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Adaptation to bile in *Salmonella enterica*

TESIS DOCTORAL

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Adaptation to bile in *Salmonella enterica*

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RESUMEN

Las bacterias pertenecientes al género *Salmonella* son patógenos Gramnegativos capaces de infectar una gran variedad de animales. En humanos, la infección por *Salmonella* suele comenzar con la ingestión de agua o alimentos contaminados, y puede dar lugar a diferentes cuadros clínicos que van desde gastroenteritis hasta fiebre tifoidea.

Durante la infección, *Salmonella* se ve expuesta a una serie de condiciones desfavorables a las que debe responder para sobrevivir. Un factor adverso, tanto en el intestino como en la vesícula biliar, es la presencia de bilis. La bilis es una secreción digestiva compuesta mayoritariamente por sales biliares, que ayudan a la digestión degradando y dispersando lípidos. Además, las sales biliares tienen actividad antimicrobiana: actúan sobre los fosfolípidos de la membrana celular a modo de detergente, desnaturalizan proteínas y producen daños en el ADN. A pesar de sus efectos nocivos, *Salmonella* es intrínsecamente resistente a la bilis, e incluso es capaz de crecer formando biofilms sobre la superficie de los cálculos biliares en el interior de la vesícula biliar. Igual que sucede con otras sustancias antimicrobianas, *Salmonella* puede adaptarse a la bilis: en presencia de concentraciones subletales de bilis, aumenta su nivel de resistencia.

El objetivo de esta Tesis ha sido el estudio de los mecanismos implicados en la resistencia de *Salmonella enterica* a la bilis, con énfasis en el fenómeno de la adaptación. En primer lugar se realizó un análisis transcriptómico en presencia de concentraciones subletales de desoxicolato (DOC), la sal biliar más abundante en la bilis. Los resultados de dicho análisis mostraron cambios en la expresión de un gran número de genes. Algunos cambios significativos eran los siguientes:

1. Activación de la respuesta general a estrés dependiente de RpoS y de otras respuestas a estrés.

2. Represión de genes de porinas y de otros genes que codifican proteínas de la envuelta bacteriana.

3. Activación de bombas de vertido.

La adaptación no es el único mecanismo que confiere resistencia a bilis en *Salmonella*. Al sembrar un cultivo de *Salmonella* en una placa que contiene una concentración letal de bilis se

obtienen colonias resistentes, y el análisis de fluctuación indica que dichos aislados proceden de células resistentes existentes en el cultivo previo. Una fracción de aislados resistentes son, como cabía esperar, mutantes. La secuenciación completa del genoma de 6 mutantes independientes reveló la existencia de diversos tipos de mutaciones, pero el tipo más común estaba relacionado con transporte de lipopolisacárido. La causa por la que mutaciones en los genes de transporte de lipopolisacárido confiere resistencia a bilis se desconoce.

Entre los aislados resistentes a bilis obtenidos con selección letal apareció un segundo tipo, *a priori* inesperado. Se trataba se aislados que perdían la resistencia a bilis si se cultivaban en ausencia de DOC (por ejemplo, en LB). La inestabilidad del fenotipo de resistencia descartaba que la resistencia se debiera a mutación. Una explicación plausible es que los aislados con resistencia inestable procedan de células que, durante el cultivo previo, habían activado accidentalmente alguna de las respuestas a estrés que confieren resistencia a bilis. Esta hipótesis es apoyada por el análisis de células individuales usando microscopía microfluídica: los cultivos de *S. enterica* en LB contienen células que tienen activada la respuesta general a estrés y otros loci de respuesta a estrés. Este patrón de expresión permitiría posteriormente la formación de colonias en placas con una concentración letal de bilis. Esta hipótesis es coherente con múltiples observaciones, realizadas por otros autores y también en nuestro laboratorio, en el sentido de que las poblaciones bacterianas clonales pueden ser fenotípicamente heterogéneas para determinados caracteres.

Entre los genes activados por bilis identificados mediante análisis transcriptómico se encontraba un gen de función desconocida anotado como *yiiU*. La pérdida de función de *yiiU* producía sensibilidad a bilis. El análisis *in silico* indicó que dicho gen era homólogo del gen *zapB* de *E. coli*, que codifica un factor no esencial de división celular. El análisis de complementación indicó que los genes *zapB* de *E. coli* y *S. enterica* son intercambiables. Por su parte, el análisis microscópico de células individuales reveló que la proteína ZapB de *Salmonella*, como la de *E. coli*, se localiza en el septo. Se desconoce la causa por la que la falta de ZapB causa sensibilidad a DOC, pero es concebible que la ausencia de anillo preseptal altere la estructura de la envuelta bacteriana haciéndola más susceptible a las sales biliares.

Usando fusiones *lacZ* y analizando los niveles de ARNm por Northern se confirmó que el gen *zapB* de *Salmonella* es activado por DOC. Ahora bien, la regulación por DOC no parece ser transcripcional, ya que se sigue observando cuando *zapB* se coloca bajo el control de un

promotor heterólogo (pL$_{tetO}$). El análisis de la estabilidad del ARNm *zapB* mediante Nothern indicó que el ARNm *zapB* era más estable en presencia de DOC, y una mutación *hfq* eliminaba dicho aumento. Estas observaciones sugieren que, en presencia de DOC, un mecanismo postranscripcional dependiente de Hfq impide la degradación del ARNm de *zapB*. No se puede descartar que la unión de Hfq proteja al ARNm *zapB* de la degradación. Ahora bien, dado que Hfq habitualmente actúa catalizando la interacción entre moléculas de ARN, parece más probable que el aumento de estabilidad del ARNm de *zapB* en presencia de DOC se deba a la interacción con un ARN pequeño regulador (sRNA). Dos candidatos son los sRNAs OxyS y RprA, cuya síntesis aumenta en presencia de bilis.

Pese al aumento de estabilidad del ARNm de *zapB* en presencia de DOC, la cantidad de proteína ZapB disminuye en presencia de DOC. Una mutación *lon* suprime el fenotipo de inestabilidad proteica, y ello sugiere que la proteasa Lon degrada la proteína ZapB en presencia de DOC. Este fenómeno puede interpretarse suponiendo que el DOC daña la proteína ZapB, ya que una de las actividades de la proteasa Lon es la degradación de proteínas anómalas.

Así como la sensibilidad a bilis por carencia de ZapB sugiere que la integridad del septo es necesaria para la resistencia a bilis, el estudio de un mutante hiperresistente a bilis proporcionó indicios de que la estructura de la pared celular también tiene un papel en la resistencia a bilis. Dicho mutante carecía de proteasa periplásmica Prc, una proteína implicada en el procesamiento de PBP3. En ausencia de Prc se detectan cambios en la actividad de PBP3 y de otras proteínas de síntesis de péptidoglicano, y ello sugirió una posible correlación entre la estructura del péptidoglicano y la resistencia a bilis.

El análisis de la estructura del péptidoglicano en presencia de una concentración subletal de DOC mostró una disminución en la cantidad de lipoproteína de Braun anclada covalentemente al péptidoglicano y una disminución de los muropéptidos entrecruzados mediante enlaces 3-3. El análisis de la estructura del péptidoglicano en presencia de una concentración letal de DOC (tras adaptación previa del cultivo) mostró cambios similares. Ello sugiere que la presencia de DOC induce una remodelación del péptidoglicano, y que dicha remodelación contribuye a la resistencia a bilis. Esta hipótesis es apoyada por observaciones adicionales: (i) un triple mutante carente de las L,D transpeptidasas YbiS, ErfK, and YcfS, que no contiene lipoproteína de Braun unida covalentemente al

péptidoglicano, resultó ser hiperresistente a bilis; (ii) la superproducción de L,D transpeptidasa YnhG, que produce un aumento de los enlaces 3-3, disminuye la resistencia a bilis. Por tanto, parece razonable añadir la remodelación de la pared celular a la lista de respuestas celulares que permiten la adaptación de *Salmonella enterica* a la bilis.

INTRODUCTION

1. *Salmonella*

1.1. The genus *Salmonella* and *Salmonella*-associated diseases

The genus *Salmonella* belongs to the family *Enterobacteriaceae*, a large group of Gram-negative bacteria, facultative anaerobic and nonspore-froming bacili. *Salmonella* is phylogenetically close to the genera *Escherichia* from which diverged approximately 100 – 160 million years ago [1].

Based on DNA relatedness, *Salmonella* is currently divided into two species, *Salmonella bongori* and *Salmonella enterica*, and the latter is subdivided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diariazonae* (IIIb) , *houtenae* (IV), and *indica* (VI) [2]. Furthermore, depending on the O (surface polysaccharide) and H (flagellar) antigens, *Salmonella* subspecies are classified into more than 2,500 serovars, most of which have been identified among *S. enterica* subspecies I isolates [3]. The current *Salmonella* nomenclature indicates the species, the subspecies, and the serovar (e. g., *Salmonella enterica* subsp. *enterica* serovar Typhimurium). However, this name is often shortened to *Salmonella* Typhimurium [4].

Salmonella serovars are pathogens capable of infecting a wide range of animals (including humans, farm animals and reptiles) and even plants. Certain serovars are able to infect a broad variety of animals while others are host-specific [5]. *Salmonella* is a facultative endopathogen and the causative agent of various human diseases, ranging from mild enteritis to typhoid fever. The majority of serovars that cause disease in humans and warm blooded animals belong to *S. enterica* subspecies *enterica*, while the remaining subspecies (and members of *S. bongori*) are commonly isolated from cold-blooded vertebrates or from the environment [6]. According to the World Health Organization, salmonellosis is the most frequent foodborne disease with around 1.5 billion infections worldwide per year (WHO 2012).

Salmonellosis in humans is associated with three distinct disease syndromes: bacteremia, typhoid fever and enterocolitis. The outcome of the infection depends on the specific serovar-host combination. For example, *S.* Typhi is a human-specific pathogen that causes enteric fever. In contrast, *S.* Typhimurium produces mild gastroenteritis in humans but causes a systemic disease similar to human typhoid fever in mice [7]. For

this reason, human infection with *Salmonella* Typhi can be modeled in the laboratory by infection of susceptible mice with *Salmonella* Typhimurium [8]. In this work, we have used the mouse-virulent strain *Salmonella* Typhimurium SL1344, from the B.A.D. Stocker collection.

1.2. *Salmonella enterica* infection and pathogenicity

Infection with *S. enterica* usually occurs as a result of consuming contaminated food or water. After ingestion, *Salmonella* must endure adverse conditions which are part of the host defence against infections, such as acid pH, low oxygen tension, high osmolarity, and the presence of bile [9]. Once in the intestine, the pathogen has the ability to penetrate inside epithelial cells. In a first step, adhesins and fimbriae are necessary to mediate the adherence [10], and afterwards the bacteria can be translocated across the intestinal epithelium via three routes **(Figure I.1)**: (i) by inducing internalization in non-phagocytic enterocytes; (ii) through specialized epithelial cells called M cells; and (iii) through dentritic cells that intercalate epithelial cells by extending protusions into the gut lumen [11, 12]. The two first routes are mediated by the virulence-associated type 3 secretion system encoded by *Salmonella* pathogenicity Island 1 (SPI-1) [13], and SPI-1-directed invasion of M cells is the predominant route of intestinal traversal [14].

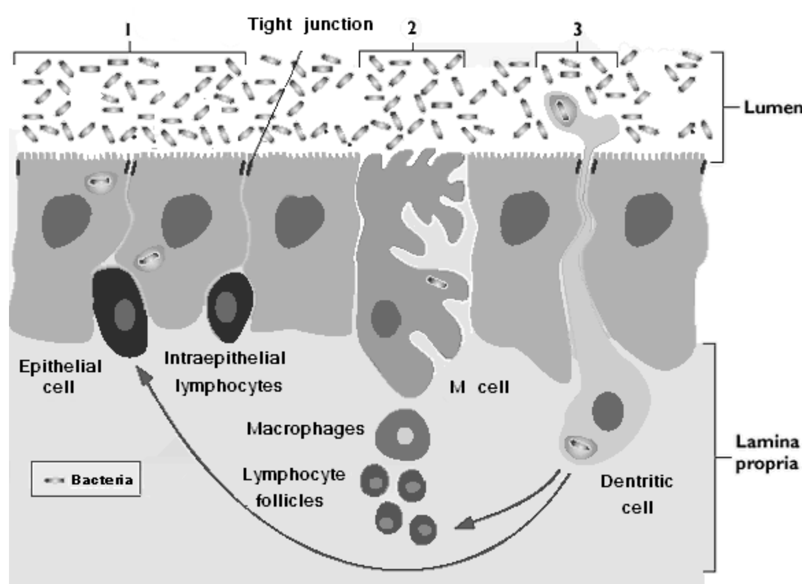


Figure I.1. Routes to cross the intestinal epithelium.- The surface of the gut is covered with epithelial cells that form intercellular tight junctions, excluding microbes. Invasive bacteria can enter

epithelial cells directly (1), through M cells (2), or using the projections that dendritic cells extend into the intestinal lumen (3). Figure modified from [15].

After invading intestinal epithelial cells, *Salmonella* can produce three main kinds of infection: gastroenteritis, systemic infection, and asymptomatic chronic carriage. **(Figure I.2)**.

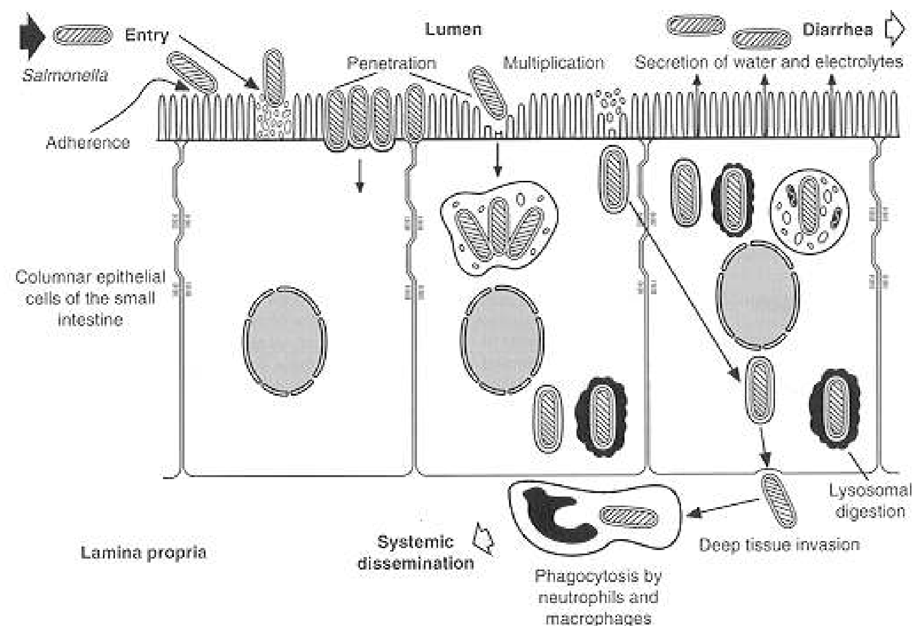


Figure I.2. *Salmonella* can produce gastroenteritis or systemic infection. Salmonellae that penetrate the intestinal mucosa induce acute inflammation and diarrhea; the latter is due to secretion of fluid and electrolytes by the small and large intestines. However, Salmonella can also undergo intracellular proliferation, spreading to mesenteric lymph nodes and throughout the body via systemic circulation. Figure from [16].

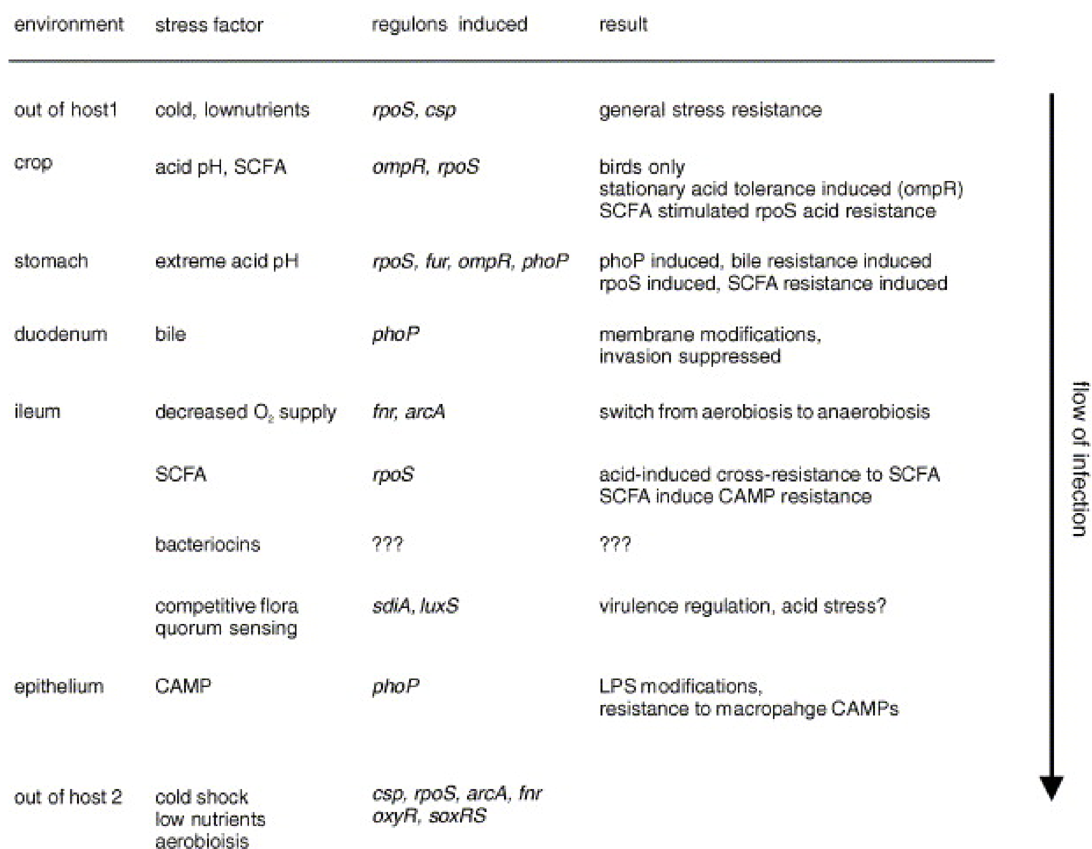
In gastroenteritis, *Salmonella* infection remains confined to the intestine where an acute inflamatory reaction is triggered in the intestinal mucosa. Inflammation leads to diarrhea due to secretion of fluid and electrolytes by the small and large intestines [17-19].

In systemic infection, *Salmonella* is able to enter inside macrophages and subsequently activate virulence mechanisms to survive and replicate in the intracellular environment. Crucial for this intracellular phase is a second type 3 secretion system encoded by *Salmonella* pathogenicity Island 2 (SPI-2). Carriage inside macrophages disseminates *Salmonella* through the lymphatic system, and permits colonization of internal organs such as the liver, the spleen, the bone marrow and the gall bladder [20].

Following systemic infection, a fraction of individuals develop chronic asymptomatic infection, becoming life-long carriers of *Salmonella* and acting as reservoirs for future infections. The mesenteric lymph nodes, the liver and the gallbladder have been proposed as potential *Salmonella* reservoirs [14]. In humans, development of chronic typhoid carriage is frequently associated with the presence of gallbladder abnormalities, especially gallstones [21]. *Salmonella* is able to form biofilms on gallstones providing a very stable matrix that results in high levels of resistance to antimicrobial agents [22]. In patients carrying both *S*. Typhi and gallstones in the gallbladder, clinically administered antibiotics are typically ineffective against the bacterial infection. Furthermore, permanent inflammation increases the risk of developing hepatobiliary carcinomas [23].

2. Conditions encountered by *Salmonella* in the host and stress responses

Bacterial pathogens have developed cellular defense systems that permit to face stress conditions in host environments.



environment	stress factor	regulons induced	result
out of host1	cold, lownutrients	*rpoS, csp*	general stress resistance
crop	acid pH, SCFA	*ompR, rpoS*	birds only stationary acid tolerance induced (ompR) SCFA stimulated rpoS acid resistance
stomach	extreme acid pH	*rpoS, fur, ompR, phoP*	phoP induced, bile resistance induced rpoS induced, SCFA resistance induced
duodenum	bile	*phoP*	membrane modifications, invasion suppressed
ileum	decreased O_2 supply	*fnr, arcA*	switch from aerobiosis to anaerobiosis
	SCFA	*rpoS*	acid-induced cross-resistance to SCFA SCFA induce CAMP resistance
	bacteriocins	???	???
	competitive flora quorum sensing	*sdiA, luxS*	virulence regulation, acid stress?
epithelium	CAMP	*phoP*	LPS modifications, resistance to macropahge CAMPs
out of host 2	cold shock low nutrients aerobioisis	*csp, rpoS, arcA, fnr* *oxyR, soxRS*	

Figure I.3. Stresses experienced by *Salmonella* upon colonization of a susceptible host. Figure reproduced from [25].

The defenses used to survive adverse conditions can be specific or can provide cross protection to a variety of hostile or stress conditions. In *Salmonella*, activation of stress responses substantially improves *Salmonella* chances of survival in unfavorable environments [24].

During infection *Salmonella* encounters stresses such as extreme acid conditions of the stomach, the detergent-like activity of bile, the elevate concentrations of osmolytes and commensal bacterial metabolites, the low oxygen supply, and the exposition to cationic antimicrobial peptides (CAMP) present on the surface of epithelial cells [25] **(Figure I.3)**.

3. Bile and *Salmonella*

3.1. Bile and bile salts.

Bile is both an excretory secretion that eliminates cholesterol, bilirubin, and waste products and a digestive secretion that aids in the absortion of fat-soluble vitamins and in the digestion of fats [26]. This yellow-green aqueous solution is synthesized in the liver by the pericentral hepatocytes, and is stored and concentrated in the gallbladder. When chyme from an ingested meal passes by the small intestine, acid and partially digested fats stimulate secretion of the enteric hormone cholecystokinin which induces contraction of the gallbladder, causing bile flow into the duodenum [27-29].

The composition of bile is complex, including both organic and inorganic compounds. Bile is a fluid poor in proteins and rich in lipids, bile salts, cholesterol, phospholipids, and the green pigment biliverdin. Bile salts constitute approximately two-thirds of the dry weight of human bile [26, 28].

The primary bile salts cholate and chenodeoxycholate are synthesized from colesterol by a multienzyme process, and all contain the same steroid nucleus [30]. Once produced in the liver, primary bile salts are conjugated to glycine or taurine to form conjugated bile salts that are soluble over a wide range of pH values. Unconjugated forms are only scarcely soluble at physiological pH, and because of this property they are usually called bile salts instead of bile acids [31]. Primary bile salts are further metabolized in the lumen of the intestine by the activity of commensal bacteria, producing the secondary bile salts

deoxycholate (DOC, from cholate) and lithocholate (from chenodeoxycholate) [31, 32]. **(Figure I.4)**

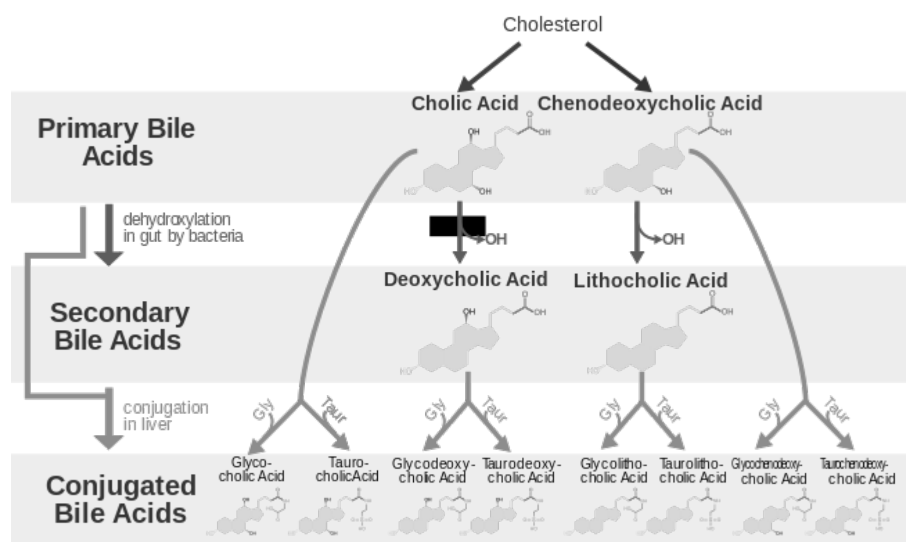


Figure I.4. Synthesis of the bile acids (secondary and conjugated) from the two primary bile acids. Figure from Wikipedia.

An adult human produces about 500 ml of bile daily [33]. Only a small amount of bile salts produced are excreted in the feces. About 95% of intestinal bile salts are reabsorbed by passive diffusion along the gut and by active transport in the distal ileum, thus returning to the liver by enterohepatic circulation [34]. The average concentration of bile salts in humans ranges from about 8% in the gallbladder to 0.2% to 2% in the intestine, and DOC accounts for about 15% of the total bile salts [35]. These values can vary from person-to-person due to dietary intake and genetic factors [36]. High intracellular concentrations of bile salts in the liver can induce necrosis and apoptosis leading to hepathocyte death [37, 38], while low bile salt intestinal concentration leads to defective micellar solubilization of dietary lipids which contributes to lipid malabsortion [39]. Moreover, decreased concentrations of bile salts in bile may result in a supersaturation of cholesterol in bile which can facilitate concretion of cholesterol to form gallstones [40].

