate GI tract colonization.

As a corollary, a majority of lactobacilli of dairy origin were negative for BSH activity (Part III). One would expect this result if this activity is an essential factor for colonization. Therefore, dairy isolates would not be under a selective pressure to harbor the activity. Genetically, BSH activity may have been lost altogether or gone cryptic. One could speculate that loss of BSH activity could be facilitated if the activity is encoded on a mobile genetic element (see below). Thus, the data obtained on the BSH phenotype in human and diary isolates supports the hypothesis that BSH is a colonization factor.

***cbsHβ* locus: a mobile genetic element.** Another conclusion altogether separate can be reached from the study of the fifty species isolates. The BSH phenotype and genotype of the *cbsHβ* locus is not always present in *Lactobacillus* strains of the same species. That is, the phenotypic and genetic trait is not constant, but variable (Part III). *Lactobacillus* strains within six different species expressed the variable BSH phenotype, and two different species encoded a variable *cbsHβ* genetic locus. It is hypothesized from these

findings that BSH activity was acquired horizontally in lactobacilli (Part III). Specifically, the *cbsHβ* locus may comprise a mobile genetic element. It is clear from the data, however, that mobile elements may extend to BSHs apart from the *cbsHβ* locus since species negative for the locus expressed a variable BSH phenotype.

In support of this hypothesis, a group II intron-encoding maturase was identified 3' but complementary to the coding strand for *cbsHβ* (Part III). Group II maturase genes are transcribed from their own promoter and mediate DNA movement by retrohoming to new sites. Little is known about group II maturases in bacteria except that the genes are associated with mobile genetic elements. The best studied examples at a functional level are from the gram positive bacterium, *Lactococcus lactis*. Group II maturases were inserted in a plasmid relaxase gene in strain ML3 and a chromosomal *mobA* gene in strain 712; both genes are involved in conjugation. In the case of strain 100-100, however, sequence analysis indicates that the maturase does not interrupt any genes. A majority of bacterial examples of maturases have been found inserted in repeat elements or insertion sequences of transposons (Edgell et al., 2000). If this were the case in strain 100-100, movement of the *cbsHβ* locus could occur under promotion of the maturase. It would require transcriptional readthrough of the maturase terminator and produce a RNA complement of the *cbsHβ* operon that could retrohome. Moreover, the BSH from *B. longum* SBT2928 may be encoded as a mobile genetic element and serves as precedent. Bile salt hydrolase activity has been cloned and characterized recently from this organism. Flanking the BSH gene in strain SBT2928 are direct and inverted repeats (Tanaka et al., 2000).

One human isolate, *L. acidophilus* strain KS-13 was characterized genetically at the *cbsHβ* locus. *L. acidophilus* is one species in which the BSH phenotype and *cbsHβ* genotype is variable (Part III). The *cbsHβ* locus from strain KS-13 was identical in genomic architecture to that of strain 100-100. The DNA sequence of the three genes, *cbsT1*, *cbsT2*, and *cbsHβ* was 85% identical to the genes in strain 100-100. In support of the hypothesis that the *cbsHβ* locus was acquired horizontally, DNA sequence similarity to strain 100-100 ended 3' of the hydrolase gene and 26 nucleotides 5' of the putative –35 promoter of the operon (Part III).

LPEA analysis. The length of the conserved DNA sequence 5' of *cbsT1* in strain KS-13 supported my data that identified the putative promoter for the *cbsHβ* operon in strain 100-100. LPEA demonstrated that the three genes in strain 100-100 at the *cbsHβ* locus formed an operon (Part III). This method was developed in our lab and constitutes a hybrid of three methods: primer extension, RACE, and LDGW PCR. The –35 and –10 elements of a putative promoter were identified with this method 78 nts 5' of *cbsT1*. It is interesting to note that DNA sequence identity to strain 100-100 ends, in strain KS-13, 26 nts 5' of the predicted –35 element (Part III). One would expect that the region of approximately 135 nts of untranslated sequence 5' *cbsT1* would be conserved in strain KS-13 if it is important for promotion and transcription of the genes.