Bile salts are amphipathic molecules that are surface active and therefore have detergent action. This detergent activity on particles of dietary fat produces fat emulsification, increasing the surface area of fat and making it available for digestion by lipases. The amphipatic nature of bile salts also allows self-association in an aqueus environment to

form micelles that can solubilize and transport lipids, which is critical for the absortion of fats and fat-soluble vitamins [32].

3.2. Antimicrobial effects of bile

A strong antimicrobial effect of bile is exerted on cellular membranes. Because of their detergent-like properties, bile salts disrupt the phospholipids and proteins that form the lipid bilayer of the cell membrane, interfering with cellular homeostasis and causing cells to lyse [26].

Inside the cell, bile salts have additional deleterious effects. Bile salts may alter the conformation of proteins causing misfolding and denaturation, and can also impair or induce secondary structure formation in RNA [28, 41]. Other adverse effects of bile are oxidative stress through the generation of oxygen free radicals and chelation of calcium and iron reducing their intracellular concentration [42, 43]. Furthermore, bile salts induce DNA damage, perhaps by oxidation of cytosine. As a consequence, the presence of bile increases the frequency nucleotide substitutions and frameshifts and stimulates DNA rearrangements [44].

3.3. Mechanisms of resistance to bile in *Salmonella*

Enteric bacteria are intrinsically resistant to bile, a trait used for more than a century to prepare selective microbiological media such as MacConkey agar [29]. To colonize the hepatobiliary tract, *Salmonella* resists the deleterious effects of bile. *Salmonella enterica* ser. Typhimurium is particulary resistant to bile, both *in vitro* and *in vivo*. Under laboratory conditions, the minimal inhibitory concentrations (MIC) of ox bile and sodium deoxycholate (DOC) are 12% and 7% respectively [45]. Upon infection of BALB/c mice, *Salmonella* cells, sometimes in very high numbers, can be easily isolated from the bile-laden gall bladder. A summary of the cell functions known to contribute to bile resistance is provided below.

3.3.1. Bacterial cell envelope integrity and surface structures

Because bile disrupts cell membranes, maintenance of membrane integrity is essential for bile resistance, and alterations of charge, hydrophobicity or lipid fluidity in the membrane

have significant consequences for bile resistance [28]. The cell envelope of Gram negative bacteria is composed by three principal layers: the outer membrane, the peptidoglycan cell wall, and the cytoplasmic or inner membrane. Several structures in the cell envelope have been described to play a role in bile resistance.

The outer membrane (OM) is an assymmetric bilayer that is composed by phospholipids in the inner leaflet and glycolipids, principally lipopolysaccharide (LPS), in the outer leaflet [46].

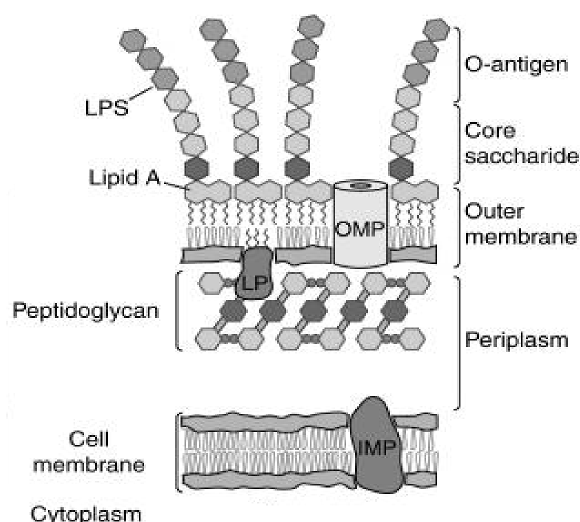


Figure I.5. The Gram-negative cell envelope. LP, lipoprotein; LPS, lipopolysaccharide; OMP, outer membrane protein; IMP, inner membrane protein. Figure adapted from [46].

The LPS is the major component of the OM and provides the main barrier to external compounds. The LPS comprises three parts: the lipid A that anchors the LPS in the membrane, the core saccharide, and the O-antigen polysaccharide that protrudes from the surface of the membrane [47]. LPS is an efficient hydrophobic barrier to bile: mutants lacking O-antigen chains in the LPS are bile-sensitive [36, 48] and the presence of very long chains have been described to increase bile resistance [49]. Furthermore, LPS modifications controlled by the two-component regulatory system PhoP-PhoQ also contribute to bile resistance [50].

Mutations in *wecD* and *wecA* genes, both involved in the biosynthesis of another outer membrane glycolipid, the enterobacterial common antigen (ECA), also cause bile sensitivity [51]. Hence, ECA may provide an additional barrier to bile salts.

3.3.2. Efflux pumps

Although the OM is an excellent hydrophobic barrier to bile, bile salts, especially if unconjugated, can enter the cell by diffusion or by passage through porins [52]. However, bile salts that enter the cell can still be removed by efflux systems that expulse bile salts before significant damage occurs [53]. The importance of efflux pumps for bile resistance is well known: for instance, the AcrAB pump is required for bile resistance [54, 55], and lack of TolC, the outer membrane component of several efflux pumps including AcrAB [53], also causes extreme sensitive to bile [56].

3.3.3. DNA repair mechanisms

Bile salts induce DNA damage [44], probably by oxidation of cytosine and perhaps by indirect effects derived from formation of reactive oxygen species. Hence, DNA repair plays a role in bile resistance. Base excision repair (BER), SOS-associated translesion synthesis, and recombinational repair by the RecBCD system are required to cope with bile-induced damage [57].

3.4. Bile and *Salmonella enterica* pathogenesis

Bile is an environmental signal that regulates virulence genes. For instance, bile inhibits *Salmonella* invasion of epithelial cells in the intestinal lumen by repressing transcription of *Salmonella* pathogenicity island 1 (SPI-1) [58].

The PhoPQ two-component system, which is activated within macrophage phagosomes [59], is absolutely required for virulence in mice [60], and is also essential for bile tolerance [61]. A recent report indicates that the PhoPQ system system is repressed by bile [62].

Bile has also been shown to promote the formation of *Salmonella* biofilms on human gallstones [22]. Biofilm formation may protect *Salmonella* from the high levels of bile found in the gall bladder. Biofilm formation may explain the reduced effectiveness of certain antibiotics in the gall bladder [63], and may contribute to establishment of the chronic carrier state.

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OBJECTIVES

When this work started, the ability of *Salmonella enterica* to survive high concentrations of bile if previously grown at sublethal concentrations had been already described in the literature. It was also a common observation in daily practice of our laboratory. However, the mechanisms underlying adaptation remained unknown.

Based on these antecedents, **Objective 1** was aimed at analyzing the changes in *S. enterica* gene expression induced by a sublethal concentration of sodium deoxycholate (DOC). High throughtput analysis of gene expression was performed by microarray technology.

Because *S. enterica* can also survive bile without adaptation, **Objective 2** was aimed at the isolation and analysis of spontaneous ('preadapted') bile-resistant mutants, including full genome sequencing of bile-resistant isolates.

During the study of bile-resistant mutants, evidence was obtained that *Salmonella enterica* is able to preadapt to bile by mechanisms other than mutation. **Objective 3** was aimed at identifying the underlying molecular mechanisms using single-cell analysis by microfluidics.

Objective 4 was aimed at studying a *S. enterica* locus of unknown function, annotated as *yiiU*. This locus was chosen for study because microarray analysis had revealed increased *yiiU* expression in the presence of a sublethal concentration of DOC.

The goal of **Objective 5** was the characterization of a *S. enterica* hyperesistant mutant isolated in a genetic screen.

Because the *S. enterica* hyperesistant mutant studied in Objective 5 turned out to lack Prc periplasmic protease, a protein involved in processing of penicillin-binding protein 3, **Objective 6** was aimed at analyzing whether cell wall remodeling occurred in the presence of bile.

RESULTS

Chapter 1: Adaptation and preadaptation of *Salmonella enterica* to bile

Adaptation and Preadaptation of *Salmonella enterica* to Bile

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Abstract

Bile possesses antibacterial activity because bile salts disrupt membranes, denature proteins, and damage DNA. This study describes mechanisms employed by the bacterium *Salmonella enterica* to survive bile. Sublethal concentrations of the bile salt sodium deoxycholate (DOC) adapt *Salmonella* to survive lethal concentrations of bile. Adaptation seems to be associated to multiple changes in gene expression, which include upregulation of the RpoS-dependent general stress response and other stress responses. The crucial role of the general stress response in adaptation to bile is supported by the observation that RpoS^{-} mutants are bile-sensitive. While adaptation to bile involves a response by the bacterial population, individual cells can become bile-resistant without adaptation: plating of a non-adapted *S. enterica* culture on medium containing a lethal concentration of bile yields bile-resistant colonies at frequencies between 10^{-6} and 10^{-7} per cell and generation. Fluctuation analysis indicates that such colonies derive from bile-resistant cells present in the previous culture. A fraction of such isolates are stable, indicating that bile resistance can be acquired by mutation. Full genome sequencing of bile-resistant mutants shows that alteration of the lipopolysaccharide transport machinery is a frequent cause of mutational bile resistance. However, selection on lethal concentrations of bile also provides bile-resistant isolates that are not mutants. We propose that such isolates derive from rare cells whose physiological state permitted survival upon encountering bile. This view is supported by single cell analysis of gene expression using a microscope fluidic system: batch cultures of *Salmonella* contain cells that activate stress response genes in the absence of DOC. This phenomenon underscores the existence of phenotypic heterogeneity in clonal populations of bacteria and may illustrate the adaptive value of gene expression fluctuations.

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Introduction

Bile is a fluid containing bile salts, cholesterol, and a variety of proteins and electrolytes [1]. Bile is synthesized by parenchymal cells (hepatocytes) in the liver. In mammals with a gall bladder, a fraction of bile is stored in the gall bladder while another fraction flows directly into the small intestine [1]. When food passes by the small intestine, gall bladder contraction releases bile into the duodenum. Bile aids in the digestion of fats, facilitates absorption of fat-soluble vitamins in the intestine, and contributes to the elimination of excess cholesterol and waste metabolic products produced in the liver [1].

About two thirds of bile (dry weight) are made of bile salts, a family of molecules with steroid structure which derive from cholesterol [2]. Bile salts dissolve membrane lipids and cause dissociation of integral membrane proteins. Inside the cell, the detergent activity of bile salts causes misfolding and denaturation of proteins [3,4]. Chelation of calcium and iron by bile salts is also a source of physiological perturbations [3,4]. Furthermore, bile salts have DNA damaging capacity, stimulate DNA rearrangements, and induce plasmid curing [4,5,6,7]. However, certain bacterial species are resistant to the antibacterial activities of bile salts [3,8]. This trait has been exploited for the design of selective microbiological media such as the one-century-old MacConkey agar used in the identification of genera of the family Enterobacteriaceae. On the other hand, bile salts regulate the expression of specific bacterial genes, some of them necessary for bile resistance and others involved in pathogenesis [3,8]. Bile salts may thus be viewed both as environmental signals used by bacteria to identify bile-containing animal environments and as antibacterial compounds [8].

An extreme example of bile-resistant pathogen is *Salmonella enterica*, which colonizes the hepatobiliary tract during systemic infection and persists in the gall bladder during chronic infection [9,10]. *Salmonella* survival in the mammalian gall bladder seems to involve several strategies. Invasion of the gall bladder epithelium may permit escape from the extremely high concentrations of bile salts present in the gall bladder lumen [11]. Formation of biofilms on gallstones may also protect *Salmonella* from the bactericidal activities of bile salts [12,13]. However, planktonic *Salmonella* cells are also found at high numbers in the bile-laden gall bladder lumen, and the mechanisms employed to thrive in such a harsh environment remain to be identified.

Bile resistance can be studied under laboratory conditions by adding ox bile or individual bile salts to microbiological media

Author Summary

This study describes mechanisms employed by the bacterium *Salmonella enterica* to survive bile: adaptation, mutation, and non-mutational preadaptation. Adaptation is easily observed in the laboratory: when a *Salmonella* culture is grown in the presence of a sublethal concentration of the bile salt sodium deoxycholate (DOC), the minimal inhibitory concentration of DOC increases. Adaptation appears to be associated to multiple changes in gene expression induced by DOC. Mutational bile resistance is also a common phenomenon: plating on agar containing a lethal concentration of bile yields bile-resistant colonies. Fluctuation analysis indicates that such colonies derive from bile-resistant cells present in the previous culture. However, selection on lethal concentrations of bile also provides bile-resistant isolates that are not mutants. Non-mutational preadaptation, a non-canonical phenomenon *a priori*, suggests that batch cultures contain rare *Salmonella* cells whose physiological state permits survival upon encountering bile. The view that non-mutational preadaptation may be a consequence of phenotypic heterogeneity is supported by the observation that *Salmonella* cultures contain cells that activate stress response genes in the absence of DOC.

[14]. Genetic and biochemical analysis in *E. coli* and *S. enterica* in the laboratory has permitted the identification of cell functions and mechanisms involved in bile resistance [3,8,14]. The relevance of these reductionist studies is supported by the fact that mutations that cause bile sensitivity *in vitro* often result in virulence attenuation in the mouse model of *S. enterica* infection [14]. The list of bile resistance factors in *Salmonella* and other enteric species includes envelope barriers such as the lipopolysaccharide [15,16] and the enterobacterial common antigen [17], the outer membrane [18,19], the cytoplasmic membrane [20], efflux pump systems [21], genes of the multiple antibiotic resistence (*mar*) and PhoPQ regulons [22,23], and DNA repair functions [5,6]. Genetic analysis has also identified cell functions whose loss increases bile resistance, probably by activating cell defense responses [20].

The bile resistance level of wild type *Salmonella* can be increased over the customary minimal inhibitory concentration by growth in the presence of sublethal concentrations of bile [8,24]. This phenomenon, henceforth called "adaptation", is easily observed in the laboratory and may be relevant during *Salmonella* colonization of the hepatobiliary tract. Below we describe studies of *Salmonella* adaptation to bile *in vitro*, and show that growth of *S. enterica* on sublethal concentrations of bile is accompanied by dramatic changes in gene expression. We also report that batch *Salmonella* cultures contain cells that show high levels of bile resistance without previous adaptation. This phenomenon, henceforth called "preadaptation", seems to involve two unrelated processes. One is mutation in specific loci, often related to lipopolysaccharide transport; another is activation of bile resistance responses in a subpopulation of bacterial cells. The latter phenomenon fits well in current views indicating that bacterial populations are heterogeneous, and that fluctuations in gene expression can have adaptive value [25,26].

Results

Viability of *S. enterica* SL1344 in the presence of sodium deoxycholate

The minimal inhibitory concentration (MIC) of sodium deoxycholate for *S. enterica* strain SL1344 grown in LB is 7%, and the MIC of ox bile is 12% under the same conditions. These MICs are similar to those previously reported for strain ATCC 14028 [5,6]. To ascertain whether inhibition of bacterial growth by bile salts involves bacterial death or merely growth arrest, we performed viability tests to distinguish live and dead *Salmonella* cells in the presence of DOC. Aliquots from *Salmonella* exponential cultures grown in LB were treated with various concentrations of DOC (1%, 3%, 5%, 7%, and 9%) for 30 min. Examination under the microscope using a commercial live/dead color-based kit was then performed, and the numbers of live/dead cells were counted. Cell counting was randomly performed, and the minimal number of bacterial cells counted was 1,700. A representative experiment is shown in Figure 1, top panel. A direct correlation was found between the percentage of dead *Salmonella* cells and the concentration of DOC.

The bactericidal capacity of bile was also monitored by plaque counts of colony-forming-units. Aliquots from *Salmonella* exponential cultures grown in LB were treated with various concentrations of DOC (1%, 3%, 5%, 7%, and 9%) for 30 min. The cultures were then diluted, plated on LB, and incubated overnight at 37°C. Colony counts confirmed that exposure to DOC renders *S. enterica* cells inviable in a dose-dependent maner (Figure 1, bottom panel).

Adaptation of *S. enterica* to lethal concentrations of sodium deoxycholate

Although the level of resistance to bile is fairly constant under given conditions, *Salmonella* can be adapted to grow at higher concentrations of bile by previous growth in the presence of sublethal concentrations [8]. To determine the concentration(s) of DOC that permit adaptation in strain SL1344, *S. enterica* cultures were grown in LB containing different concentrations of DOC (from 1% to 7%). Aliquots from the cultures were then transferred to microtiter plates containing DOC at concentrations ranging from 1% to 14%. As shown in the diagram of Figure 2, growth at concentrations slightly lower than the MIC (4% and 5%) increased the MIC of DOC to ≥14%. A smaller increase of the MIC was likewise observed after growth in 3% DOC.

The bacterial cells that had survived bile in the microtiter plate were transferred to LB and cultured overnight. Aliquots from these cultures were then used to determine the MIC of DOC in microtiter plates. As shown in Figure 2, a MIC of 7% DOC was determined for all cultures, indicating that resistance had decreased back to the level characteristic of strain SL1344. Hence, adaptation to DOC by growth at concentrations of 3–5% is reversible, and does not involve selection of bile-resistant mutants, at least under the conditions tested.

Transcriptomic analysis of *S. enterica* gene expression during growth on sublethal concentrations of sodium deoxycholate

The capacity of bile salts to induce changes in gene expression is well known in *S. enterica* [22,27] and in other bacteria [3], as well as in eukaryotes [28]. On these grounds, we hypothesized that the reversible increase of MIC observed when *S. enterica* is grown in the presence of sublethal concentrations of DOC might involve changes in gene expression. This hypothesis was tested by transcriptomic analysis using the Salgenomics microarray [29].

RNA extraction was performed in exponential and stationary cultures grown in LB with and without 5% DOC ($O.D._{600} = 0.4$ and $O.D._{600} \geq 1$, respectively). *S. enterica* grew at slower rates in LB+DOC than in LB, and the concentration of bacterial cells in LB+DOC reached a plateau well below the LB control. However, growth did occur, thus indicating that 5% is a sublethal

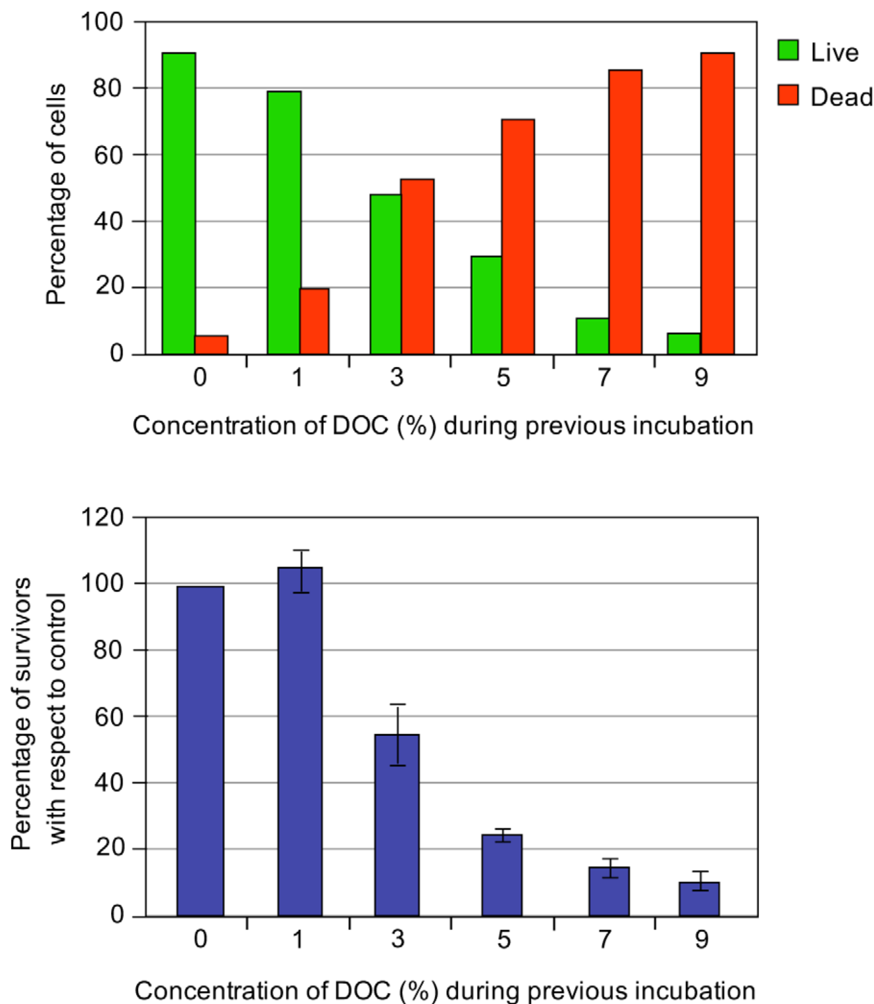

Figure 1. Percentages of live and dead bacteria and relative numbers of colony-forming units. Top panel: Percentages of live and dead bacteria (green and red histograms, respectively) found in 1 ml aliquots of an exponential culture of *S. enterica* SL1344 incubated in the presence of different concentrations of sodium deoxycholate (1%, 3%, 5%, 7% and 9%) during 30 minutes at 37°C. Bottom panel: Relative numbers of colony forming-units (CFU) after incubation of *S. enterica* SL1344 in the presence of different concentrations of sodium deoxycholate (1%, 3%, 5%, 7% and 9%) during 30 minutes at 37°C. The number of CFU in the absence of DOC is shown as 100%.
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concentration of DOC under such conditions (LB medium, 37°C). Note that these conditions are different from those used in the viability tests described above. This does not exclude, of course, that a fraction of bacterial cells may have been killed, and that the culture derives from the surviving subpopulation. Under these conditions, a large number of *Salmonella* loci showed differences in their RNA levels depending on whether DOC was present or absent. Raw data from transcriptomic analysis in the presence of 5% DOC have been deposited at the Array Express database (http://www.ebi.ac.uk/miamexpress) with accession number E-MTAB-637. Relevant data are summarized in Table 1 and Table 2. The main conclusions from these experiments can be summarized as follows:

(i) The RpoS-dependent genes *osmY*, *dps*, *uspB*, and *ecnB* [30] were found to be strongly upregulated by DOC in exponential cultures. The poorly characterized RpoS-dependent gene *ybiF*, located next to *dps* on the *Salmonella* chromosome, showed also >3 fold upregulation. In stationary cultures, *dps* and *osmY* were also upregulated by DOC. Their lower upregulation under such conditions is consistent with the fact that the RpoS-dependent general stress response is activated in non dividing cells [31]. These observations suggested that activation of the RpoS-dependent general stress response might play a role in adaptation to bile. A previous transcriptomic study in strain ATCC 14028 did not detect RpoS activation upon exposure to DOC [22]. However, the concentration of DOC used by Prouty et al. (3% ox bile) [22] may have been insufficient to trigger RpoS activation. In fact, under the conditions used in our study, concentrations of DOC much higher than those expected to be present in 3% ox bile (around 1% bile salts [1]) did not adapt *S. enterica* to bile (Figure 2).

(ii) The general stress response is not the only stress response activated by sublethal concentrations of bile salts: the stress-inducible *cspD* gene [32,33] was found to be upregulated by DOC in both exponential and stationary cultures, and the *uspA* gene [34] in stationary cultures only.

(iii) The outer membrane protein (OMP) genes *ompC* and *ompD* [35] were downregulated by DOC during exponential

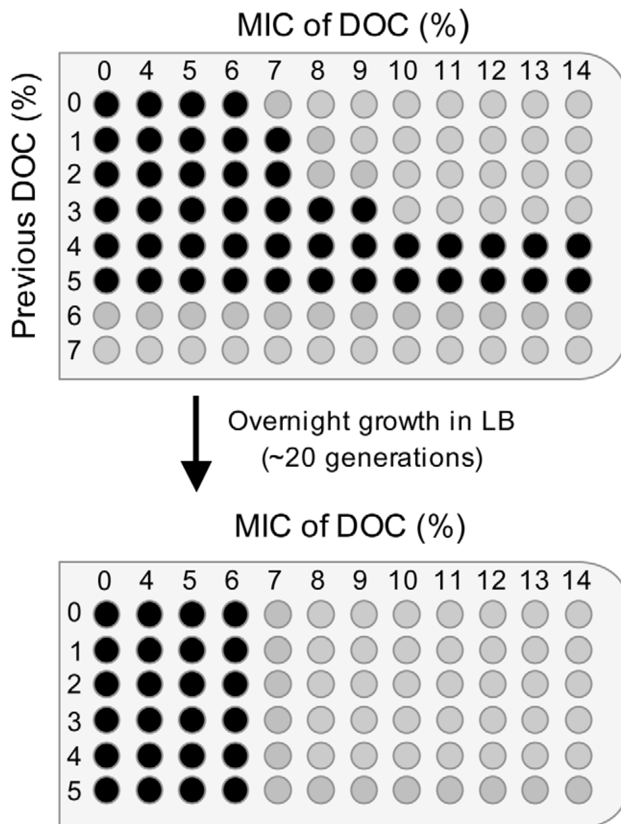

Figure 2. Minimal inhibitory concentrations (MICs) of sodium deoxycholate (DOC) for *Salmonella* cultures pre-exposed to various concentrations of DOC, and MICs for the same cultures after overnight growth in LB.
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growth. Because porins provide passage to bile salts [8], downregulation of *ompC* and *ompD* may be tentatively interpreted as a defensive modification of the outer membrane to decrease uptake of bile salts.

(iv) Modification of the cell envelope may not be limited to outer membrane remodeling. Downregulation of *mltB*, a gene involved in peptidoglycan recycling [36], may provide evidence for cell wall changes produced in response to DOC. The crucial role of the cell envelope in bile resistance is well established in the literature [15,17,18,19,37].

(v) A sublethal concentration of DOC upregulated *acrD*, which encodes a component of a multidrug resistance efflux pump [38]. Because efflux systems are known to transport bile salts outside the cell [21], upregulation of *acrD* may be viewed as another defensive response. Upregulation of other transport genes (*ugpB* and *pnuC*) was also observed but it is difficult to interpret.

(vi) *Salmonella* pathogenicity islands SPI-1 and SPI-2 were strongly downregulated by DOC, as previously described by other authors [27,39]. Downregulation was observed in stationary cultures only, a result consistent with the fact that neither SPI-1 nor SPI-2 are expressed in exponential cultures [40].

(vii) Miscellaneous gene expression changes of difficult interpretation are also presented in Table 1 and Table 2. Changes in the synthesis of cytochrome components may suggest alterations in electron transport. The possibility that chemotaxis is altered in the presence of DOC may be also considered, as previously proposed [22]. Altered expression of numerous metabolic genes is also observed.

Table 1. *S. enterica* loci showing altered expression (>3 fold) in the presence of 5% DOC during exponential growth.

Locus	Function of product	Fold change
aroG	Phenylalanine biosynthesis	+9.94
osmY	RpoS-dependent general stress response	+7.10
dps	RpoS-dependent general stress response	+7.04
ecnB	Entericidin synthesis	+6.28
ugpB	ABC transporter	+5.02
yiiU	Unkown function	+4.58
cspD	Stress response	+3.55
cyoA	Cytochrome oxidase	+3.39
cfa	Fatty acid synthesis	+3.22
pnuC	Nucleoside transport	+3.11
acrD	Efflux pump	+3.00
sseA	SPI-2 virulence effector	−12.01
malZ	Maltose catabolism	−5.28
ssaS	Component of the SPI-2 secretion apparatus	−4.48
ompC	Outer membrane	−3.42
ompF	Outer membrane	−3.39
ibpA	Heat shock	−3.02
cheM-cheW	Chemotaxis	−3.01
mltB	Peptidoglycan synthesis	−3.00

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Validation of microarray analysis using *lac* fusions

Data provided by transcriptomic analysis were validated by monitoring the effect of DOC on the expression of transcriptional *lac* fusions in 14 *S. enterica* genes. As a general rule, we chose genes which had shown strong expression changes in the presence of DOC. The sample included genes of the RpoS regulon (*osmY*, *dps*, and *ecnB*), a transport gene (*ugpB*), a metabolic gene (*aroG*), and two SPI-1 genes (*hilA* and *prgH*). Genes of unknown function, present in all enterics (*yiiU*, *yceK*, *ytfK*, *ybjM*, and *yajI*) or *Salmonella*-specific (*STM1441* and *STM1672*), were also included. The selection of these loci was based on the bile-sensitive phenotype of their mutants (e. g., *yiiU*), their DNA sequence relatedness to known bile resistance genes (e. g., *STM1441*, encoding a putative efflux pump) or the cellular location of their products (e. g., *yceK*, *yajI* and *STM1642*, which may encode outer membrane proteins, and *ybjM*, which may encode a cytoplasmic membrane protein). ß-galactosidase activities were measured in LB and in LB containing 5% DOC. Raw data are shown in Tables S1 and S2. Figure 3 is an elaboration that compares the ß-galactosidase activities of the *lac* fusions and the expression levels of the corresponding mRNAs detected by microarray analysis. Although differences in expression levels are observed, a correlation between mRNA content and ß-galactosidase activity is found in all cases.

Activation of the RpoS-dependent general stress response in the presence of sublethal concentrations of DOC was further analyzed by monitoring expression of RpoS-dependent genes other than *dps* and *osmY*. An additional goal of these experiments was to confirm

Table 2. *S. enterica* loci showing altered expression (>3 fold) in the presence of 5% DOC during stationary phase.

Locus	Function of product	Fold change
cyoA	Cytochrome oxidase	+26.33
mdh	Central metabolism	+12.02
tsr	Chemotaxis	+10.38
shdB	Central metabolism	+8.23
sucA	Central metabolism	+8.23
ugpB	ABC transporter	+7.40
ompF	Outer membrane	+7.34
uspA	Stress response	+6.50
dps	RpoS-dependent general stress response	+5.42
filE-fliF	Flagellum	+5.08
narG-narK	Nitrate reduction	+4.63
ahpC-ahpF	Stress response	+4.62
nagB-nagE	PTS system	+4.56
uspB	RpoS-dependent general stress response	+4.06
cspD	Stress response	+3.70
osmY	RpoS-dependent general stress response	+3.35
nirD-nirC	Nitrite reduction	+3.26
phdR	Central metabolism	+3.24
ppa	Central metabolism	+3.19
fpb	Central metabolism	+3.18
rrmA	Ribosomal RNA modification	+3.17
cydA	Cytochrome oxidase	+3.17
pipA-pibB	SPI-1 virulence effectors	−119.08
sseA	SPI-2 virulence effector	−68.34
sopE	SPI-1 virulence effector	−63.96
sifB	SPI-2 virulence effector	−54.29
ssaS-ssaT	SPI-2 secretion apparatus	−39.73
ssaB	SPI-2 secretion apparatus	−17.48
hilC	SPI-1 regulatory protein	−12.85
prgH	SPI-1 regulatory protein	−10.51
hilD	SPI-1 regulatory protein	−10.50
pagC	Intracellular survival in macrophages	−9.94
iroN	Siderophore	−9.23
sseJ	SPI-2 virulence effector	−7.91
valW	tRNA	−6.41
potC	Spermidine/putrescine transporter	−6.08
sifA	SPI-2 virulence effector	−6.08
sifB	SPI-2 virulence effector	−5.17
invH	SPI-1-encoded outer membrane lipoprotein	−5.10
cheM-cheW	Chemotaxis	−4.33
hilA	SPI-1 regulatory protein	−4.14
tnpA	IS*200* transposase	−4.09
traX	Conjugal transfer of the virulence plasmid	−3.39
pagD	Resistance to antimicrobial peptides	−3.18
pyrI	Central metabolism	−3.15

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that DOC upregulated the RpoS regulon in both exponential and stationary cultures. For these experiments, *lac* fusions in *katE*, *xthA*, *ots*, *dps*, and *osmY* were used. ß-galactosidase activities were measured in LB and in LB containing 5% DOC. Expression in exponential cultures was tested at an $O.D._{600} = 0.4$. Stationary cultures were tested at an $O.D._{600} \geq 1$. Data shown in Table 3 and Table 4 confirmed that the RpoS regulon is upregulated by DOC. Upregulation occurs in both exponential and stationary cultures, and the induction ratios vary depending on the gene under study.

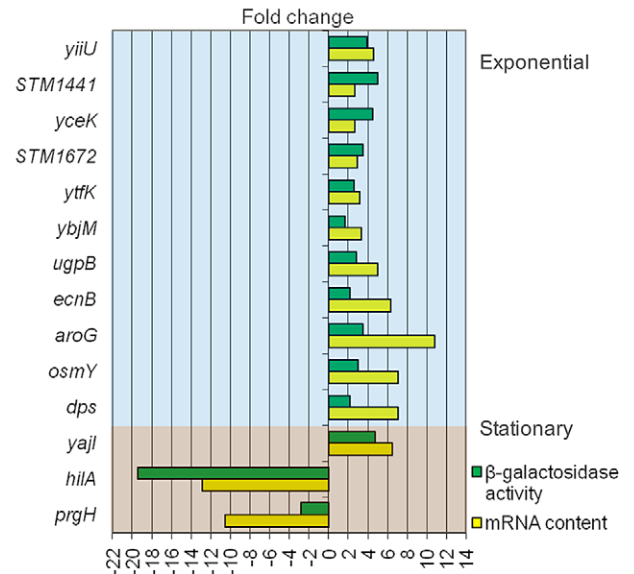

Figure 3. Validation of transcriptomic analysis: comparison of gene expression differences between LB and LB+5% deoxycholate as measured by RNA content (microarray analysis) and activity of *lac* fusions.
doi:10.1371/journal.pgen.1002459.g003

Identification of bile-responsive genes necessary for bile resistance

The identification of genes whose expression was altered in the presence of sublethal concentrations of DOC raised the possibility that such loci might be necessary for bile resistance. We thus tested the MIC of sodium deoxycholate for mutants carrying loss-of-function mutations in 16 genes identified above as upregulated by bile: *acrD*, *yiiU*, *STM1441*, *yceK*, *STM1672*, *ytfK*, *ybjM*, *ugpB*, *ecnB*, *aroG*, *osmY*, *dps*, *yajI*, *katE*, *ots*, and *xthA*. Results from these trials are shown in Table S3, and can be summarized as follows:

Table 3. ß-galactosidase activities of *lac* fusions in RpoS-regulated genes in the presence and in the absence of 5% sodium deoxycholate during exponential growth.

Strain	Gene fusion	LB	LB+DOC
SV6065	*katE::lac*	104±33	758±121
SV6888	*katE::lac rpoS*	10±3	18±3
SV6066	*ots::lac*	66±5	267±34
SV6067	*xthA::lac*	5±1	13±5
SV6068	*osmY::lac*	17±4	95±9
SV6069	*dps::lac*	5±2	12±2

ß-galactosidase activities are shown in Miller units. Data are averages and standard deviations from 3–5 independent experiments.
doi:10.1371/journal.pgen.1002459.t003

Table 4. ß-galactosidase activities of *lac* fusions in RpoS-regulated genes in the presence and in the absence of sodium deoxycholate in stationary phase.

Strain	Gene fusion	LB	LB+DOC
SV6065	*katE::lac*	512±88	1204±104
SV6888	*katE::lac rpoS*	14±6	22±5
SV6066	*ots::lac*	200±74	1575±220
SV6067	*xthA::lac*	32±4	63±11
SV6068	*osmY::lac*	105±27	370±49
SV6069	*dps::lac*	30±10	71±14

ß-galactosidase activities are shown in Miller units. Data are averages and standard deviations from 3–5 independent experiments.
doi:10.1371/journal.pgen.1002459.t004

(i) The only bile-sensitive mutant was $YiiU^{-}$ (MIC of DOC≅1.5%, compared with 7% in the wild type). Because this locus is virtually unknown, no explanation can be offered for its role in bile resistance.

(ii) The observation that the $AcrD^{-}$ mutant was not bile-sensitive is in agreement with previous observations made in strain ATCC 14028 [41], and can be explained by redundancy: *S. enterica* possesses multiple efflux systems, many of them versatile and with overlapping substrate specificity. Hence, bile sensitivity occurs only if multiple efflux systems are eliminated [41]. In agreement with this view, a $TolC^{-}$ mutant (strain SV6629), which lacks an outer membrane protein of all RND efflux pumps [38,41], showed extreme sensitivity to DOC (MIC≅0.02%, 350 fold lower than the MIC for the wild type). Extreme bile sensitivity of a $TolC^{-}$ mutant has been likewise described in strain ATCC 14028 [41].

(iii) Redundancy may also explain why mutants lacking individual RpoS-dependent genes (*osmY*, *dps*, *xthA*, *katE*, and *ots*) are not bile-sensitive. However, an $RpoS^{-}$ derivative of SL1344 (strain SV5561) showed a MIC of DOC≅3%. Hence, an active RpoS regulon appears to be necessary for bile resistance but the individual RpoS-dependent functions tested in this study (OsmY, Dps, XthA, KatE, and Ots) are dispensable.

Isolation of bile-resistant derivatives of *S. enterica* SL1344

Non adapted *Salmonella* populations (e. g., laboratory cultures in LB) are unable to grow on lethal concentrations of bile. However, bile resistance can be acquired by mutation, and bile-resistant mutants can be easily isolated upon plating on lethal concentrations of bile [14,37]. However, previous descriptions of bile-resistant mutants had involved transposon insertions, which usually cause loss of function and are lethal if they occur in essential genes. To avoid these constraints, we isolated bile-resistant mutants of *S. enterica* of spontaneous origin. Aliquots from a *S. enterica* culture grown in LB were plated on LB supplemented with a lethal concentration of ox bile (180 g/l). Use of ox bile instead of DOC was justified by the fact that high concentrations of DOC prevent agar solidification. Bile-resistant colonies appeared at frequencies ranging between 10^{-6} and 10^{-7} per cell and generation. Because high concentrations of bile salts are bactericidal (Figure 1), we expected that bile-resistant colonies would derive from bile-resistant cells present in the previous culture. This hypothesis was supported by Luria-Delbrück fluctuation analysis [42]: the averages of bile-resistant colonies obtained from independent cultures showed a variance much higher than the averages from a single culture (Table S4).

Bile-resistant colonies were purified in LB and plated again on LB+bile to confirm bile resistance. During these routine purification procedures, we made the unexpected observation that a relatively large number of bile-resistant isolates had become bile-sensitive. A systematic analysis of the phenomenon was then carried out. Bile resistant isolates were obtained by plating strain SL1344 on LB+ox bile. Colonies were transferred to LB and grown overnight. The MIC of DOC for each isolate was then determined in microtiter plates. These trials confirmed that bile-resistant isolates were of two types: (i) stable, putatively carrying mutations that confer bile resistance; (ii) unstable isolates that lose bile resistance, either partially or completely, upon nonselective growth in LB. The frequencies of mutants and unstable isolates varied from one trial to another (data not shown). A representative experiment involving 59 independent bile-resistant isolates is shown in Figure 4. In this case, 10 isolates turned out to be mutants while the other 49 were unstable bile-resistant isolates.

Characterization of bile-resistant mutant derivatives of *S. enterica* SL1344

Six spontaneous bile-resistant mutants (MIC of DOC ≥14%) of independent origin were chosen for full genome sequencing with the SOLiD platform [43]. Genome sequencing was followed by alignment with the *S. enterica* SL1344 genome sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs) to identify DNA sequence differences. The genome of the laboratory stock of *S. enterica* SL1344 was also sequenced as a control. The mutations found are described in Table 5. Relevant observations are as follows:

(i) Four bile-resistant strains were single mutants. Mutants #1 and #6 harbored a nucleotide substitution and an in-frame deletion, respectively, in the *S. enterica yrbK* gene. YrbK (recently renamed LptC) is a lipopolysaccharide transport protein in *E. coli* [44]. Mutant #3 harbored a nucleotide substitution in the *rlpB* gene (also known as *lptE*), which encodes the *Salmonella* homolog of *E. coli* RlpB, a lipopolysaccharide assembly protein [45]. Mutant #4 harbored a nucleotide substitution in the poorly known *deaD* gene, which in *E. coli* encodes a putative ATP-dependent RNA helicase [46].

(ii) Mutants #2 and #5 were double mutants. Interestingly, one of the mutations found in strain #2 mapped in the lipopolysaccharide transport gene *yrbK*. Strain #2 carried

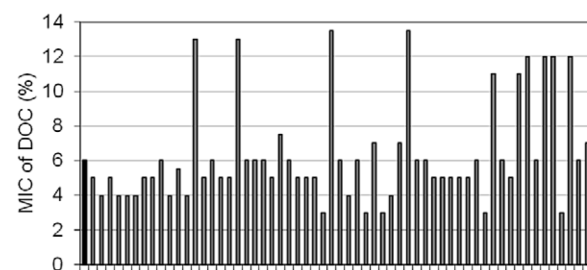

Figure 4. Minimal inhibitory concentration of sodium deoxycholate for bile resistant isolates after non-selective growth in LB. The isolates had been originally obtained on plates containing 18% ox bile.
doi:10.1371/journal.pgen.1002459.g004

Table 5. Mutations present in the genomes of bile-resistant derivatives of *S. enterica* SL1344.

Mutant	Locus affected	Mutation[a]	Location of the mutation[b]	Predicted mutational change	Cellular function affected
1	*yrbK*	G→C substitution	Nucleotide 182	Arg→Pro	Lipopolysaccharide transport
2	*yrbK*	+1 frameshift	After nucleotide 399	Premature stop codon after amino acid 134	Lipopolysaccharide transport
2	Putative intergenic region on plasmid 2	A→G substitution	Base pair 13926	Unknown	Unknown
3	*rlpB*	C→A substitution	Nucleotide 287	Ala→Glu	Lipopolysaccharide transport
4	*deaD*	C→G substitution	Nucleotide 923	Ala→Gly	Putative ATP-dependent RNA helicase
5	*strA* locus on plasmid 3	+1 frameshift	Base pair 7949	Unknown	Unknown
5	*sul2* locus on plasmid 3	+1 frameshift	Base pair 38	Unknown	Unknown
6	*yrbK*	Deletion of 30 nucleotides	Base pairs 415–444	Loss of 10 amino acids	Lipopolysaccharide transport

[a]Nucleotide change is indicated for the coding sequence, when known.
[b]Base pair numbers are those of the annotated genome of *S. enterica* strain SL1344 (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs).
doi:10.1371/journal.pgen.1002459.t005

an additional mutation in a putative intergenic region of plasmid 2. In turn, mutant #5 carried frameshifts in two putative loci of unknown function (annotated as *strA* and *sul2*) on plasmid 3. These plasmids are specific of strain SL1344 and have not been described in other strains of serovar Typhimurium. A potential role of these plasmids in adaptation to bile seems unlikely, because strain ATCC 14028, which lacks the plasmids, shows MICs of DOC and bile virtually identical to those of SL1344 [5,17,19]. The occurrence of plasmid-borne mutations that cause bile resistance is thus interesting but difficult to interpret.

Bile-resistant mutants carrying mutations in known genes (*yrbK*, *rlpB*, and *deaD*) were reconstructed (see Materials and Methods for reconstruction procedures). All reconstructed mutants showed a MIC of DOC ≥14%, thus confirming that single mutations in *yrbK*, *rlpB*, and *deaD* caused the bile-resistant phenotype of these isolates. The cause of bile resistance in the DeaD$^-$ mutant was not further investigated since *deaD* is a poorly known gene [46]. In contrast, the high frequency of mutations found in lipopolysaccharide transport genes (3 in *yrbK* and 1 in *rlpB*) provided evidence that alterations in lipopolysaccharide transport can cause bile resistance. LPS transport genes are known to be essential in *E. coli* [47,48]. If such is also the case in *S. enterica*, the mutations detected must be leaky. Leakiness may seem normal for the G→C (*yrbK*) and C→A (*rlpB*) substitutions detected in mutants #1 and #3, and even for the *yrbK* in-frame deletion detected in mutant #6. The mutation detected in strain #2 (*yrbK*), however, is a frameshift, a mutation type that often causes loss of function. In fact, the frameshift consists of a C insertion, and results in the formation of a premature stop codon (TAA) eight nucleotides downstream. However, these changes map near the C-terminal region, suggesting that a truncated YrbK protein may be leaky. The view that the C-terminal region of YrbK is dispensable is further supported by the observation that the in-frame deletion found in mutant #6 maps in the same region (Table 5).

Analysis of lipopolysaccharide in bile-resistant mutants

Because the *rlpB* and *yrbK* genes have been described in *E. coli* as involved in LPS transport across the periplasm and LPS assembly at the outer membrane [49], we examined whether the bile-resistant mutants under study showed LPS alterations. The LPS of the DeaD$^-$ mutant was also examined. Migration of the LPS in polyacrylamide gel is known to be affected by the number and size of repeating oligosaccharide units in long-chain LPS, such that bands in the profile represent progressively larger concatemers of the repeating oligosaccharide units [45]. Comparison of the LPS profiles of the mutants and the wild type shows that the *rlpB*, *yrbK*, *and deaD* mutations under study do not visibly alter the amount of LPS. However, structural differences are clearly observed between the wild type and mutants #1, #2, and #6, which carry *yrbK* mutant alleles (Figure 5). Reconstructed YrbK$^-$ mutants showed LPS profiles identical to those of their parental mutants (Figure S1). The profiles found in these mutants may indicate differences in the oligosaccharide units that form long-chain LPS [48].

Single cell analysis of gene expression in the presence and in the absence of sodium deoxycholate

The observation that preadaptation of *S. enterica* to bile can occur by reversible, non mutational mechanisms raised the possibility that the bacterial population might contain cells which activate bile resistance responses in the absence of bile. This hypothesis was initially tested by examining the expression level of *osmY*, a gene of the RpoS regulon, in single *Salmonella* cells grown in the presence and in the absence of a sublethal concentration of DOC (5%, the same concentration used for transcriptomic analysis). To monitor *osmY* expression, a green fluorescent protein (GFP) fusion was constructed at the 3′ end of the *osmY* coding sequence (strain SV6562). Expression of the *osmY*::GFP fusion in individual *Salmonella* cells was monitored using a microscope automated fluidic system [50]. These experiments were of two kinds:

(i) Single point experiments: *S. enterica* cultures grown in LB and LB+5% DOC were diluted and transferred to agar pads. Ten fields, each containing ≥30 cells, were manually defined, and the fluorescence level of individual cells was measured [50]. Two representative, independent experiments are shown in Figure 6A and 6B. Exposure to 5% DOC increased *osmY*::GFP expression in an heterogeneous but consistent manner (red histograms). Cells grown in the absence of DOC (grey histograms) showed lower and more homogeneous levels of *osmY*::GFP expression. However, a significant degree of heterogeneity was observed, indicating that some cells activate the RpoS general stress response in the absence of DOC. Comparison of panels A and B reveals that the

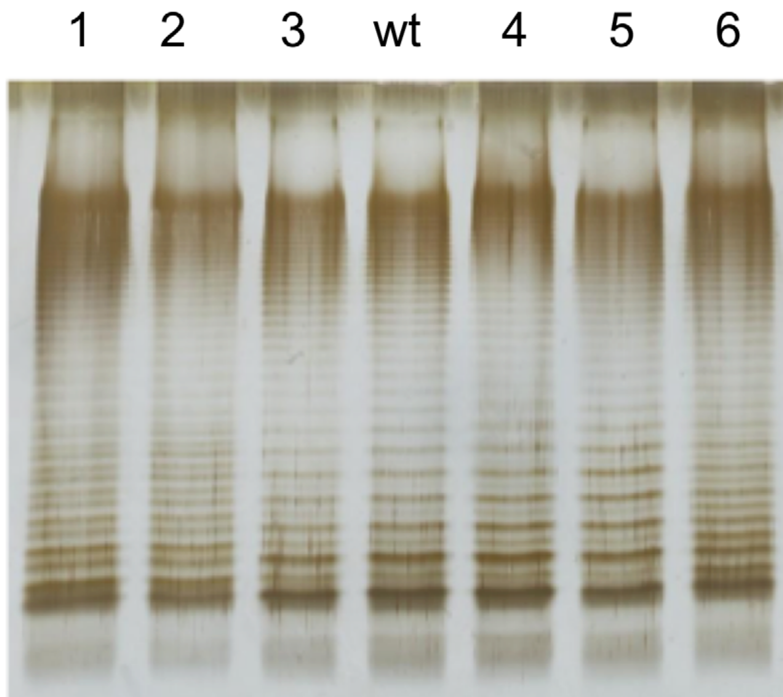

Figure 5. Lipopolysaccharide profiles of bile-resistant derivatives of *S. enterica* SL1344, as observed by electrophoresis and silver staining. The lane marked "wt" shows the LPS profile of the wild type strain. Lanes 1–6 show the LPS profiles of bile-resistant mutants #1, #2, #3, #4, #5, and #6.

doi:10.1371/journal.pgen.1002459.g005

number of cells that activate the RpoS general response in the absence of DOC varies from one experiment to another, thus providing further evidence that phenotypic heterogeneity occurs in batch cultures. As a control, we analyzed *osmY*::GFP expression in an RpoS$^-$ background (strain SV6780). As expected, the level of expression of *osmY* was significantly lower in the absence of RpoS, and *osmY*::GFP induction by DOC was very modest in the RpoS$^-$ mutant (Figure 6C).

(ii) Time lapse experiments: *S. enterica* cultures grown in LB were diluted, transferred to microscope slides, and covered with agar pads containing either LB or LB+5% DOC. The fluorescence of individual cells was then monitored during 90 minutes. A representative experiment involving 14 *Salmonella* cells is shown in Figure 7. The presence of DOC increased the level of fluorescence, albeit at different levels in different cells (perhaps reflecting differences in the initial level of *osmY*::GFP induction). Lysis of some cells was observed in the presence of DOC. In the absence of DOC, the fluorescence levels were lower. However, heterogeneous expression of *osmY*::GFP was observed in the absence of DOC, as in the single point experiments shown in Figure 6.