Lundeen and Savage demonstrated at a physiological level that induction of BSH expression corresponds with entry of strain 100-100 into stationary phase (Lundeen & Savage, 1990). LPEA analysis supported this conclusion as well. There are no

conserved operator/enhancer elements since sequence identity between strain KS-13 and 100-100 ends 5' of the –35 promoter element. Thus, the RNA analysis supports simple sigma-dependent stationary phase induction of BSH activity (Part III). This is in contrast to 7α-dehydroxylation operons in *Eubacterium* sp. strain VPI 12708 and *Clostridium* sp. strain TO-931. RNA analysis in these organisms revealed that transcripts encoding these operons were induced upon addition of 50 μM cholic acid (White et al., 1988; Wells & Hylemon, 2000). In addition, several DNA sequences upstream of the promoter region were highly conserved between the two organisms and may be specific to bile acid regulation (Wells & Hylemon, 2000).

Transport of Bile Acids

My initial observations of taurocholic acid uptake by cells expressing CbsT2 suggested that uptake was dependent on EF (Elkins & Savage, 1998). The level of uptake of taurocholic acid with such cells was modest, only threefold higher than in control cells. Attempts to increase this threefold accumulation in cells expressing CbsT2 proved unsuccessful. I was confident in these data since they resembled the levels of deconjugated bile acid uptake with BaiG from *Eubacterium* sp. strain VPI 12708 (Mallonee & Hylemon, 1996). This transporter confers the capacity to accumulate five- to sevenfold more deconjugate in *E. coli* DH5α cells than control cells. Furthermore, AcrAB and EmrAB efflux pumps in *E. coli* confer modest levels of bile acid efflux when compared to a strain disrupted for these activities (HN971; Thanassi et al., 1997). Therefore, the known examples of bile acid transporters were similar empirically.

The transporters and EF were studied further for uptake of bile acids (Part IV). From such studies, I conclude that they function as facilitated exchangers of taurocholic and cholic acids. They act as facilitators since taurocholic acid was accumulated in the presence of EF^+ in a saturable, energy independent fashion. Similar to facilitators, CbsT2 demonstrated a concentration dependent accumulation of [^{3}H]taurocholic acid in the presence of EF^+ when supplied at external levels 1,000-fold lower than typically used with [24-^{14}C]taurocholic acid. Furthermore, the "EF^+-effect" on taurocholic acid uptake in *E. coli* cells could be duplicated in EF^- solution containing unlabeled cholic acid (at the same concentration as taurocholic acid that was added to produce EF^+ supernatant). Moreover, studies of uptake with taurocholic acid labeled on different parts of the molecule (^{3}H or ^{14}C) and in the presence of the BSH suggested that the taurine remains intracellular while cholic acid leaves the cell (Part IV). Taken in aggregate, the function associated with CbsT2 (and CbsT1) is one of exchange.

Whether the exchange is electrogenic or electroneutral was determined by measuring growth rates in *E. coli* expressing constructs of the *cbsHβ* operon. There was no measurable difference in growth rate (as measured by size of deoxycholic acid halo) in cells expressing transporter(s) and BSH or cells expressing BSH alone. The ATP levels in strain 100-100 exposed to taurocholic acid were compared to the levels in cells unexposed to such molecules. I observed no difference, as measured by luminescence, in ATP levels between the cells within 30 min (Part IV). These data suggest that the exchange is electroneutral such that taurocholic acid^{-1} is exchanged for cholic acid^{-1}.

Role of bile acid exchange in BSH activity. The facilitated exchange of taurocholic for cholic acid is interesting when analyzed in reference to the BSH isozymes. These enzymes have a range in pH optima of approximately 4.2 to 4.5. BSH activity decreases rapidly once outside this range (Lundeen & Savage, 1990; 1992a). How these enzymes function in the relatively neutral pH homeostatic environment within cells can be addressed, I suggest, via the electroneutral exchange bile acids. If cholic $acid^{-1}$ is facilitated out of cells by the transporters, then pools of protonated cholic acid would reequilibrate, and hence, become partially deprotonated. This process may cause a local drop in pH such that the isozymes could function within their optimal range. This would also suggest that the BSHs are in proximity to the transporters.