These experiments suggest that *S. enterica* cultures grown in LB contain cells with elevated expression of the RpoS-dependent general stress response. This observation offers a tentative explanation for non mutational preadaptation to bile: when an aliquot from an LB culture is plated on a lethal concentration of bile, sustainment and/or amplification of the general stress response in certain cells may permit the formation of bile-resistant colonies.

To investigate whether heterogeneous gene expression occurred also in bile-responsive loci that do not belong to the RpoS regulon, we monitored expression of *cspD* [32,33], a stress response gene which is upregulated by exposure to a sublethal concentration of DOC (Table 1 and Table 2). For this purpose, a GFP fusion was constructed at the 3′ end of the *cspD* coding sequence (strain SV6802). Single point experiments in LB and LB+5% DOC were carried out as above, and the fluorescence level of individual cells was measured [50]. Exposure to 5% DOC increased *cspD*::GFP expression in an heterogeneous but consistent manner (Figure 8). However, heterogeneity was also observed in LB (Figure 8), indicating that some cells activate *cspD* expression in the absence of DOC. Hence, phenotypic heterogeneity in gene expression is not restricted to RpoS regulon. This observation suggests that non mutational preadaptation to bile may require specific gene expression patterns, perhaps involving multiple loci.

Discussion

Bacteria live in a changing environment, devoid of the homeostatic mechanisms that create stable conditions in the tissues of multicellular eukaryotes. Except for obligate parasites that have adapted to stable environments, survival of bacteria depends on ceaseless adaptation. This study describes mechanisms employed by *Salmonella enterica* to survive bile, a fluid with antibacterial capacity due to the presence of bile salts [3,4,8]. The adaptation mechanisms have been investigated using either ox bile, which contains a mixture of bile salts [1], or sodium deoxycholate, the most abundant and well known bile salt [2].

The concentrations of bile and DOC that inhibit *S. enterica* growth depend on the conditions used. For instance, it is well known that dividing cells are more sensitive to bile salts than non dividing cultures [19]. Furthermore, even under specific conditions, the minimal inhibitory concentration of bile increases if the *S. enterica* culture is previously grown on sublethal concentrations

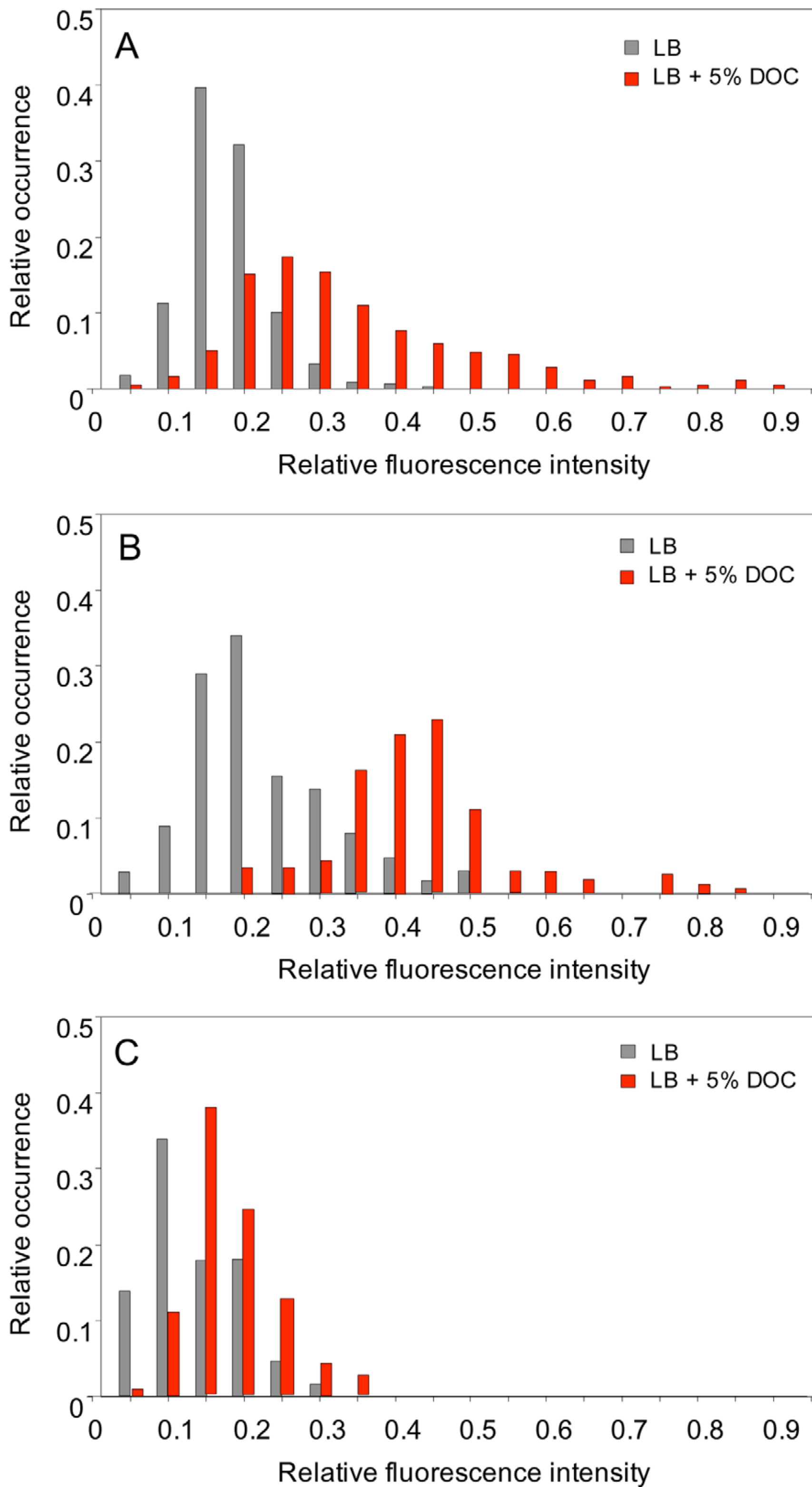

Figure 6. Levels of *osmY* gene expression in individual bacterial cells. Panels A and B show the distribution of fluorescence intensity in individual cells ($N>300$) of *S. enterica* SV6562 (*osmY*::GFP) in two independent experiments. In both cases, strain SV6562 was grown during 5 h in LB with or without 5% sodium deoxycholate. Histograms represent the proportion of bacterial cells showing distinct fluorescence levels in LB (grey) and LB+DOC (red). Fluorescence intensities are shown in an arbitrary scale (0–1). Panel C shows the distribution of fluorescence intensity in individual cells ($N>300$) of *S. enterica* SV6780 (*osmY*::GFP RpoS^{-}) under conditions identical to those of experiments A and B.
doi:10.1371/journal.pgen.1002459.g006

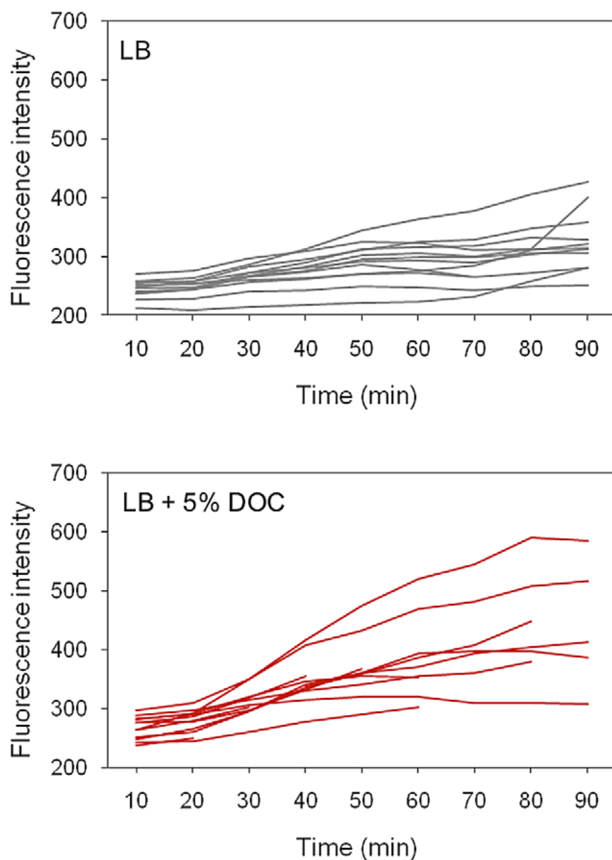

Figure 7. Time course of *osmY*::GFP expression in individual cells in the presence and in the absence of DOC. Aliquots from an exponential culture (O.D.$_{600}$ = 0.5) of *S. enterica* SV6562 (*osmY*::GFP) grown in LB were transferred to agar pads containing or not 5% sodium deoxycholate. Bacterial cells were fixed *in situ*, and GFP fluorescence intensity was measured at 10 min intervals during 90 min.
doi:10.1371/journal.pgen.1002459.g007

[8]. For instance, minimal inhibitory concentration analyses shown in Figure 2 indicate that growth on 4–5% DOC, a concentration which does not inhibit *Salmonella* growth, increases the MIC to ≥14%. Growth at DOC concentrations below 3% does not increase the MIC, and concentrations higher than 5% are inhibitory (Figure 2). Hence, sublethal (but relatively high) concentrations of DOC are necessary to increase the MIC above the standard inhibitory concentration.

A conceivable explanation for *Salmonella* adaptation to bile was that sublethal concentrations of bile might trigger changes of gene expression that could facilitate survival. Transcriptomic analysis in the presence of 5% DOC revealed indeed multiple changes in gene expression, some of which provided tentative explanations for the observed increase in bile resistance. Especially relevant was the observation that exposure to DOC activates the RpoS-dependent general stress response (Table 1, Table 2, Table 3, Table 4, and Figure 3). This response facilitates survival of *E. coli* and other gamma-proteobacteria under conditions that do not support active growth, and is also known to be activated by multiple stress conditions [31]. Hence it is not surprising that bile salts, which denature proteins and cause DNA damage [3,4], can trigger the general stress response. The conclusion that the RpoS-dependent general stress response plays a crucial role in bile resistance is supported by two additional observations: (i) Lack of RpoS causes bile sensitivity (Table S3); (ii) *S. enterica* stationary cultures, in which the RpoS response is physiologically active, are more resistant to both DOC and ox bile [19]. The occurrence of extreme levels of resistance (≥14% DOC) in adapted cultures admits several explanations (not mutually exclusive). Activation of the RpoS regulon by sublethal concentrations of bile may be stronger than physiological activation in stationary cells. It is also possible that high levels of bile resistance result from simultaneous activation of more than one bile-resistance response. This latter possibility is supported by the observation that stress-inducible genes (*cspD*, *uspA*, *aphC*, etc.) that do not belong to the RpoS regulon are also activated by exposure to 5% DOC (Table 1 and Table 2). Furthermore, exposure to bile upregulates the expression of genes of unknown function, some of which might be part of stress

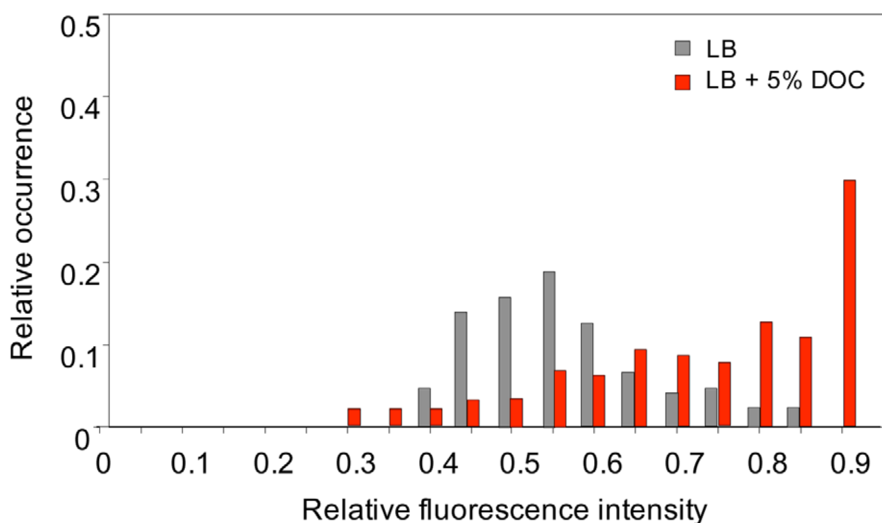

Figure 8. Levels of *cspD* gene expression in individual bacterial cells. The distribution of fluorescence intensity was measured in individual cells (*N*>300) of *S. enterica* SV6802 (*cspD*::GFP) after growth during 5 h in LB with or without 5% sodium deoxycholate. Histograms represent the proportion of bacterial cells showing distinct fluorescence levels in LB (grey) and LB+DOC (red). Fluorescence intensities are shown in an arbitrary scale (0–1).
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response networks. An example is *yiiU*, which is activated by DOC (Table 1 and Figure 3) and is essential for bile resistance (Table S3).

High throughput analyses of gene expression also indicated that adaptation to bile may involve downregulation of porin genes and other genes encoding envelope structures, as well as upregulation of efflux pumps (Table 1 and Table 2). All these gene expression changes fit well in the literature: porins provide passage to bile salts [8], envelope structures are major barriers for bile salt uptake [8], and efflux pumps can transport bile salts outside the cell [21]. Hence, growth on sublethal concentrations of bile may permit *Salmonella* adaptation to lethal concentrations by triggering multiple changes in gene expression. Downregulation of pathogenicity island 1 by sublethal concentrations of DOC (Table 2), a phenomenon previously described [27], may be viewed as a signalling system used by *Salmonella* to identify environments that are not appropriate for epithelial cell invasion (e. g., the duodenum). In turn, downregulation of pathogenicity island 2 in the presence of bile (Table 2) may be viewed as a response that prevents activation of *Salmonella* functions involved in intracellular survival.

Preadaptation of *S. enterica* to bile is a completely different phenomenon, which does not pertain to *Salmonella* populations but to individual bacterial cells. Preadaptation is easily observed under laboratory conditions: when an aliquot of a *S. enterica* batch culture is plated on LB agar containing a lethal concentration of ox bile, bile-resistant colonies appear at frequencies ranging from 10^{-6} to 10^{-7} per cell and generation. These numbers fall in the known range of bacterial mutation frequencies [51]. Not surprisingly, Luria-Delbrück fluctuation analysis confirms that such colonies arise from bile-resistant cells found in the culture aliquot used for inoculation (Table S4). However, we were surprised to find that the majority of bile-resistant isolates obtained under such conditions were unstable, and lost bile resistance if grown overnight in LB without bile. Hence, preadaptive bile resistance seems to involve two distinct phenomena: mutation and non mutational preadaptation.

Full genome sequencing of 6 bile-resistant mutants revealed that 3 mutants carried mutations in *yrbK* and one in *rlpB*. The high frequency of mutations in lipopolysaccharide transport genes leaves little doubt that altered LPS transport can cause bile resistance. However, the identification of such mutants has intriguing aspects. One comes from the fact that LPS transport genes are known to be essential in *E. coli* [47,48]. If such is also the case in *Salmonella*, the mutations found (two nucleotide substitutions, one in-frame deletion and one premature stop codon relatively close to the 3′ end of the coding sequence) must all be leaky. Another intriguing question is related to the fact that LPS is a major barrier against bile salts [15]. However, it is conceivable that transport of LPS components across the envelope might sensitize the cell to bile salts, thus explaining why altered transport may confer bile resistance. An alternative, speculative explanation is that the LPS transport proteins altered in bile-resistant mutants might be also involved in LPS assembly and/or modification, and that specific mutations might boost bile resistance. This view may be tentatively supported by the observation that some of the bile-resistant mutants described in this study have altered LPS profiles (Figure 5 and Figure S1).

Non mutational preadaptation to bile was at first sight an intriguing phenomenon. How can a *Salmonella* batch culture contain cells that are bile-resistant without previous adaptation by growth at sublethal concentrations? However, it is well known that bacterial cultures, albeit genetically clonal, can contain subpopulations of cells with distinct patterns of gene expression [25], either as a consequence of epigenetic control [52] or as the result of stochastic fluctuations in gene expression [25,53]. We thus hypothesized that non mutational preadaptation might be caused by activation of bile-resistance responses in the absence of bile. This hypothesis was tested by analyzing expression of *osmY*, an RpoS-dependent gene, and *cspD*, a stress response gene that does not belong to the RpoS regulon [32,33], in individual *Salmonella* cells grown in the presence and in the absence of a sublethal concentration of DOC. Microscopic microfluidics [50] confirmed that exposure to DOC activates *osmY* and *cspD* expression in most *Salmonella* cells (Figure 6 and Figure 7). However, upregulation of *osmY* and *cspD* expression was also observed in subpopulations of *Salmonella* cells in the absence of DOC (Figure 6, Figure 7, and Figure 8). Non mutational preadaptation may thus result from activation of the RpoS-dependent general stress response and/or other stress responses in a cell subpopulation. Stress response activation may either be triggered by a stress situation encountered by individual cells (e. g., increased concentrations of harmful metabolic products) or be accidental. Repression of specific loci, which has not been addressed in this study, may also contribute to preadaptation. It is possible that non mutational preadaptation to environmental challenges is a common phenomenon, and that the acquisition of bile resistance described in this study is merely one example among many others. The widespread occurrence of phenotypic polymorphism in clonal populations of bacteria [25,52,53] may support this possibility.

At this stage, it is impossible to ascertain whether the three distinct modes of *Salmonella* adaptation to bile described in this study may occur or not upon infection of animals. Current evidence obtained in the mouse model of typhoid fever indicates that *Salmonella* cells can escape the high concentrations of bile salts found in the gall bladder lumen by invading the gall bladder epithelium [11] and by forming biofilms on the surface of gallstones [12,13]. However, planktonic *Salmonella* cells are also found in the gall bladder lumen, and little is known about the mechanisms that permit their survival and multiplication. Our model envisions that planktonic *Salmonella* cells may adapt to the gall bladder lumen by changing their gene expression pattern. Passage by the small intestine and the liver, which contain bile concentrations much lower than those found in the gall bladder [1,2], might facilitate adaptation in an analogous manner as growth on sublethal concentrations of DOC in the laboratory. Phenotypic heterogeneity and subpopulation formation may additionally contribute to adaptation by activating bile resistance responses prior to colonization of the hepatobiliary tract. Lastly, appearance of bile-resistant mutants may provide an alternative mechanism for *Salmonella* adaptation to the gall bladder. Because bile salts are mutagenic [5,6] and the dose of a mutagen is the product of its concentration by the time of exposure [54], mutational adaptation of *Salmonella* to bile might be speeded up by bile itself, especially during long term infection of the bile-laden gall bladder. This phenomenon might be relevant during persistent and chronic infections, as found for instance in human carriers of *Salmonella* Typhi [10].

Materials and Methods

Bacterial strains, plasmids, bacteriophages, media, and culture conditions

Strains of *Salmonella enterica* serovar Typhimurium (often abbreviated as *S. enterica*) used in this study (Table 1) derive from the mouse-virulent strain SL1344. Strains SV6065, SV6066, SV6067, SV6068, SV6069, SV6629, and SV6745 were constructed by transducing alleles from ATCC 14028 or LT2 to SL1344.

Transduction was performed with phage P22 HT 105/1 *int201* ([55] and G. Roberts, unpublished data). The P22 HT transduction protocol was described elsewhere [56]. To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan et al. [57], except that methyl blue (Sigma Chemical Co., St. Louis, Missouri) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Luria-Bertani broth (LB) was used as standard rich medium and E medium [58] as minimal medium. Solid media contained agar at 1.5% final concentration. Cultures were grown at 37°C. Aeration of liquid cultures was obtained by shaking in an orbital incubator. Deoxycholic acid (sodium salt) and sodium choleate (ox bile extract) were both from Sigma. Antibiotics were used at the final concentrations described previously [5]. A strain list is provided as Table 6.

Gene disruption and directed construction of *lac* fusions

Targeted gene disruption was achieved using plasmids pKD3, pKD4, and pKD13 [59]. Antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 [59]. The oligonucleotides used for disruption (labeled "FOR" and "REV") are listed in Table S5 together with the oligonucleotides (labeled "E") used for allele verification by the polymerase chain reaction. For the construction of transcriptional and translational *lac* fusions in the *Salmonella* chromosome, FRT sites generated by excision of Km^r cassettes were used to integrate either plasmid pCE36 or pCE40 [60].

Table 6. Strain list.

Strain	Genotype
SV5561	*rpoS*::Ap^r
SV6065	*katE*::MudK (Km^r)
SV6066	*ots*::MudJ (Km^r)
SV6067	*xthA::lacZ*
SV6068	*osmY::lacZ*
SV6069	*dps::lacZ*
SV6088	*hilA::lacZ*
SV6090	*prgH::lacZ*
SV6109	*STM1441::lacZ*
SV6112	*ybjM::lacZ*
SV6115	*ecnB::lacZ*
SV6118	*STM1672::lacZ*
SV6124	*yajI::lacZ*
SV6127	*ugpB::lacZ*
SV6261	*aroG::lacZ*
SV6267	*ytfK::lacZ*
SV6270	*yiiU::lacZ*
SV6292	*yceK::lacZ*
SV6435	*rlpB* (287 C→A)
SV6562	*osmY*::GFP (Cm^r)
SV6629	*tolC*::Cm^r
SV6745	*acrD*:: Km^r
SV6780	*osmY*::GFP (Cm^r) *rpoS*::Ap^r
SV6802	*cspD*::GFP (Cm^r)
SV6880	*yrbK* (182 G→C) *yrbL*–Km^r–*mtgA*
SV6883	*yrbK* (399+) *yrbL*–Km^r–*mtgA*
SV6884	*yrbL*–Km^r–*mtgA*
SV6888	*katE*::MudK *rpoS*::Ap^r
SV6889	*deaD* (923 G→C)

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Construction of *osmY*::GFP and *cspD*::GFP fusions

An *osmY::gfp* fusion was constructed as described by Hautefort et al. [61]. A fragment containing the promoterless green fluorescent protein (*gfp*) gene and the chloramphenicol resistance cassette was amplified from the pZEP07 plasmid with primers 5′ AAG CCG TTG ATG GCG TAA AAA GTG TTA AAA ACG ATC TGA AAG TTC AGT AAT AAG AAG GAG ATA TAC ATA TGA G 3′, and 5′ GGT GCA CAT TAC GCC TCC CGA CAA ACG TCG GGA GGA CGA ATT ACG ACG AAT TAT CAC TTA TTC AGG CGT A 3′. Primers used for *cspD* amplification were 5′ GCA ATC ACG CCA GCG TCA TCG TGC CCA TCG AAG CAG AGG CCG TTG CAT AGT AAG AAG GAG ATA TAC ATA TGA G 3′, and 5′ CGA TCG GGC TGG CAT TTT GCC TCC TGG ATG TAC ACA ATG AGA CAG AGG AGT TAT CAC TTA TTC AGG CGT A 3′. The 5′ regions of these primers are homologous to the 3′ end of the *osmY* and *cspD* coding sequences, so that the fusion is formed immediately after the *osmY* and *cspD* stop codons. The constructs were integrated into the chromosome of *S. enterica* using the Lambda Red recombination system [59].

Directed construction of *rlpB* and *deaD* point mutations

The *rlpB* allele from mutant #3 and the *deaD* allele from mutant #4 were PCR-amplified using pairs of 30-nucleotide primers that contained XbaI and SacI targets. The primers for *rlpB* amplification were 5′ TTT TGA GCT CGA AGG TGA TAT CGA CAA CGC 3′, and 5′ TTT TTC TAG ACT CAT TCA TTG CCG CGT TAG 3′; for *deaD* amplification, 5′ TTT TGA GCT CCG TCT GCT TGA TCA CTT AAA 3′, and 5′ TTT TTC TAG AAC GAC GTT CAC GAC GCG GAC 3′. The resulting fragments were digested with XbaI and SacI, cloned onto pDMS197 [61] and propagated in *E. coli* CC118 lambda *pir* [62]. Plasmids derived from pMDS197 were transformed into *E. coli* S17-1 lambda *pir* [63]. The resulting strains were used as donors in matings with *S. enterica* SL1344 as recipients, selecting Tc^r transconjugants on E plates supplemented with tetracycline and histidine. Several Tc^r transconjugants were grown in nutrient broth (without NaCl) containing 5% sucrose. Individual tetracycline-sensitive segregants were then examined for the incorporation of the mutant allele by DNA sequencing.

Reconstruction of bile-resistant mutants by P22-mediated transduction of a linked marker

The kanamycin-resistant cassette of plasmid pKD4 was inserted at a region close to the mutation under study, using lambda Red recombination [59]. The oligonucleotides employed for gene targeting are listed in Table S5. To reconstruct *yrbK* mutations, the Km^r cassette was inserted at an intergenic region between *yrbL* and *mtgA*, 37 bp downstream the *yrbL* stop codon and 28 bp downstream the *mtgA* stop codon (note that *yrbL* and *mtgA* undergo divergent transcription). The distance from the Km^r cassette and the *yrbK* mutations under study is 6,135 bp for mutant #1, and 5,918 bp for mutant #2. To reconstruct the *rlpB* mutation of mutant #3, the Km^r cassette was introduced at an intergenic region between the putative ORFs *ybeL* and *ybeQ* (21 bp downstream the putative *ybeL* stop codon, and 8,253 bp away from the *rlpB* mutation). DNA sequence analysis employed the *S.*

Typhimurium SL1344 genome database (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs).

The presence of the original mutation in the reconstructed mutants was verified by PCR amplification using primers designed *ad hoc* (Table S5), followed by DNA sequencing. In all constructions, the distance between the Km^r cassette and the mutation under study permitted >90% cotransduction of the Km^r cassette and the point mutation under study, fulfilling calculations made with the formula of Wu [62].

Determination of minimal inhibitory concentrations of sodium deoxycholate

Exponential cultures in LB broth were prepared, and samples containing around 3×10^2 colony-forming-units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of sodium deoxycholate (DOC). After 12 h incubation at 37°C, growth was visually monitored. Assays were carried out in triplicate. Student's *t*-test was used to analyze every MIC. The null hypothesis was that MICs were not significantly different from the MIC for the wild-type. *P* values of 0.01 or less were considered significant.

Assessment of bacterial viability using a cell staining kit

One ml aliquots of an exponential culture of *S. enterica* SL1344 grown in LB were incubated in the presence of different concentrations of sodium deoxycholate (1%, 3%, 5%, 7% and 9%) during 30 minutes at 37°C. The cells were then washed three times with 0.85% NaCl and stained using the Viability/Cytotoxicity Assay Kit for Bacteria (Biotium Inc., Hayward, California). Control suspensions of live and dead cells were prepared as described in the kit protocol. Live and dead cells were distinguished using a Leica DMR 020-525.024 fluorescence microscope (Leica Camera AG, Solms, Germany). Live and dead bacteria were counted as the green and red cells (respectively) found in randomly selected 5×5 mm squares painted on a micro cover glass.

Assessment of bacterial viability by plate counts

Aliquots of *Salmonella* exponential cultures grown in LB, each containing 2×10^6 cells, were treated with various concentrations of DOC (1%, 3%, 5%, 7%, and 9%) for 30 min. The cultures were then diluted, plated on LB and incubated at 37°C. Counts of colony-forming-units were performed after overnight growth.

Isolation of bile-resistant mutants and Luria-Delbrück fluctuation assays

Bile-resistant derivatives of *S. enterica* SL1344 were isolated by plating 0.1 ml aliquots (approximately, 2×10^8 cells) from an overnight LB culture onto LB plates containing 18% ox bile (Sigma-Aldrich, St. Louis, Missouri). Fluctuation analysis was performed as described by Luria and Delbrück [42], and the number of independent cultures was 40.

DNA isolation and full-genome sequencing

Whole genome DNA samples from bile-resistant mutants and from the parent strain SL1344 were prepared by phenol extraction and ethanol precipitation. Whole genome sequencing was performed using the oligonucleotide ligation and detection (SOLiD, v2) platform [43] at the facilities of Sistemas Genómicos S.L., Parque Tecnológico de Valencia, Paterna, Spain, using mate-pair libraries and reads of 25 nucleotides [63]. DNA sequences were aligned with the genome sequence of *Salmonella* Typhimurium SL1344 available at the Wellcome Trust Sanger Institute, Hinxton, England (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs). Two mismatches or mispairs per reading were permitted.

RNA isolation, microarray procedures, and data analysis

To prepare cells for RNA extraction, 25 ml of fresh LB and LB+DOC 5% in a 250 ml flask was inoculated with a 1:100 dilution from an overnight bacterial culture, and incubated with shaking at 250 rpm in a New Brunswick Innova 3100 waterbath at 37°C. Three biological replicates were performed for each strain, and RNA was extracted at an optical density (OD_{600})~0.4 (exponential phase) and >1 (stationary phase). RNA extractions were performed as described by Mangan et al. [64], and their quality was assessed on an Agilent 2100 Bioanalyzer. Transcriptomic analyses were performed with the Salgenomics microarray [29]. Hybridization and microarray scanning were performed at the Genomics Service of the Centro Nacional de Biotecnología, C.S.I.C., Cantoblanco, Madrid, Spain (http://www.cnb.uam.es/content/services/genomics). For normalization of the two-color microarray data, LiMMA software [65] was used. Further bioinformatic analysis was carried out with the FIESTA programme (http://bioinfogp.cnb.csic.es/tools/FIESTA/index.php). Raw transcriptomic data were deposited at the Array Express database (http://www.ebi.ac.uk/miamexpress) under accession number E-MTAB-637.

ß-galactosidase assays

Levels of ß-galactosidase activity were assayed as described by Miller [66], using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

Electrophoretic visualization of lipopolysaccharide profiles

To investigate lipopolysaccharide (LPS) profiles, bacterial cultures were grown in LB. Bacterial cells were harvested and washed three times with 0.9% NaCl. The $O.D._{600}$ of the washed bacterial suspension was measured to calculate cell concentration. A bacterial mass containing about 3.2×10^8 cells was pelleted by centrifugation. Treatments applied to the bacterial pellet, electrophoresis of crude bacterial extracts, and silver staining procedures were performed as described by Buendía-Clavería *et al.* [67]. Three replicates per strain were performed.

Microscopy

Cells were inoculated onto a microscope cover slip and covered with a thin (2 mm thick) semisolid LB agar (1.5%) matrix with or without DOC. In the DOC-containing samples, the final concentration of DOC was 5%. The cover slip and the agar pad were then mounted in a seal flow chamber allowing constant aeration and reduced desiccation. Flow chambers were incubated in a temperature-controlled automated microscope (Nikon TE2000-E-PFS, Nikon, Champigny-sur-Marne, France) at 37°C. For single point experiments, 10 fields, each containing at least 30 cells treated or not with DOC, were manually defined. For 90 min time lapse experiments, a single field was examined, and time points were taken every 10 min under agar pads with or without DOC. Images were recorded using a CoolSNAP HQ2 high resolution camera (Roper Scientific, Evry, France) and a 100x/1.4 DLL objective. Digital analysis and image treatment were performed with Metamorph software 7.5 (Molecular Devices, Sunnyvale, California) as previously described [50].

Supporting Information

Figure S1 LPS profiles of bile resistant mutants #1 and #2 (both carrying *yrbK* mutations), reconstructed by P22-mediated transduction of a linked Km^r marker. Lanes are as follows: 1, $YrbK^+$ Km^r transductant obtained with a P22 HT lysate grown on SV6880 (*yrbK* G→C Km^r); 2, $YrbK^-$ Km^r transductant obtained with a P22 HT lysate grown on SV6880; wt, wild type; 3, $YrbK^-$ Km^r transductant obtained with a P22 HT lysate grown on SV6883 (*yrbK* +1 frameshift Km^r); $YrbK^+$ Km^r transductant obtained with a P22 HT lysate grown on SV6883. Transductants 1 and 4 were bile-sensitive, while transductants 2 and 3 were bile-resistant. The *yrbK* mutations carried by transductants 2 and 3 were confirmed by PCR amplification and DNA sequencing of the amplified fragments.
(PDF)

Table S1 ß-galactosidase activities of *lac* fusions in bile-responsive genes during exponential growth in the presence and in the absence of DOC.
(DOC)

Table S2 ß-galactosidase activities of *lac* fusions in bile-responsive genes in stationary cultures grown in the presence and in the absence of DOC.
(DOC)

Table S3 Minimal inhibitory concentrations (g/100 ml) of sodium deoxycholate for *S. enterica* strains mentioned in this study, all derived from SL1344.
(DOC)

Table S4 Fluctuation in the frequencies of bile-resistant mutants obtained upon plating of *S. enterica* SL1344 on LB+18% ox bile.
(DOC)

Table S5 Oligonucleotides.
(DOC)

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Author Contributions

Conceived and designed the experiments: JC LA. Performed the experiments: SBH IC AD LA. Analyzed the data: SBH IC AD LA JC. Contributed reagents/materials/analysis tools: SBH IC AD LA JC. Wrote the paper: SBH IC AD LA JC.

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Supplemental online material

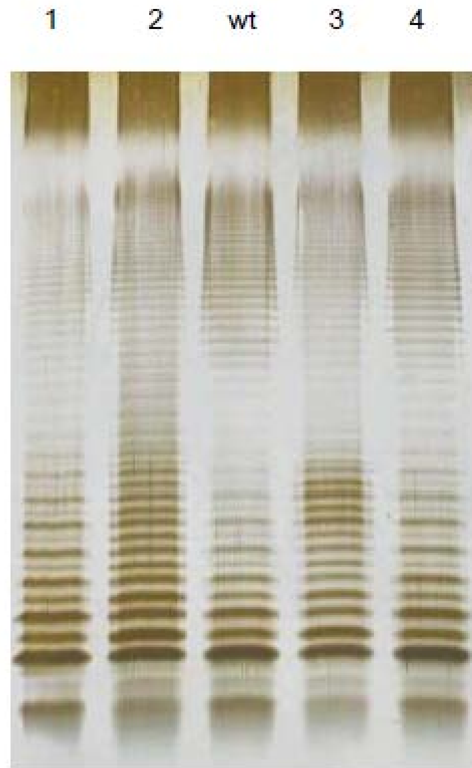

Figure S1. LPS profiles of bile resistant mutants #1 and #2 (both carrying *yrbK* mutations), reconstructed by P22-mediated transduction of a linked Km^r marker. Lanes are as follows: 1, $YrbK^+$ Km^rtransductant obtained with a P22 HT lysate grown on SV6880 (*yrbK* G→C Km^r); 2, $YrbK^-$ Km^rtransductant obtained with a P22 HT lysate grown on SV6880; wt, wild type; 3, $YrbK^-$ Km^rtransductant obtained with a P22 HT lysate grown on SV6883 (*yrbK* +1 frameshift Km^r); $YrbK^+Km^r$ transductant obtained with a P22 HT lysate grown on SV6883. Transductants 1 and 4 were bile-sensitive, while transductants 2 and 3 were bile-resistant. The *yrbK* mutations carried by transductants 2 and 3 were confirmed by PCR amplification and DNA sequencing of the amplified fragments.

Table S1. ß-galactosidase activities of *lac* fusions in bile-responsive genes in exponential cultures grown in the presence and in the absence of sodium deoxycholate (DOC)

Strain	Gene fusion	LB	LB + DOC
SV6068	*osmY::lacZ*	19± 0.7	80 ± 24
SV6069	*dps::lacZ*	5 ± 0.2	11.5 ± 1.2
SV6088	*hilA::lacZ*	38 ± 3	14 ± 2.5
SV6090	*prgH::lacZ*	555 ± 60	208 ± 48
SV6109	*STM1441::lacZ*	2.8 ± 0.1	14.8 ± 3.1
SV6112	*ybjM::lacZ*	9.5 ± 3.5	15 ± 1.4
SV6115	*ecnB::lacZ*	2480 ± 143	6700 ± 950
SV6118	*STM1672::lacZ*	370 ± 14	1400 ± 124
SV6124	*yajI::lacZ*	1.4 ± 0.07	6.2 ± 0.4
SV6127	*ugpB::lacZ*	2.3 ± 05	4.8 ± 1
SV6261	*aroG::lacZ*	97 ± 6	330 ± 100
SV6267	*ytfK::lacZ*	360 ± 80	650 ± 140
SV6270	*yiiU::lacZ*	1332 ± 66	7676 ± 835
SV6292	*yceK::lacZ*	11 ± 0.2	45 ± 10

Cultures were prepared in LB and LB containing 5% DOC. Aliquots were extracted at O.D.$_{600}$ = 0.4. ß-galactosidase activities are shown in Miller units. Data are averages and standard deviations from 3 experiments.

Table S2. ß-galactosidase activities of *lac* fusions in bile-responsive genes in stationary cultures grown in the presence and in the absence of sodium deoxycholate (DOC)

Strain	Gene fusion	LB	LB + DOC
SV6068	*osmY::lacZ*	136 ± 12	344 ± 35
SV6069	*dps::lacZ*	35.6 ± 0.36	64 ± 1.4
SV6088	*hilA::lacZ*	330 ± 24	17 ± 1.4
SV6090	*prgH::lacZ*	1026 ± 50	360 ± 28
SV6109	*STM1441::lacZ*	0.9 ± 0.2	6.6 ± 2.6
SV6112	*ybjM::lacZ*	22.3 ± 1.2	34.4 ± 2.8
SV6115	*ecnB::lacZ*	10095 ± 504	12140 ± 689
SV6118	*STM1672::lacZ*	1280 ± 42	1440 ± 130
SV6124	*yajI::lacZ*	1.8 ± 0.5	8 ± 2
SV6127	*ugpB::lacZ*	5.3 ± 0.7	6.5 ± 1.8
SV6261	*aroG::lacZ*	46 ± 3	282 ± 25
SV6267	*ytfK::lacZ*	960 ± 20	1020 ± 192
SV6270	*yiiU::lacZ*	1278 ± 36	3957 ± 226
SV6292	*yceK::lacZ*	7.2 ± 0.1	48 ± 4.2

Cultures were prepared in LB and LB containing 5% DOC. Aliquots were extracted at O.D.$_{600}$ $\geq$ 1. ß-galactosidase activities are given in Miller units. Data are averages and standard deviations from 3 experiments.

Table S3. Minimal inhibitory concentrations (g/100 ml) of sodium deoxycholate for *S. enterica* strains mentioned in this study

Strain	Genotype	M.I.C.
SV5561	*rpoS*::Ap^r	3
SV6065	*katE*::Mu*d*K (Km^r)	7
SV6066	*ots*::Mu*d*J (Km^r)	7
SV6067	*xthA::lacZ*	7
SV6068	*osmY::lacZ*	7
SV6069	*dps::lacZ*	7
SV6109	*STM1441::lacZ*	7
SV6112	*ybjM::lacZ*	7
SV6115	*ecnB::lacZ*	7
SV6118	*STM1672::lacZ*	7
SV6124	*yajI::lacZ*	7
SV6127	*ugpB::lacZ*	7
SV6261	*aroG::lacZ*	7
SV6267	*ytfK::lacZ*	7
SV6270	*yiiU::lacZ*	1.5
SV6292	*yceK::lacZ*	7
SV6435	*rlpB* (287 C → A)	>12
SV6562	*osmY*::GFP (Cm^r)	7
SV6629	*tolC*::Cm^r	0.02
SV6745	*acrD*:: Km^r	7
SV6780	*osmY*::GFP (Cm^r) *rpoS*::Ap^r	7
SV6880	*yrbK* (182 G → C) *yrbL*–Km^r–*mgtA*	>12
SV6802	*cspD*::GFP (Cm^r)	7
SV6883	*yrbK* (399+) *yrbL*–Km^r–*mgtA*	>12
SV6884	*yrbL*–Km^r–*mgtA*	7
SV6888	*katE*::Mu*d*K *rpoS*::Ap^r	3
SV6889	*deaD* (923 G → C)	>12

Table S4. Fluctuation in the frequencies of bile-resistant colonies obtained upon plating of *S. enterica* SL1344 on LB + 18% ox bile

Number of colonies	
Independent cultures	Single culture
12	6
5	9
6	8
18	7
1	4
25	1
310	1
32	22
2	10
7	12
1	10
3	10
4	8
5	4
9	8
1	14
2	10
4	6
12	7
5	6

23	9	
2	11	
5	8	
8	5	
20	7	
5	7	
8	4	
2	4	
8	6	
5	4	
12	7	
3	6	
11	6	
14	8	
6	12	
2	17	
18	8	
18	6	
23	4	
4	8	
Average	16.52	7.75
Standard deviation	48.20	3.94
Coefficient of variation	2.91	0.50

Table S5. Oligonucleotides

Oligonucleotide name	Sequence (5'-3')
aroG-E1	aacgcagcagagaatcttgc
aroG-E2	caggaagtacctaagtacgc
aroG-P1-REV	cccgctttggtgaggccttctttcacctcagcaacatgctgtgtaggctggagctgcttc
aroG-P4-FOR	tctgctggtggtgatcggtccttgttcaattcatgatccgattccggggatccgtcgacc
ecnB-E1	ctggttcttcttaccagcac
ecnB-E2	cacgcctttacatgagcttg
ecnB-P1-REV	gttgcagcgccagagatcgcactaccaccgtccgagatgtgtgtaggctggagctgcttc
ecnB-P4-FOR	gacaattgcagcgatcttttctgttttggtactttccactattccggggatccgtcgacc
osmY-E1	accgcattcagcaatgcaac
osmY-E2	aatatgagcgctcaggctac
osmY-P1-FOR	actctgctggccgtaatgttgacctctgctgttgcgacaggtgtaggctggagctgcttc
osmY-P2-REV	acgccatcaacggctttcgcgatgctttcagcgcggtcgccatatgaatatcctccttag
STM1441-E1	caaagcgtggaaagtgactc
STM1441-E2	cacattcatgccaacggtag
STM1441-P1-REV	tgcgagcagttcagcagcaccactccccaacgtaataaccgtgtaggctggagctgcttc
STM1441-P4-FOR	cgcgttcccgatggttaatattgtagaaatcacgcagttgattccggggatccgtcgacc
STM1672-E1	cagtactgtacggtgtagtg
STM1672-E2	ctggtaatgcgctaaagcag
STM1672-P1-REV	gcaaaatgccgggtagcgctggcccagggcattcgcatcagtgtaggctggagctgcttc
STM1672-P4-FOR	aatggtagcttgtttttcgtatatcgcacatgccagtaccattccggggatccgtcgacc
ugpB-E1	ctctctgtcgccttactatc
ugpB-E2	ctttgcagcgaataccacag
ugpB-P1-REV	gcaccggggtttttatcgtaataaccctgctcgcgggtcagtgtaggctggagctgcttc
ugpB-P4-FOR	caacgcgccggcaatattgcaggtttatgaggttgggacgattccggggatccgtcgacc
yajI -E1	gttcattacacaccaggcac

yajI -E2	cgtaatcgattgcgttcacg
yajI -P1-REV	aacggtaacgaaatattgacatcactgggcgccagcaagcgtgtaggctggagctgcttc
yajI -P4-FOR	ccagatgcatcaaagcattagcaccctgaataaaagagatgattccggggatccgtcgacc
ybjM-E1	gtaacttgccaagacacagg
ybjM -E2	cggcatgataacacctgttc
ybjM -P1-REV	aacgcacaccagaatacggcgctaaataaccacgcgagttgtgtaggctggagctgcttc
ybjM -P4-FOR	tatgcatgtacaaggcgcttttcgcgcggcagggcatcctattccggggatccgtcgacc
yceK-E1	gtttacactatcgggtctgg
yceK-E2	tgtgcagcagttcaacgttc
yceK-P1-REV	agtagcagtgtatcgaagatcagtgagaagggcagatcgagtgtaggctggagctgcttc
yceK-P4-FOR	gattaccctgagcggctgcggcagtattatcagcagaacgattccggggatccgtcgacc
yiiU-E1	gtttacactatcgggtctgg
yiiU-E2	tgtgcagcagttcaacgttc
yiiU-P1-REV	agtagcagtgtatcgaagatcagtgagaagggcagatcgagtgtaggctggagctgcttc
yiiU-P4-FOR	gattaccctgagcggctgcggcagtattatcagcagaacgattccggggatccgtcgacc
ytfK-E1	cgtaaggtcatggtcattcc
ytfK-E2	ctaccgtatcgatcagttgc
ytfK-P1-REV	ataacctgacggttaacttcagacatcacagacaagtgctgtgtaggctggagctgcttc
ytfK-P4-FOR	taatactctgcaggagacaacaatgaaaattttccaacgcattccggggatccgtcgacc
yrbK-FOR	gactatgtgacgcacattgc
yrbK-REV	ctcgtagtgcatatgggaag
acrD-P4-FOR	catttttgcctgggtgctggctatcctgttgtgtctgacaattccggggatccgtcgacc
acrD-P1-REV	cgtatcagcacgaaaaacaggggtacaaagaagatagccatgtaggctggagctgcttcg
acrD-E1	ccaacaaggaagagagtcag
acrD-E2	ttgaacgtgaactggggaac

Chapter 2: Role for the ZapB cell division factor in bile resistance

Role for the ZapB cell division factor in bile resistance

Summary

Resistance to bile is a crucial factor in all types of *Salmonella enterica* infections: the pathogen encounters bile in the animal gut during intestinal infection, in the hepatobiliary tract during systemic infection, and in the gall bladder during chronic infection. Bile resistance involves multiple mechanisms including changes in gene expression. Previous transcriptomic analysis has shown that a sublethal concentration of sodium deoxycholate (DOC), the archetypal and most abundant bile salt, alters the expression of >100 *Salmonella* loci. One such locus is the poorly known gene *yiiU*. The *S. enterica* YiiU product is homologous to the ZapB protein of *E.coli*, a non essential cell division factor involved in Z-ring assembly. ZapB⁻ null mutants of *S. enterica* are bile sensitive. An increased amount of *zapB* mRNA is found in the presence of a sublethal concentration of DOC. This increase is not caused by upregulation of *zapB* transcription but by increased stability of *zapB* mRNA. This increase is suppressed by an *hfq* mutation, suggesting the involvement of a small regulatory RNA (sRNA). Even though *zapB* expression is upregulated by bile, the amount of ZapB protein decreases in the presence of a sublethal concentration of DOC. Stability assays show that the ZapB protein is degraded in the presence of DOC, and degradation seems to involve the Lon protease. We propose that increased stability of *zapB* mRNA in the presence of DOC might contribute to maintain a basal level of ZapB protein.

Introduction

During infection of the gastrointestinal tract, enteric pathogens must endure harsh environments such as pH variations, low oxygen levels, elevated osmolarity, and nutrient limitation [1]. In the small intestine, periodical secretion of bile, aside from playing a major role in the emulsification and solubilization of lipids, poses another challenge due to the antibacterial activity of bile salts [2]. *Salmonella* serovars that cause systemic and chronic infections also encounter bile in the gall bladder, at concentrations higher and more steady than in the intestine [3]. Bile acids are the main components of bile, and act as a detergent on bacterial cell membranes disrupting phospholipids and proteins [4]. Bile salts have additional antibacterial activities including misfolding and denaturation of proteins, DNA damage, and interference with secondary structure formation in RNA [4-7].

Resistance to bile can be studied under laboratory conditions by adding ox bile or individual bile salts to microbiological culture media. In both *E. coli* and *Salmonella*, this reductionist approach has inspired biochemical or genetic analyses aimed to identify cell functions and mechanisms involved in bile resistance. For instance, isolation of bile-sensitive mutants has proven useful to identify loci required for bile resistance, and suppressor analysis has contributed to understand the mechanisms involved. In *Salmonella*, the significance of this kind of studies is outlined by the fact that a significant proportion of bile-sensitive mutants are impaired for infection in the mouse model of typhoid. In the last decade, bacterial responses to bile have been also investigated by high throughput analysis of gene expression, and the combination of genetics, biochemistry, and transcriptomics provides a picture of the cell components and bacterial responses that permit bile resistance. Envelope structures such as the lipopolysaccharide [8-10] and the enterobacterial common antigen [11] may provide barriers that reduce bile salts intake [8-10]. In addition, bile salts are transported outside the cell by efflux pumps, especially AcrAB-TolC [12, 13]. Exposure to bile also activates stress responses [5, 12, 14] and DNA repair functions [15].

Among the bile-upregulated genes identified by transcriptomic analysis, a *Salmonella enterica* locus annotated as *yiiU* was found [16]. *S. enterica* YiiU is an homolog of the *E. coli* ZapB protein, a cell division factor involved in Z-ring formation [17]. Below we provide

evidence that *S. enterica* ZapB is a cell division factor like its *E.coli* counterpart, and show that ZapB is essential for bile resistance in *Salmonella*. We also show that the increased level of *zapB*_mRNA in the presence of bile is the consequence of postranscriptional regulation. However, in the presence of bile the ZapB protein is degraded by a mechanism involving the Lon protease.

Results

S. enterica YiiU is a homolog of the *E.coli* cell division factor ZapB

The *yiiU* gene of *E.coli* encodes a 81 amino acid protein that acts as a non-essential cell division factor involved in formation of the Z-ring. The name *zapB* (for 'Z ring-associated protein B') was proposed by Ebersbach *et* al. [17]. Clustal alignment of the amino acid sequences of the *yiiU* loci of *E. coli and S. enterica* shows a high identity value (91%). For this reason, the *S. enterica yiiU* locus will be henceforth named *zapB*.

To detect *S. enterica* ZapB by Western immunoblot analysis, the ZapB protein was tagged with a 3xFLAG epitope. Electrophoretic separation of cell fractions (cytosol, cytoplasmic membrane and outer membrane) and Western analysis of the resolved protein extracts were carried out. Like its *E. coli* homolog, the *Salmonella* ZapB protein was found to be a cytoplasmic protein (Fig. 1).

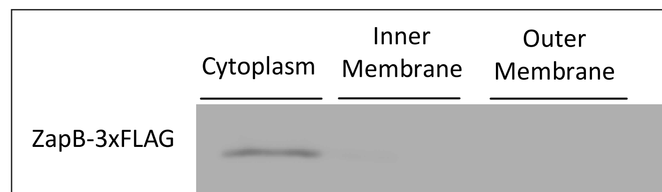

Figure 1. Distribution of ZapB protein tagged with a 3×FLAG epitope in subcellular fractions of *S. enterica* serovar Typhimurium. Anti-FLAG western hybridization is shown for three fractions: cytoplasm, inner membrane, and outer membrane. The volumes loaded for all fractions were normalized to the same number of bacteria (5×10^8 CFU).