Transport of bile acids, in general, may be important to the bacterium from an ecological perspective. Since lactobacilli associate with the upper gastric epithelium, they constantly slough, pass through the lower GI, and reestablish subpopulations within the cecum and colon. Therefore, they are exposed to and survive extremely high concentrations of bile acids in the small intestine. The transporters provide the bacterium with a mechanism to mediate uptake and concomitant efflux of such molecules. It is consistent with the capacity of strain 100-100 to produce an unprecedented four BSH isozymes. The bacterium can utilize transient increases in conjugated bile for an advantageous gain or can become hyperresistant to such increases.

Future Research Objectives

The idea that bacteria can exert a positive influence on the health of the host organism has been investigated for many years. Research, with this focus, has been either to improve the beneficial or probiotic qualities of indigenous GI tract bacteria or to engineer strains that can survive passage through the GI. Such engineered strains, if ingested continuously, would be capable of exerting this positive influence. However, factors that determine whether organisms pass slowly or even multiply in the GI are multifaceted (Savage, 2000). For example, the ability to survive passage through stomach and bile acids or the ability to adhere to intestinal epithelial cells is multigenic (Savage, 2000). It is for these reasons that molecular genetic study has been undertaken of intestinal bacteria to identify such traits. This thesis describes one such trait at the molecular level.

Investigation that pursues whether BSH activity is a colonization factor for GI tract bacteria will likely serve as the focus of future study. Such study was initiated in the survey of fifty lactobacilli for the BSH phenotype and *cbsHβ* genotype that is reported in this thesis (Part III). Lundeen and Savage generated a series of BSH mutants by ultraviolet radiation (unpublished data). However, these mutants were undefined genetically because precise knowledge of the BSH loci within the genome of strain 100-100 was not available at the time. The genetic analysis of BSH activity in strain 100-100, reported in this thesis, will permit study with defined mutants for the activity. Using engineered strains containing commercially available biological markers such as bioluminescence or fluorescent protein can permit quantification or microscopic

visualization of these bacteria as they are associated with the GI tracts of host organisms. Certainly, questions that address whether BSH activity is required for persistent colonization can be addressed in this fashion.

The existence of a putative maturase gene 3' of the *cbsHβ* operon is an interesting aspect to this work and may also warrant further investigation. Whether the operon can be mobile under the promotion of the maturase could be tested at the RNA level. This process would, or course, require transcriptional readthrough of the maturase terminator and extension through the DNA complement of the operon. Research aimed at improving the probiotic effect of indigenous bacteria would benefit if the hypothesis that BSH genes are transferred horizontally is supported by future work. An engineered strain could be introduced orally and generate a global genetic effect *in trans* on the autochthonous microbiota via conjugal transfer. Such an effect could either increase or decrease BSH activity accordingly depending on the desired outcome. It seems more scientifically sound to investigate this approach than to undertake the task of creating strains to survive and multiply in the GI tract. Of course, more research on GI tract organisms at the molecular level is needed to assess the efficacy of altering other probiotic traits (e.g., enhanced lactase levels).

Recent efforts to sequence the *L. acidophilus* genome have been undertaken (Abstracts of the 99th General Meeting of the American Society for Microbiology). One rationale for undertaking the project focused on gathering genetic information on BSH activity. Such information is reported in this thesis for strain KS-13. However, with the advent of DNA array technologies that are commercially available, it seems plausible to champion this technology and study unidentified genes from this organism. Such genes

may be factors that are important for colonization of the GI tract and could be tested for the activity. Alternatively or in addition, expression of genes involved in nitrogen regulation or amino acid catabolism could be studied relative to BSH activity. The bile salt hydrolase of *B. longum* SBT2928 is coordinately regulated with *glnE*, a gene in the nitrogen regulation cascade (Tanaka et al., 2000). Such coordinate regulation in SBT2928 supports a hypothesis the BSH activity is important for obtaining an extra amino acid source with available nitrogen.

Bile acid transport by CbsT1 and CbsT2 could be studied further. Amino acid residues in the transporter proteins important for function or that uncouple exchange of conjugated for unconjugated bile acids could be identified via mutagenesis. These two transporters provide some inherent information on conserved residues important for function since they are duplicates. A defined background to study such functional mutants of the transporters would be required. Artificial membranes, liposomes, would be suitable and represent the only alternative for a background deficient in bile acid flux (except for diffusion). CbsT1 or CbsT2 could be tagged with a hexahistidine at the C terminus, purified by nickel column chromatography, associated with liposomes, and assayed for function. Whether these transporters act as flippases could be addressed similar to studies of phosphatidylcholine (PC) translocation mediated by MDR2 (Ruetz & Gros, 1995). In these studies, a fluorescent PC was used in concert with dithionite that could reduce PC to a nonfluorescent product. Only those fluorescent groups in the outer leaflet would be reduced since dithionite is membrane impermeant. Therefore, asymmetric distribution of PC between the inner and outer leaflets of the membrane could be determined. This method could be applied to my system with CbsT1 and

CbsT2, but would depend on whether fluorescent conjugates of taurocholic and cholic acids could be obtained and whether CbsT1 and CbsT2 could utilize such conjugates as substrates.

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APPENDICES

Appendix A:

Genbank report of *cbsHβ* operon in *Lactobacillus johnsonii* strain 100-100

```
LOCUS       AF054971     4272 bp    DNA           BCT       21-AUG-2000
DEFINITION  Lactobacillus johnsonii conjugated bile salt hydrolase
            operon, complete sequence.
ACCESSION   AF054971 AF008584 U90066 U90067
VERSION     AF054971.2  GI:6457644
KEYWORDS    .
SOURCE      Lactobacillus johnsonii.
ORGANISM    Lactobacillus johnsonii
            Bacteria; Firmicutes; Bacillus/Clostridium group;
            Lactobacillaceae; Lactobacillus.
REFERENCE   1  (bases 1 to 4272)
  AUTHORS   Elkins,C.A. and Savage,D.C.
  TITLE     Identification of genes encoding conjugated bile salt
            hydrolase and transport in Lactobacillus johnsonii 100-100
  JOURNAL   J. Bacteriol. 180 (17), 4344-4349 (1998)
  MEDLINE   98389644
  PUBMED    9721268
REFERENCE   2  (bases 1 to 4272)
  AUTHORS   Savage,D.C. and Elkins,C.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-MAR-1998) Microbiology, University of
            Tennessee, M409 Walters Life Sciences Building, Knoxville,
            TN 37996-0845, USA
REFERENCE   3  (bases 1 to 4272)
  AUTHORS   Savage,D.C. and Elkins,C.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-NOV-1999) Microbiology, University of
            Tennessee, M409
            Walters Life Sciences Building, Knoxville, TN 37996-0845,
            USA
  REMARK    Sequence update by submitter
COMMENT     On Nov 22, 1999 this sequence version replaced gi:2997722.
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                     /note="BSH"
     gene            134..1492
                     /gene="cbsT1"
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BASE COUNT     1172 a    770 c    831 g   1495 t      4 others
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4261 tgccaatgna ng
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Appendix B:

Genbank report of *cbsHα* locus in *Lactobacillus johnsonii* strain 100-100

```
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DEFINITION  Lactobacillus johnsonii conjugated bile salt hydrolase
            alpha peptide gene, complete cds.
ACCESSION   AF297873
VERSION     AF297873.1  GI:10732792
KEYWORDS    .
SOURCE      Lactobacillus johnsonii.
ORGANISM    Lactobacillus johnsonii
            Bacteria; Firmicutes; Bacillus/Clostridium group;
            Lactobacillaceae; Lactobacillus.
REFERENCE   1  (bases 1 to 1200)
  AUTHORS   Elkins,C.A. and Savage,D.C.
  TITLE     Genetic Characterization of Bile Salt Hydrolase Activity in
            Lactobacillus johnsonii 100-100
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1200)
  AUTHORS   Elkins,C.A. and Savage,D.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-AUG-2000) Microbiology, University of
            Tennessee, M409 Walters Life Sciences Bldg., Knoxville, TN
            37996-0845, USA
FEATURES             Location/Qualifiers
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                     /protein_id="AAG22541.1"
                     /db_xref="GI:10732793"