The growth kinetics of a *S. enterica* Δ*zapB* strain was monitored in Luria-Bertani (LB) and in M9 glucose media at different temperatures. Deletion of *zapB* did not alter *S. enterica* growth (data not shown), an observation previously made in *E. coli* [17].

ZapB is required for bile resistance

Minimal inhibitory concentration (MIC) analysis was performed to determine whether the *S. enterica* ZapB protein is necessary for bile resistance. A null ZapB⁻ mutant was found to be sensitive to DOC (Table 1). This conclusion was confirmed by complementation analysis with plasmid-borne *E. coli* and *S. enterica zapB* genes expressed from the arabinose-inducible promoter of pBAD18 (Table 1).

Table 1. Minimal inhibitory concentrations of DOC

Strain	Genotype	MIC of DOC (%)
SL1344	Wild type	7
SV6269	Δ*zapB*	2
SV7264	SL1344 / pBAD18	7
SV7265	Δ*zapB* / pBAD18	2
SV7267	SL1344 / pBAD::*zapB*	7
SV7268	Δ*zapB* / pBAD18::*zapB*	7
SV7432	Δ*zapB* / pBAD18::*zapB-E.coli*	7
SV6898	*zapB::*3xFLAG	7
SV7571	*zapB::mCherry*	2
SV7016	*lon::*Tn*10*	9
SV7022	*zapB::Km lon::*Tn*10*	2

MIC analysis was also performed to determine whether lack of ZapB sensitized *S. enterica* additional antimicrobial substances. No differences were found in the sensitivity to kanamycin, chloramphenicol, tetracycline, nalidixic acid, ampicillin and malachite green (data not shown), suggesting that the bile sensitivity defect of the ZapB⁻ mutant is unlikely to be caused by increased membrane permeability. The lipopolisaccharide (LPS) structure also remained intact in the ZapB⁻ mutant (data not shown). Visualization of *S. enterica* Δ*zapB* cells by electron microscopy also failed to show differences with the wild type, both in LB and in LB with 5% of DOC (Fig. 2).

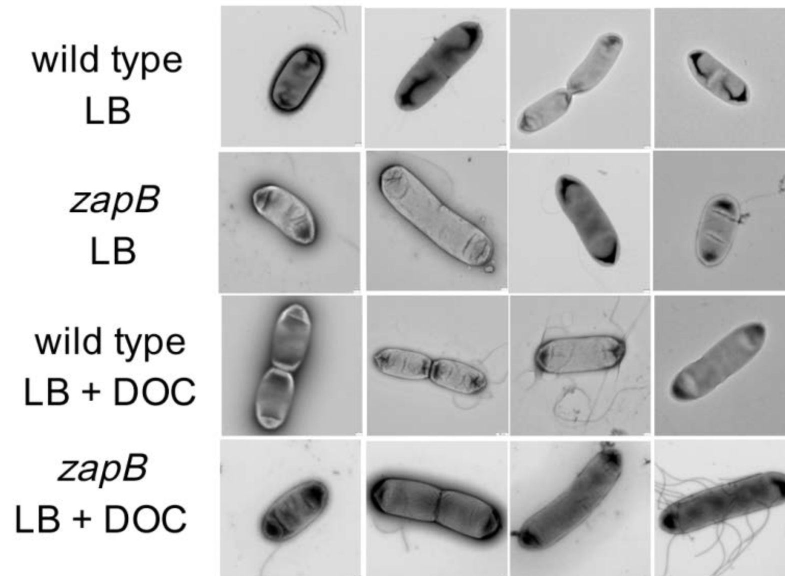

Figure 2. Morphology of *zapB* mutants. Electron microscopy visualization of wild type and Δ*zapB* cells of *S. enterica*.

Subcellular localization of *S. enterica* ZapB

To investigate the involvement of *S. enterica* ZapB, a *zapB*-mCherry fusion (*zapB::mCherry*) was constructed on the chromosome. Fluorescence microscopy images showed that the mCherry signal was present at the constriction site, as previously reported for *E.coli* ZapB [17, 18]. However, in certain cells the mCherry signal was also found localized at the poles (Fig. 3a). A caveat in these experiments was that the *zapB*-mCherry construction does not seem to be functional as the strain is bile-sensitive (Table 1). Hence, we also determined the subcellular localization of the ZapB protein in an immunostaining assay with a Cy3 Anti-Rabbit conjugate antobody using the ZapB-3xFLAG construction which is bile-resistant and therefore *bona fide* wild type (Table 1). Immunostaining showed ZapB foci at the constriction site, and in certain cells also at the poles (Figure 3b). These observations strengthen the evidence that ZapB may be a non-essential cell division factor involved in Z-ring formation, like its *E. coli* homolog.

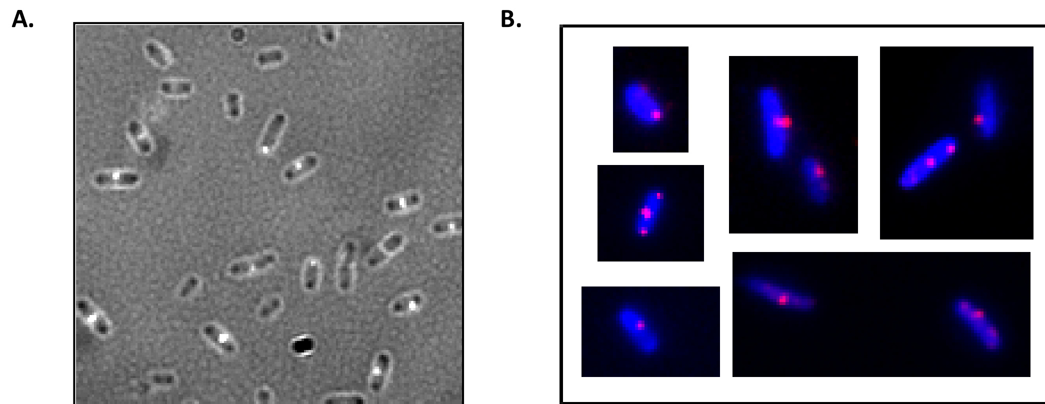

Figure 3. Cellular localization of *zapB*. **A.** Combined phase-contrast and fluorescence microscopy of the *zapB::mCherry* fusion in *S. enterica* cells harvested from an exponential culture. **B.** Fluorescence images of *zapB::*3xFLAG immunostaining with Hoechst 33342 under the same conditions.

Identification of the *zapB* promoter in *S. enterica*

The transcriptional start site of the *zapB* gene was determined by 5´-RACE. Twenty four out of twenty nine clones analyzed from two independent 5´-RACE assays showed a single 5'-end A residue located 56 nucleotides upstream of the *zapB* translational start codon (data not shown). *In silico* analysis of the region revealed the occurrence of nucleotide sequences compatible with the consensus sequences of −35 and −10 regions in σ^{70}-dependent promoters, and with appropriate spacing [19]. The proposed structure of the *zapB* promoter is presented in Fig. 4.

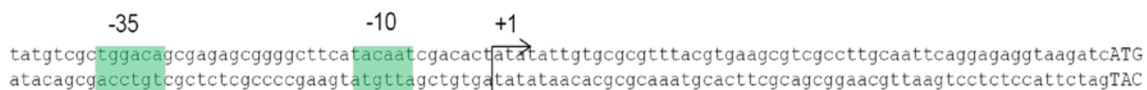

Figure 4. Diagram of the putative promoter region of *zapB*. The transcription initiation site (arrow), and the putative −35 and −10 modules (green boxes) are shown. Capital letters are nucleotides of the putative *zapB* coding sequence.

To confirm the existence of the predicted *zapB* promoter, the putative promoter region was cloned on the promoter-probe vector pIC552 to generate a transcriptional fusion with the *lacZ* gene. Details of the construction are provided in supplementary Fig. S1. The pIC552 derivative was introduced in strain SL1344, and β-galactosidase activity measurements confirmed that the cloned region was able to drive *zapB* expression.

Postranscriptional regulation of *zapB* in *S. enterica*

Transcriptomic analysis had shown upregulation of *zapB* expression in both exponential and stationary cultures grown in the presence of DOC [16]. Increased expression of *zapB* in the presence of 5% DOC was confirmed by β-galactosidase assays using a translational *zapB::lacZ* fusion and by Northern blotting analysis (Fig. 5).

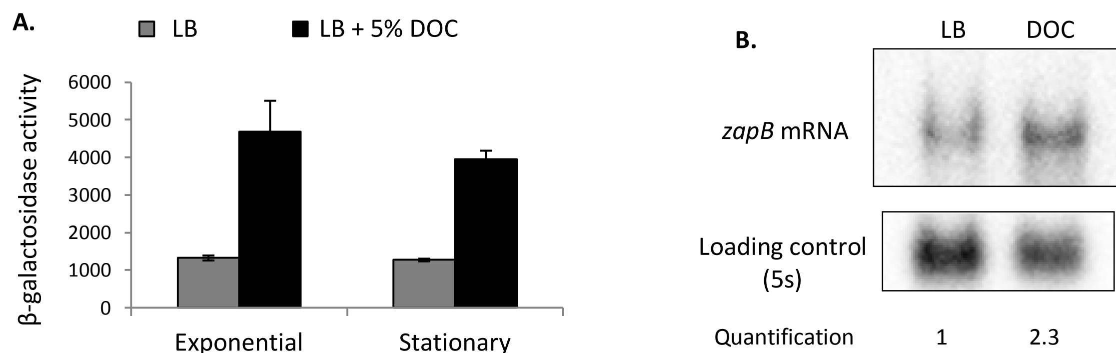

Figure 5. Regulation of *zapB* expression by bile. **A.** β-galactosidase activity of a *zapB::lac* fusion in exponential and stationary cultures in LB (grey histograms) and LB + 5% DOC (black histograms). **B.** Northern blot analysis of the levels of *zapB* mRNA in extracts from stationary cultures in LB and LB + 5% DOC.

To investigate whether regulation of *zapB* by bile was transcriptional or postranscriptional, the translational *zapB::lac* fusion was placed under the control of an heterologous promoter, pL_{tetO}. In this construct, which was engineered on the *S. enterica* chromosome, the native, σ^{70}-dependent *zapB* promoter was replaced with pL_{tetO} in a way that left the 5'UTR intact. A diagram of this construct is shown in Fig. 6A. Measurements of β-galactosidase activity are shown in Fig. 6B. The presence of DOC increased *zapB* expression from both the native promoter and the heterologous

promoter, thereby suggesting that DOC-dependent regulation of *zapB* is not transcriptional.

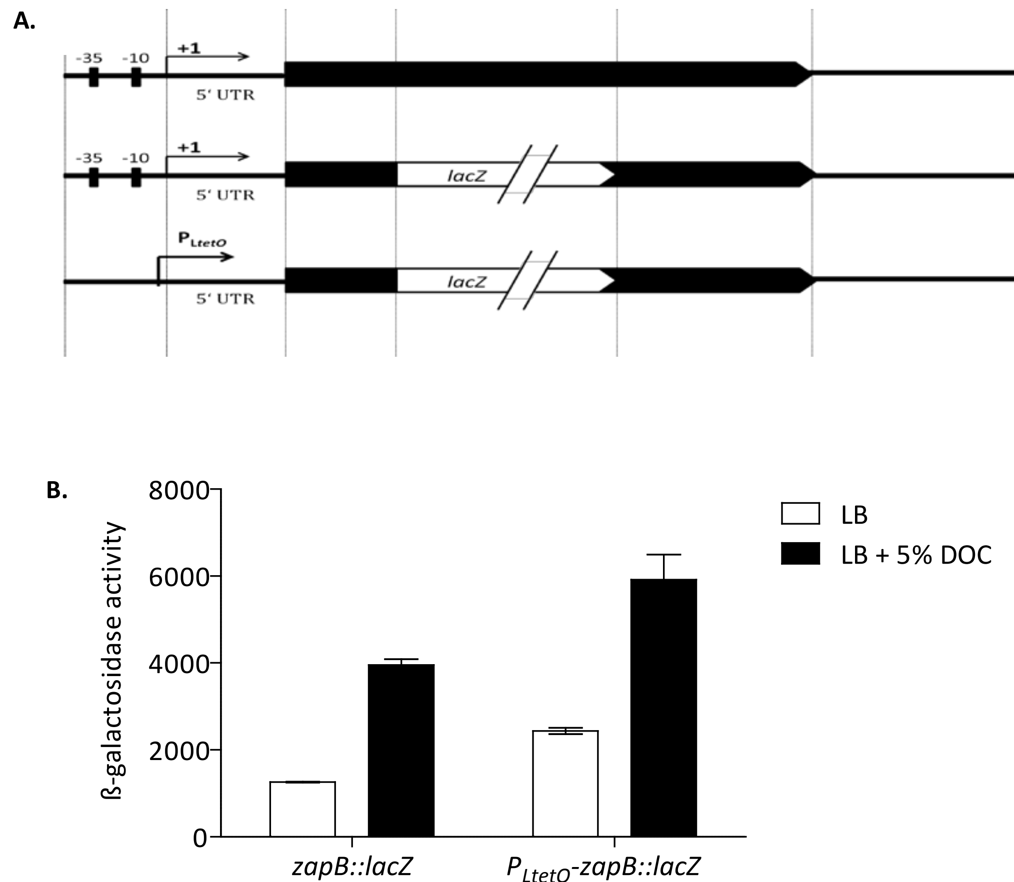

Figure 6. A. Diagram of the *zapB* gene of *S. enterica*. The promoter region, the 56 bp long 5'UTR, the *zapB* coding sequence, and the 84 bp long 3'UTR are shown. Diagrams of the *lacZ* fusions constructed for this study are also shown. **B.** β-galactosidase activities of *zapB::lacZ* fusions under the control of the *zapB* native promoter and the pL_{tetO} promoter in LB (white histograms) and LB + DOC (black histograms).

Bile stabilizes *zapB* mRNA by a mechanism involving Hfq

Evidence that upregulation of *zapB* in the presence of bile involved a postranscriptional mechanism prompted a comparison of the stability of the *zapB* transcript in LB and LB + 5% DOC. For this purpose, Northern blot analysis was performed using the ZapB oligoprobe (see Experimental Procedures). Results presented in Figure 7 show that *zapB*

mRNA decays faster in LB than in LB + DOC. Hence, the increased level of *zapB* mRNA found in the presence of bile seems to result from increased mRNA stability.

Among the mechanisms that control bacterial mRNA stability, interaction with small regulatory RNAs (sRNAs) has received increasing attention in the last decade [20]. Hence, we considered the possibility that the stability of *zapB* mRNA might be controlled by a sRNA, either destabilizing the transcript in LB or stabilizing the transcript in the presence of DOC. Because most bacterial sRNAs require the RNA chaperone Hfq for both target interaction and intracellular stability [21], we analyzed the rate of decay of *zapB* mRNA in an Hfq⁻ strain using Northern blots (Fig. 7). In the Hfq⁻ background, the half-life of the *zapB* transcript in presence of DOC was similar to the half life in LB of the wild-type and the Hfq⁻ strains. So, in an Hfq⁻ background, the stabilization of the *zapB* transcript in the presence of DOC was not observed, suggesting that stabilization requires the Hfq RNA chaperone.

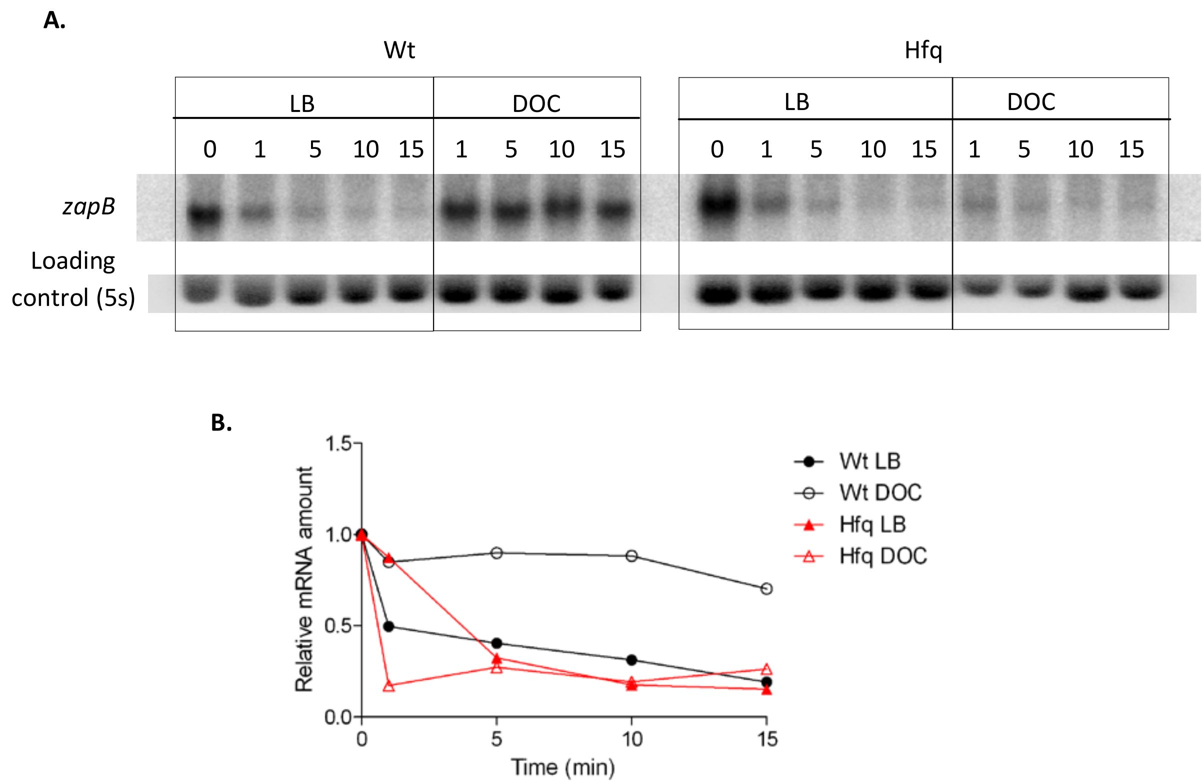

Figure 7. Stability of *zapB* mRNA. **A.** Levels of *zapB* mRNA in RNA extracts from exponential cultures in LB and LB + 5% DOC. Samples were taken 1, 5, 10, and 15 min.

after the addition of rifampicin. **B.** Quantification of the level of *zapB* mRNA relative to the loading control and to time zero.

Search for a potential sRNA that might stabilize *zapB* mRNA in the presence of DOC

Although we cannot rule out the possibility that Hfq binding might be sufficient to stabilize *zapB* mRNA, this possibility seemed unlikely as Hfq typically acts in concert with sRNAs. A computational search for potential interactions between *Salmonella* sRNAs candidates and *zapB* using the program 'IntaRNA' [22] provided a list of potential sRNAs interacting with *zapB* (Fig. 8). Noteworthy candidates in the list are the RprA and OxyS sRNAs since both are induced by bile (J. Hinton lab, unpublished data).

sRNA	Energy [kcal/mol]	Position - sRNA	Position – mRNA
ryhB-2	-14.593	67 -- 77	124 – 134
isrH	-14.177	348 -- 379	347 – 378
ryhB-1	-13.432	51 -- 61	124 – 134
isrA	-11.640	18 -- 28	286 – 296
isrM	-11.510	254 -- 260	290 – 296
csrC	-11.326	169 -- 182	156 – 168
isrO	-11.296	99 -- 171	229 – 313
cyaR	-10.538	24 -- 35	137 – 148
isrJ	-10.340	23 -- 42	318 – 339
csrB	-10.223	224 -- 242	299 – 314
oxyS	-10.074	79 -- 87	367 – 375
isrK	-10.041	40 -- 70	301 – 331
isrG	-9.994	109 -- 120	234 – 244
isrI	-9.602	133 -- 149	70 – 88
micF	-9.591	30 -- 59	89 – 120
rygD	-9.280	2 -- 23	320 – 349
isrL	-9.144	99 -- 109	205 – 215
invR	-9.113	3 -- 38	78 – 115
isrF	-8.839	78 -- 89	249 – 261
tpke	-8.733	227 -- 238	142 – 153
micA	-8.708	4 -- 13	52 – 61
gcvB	-8.685	135 -- 144	214 – 223
rseX	-8.506	7 -- 58	172 – 224
isrN	-8.502	7 -- 25	234 – 254
isrQ	-8.479	68 -- 80	143 – 154
spf	-8.286	20 -- 34	188 – 203
rprA	-8.267	46 -- 69	230 – 253

Figure 8. Predicted sRNAs with *zapB* mRNA as target. List of sRNAs containing potential hybridization regions with the *zapB* transcript, predicted by the program "IntaRNA".

ZapB protein is degraded by the Lon protease in presence of bile

Western blotting analyses using the wild type (bile-resistant) allele *zapB*::3X FLAG, revealed that the amount of ZapB protein was around twice lower in LB + 5% DOC than in LB (Fig. 9). Because *zapB* mRNA had been found to increase in presence of bile (Fig. 5), degradation of the ZapB protein in the presence of DOC was a tentative explanation for the ZapB drop. To investigate this possibility, protein stability assays were carried out to monitor the half-life of the ZapB protein upon addition of DOC. Data shown in Fig. 10 (top) reveal that ZapB is stable in LB (half-life >90 min) while it is degraded fast in presence of DOC (half-life around 30 min).

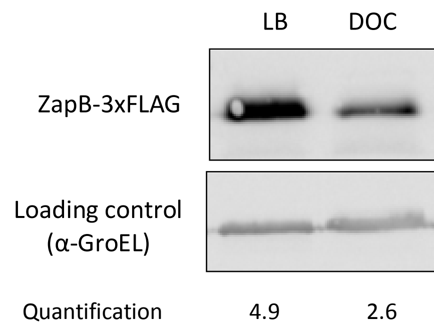

Figure 9. Decrease of ZapB protein level in the presence of bile**.** Western blot analysis of the effect of DOC on the levels of ZapB-3xFLAG protein.

Because the Lon protease has been described to be involved in the degradation of unfolded and misfolded proteins [23] and bile can cause misfolding and denaturation of proteins [5], ZapB protein stability assays were carried out in a Lon$^-$ background. Results shown in Fig. 10 (bottom) provided evidence that the ZapB stability in the presence of DOC increases in the absence of Lon protease. Hence, we tentatively conclude that Lon is involved in the degradation of ZapB in the presence of DOC.

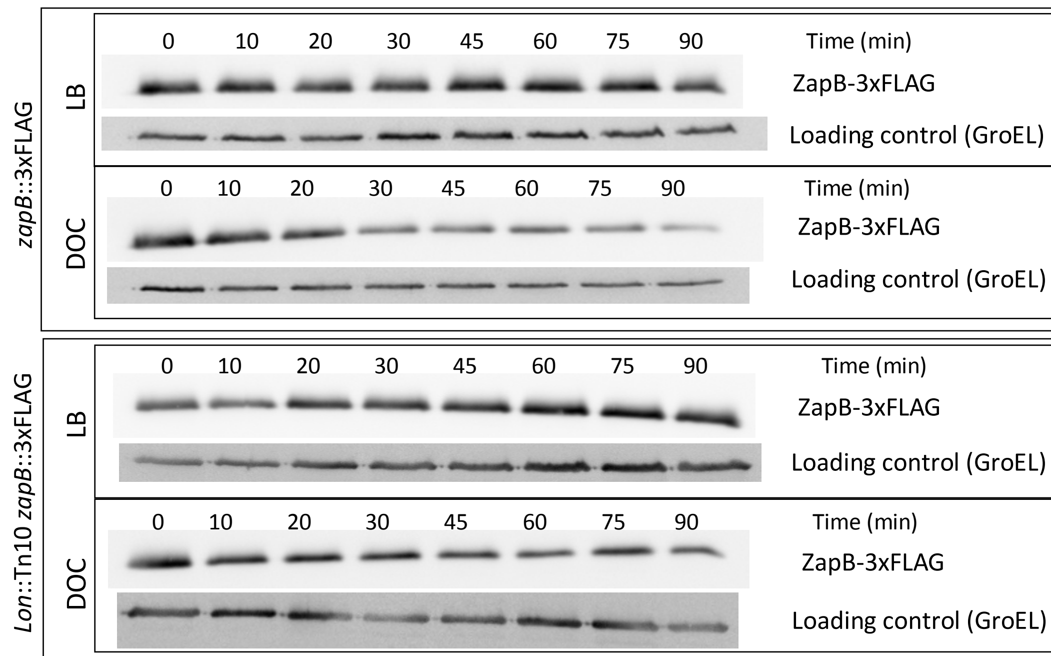

Figure 10. Degradation of ZapB protein by the Lon protease in the presence of bile. Protein stability assays were performed in enponential culture aliquots extracted 10, 20, 30, 45, 60, 75, and 90 min after adding 5% DOC and chloramphenicol. As a control, extractions were also performed upon addition of chloramphenicol to LB.

Discussion

Initial evidence that the ZapB cell division factor might be involved in bile resistance was provided by transcriptomic analysis: the *zapB* gene, which in *Salmonella* had not been characterized previously and was still annotated as a locus of unknown function (*yiiU*), was found to be upregulated in the presence of a sublethal concentration of DOC [16]. In this study, we show that disruption of the *yiiU* locus causes sensitivity to DOC (Table 1). Hence *yiiU* is not merely a bile-induced locus but also a gene necessary for bile resistance. Change of the *yiiU* gene designation to *zapB* is supported by the 91% identity between the predicted *Salmonella* YiiU gene product and the *E. coli* ZapB protein, and by the ability of *E. coli* ZapB to complement DOC sensitivity in a *S. enterica* YiiU$^-$ null mutant (Table 1). Furthermore, the *Salmonella* ZapB protein appears to be localized at the septum like its *E. coli* counterpart (Fig. 3). Lack of ZapB does not cause visible defects in *S. enterica* cells, neither in the absence nor in the presence of DOC (Fig. 2).

Upregulation of *S. enterica zapB* expression in the presence of DOC is still observed when *zapB* transcription is driven by a heterologous promoter (Fig. 6), thereby suggesting the involvement of a postranscriptional mechanism. Comparison of *zapB* mRNA decay in LB and LB + 5% DOC reveals that *zapB* mRNA is more stable in the presence of DOC, thus explaining the higher mRNA level detected both in the initial transcriptomic analysis and in Northern blots (Fig. 5). Stabilization of the *zapB* transcript in the presence of DOC requires the Hfq RNA chaperone (Fig. 7), an effect that admits two alternative explanations: (i) Hfq binding might protect *zapB* mRNA from degradation; (ii) Hfq might catalyze the interaction of *zapB* mRNA with a small regulatory RNA, and the mRNA:sRNA interaction might increase *zapB* mRNA stability. The latter hypothesis seems more likely considering that Hfq typically catalyzes mRNA:sRNA interactions [21]. *In silico* search for sRNAs containing regions that might interact with *zapB* mRNA by base pairing have identified two relevant candidates, OxyS and RprA, two sRNAS which are known to be produced at higher levels in the presence of bile (J. Hinton, pers. comm.).

A paradox is that the increased *zapB* mRNA level found in the presence of bile does not result in higher amounts of ZapB protein. Actually, the ZapB protein level decreases in the presence of DOC, and ZapB protein analysis in a Lon$^-$ background provides

evidence that degradation of ZapB in presence of bile is mediated by the Lon protease. In *E. coli* Lon protease has been shown to play a main role in degradation of abnormally folded proteins [24]. Because bile salts are known to cause misfolding and denaturation of proteins [5], we tentatively propose that DOC may cause ZapB misfolding, and that misfolding may trigger degradation by the Lon protease. If this view is correct, increased *zapB* mRNA stability in the presence of bile might provide a mechanism to compensate Lon-mediated degradation and to maintain an intracellular amount of ZapB needed for survive in the presence of bile.

The cause of bile sensitivity in the absence of ZapB remains unknown. However, it is remarkable that another non-essential cell division factor, DamX, is also necessary for bile resistance in both *S. enterica* [25] and *E. coli* [26]. It is thus conceivable that perturbations in Z-ring assembly might render the *Salmonella* cell bile-sensitive. This view is consistent with the major role played by the bacterial envelope as a protective barrier against bile salts [27]. Besides the protective roles played by the lipopolysaccharide and the enterobacterial common antigen [8-11], recent studies in our laboratory have provided evidence that bile salts induce peptidoglycan remodeling (manuscript in preparation). If the cell wall contributes to bile resistance, it may be not surprising that perturbation of Z-ring assembly renders *Salmonella* bile-sensitive.

Experimental procedures

Bacterial strains, bacteriophages, media, and culture conditions

The strains of *Salmonella enterica* used in this study (Table S1) belong to serovar Typhimurium, and derive from the mouse-virulent strain SL1344 (SV5015). *Escherichia coli* K12 (RK4353) was used to clone the *zapB* of *E.coli* into the p$_{BAD}$ plasmid. For cloning procedures, *Escherichia coli* strains DH5α was used. Transduction was performed with phage P22 HT 105/1 *int201*[28] (and G. Roberts, unpublished data). The P22 HT transduction protocol was described elsewhere [29]. To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan et al. [30], except that methyl blue (Sigma Chemical Co., St. Louis, Missouri) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Luria–Bertani (LB) broth and M9 minimal medium [31] supplemented with 0.2% of casa amino acids and 0.2% of glucose was used as liquid medium. To get 5% DOC media, the appropriate volume from a 25% stock of sodium deoxycholate (DOC, from Sigma) was added. Solid LB contained agar at 1.5% final concentration. Liquid cultures were grown with aeration by shaking in an orbital incubator. If necessary for the maintenance of plasmids or selection of recombinant strains appropriate antibiotics: kanamycin sulfate (Km), chloramphenicol (Cm), tetracycline (Tc) or ampicillin (Ap) were added to broth or agar plates at a final concentration of 50 μg ml^{-1}, 20 μg ml^{-1}, 20 μg ml^{-1} and 100 μg ml^{-1}, respectively.

Subcellular fractionation

Subcellular fractionation was performed as previously described [32], with some modifications. Briefly, bacteria were grown in LB medium at 37°C and spun down by centrifugation at 15,000× g for 5 min at 4°C, then resuspended twice in cold phosphate-buffered saline (PBS, pH 7.4). The bacterial suspension were then disrupted by sonication. Unbroken cells were further removed by low-speed centrifugation (5,000× g, 5 min, 4°C). The supernatant was centrifuged at high speed (100,000× g, 30 min, 4°C) and the new supernatant was recovered as the cytosol fraction. The pellet containing envelope material was suspended in PBS with 0.4% Triton X-100 and incubated for 2 h at 4°C. The sample was centrifuged again (100,000× g, 30 min, 4°C) and divided into the

supernatant containing mostly inner membrane proteins and the insoluble fraction corresponding to the outer membrane fraction. An appropriate volume of Laemmli buffer was added to each fraction. After heating (100°C, 5 min) and clearing by centrifugation (15,000× g, 5 min, room temperature), the samples were analyzed for protein content by SDS-PAGE.

Microscopy

For phase-contrast and fluorescence microscopy 2 to 3 µl of the exponential culture samples (grown at 30ºC in M9 medium) were placed on a microscope slide where 1 µl of Poly-L-Lysine solution 0.1% was previously spread out to immobilize the cells. For DNA staining, samples suspended in 100µl of Phosphate-Buffer Saline (PBS) were mixed with 2 µl of Hoechst 33342 (500 µg ml^{-1}), incubated 20-30 min at 30°C and washed with PBS. Images were acquired with a Leica DMR fluorescent microscope using the 100× oil-objective lens and analyzed with the Leica IM50 software. Final image preparation was performed in Adobe Photoshop. For electron microscopy (EM) sodium deoxycholate (DOC) treatments were carried out from 15 to 45 minutes of exposition. For EM observations copper grids (200 mesh) covered with Pieloform and carbon were floated on small drops of cultures laid on a piece of Parafilm for 2 min. Grids were then retired, and excess liquid removed touching with filter paper. Grids were washed four fold by floating onto water drops (bi-distilled in glass) and fixed-stained by floating 1 min on 1% (w/v) uranyl acetate in water. Grids were briefly washed in a drop of water and air dried. Pictures were captured in a JEOL JEM1010 transmision electron microscope equipped with a Tem Cam-F416 (TVIPS) digital camera, at an acceleration voltage of 60 kV.

Inmunostaining

Cell preparation and staining were performed as described previously [33]. Briefly, cells from 1.5 ml of an exponential culture (OD_{600} ~0.4) of the *zapB::*3xFLAG strain (grown in M9 media at 30°C) were collected by centrifugation, washed, resuspended in 1 ml TE buffer and fixed by adding the same volume of cold 74% ethanol. Ethanol-fixed cells (100 µl) were stained with mouse monoclonal anti-FLAG M2-Cy3 antibody (Sigma-Aldrich). Immunostained cells were stained with Hoechst 33342, in 10 µl mounting medium (40% glycerol in 0.02 M phosphate buffered saline, pH 7.5). 5-10 µl of ethanol-

fixed cells was spread onto a poly-L-lysine-coated slide, and dried at room temperatures. Slides of stained samples were stored at room temperature in the dark.

Determination of antibiotic minimal inhibitory concentrations

Exponential cultures in LB broth were prepared, and samples containing around 3×10^2 colony-forming-units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of the antibiotic tested. After 12 h incubation at 37°C, growth was visually monitored. Assays were carried out in triplicate. Student's *t*-test was used to analyze every MIC. The null hypothesis was that MICs were not significantly different from the MIC for the wild-type. *P* values of 0.01 or less were considered significant.

β-galactosidase assays

Levels of β-galactosidase activity were assayed as described previously [34], using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure. β-galactosidase activity data are the averages and standard deviations from ≥3 independent experiments.

5´RACE

RNA was isolated as described below from *S.enterica* stationary cells (DO_{600} ~2) grown in LB medium. Fifteen micrograms of RNA were used to determine cDNA ends using a protocol similar to that described previously [35, 36]. The RNAs were prepared either with or without tobacco acid pyrophosphatase (TAP) to distinguish primary transcript 5´ ends from internal 5´ processing sites. The sequences of all of the primers used to this experiment are listed in Table S2. The DNA primer for cDNA synthesis, Racer2-zapB, is complementary to the *ZapB* RNA. A second DNA primer for subsequent PCR amplification of cDNAs, Racer-out, is homologous to the adaptor RNA primer: Racer-RNA-adaptor used for 5´ RACE. PCR products that were detected both with and without tobacco acid pyrophosphatase treatment were again amplificated with the internal primers: Racer-nested and Racer1-zapB, and then purified. This PCR products were cloned by using the pGEM-T Easy vector (Promega), and total of 29 clones were sequenced from two different assays.

RNA extraction and Northern analysis

Aliquots of 4 ml or 2 ml from exponential cultures of LB or DOC 5% respectively (O.D.$_{600}$~0.4 and ~0.7) were centrifuged at 16,000x g, 37°C, during 2 min, and washed twice with 500ml of NaCl 0.9%. The pellets were resuspended in 100 µl of a solution of lysozyme (Sigma), 3 mg ml^{-1}. Cell lysis was facilitated by freeze at -20°C for more that 2 hours. After lysis, RNA was extracted using 1 ml of TRIsure reagent (Bioline), according to manufacture's instructions. Lastly, total RNA was resuspended in 25 µl of RNase-free water. The quality of the preparation and the RNA concentration were determined using a ND-1000 spectrophotometer (NanoDrop Technologies). For Northern blot analysis, 10 µg of total RNA was loaded per well and electrophoresis in denaturing 1% agarose formaldehyde gels. Vacuum transfer and fixation to Hybond-N^{+} membranes (GE Healthcare) were performed using 0.05 M NaOH. UV crosslinking was used to immobilize the RNAs on the membrane. For stability experiments, rifampicin (500 mg ml^{-1}) was added to exponential cultures grown in LB at 37°C (zero-time control) and at this point the culture was divided in two: the DOC sample, to which 25% of DOC was added to reach a final concentration of 5%; and the LB sample, to which LB was added to equalize the volume with the DOC sample. Then incubation was continued and culture aliquots were taken at the times indicated in the respective figures for RNA isolation. DNA oligonucleotides (Racer2-zapB for *zapB* mRNA and 5S-probe for the loading control) were labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase (Biolabs). Labeled probes were purified over microspin G-25 columns (GE Healthcare) to remove unincorporated nucleotides. As a control of RNA loading and transfer efficiency, the filters were hybridized with a oligoprobe for the rRNA-5s ribosomal RNA. Membranes were hybridized at 42°C with oligoprobes. Signals were visualized with a FLA-5100 image system (Fujifilm), and quantification was performed using the MultiGauge software (Fujifilm).

Preparation of protein extracts and Western blot analysis

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB medium or LB with 5% of DOC until stationary phase (OD$_{600}$ >1). Around $3 \cdot 10^{9}$ cells from the bacterial cultures were collected by centrifugation (16,000 g, 2 min), suspended in 50 µl of Laemmli sample buffer [1.3% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl,

1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8] and boiled (100°C; 5 min). Before be loaded into gels the samples were cleared by centrifugation (15,000 × *g*; 5 min). For analysis of protein stability, overnight bacterial cultures were diluted and grown at 37°C in LB medium with shaking until obtain an OD_{600} of 0.6 to 0.8. Chloranphenicol (40 μg ml^{-1}) was added to the culture and at this point the culture was divided in two: the DOC sample, to which 25% of DOC was added to reach a final concentration of 5%; and the LB sample, to which LB was added to equalize the volume with the DOC sample in order to not change the cell concentration of the samples. Then a 1ml aliquot was removed as zero-time control, and the samples were incubated again at 37°C (to avoid DOC precipitation) without shaking, and 1 ml samples were removed after 10, 20, 30, 45, 60, 75 and 90 minutes and treated as mentioned above. Proteins were resolved by Tris-Tricine-PAGE (12%) electrophoresis and transferred onto polyvinylidene-difluoride membranes using a semidry electrophoresis transfer apparatus (Bio-Rad). Specific proteins were detected with the following primary antibodies: anti-Flag M2 monoclonal antibody (1:5,000, Sigma-Aldrich) and anti-GroEL polyclonal antibody (1:20,000; Sigma-Aldrich). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5,000; Bio-Rad) or goat anti-rabbit horseradish peroxidase-conjugated antibody (1:20,000; Santa Cruz Biotechnology) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin–luminol reagents (Thermo Scientific) in a LAS 3000 Mini Imaging System (Fujifilm). For quantification, the intensity of the bands was determined using MultiGauge software (Fujifilm). GroEL was used as loading control.

Supplemental material

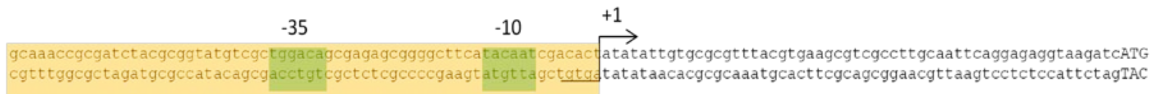

Figure S1. Diagram of the putative promoter region of zapB. The transcription initiation site (arrow), and the putative −35 and −10 modules (green boxes) are shown. Capital letters are nucleotides of the putative *zapB* coding sequence. The region that was cloned on the promoter-probe vector pIC552 to generate a transcriptional fusion with the *lacZ* gene is marked in yellow.

Table S1. Strains of Salmonella enterica serovar Typhimurium.

Strain	Genotype	Reference or source
SV6268	*zapB::Km*	This study
SV6269	Δ*zapB*	This study
SV6270	*zapB::lacZ*	Ref chap1
SV6898	*yiiU*::3Xflag	This study
SV7016	*lon::*Tn*10*	This study
SV7017	*yiiU*::3xFLAG *lon::*Tn*10*	This study
SV7022	*yiiU::Km lon::*Tn*10*	This study
SV7264	SL1344 pBAD18	This study
SV7265	Δ*zapB* pBAD18	This study
SV7267	SL1344 pBAD::*zapB*	This study
SV7268	Δ*zapB* pBAD18::*zapB*	This study
SV7338	SL1344 pIC552	This study
SV7339	SL1344 pIC552-*zapB*Promoter	This study
SV7429	pL*tetO::zapB::lacZ*	This study
SV7432	Δ*zapB* pBAD18::$zapB_{E. coli}$	This study
SV7571	*zapB::mCherry*	This study

Strains and plasmids construction.

Targeted gene disruption was achieved using pKD13 plasmid [37] and the oligonucleotides zapB-FOR and zapB-REV. Antibiotic resistance cassette introduced during strain construction was excised by recombination with plasmid pCP20 [37]. For the construction of the *zapB::lacZ* fusion in the *Salmonella* chromosome, FRT sites generated by excision of the Km^r cassette were used to integrate the plasmid pCE40 [38].

Strain producing ZapB-mCherry fusion from the native chromosomal location was constructed by the λ Red recombination method [37] using the oligonucleotides ZapB-mChe-FOR and ZapB-mChe-REV and as template the pDEX-mCherry plasmid, a derivative of the pDEX-G [39] carrying *mCherry* gene.

Addition of 3xFLAG tag to protein-coding DNA sequences was carried out using the pSUB11 plasmid as a template [40] and the oligonucleotides zapB-FLAG-FOR and zapB-FLAG-REV.

Flanking gene specific primers zapB-E1 and zapB-E2 were used for verification of the mutated alleles constructed by the polymerase chain reaction.

Allele *lon::*Tn*10* was transducted from the ATCC 14028 mutant strains SV5663 [41] to the SL1344.

Plasmids p_{BAD}::*zapB* (pIZ2003) and p_{BAD}::*zapB, zapB* from *E.coli* (pIZ2004) were constructed by cloning the respective gene or fusion under the control of the arabinose-dependent p_{BAD} promoter of the pBAD18 plasmid using the pairs of oligonucleotides: zapB-pBAD-FOR and zapB-pBAD-REV; zapBEcoli-pBAD-FOR and zapBEcoli-pBAD-REV respectively.

To clone the *zapB* promoter with its 5´ UTR upstream the *lacZ* of the pIC552 plasmid [42] the primers pIC-zapB-FOR and pIC-zapB-REV were used. The plasmid constructed was named pIZ2002.

$P_{L\,tetO}$-*zapB* construction was engineered by inserting the $P_{L\,tetO}$ promoter from the pXG1 plassmid [43] upstream the transcriptional start site of *zapB::lacZ* on the *Salmonella* chromosome as was described by Lopez-Garrido [44] using the primers labeled zapB-$P_{L\,tetO}$-FOR and zapB-$P_{L\,tetO}$-REV.

All the primers used are listed in table S2.

Table S2. Oligonucleotides used in this study.

Name	Sequence
pIC-zapB-FOR	tacgagatctgcaaaccgcgatctacgcggtatgtc
pIC-zapB-REV	tagcctcgagtgtcgattgtatgaagccccgctctc
Racer1-zapB	gggtgatggtgtcaatcgcctgc
Racer2-zapB	caatcgcctgctgtacttttgcttcc
Racer-nested	ggacactgacatggactgaaggagta
Racer-Out	cgactggagcacgaggacactga
Racer-RNA-adaptor	cgacuggagcacgaggacacugacauggacugaaggaguagaaa
zapB-E1	tgagacagcagaagagagtg
zapB-E2	ccgcttgatatcgagtgatc
zapBEcoli-pBAD-FOR	atcggaattccaattcaggagaggtatgacaatgtc
zapBEcoli-pBAD-REV	tcgagtcgaccggaagatgaagcgtaatcagacctc
zapB-FLAG-FOR	ggaacgtttgcaggcgctgcttggtcgtatggaagaagtcgactacaaagaccatgacgg
zapB-FLAG-REV	cacgcagccaacgccgccataacgtgaaatatgacgaggacatatgaatatcctccttag
zapB-MChe-FOR	ggaacgtttgcaggcgctgcttggtcgtatggaagaagtcatggtgagcaagggcgagga
zapB-MChe-REV	gcagccaacgccgccataacgtgaaatatgacgaggattaagtcacgacgttgtaaaacg
zapB-P1-REV	aaacgttcctgccagccgctctgctgttccttcagagaatgtgtaggctggagctgcttc
zapB-P4-FOR	ggaagcaaaagtacagcaggcgattgacaccatcaccctgattccggggatccgtcgacc
zapB-pBAD-FOR	atcggaattccaggagaggtaagatcatgtctttag
zapB-pBAD-REV	tcgagtcgacgtgaaatatgacgaggattagacttc
zapB-PLtetO-FOR	tacgtgaagcgtcgccttgcaattcaggagaggtaagatcaggcttacccgtcttactgtc
zapB-PLtetO-REV	cacgcagccaacgccgccataacgtgaaatatgacgaggaatgtgctcagtatctctatcactgatag
5S-probe	ctacggcgtttcacttctgagttc

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Chapter 3: Increased bile resistance in *Salmonella enterica* mutants lacking Prc periplasmic protease

RESEARCH ARTICLE (Ref. 13-048)

Increased bile resistance in *Salmonella enterica* mutants lacking Prc periplasmic protease

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Summary

Prc is a periplasmic protease involved in processing of penicillin-binding protein 3 (PBP3). Lack of Prc suppresses bile sensitivity in Dam$^-$, Wec$^-$, PhoP$^-$, DamX$^-$, and SeqA$^-$ mutants of *Salmonella enterica*, and increases bile resistance in the wild type. Changes in the activity of penicillin binding proteins PBP3, PBP4, PBP5/6 and PBP7 are detected in a Prc$^-$ background, suggesting that peptidoglycan remodeling might contribute to bile resistance.

Introduction

Salmonella enterica is a bacterial pathogen that infects humans and livestock animals causing intestinal, systemic, and chronic infections [9]. In the intestine and in the hepatobiliary tract, *Salmonella* is exposed to bile, a fluid containing cholesterol, bile salts, phospholipids, proteins, bilirubin, and electrolytes [15]. About two thirds of bile (dry weigth) are made of bile salts, a family of amphipathic molecules derived from cholesterol [16]. The relationship between intestinal bacteria and bile salts is complex. On one hand, bile salts control the expression of certain genes, and can be considered environmental signals used by the bacterium to identify the intestinal milieu [3]. On the other hand, bile salts are antibacterial compounds that disrupt membranes, denature proteins, and damage DNA [3,10]. Enteric bacteria are able to resist the antibacterial activities of bile salts, and an extreme example is *Salmonella enterica* which colonizes the bile-laden gall bladder during systemic and chronic infection [7,10]. In asymptomatic human carriers of *Salmonella* Typhi, persistence in the gall bladder can last for decades or even for a lifetime [7].

The mechanisms that permit *Salmonella* survival in the presence of bile are partially understood. Envelope structures such as the lipopolysaccharide and the enterobacterial common antigen serve as barriers that reduce intake of bile salts [3]. However, the protection provided by these barriers is incomplete, making other mechanisms necessary. Intake of bile salts induces the RpoS general stress response and other stress responses that facilitate survival [14]. In turn, activation of the SOS system helps to cope with bile-induced DNA injuries [26]. In addition, the intracellular concentration of bile salts is reduced by active transport of bile from the cytoplasm, especially by the AcrAB efflux pump [22].

Genetic analysis has proven useful for the identification of bile resistance functions. Isolation of bile-sensitive mutants has permitted the identification of cellular functions necessary for bile resistance, and searches for suppressors of bile sensitivity have helped to outline the responses or "pathways" involved. Especially productive has been the use of *Salmonella* Dam$^-$ mutants, which are extremely sensitive to bile [28]. Certain suppressors of bile sensitivity in the Dam$^-$ background have been found to suppress bile

sensitivity caused by mutations other than *dam* [24,26,27]. Broad suppressor capacity usually indicates that a cellular defense response has been activated by the suppressor mutation, thus permitting the identification of bile defense responses [24].

Below, we describe a novel class of suppressors of bile sensitivity in *Salmonella* Dam⁻ mutants. Loss of function in the *S. enterica prc* gene restores bile resistance in Dam⁻ mutants and in other bile-sensitive mutants, and increases bile resistance in the wild type. Penicillin-binding proteins PBP3, PBP4, PBP5/6, and PBP7 show altered activity in *S. enterica* Prc⁻ mutants, suggesting that changes in PBP activity can modulate bile resistance, perhaps by modification of peptidoglycan structure.

Materials and methods

Bacterial strains, bacteriophages, media and growth conditions. The strains of *Salmonella enterica* used in this study belong to serovar Typhimurium, and derive from the mouse-virulent strain SL1344 (His^+). An exception is TH3468 (*proAB47*/F'128 [*pro-lac*] *zzf-3834*::Tn10dTc[*del-20 del-25*] [T-POP3]), an LT2 derivative provided by K. T. Hughes, Univ. Utah, Salt Lake City. *Escherichia coli* DH5α [11] was used as the host of plasmids. Transductional crosses using phage P22 HT 105/1 *int201* [33] were used for strain construction in *S. enterica*. The P22 HT transduction protocol was described elsewhere [6]. To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan et al. [4] except that methyl blue (Sigma Chemical Co., St. Louis, Missouri) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Luria-Bertani broth (LB) was used as standard rich medium. Liquid cultures were grown with aeration by shaking in an orbital incubator. Solid LB contained agar 1.5 % final concentration. When specified, sodium deoxycholate (DOC) (Sigma Chemical Co.) was added.

Mutagenesis with T-POP and characterization of T-POP insertions. Pools of random T-POP3 [30] insertion mutants were generated using a P22 lysate grown on TH3468. The pools were then used to transduce strain SV6100 (Δ*dam-231*). Transductants were selected on LB plates supplemented with 20 μg/ml Tc and 2.5 % DOC. Putative suppressor-carrying isolates were lysed with P22 HT, and the lysates were used to transduce SV6100, selecting Tc^r transductants. A 100% linkage between tetracycline resistance and DOC resistance confirmed the existence of a suppressor mutation generated by T-POP insertion. Chromosomal DNA from bile resistant mutants carrying T-POP insertions was digested with *Pst*I, which does not cleave within the T-POP element, and ligated to the *Pst*I site of plasmid pBlueScript II. Ligation mixtures were electroporated into *E. coli* DH5α, and transformants were selected on LB plates supplemented with 100 μg/ml Ap. Upon plasmid DNA purification, T-POP boundaries were sequenced with primers 5´ GAT CAC CAA GGT GCA GAG CC 3´, and 5´ TCT TGA TAA CCC AAG AGG GC 3´.