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GMDSASRFVKIAFVRAHSPQGNNELSSVTNYFHILHSVEQPKGTDEVGPNSYEYTIYS
DGTNLETGTFYYTNYENNQINAIELNKENLNGDELTDYKLIEKQTINYQN"
BASE COUNT      430 a    173 c    211 g    386 t
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241  taccaaataa aaaggcaaaa tatgccatgg ttgggatggc catcgtagag gataactatc
301  ctttgtattt tgatgcatca aatgaagaag gactaggaat tgctggcctt aattttgatg
361  gtccgtgtca ttatttttcca gaaaatgcgg agaaaaataa tgttacacca tttgaattaa
421  ttccttattt gctaagtcaa tgtactacgg ttgctgaagt aaaagatgca ttgaaagatg
481  ttagcttagt aaatataaac ttttcagaaa aactaccact ttctccactt cactggttaa
541  tggctgataa gactggtgag tcgatcgttg tagaatcgac tttaagtgga ttacacgttt
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841  taagtagtgt aacaaattat ttccatattt tacattcggt tgaacagcca aagggaacag
901  atgaagtcgg accaaattct tatgagtaca caatttactc tgatggaact aacttggaga
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Appendix C:

Genbank report of *cbsHβ* operon in *Lactobacillus acidophilus* strain KS-13

```
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DEFINITION  Lactobacillus acidophilus putative bile salt hydrolase
            operon, complete sequence.
ACCESSION   AF091248
VERSION     AF091248.3  GI:6532002
KEYWORDS    .
SOURCE      Lactobacillus acidophilus.
ORGANISM    Lactobacillus acidophilus
            Bacteria; Firmicutes; Bacillus/Clostridium group;
            Lactobacillaceae; Lactobacillus.
REFERENCE   1  (bases 2749 to 4027)
  AUTHORS   Savage,D.C. and Moser,S.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-SEP-1998) Microbiology, University of
            Tennessee, M409 Walters Life Sciences Building, Knoxville,
            TN 37996-0845, USA
REFERENCE   2  (bases 2749 to 4027)
  AUTHORS   Savage,D.C. and Moser,S.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-NOV-1999) Microbiology, University of
            Tennessee, M409 Walters Life Sciences Building, Knoxville,
            TN 37996-0845, USA
  REMARK    Sequence update by submitter
REFERENCE   3  (bases 1 to 4027)
  AUTHORS   Moser,S.A., Elkins,C.A. and Savage,D.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-DEC-1999) Microbiology, University of
            Tennessee, M409 Walters Life Sciences Building, Knoxville,
            TN 37996-0845, USA
  REMARK    Sequence update by submitter
COMMENT     On Dec 7, 1999 this sequence version replaced gi:6457642.
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4021 ttcaaca

VITA

Christopher Elkins was born in Hershey, Pennsylvania on December 20, 1972. He was a ward of Catholic Charities and was adopted by his present family on February 14, 1973. For eight years, he attended Annunciation of the Blessed Virgin Mary primary school in McSherrystown, Pennsylvania. His secondary education was completed at Delone Catholic High School in McSherrystown where he graduated salutatorian in May 1990. He enrolled at Case Western Reserve University in Cleveland, Ohio in the fall semester of 1990 and received a Bachelor of Arts degree in Biology and History in May 1994. In the fall semester of 1995, he entered the Department of Microbiology at the University of Tennessee, Knoxville. He worked in the lab of Dr. Dwayne Savage and graduated with a Ph.D. in Microbiology in May 2001. He is a member of the American Society for Microbiology. His work on bile acid transport led him to assume a postdoctoral fellowship in the lab of Dr. Hiroshi Nikaido at the University of California, Berkeley. He will work on drug transport systems across microbial membranes.