Construction of a Prc⁻ mutant by site-directed mutagenesis. The *S. enterica prc* gene was disrupted by lambda Red recombination using plasmid pKD4 [5] and oligonucleotides 5´ CAC CTG GTG TTC TGA AAC GGA GGC CAG GCC TGG CAT GAA CTG TAG GCT GGA GCT GCT TCG 3´ and 5´ CCT GTT TAG CGT TAC TTA TTG GCT GCC GCC TGC TCC GCT GCA TAT GAA TAT CCT CCT TAG 3´. The external primers 5´ GTA GCG CGT CGT AAA GAA GG 3´and 5´ CCA TGA TCA GCA AGC CTT GC 3´ were used for allele verification. The antibiotic resistance cassette introduced during strain construction was excised by recombination with plasmid pCP20 [5].

Determination of the minimal inhibitory concentration [MIC]. An aliquot from an exponential culture, containing approx. 3×10^3 colony-forming-units/ml, was transferred to a polypropylene microtiter plate (Greiner, Frickenhausen, Germany) containing increasing concentrations of the antibacterial substance to be tested (DOC, antibiotic). After overnight incubation at 37°C, the MIC was determined by visual inspection.

Growth curves. To monitor growth rate, 200 µl from an overnight culture grown in LB was diluted in 20 ml of salt-free LB (0% NaCl) or LB (0.5% NaCl), and grown at 30ºC or 37ºC with aeration by shaking. Growth was monitored by measuring the OD_{600} at 1 hour intervals. Experiments were performed in triplicate.

Microscopic observation of cells. Cultures were grown at 37°C to exponential phase. For DNA staining, samples suspended in 100 µl of phosphate-buffer saline (PBS) were mixed with 2 µl of Hoechst 33342 (500 µg µg/ml), incubated 20–30 min at 37°C, and washed with PBS. About 2-3 µl of the culture samples were placed on a microscope slide. Images were acquired with a Leica DMR fluorescence microscope using the 100 × oil-objective lens, and were analyzed with the Leica IM50 software.

Preparation of cell envelopes. Envelopes were prepared as described elsewhere [28]. Briefly, *ca.* 10^{10} cells were rapidly cooled in an ice-salt mix and harvested by low-speed centrifugation (15 min,15,000 ×*g,* 4ºC). Bacterial pellets were resuspended in 1 ml of PBS pH 8.0. Cell suspensions (0.5 ml, approx.) were subjected to three bursts of sonication (30 s pulses) with a Branson sonifier, mod. 250 (Branson Ultrasonics Co., Danbury, Connecticut). Unbroken cells were discarded by centrifugation at 5,000 ×*g,* 10 min, 4ºC. Cell envelopes were recovered by high speed centrifugation (200,000 ×*g,* 20 min, 4ºC)

and resuspended in 100 μl of PBS pH 8.0.

***In vitro* assays of PBP activity.** The assays were performed upon modification of previously described procedures [8]. Envelope fractions were prepared from exponential and stationary cultures grown in LB and LB without NaCl. The protein concentration was determined with a D-C protein assay kit (Bio-Rad, Hercules, California) and adjusted to 6 mg/ml in PBS, pH 8.0. Samples for binding assay were diluted 1/10 with PBS, pH 8.0, and 3 μl of bocillin FL (Molecular Probes, Eugene, Oregon) was added (10 μM final concentration) in a final volume of 75 μl. The mixtures were incubated for 30 min at 37°C. Twenty five μl of NUPAGE sample buffer 4X (Life Technologies, Alcobendas, Spain) was added and samples were boiled for 10 min. Insoluble materials were removed by centrifugation at an Eppendorf centrifuge (14,500 rpm, 10 min, 20°C). Proteins in the sample (50 μl) were separated by SDS-PAGE in a NUPAGE 10 % BIS-TRIS acrylamide gel run in MOPS 1X buffer at a constant voltage of 75 V. The gel was washed in distilled water and fluorescence was detected directly on the gels using a Thyphon 9410 variable-mode imager (General Electric, Madrid, Spain) with an excitation wavelength of 588 nm and a 520BP40 emission filter.

Results

A genetic screen with the T-POP3 transposon [30] was used to search for supressors of bile sensitivity in a *S. enterica* Dam⁻ mutant. Cloning and sequencing of T-POP3 boundaries provided eight independent candidates in which T-POP3 had inserted at the *S. enterica prc* gene [2]. In *E. coli*, *prc* encodes a periplasmic protease also known as tail-specific protease [13,35].

Additional confirmation that loss of Prc function suppressed bile sensitivity in a Dam⁻ mutant was obtained by disrupting the *prc* gene with the lambda Red recombination procedure, and introducing the mutant allele into the Dam⁻ background. MIC analysis confirmed that bile sensitivity was suppressed by the *prc* mutation (data not shown). Further work was carried out with the *prc* deletion allele constructed by site-directed mutagenesis (strain SV6278).

To ascertain whether the ability of a *prc* mutation to suppress bile sensitivity was specific for Dam⁻ mutants or broader, Prc⁻ derivatives were constructed in other bile-sensitive mutants of *S. enterica* such as PhoP⁻ [36], WecD⁻ [29], DamX⁻ [20] and SeqA⁻ [25]. MIC determinations unambiguously showed that a *prc* mutation suppressed bile sensitivity in all genetic backgrounds under study (Table 1). We interpret broad suppression capacity as an indication that the *prc* mutation causes some structural or physiological change that increases bile resistance. This view is supported by an additional observation: introduction of a null *prc* allele in the wild type increased the MIC of DOC from 7% to 12% (Table 1).

Table 1. MICs of sodium deoxycholate

Strain	Genotype	MIC of DOC (%)[a]
SL1344	Wild type	7
SV6100	Δ*dam*	0.2
SV6278	Δ*prc*	12
SV6946	Δ*prc* Δ*dam*::*Km*^{r}	6
SV6934	Δ*phoP*::*MudJ*	0.5
SV6940	Δ*prc* Δ*phoP*::*MudJ*	6
SV6947	Δ*damX*::*Km*^{r}	0.5
SV6953	Δ*prc* Δ*damX*::*Km*^{r}	6
SV6954	Δ*wecD*::*MudJ*	2
SV6960	Δ*prc* Δ*wecD*::*MudJ*	6
SV6961	Δ*seqA*::*Tn10*	0.5
SV6967	Δ*prc* Δ*seqA*::*Tn10*	7

[a] Median of >5 independent experiments.

Other phenotypes of *S. enterica* Prc⁻ mutants. Growth curves of the *S. enterica* wild type strain and a Prc⁻ derivative (SV6278) were constructed under various osmolarity and temperature conditions. The Prc⁻ mutant showed a growth defect at low osmolarity, irrespective of the incubation temperature (Fig. 1). Similar observations were made when a Prc⁻ mutant was streaked for single colonies on a salt-free LB plate (data not shown). Unlike their *E. coli* counterparts [13], *S. enterica* Prc⁻ mutants appeared to be sensitive to low osmolarity in a temperature-independent fashion. Growth in low osmolarity medium results in the formation of cell filaments, a morphological alteration previously described

in *E. coli* [13]. Filament formation was observed in a fraction of cells only, and typically produced filaments of 3–6 cells (Fig. 1). Like sensitivity to low osmolarity, filament formation turned out to be temperature-independent in *S. enterica*.

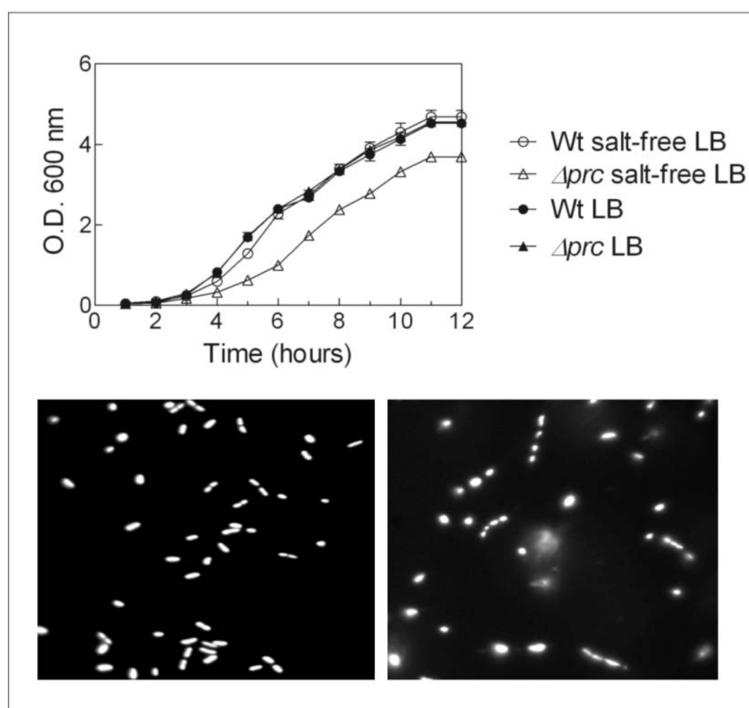

Fig. 1. Top: Growth of the wild type strain and its Prc⁻ derivative SV6278 in LB and salt-free LB. Bottom: Microscopic photographs of wild type (SL1344, left) and Prc⁻ (SV6278, right) *S. enterica* cells grown in salt-free LB.

To investigate whether inactivation of the *prc* gene altered the susceptibility of *S. enterica* to antibiotics, the MICs of selected drugs were determined for the wild type and for SV6278 (Pcr⁻). The Pcr⁻ mutant showed increased sensitivity to nalidixic acid and chloramphenicol, as previously described in *E. coli* [34]. Increased sensitivity to malachite green and polymixin B (not tested in the *E. coli* study) was also detected. However, unlike *E. coli*, the levels of resistance to ampicillin and kanamycin remained unaltered in the *S. enterica* Prc⁻ mutant (data not shown).

Analysis of penicillin-binding proteins in Prc⁻ strains. Cell envelopes were prepared from the wild type and from a Prc⁻ mutant (SV6278). Bacteria were grown in LB and

salt-free LB. The activity of PBPs was analyzed by detecting their capacity to bind bocillin FL [8]. A representative experiment is shown in Fig. 2. Under low osmolarity, differences were found between the wild type and the Prc⁻ mutant: (i) Bocillin binding bands corresponding to PBP3, PBP4, and PBP7 were detected in stationary cultures of Prc⁻ mutant but not in the wild type when grown in low osmolarity media; (ii) Subtle differences in the PBP5/PB6 levels were also observed, and PBP5 was found to increase in stationary cultures of the Prc⁻ strain under low osmolarity conditions; and (iii) PBP7 activity increased in the Prc⁻ mutant in both exponential and stationary cultures.

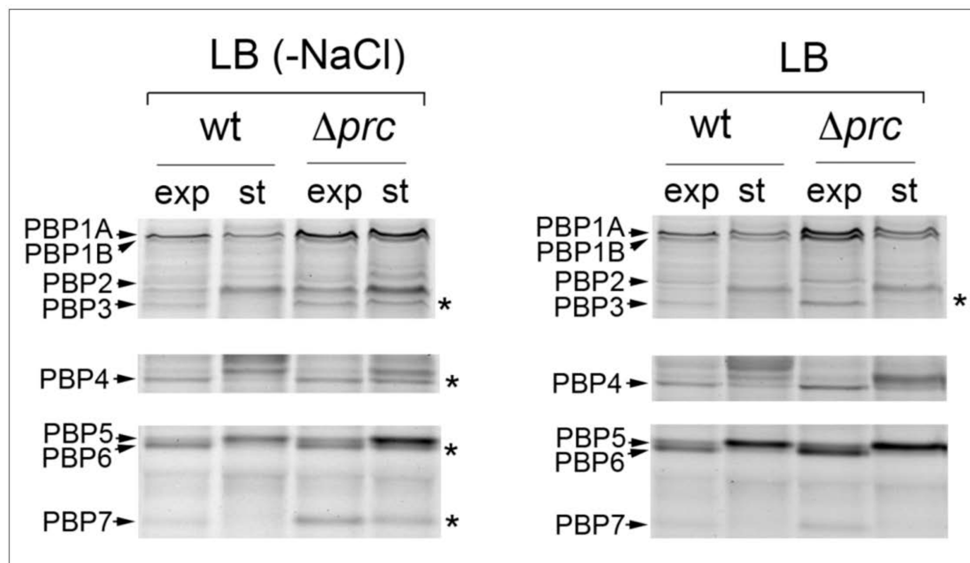

Fig. 2. Binding of bocillin FL to cell envelopes of stationary (st) and exponential (exp) cultures from wild-type *S. enterica* (wt) and from the Prc⁻ mutant SV6278 (Δ*prc*) grown in LB and salt-free LB (LB – NaCl).

Discussion

Mutations that increase the wild type level of bile resistance in *Salmonella enterica* have been described previously [14,24], and this study adds *prc* to the list. In *E. coli*, the *prc* gene encodes a periplasmic protease (also known as Tsp protease, for tail-specific protease) [35]. Prc/Tsp is involved in C-terminal processing of PBP 3 [13], in the degradation of abnormal proteins [17,18], and perhaps in fatty acid transport [1]. It seems likely that Prc may play similar roles in *S. enterica*, as the predicted gene product shows a 94% identity in amino acid sequence with its *E. coli* counterpart. Furthermore, the phenotypes of the *S. enterica* Prc⁻ mutants described in this study resemble those previously described in *E. coli* [13], with minor variations (Fig. 1).

The capacity of *prc* mutations to act as enhancers of bile resistance in the wild type and as general suppressors of bile sensitivity in a variety of mutant backgrounds (Table 1) suggests that bile resistance may result from a response triggered by Prc absence. One possibility is that enhanced bile resistance may be the consequence of the changes in activity of several PBPs such as PBP3, PBP4 and PBP7 that were detected in Prc⁻ mutants (Fig. 2). PBPs are membrane enzymes involved in polymerization and restructuring of peptidoglycan in the final steps of peptidoglycan biosynthesis [32]. PBP3 is an essential transpeptidase that catalyzes crosslink of the peptidoglycan strands during formation of the cell division septum [21]. PBP3 processing by Prc is not required for cell viability [12]. In turn, PBP7 and PBP4 are DD-endopeptidases that break the peptide cross-bridges between glycan chains in high-molecular-mass murein sacculi [31]. This study does not prove that the bile resistance phenotype of Prc⁻ mutants is a consequence of peptidoglycan remodeling. However, the increase in PBP7 and PBP4 activities observed in Prc⁻ mutants fits well in the view that these PBPs may produce a peptidoglycan structure necessary for cell survival under certain adverse conditions such as starving or exposure to oxidative damaging agents [19]. In fact, one of the antibacterial actions of bile salts is DNA oxidative damage [27].

Prc⁻ mutants are unlikely to be found in nature: during *Salmonella* infection, the potential advantage of acquiring a *prc* mutation would be compensated by its negative consequences, which include sensitivity to low osmolarity and impaired cell division. In fact, *S. enterica* Prc⁻ mutants show reduced survival within macrophages [2]. A parallel

case is found in *S. enterica* AsmA⁻ mutants, which are hyperesistant to bile but show impaired capacity to invade epithelial cells [24]. However, hyperesistant mutants should not be merely viewed as laboratory curiosities as their physiological defects can unveil mechanisms that operate in the wild type. In the case of Pcr⁻ mutants, their defects raise the possibility that alterations in the machinery for peptidoglycan synthesis may contribute to bile resistance. In support of this hypothesis, other components of the cell envelope are known to play roles in bile resistance [3,10]. A reason to overlook the cell wall in previous studies may have been the essential nature of most functions involved in cell wall biogenesis, which makes classical genetic analysis difficult.

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Competing interests. None declared.

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Chapter 4: Bile-induced peptidoglycan remodeling in *Salmonella enterica*

Bile-induced peptidoglycan remodeling in *Salmonella enterica*

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Running title: Bile-induced peptidoglycan remodeling

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Summary

Changes in the peptidoglycan structure of *Salmonella enterica* are detected in the presence of a sublethal concentration of sodium deoxycholate (DOC): (i) lower proportions of Braun lipoprotein-bound muropeptides; (ii) low amounts of muropeptides crosslinked by L(meso)-diaminopimelyl-D(meso)-diaminopimelic acid (L-D) peptide bridges (3-3 crosslinks). Similar structural changes are found in *S. enterica* cultures adapted to grow in the presence of a lethal concentration of DOC, suggesting that reduced anchoring of Braun protein to peptidoglycan and low occurrence of 3-3 crosslinks may increase *S. enterica* resistance to bile. This view is further supported by additional observations: (i) A triple mutant lacking L,D-transpeptidases YbiS, ErfK, and YcfS, which does not contain murein lipoprotein anchored to peptidoglycan, is hyperesistant to bile; (ii) Enhanced 3-3 crosslinking upon overexpression of YnhG transpeptidase causes a decrease in bile resistance. These observations suggest that remodeling of the cell wall may be added to the list of bacterial responses that permit survival of *Salmonella enterica* in the presence of bile.

Introduction

Bile is a fluid containing bile salts, cholesterol, phospholipids, proteins, bilirubin, and electrolytes [1]. Bile is synthesized in the liver and stored and concentrated in the gall bladder. During food passage, bile is released through the bile duct into the intestine. Bile salts aid in the digestion and absorption of fats and fat-soluble vitamins in the small intestine [1]. Bile salts are amphipathic molecules derived from cholesterol, and constitute about two thirds of bile (dry weight). The most abundant bile salts in humans are cholate and deoxycholate [2].

Bile salts control the expression of certain genes in intestinal bacteria, and may be considered environmental signals used by the bacterium to identify the intestinal environment [3, 4]. However, bile salts are also antibacterial compounds that disrupt membranes, denature proteins, and cause oxidative damage to DNA [3-7]. At high concentrations, bile salts are bactericidal [8]. However, enteric bacteria are able to resist the antibacterial activities of bile salts, and an extreme example is *Salmonella enterica* which colonizes the hepatobiliary tract, including the bile-laden gall bladder, during systemic infection [9]. The gall bladder is actually the major niche for *Salmonella* in chronic carriers of typhoid [9].

Bile resistance can be studied under laboratory conditions by adding ox bile or individual bile salts to microbiological media [10]. Genetic and biochemical analysis in *E. coli* and *S. enterica* in the laboratory has permitted the identification of cell functions and mechanisms involved in bile resistance [3, 10]. Envelope structures that contribute to bile resistance in enteric bacteria include the lipopolysaccharide (LPS) [11-13] and the enterobacterial common antigen [14]. Reduction of porin synthesis also increases bile resistance. Furthermore, efflux systems reduce the intracellular concentration of bile salts [15, 16]. While the role of these envelope structures in bile resistance have been confirmed by numerous studies, the potential involvement of the cell wall in bile resistance has not been investigated previously.

The cell wall structure is similar in *S. enterica* and *E. coli* [17, 18]. A major component is peptidoglycan (murein) which is located in the periplasmic space providing mechanical strength and serving as anchor for covalent linkage of surface proteins [19, 20]. Peptidoglycan is a heteropolymer, consisting of glycan strands of N-acetylmuramic acid

and N-acetylglucosamine coupled through short peptide cross-links [21]. Two different types of peptide cross-links can be distinguished: the most common 3-4 cross-links formed by D,D-transpeptidases and 3-3 cross-links formed by L,D-transpeptidases [17, 22]. The peptidoglycan is linked covalently to the outer membrane by the Braun lipoprotein (also known as murein lipoprotein or Lpp) which is also found freely associated to the outer membrane [23, 24]. In *E.coli,* lack of Lpp impairs the permeability barrier of the outer membrane increasing bacterial susceptibility to detergents [25]. In *Salmonella enterica* serovar Typhimurium, two *lpp* genes exist in the chromosome, and mutants lacking both Lpp species are severely attenuated upon mouse infection [26].

Previous studies have shown that the chemical structure of the peptidoglycan can change depending on the growth phase of the culture [27] and also in response to environmental stimuli such as the concentration of NaCl [28] and D-amino acids [29, 30]. In this study we show that exposure to sodium deoxycholate (DOC), the archetypal and most abundant bile salt in the animal gut, is associated with changes in the peptidoglycan structure of *S. enterica* serovar Typhimurium. We also provide evidence that peptidoglycan remodeling may contribute to bile resistance.

Results

Growth of S. enterica in the presence of a sublethal concentration of sodium deoxycholate alters peptidoglycan structure

The effect of sodium deoxycholate on peptidoglycan structure was analyzed in *S. enterica* cultures grown in LB contaning 5% DOC, a sublethal concentration that adapts *S. enterica* to resist lethal concentrations [8]. Muropeptide composition was determined by high-performance liquid chromatography (HPLC). Quantification of each muropeptide species was performed by integration of the peaks of the HPLC profile, and the muropeptides were grouped into classes according to structural similarities [31]. Because *S. enterica* shows different levels of bile sensitivity during exponential growth and in the stationary phase [32], peptidoglycan analysis was performed under both growth conditions. The main observations were as follows:

(i) A reduction was detected in the peaks annotated as M3L and D43L, which correspond to muropeptides containing a C-terminal dipeptide fragment of the Braun lipoprotein [33]. During exponential growth, around 30% less lipoprotein anchored to the peptidoglycan was detected in the presence of 5% DOC (Table 1 and Fig. S1). In stationary cultures, around 40% less lipoprotein anchored to the peptidoglycan was detected in the presence of 5% DOC (Table 1 and Fig. S2).

(ii) Reduction of *meso*DAP-*meso*DAP (DAP=diaminopimelic acid) 3-3 crosslinks [17] was detected. The crosslink reduction was of 50% in exponential cultures (Table 1 and Fig. S1) and of 30% in stationary cultures (Table 1 and Fig. S2).

A tentative interpretation of these observations was that the presence of DOC had caused a decrease both in the amount of Braun lipoprotein anchored to peptidoglycan and in 3-3 (Dap-Dap) crosslinking.

Table 1. Muropeptide composition of peptidoglycan from *S. typhimurium* SL1344 grown in LB and LB containing 5% DOC

Muropeptide group	Exponential		Stationary	
	LB	DOC	LB	DOC
Monomers	67.8	68.4	59.7	68.0
Dimers	30.3	29.5	37.8	30.2
Trimers	1.9	2.1	2.5	1.8
Lipoprotein	9.2	6.5	17.0	10.1
Anhydro	1.3	1.5	2.5	1.9
Dap-dap	3.6	1.6	9.1	3.1
Pentapeptide	0.2	0.0	0.3	0.6
Penta-glycine	0.3	0.3	0.0	0.0

Adaptation of S. enterica *to survive a lethal concentration of bile is accompanied by changes in Lpp anchoring and crosslinking*

Growth in the presence of sublethal concentrations of bile (e. g., 5% DOC) adapts *S. enterica* to survive a lethal concentration (e. g., 11% DOC) [8]. We thus analyzed the muropeptide composition of *S. enterica* grown in LB containing 11% DOC. In these experiments, stationary cultures were used because bile resistance is known to be higher under such conditions [32]. Results from a representative experiment are shown in Table 2. A 50% reduction in the amount of Braun lipoprotein was detected. Dap-Dap crosslinking was also found to decrease twofold. These observations further supported the existence of a correlation between bile resistance and peptidoglycan remodeling.

Comparison of data from Tables 1 and 2 also suggest that an inverse correlation may exist between anchoring of Braun lipoprotein to peptidoglycan and bile resistance: 30% reduction in 5% DOC (exponential culture), 40% reduction in 5% DOC (stationary culture), and 50% reduction in 11% DOC (stationary culture).

Table 2. Muropeptide composition of peptidoglycan from bile-adapted *S. enterica* grown in LB and LB containing 11% DOC

Muropeptide group	Relative abundance (mol %)	
	LB	DOC
Monomers	63.4	69.1
Dimers	34.5	28.5
Trimers	2.1	2.3
Lipoprotein	14.8	7.1
Anhydro	3.5	3.5
Dap-dap	6.8	3.1
Pentapeptide	0.4	0.0
Penta-glycine	0.0	0.0

Increased bile resistance in S. enterica *mutants lacking ErfK, YcfS and YbiS L,D-transpeptidases*

In *E.coli,* anchoring of Braun lipoprotein to the peptidoglycan and 3-3 crosslinks are catalyzed by the activity of the L,D-transpeptidases [22]. Five L,D-transpeptidases exist in *E.coli*: ErfK, YcfS and YbiS, which anchor Braun lipoprotein to the peptidoglycan, and YcbB and YnhG which catalyze 3-3 crosslinking [22, 34]. To further investigate the tentative relationship found between muropeptide alteration and bile resistance, *S. enterica* homologs of the *E. coli* L,D-transpeptidase genes (*erfK, ycbB, ycfS, ybiS and ynhG*) were deleted in strain SL1344. The minimal inhibitory concentration (MIC) of mutants carrying individual or multiple deletions was then determined. Results shown in Table 3 can be summarized as follows:

(i) Deletion of individual genes *erfK* or *ycfS* did not alter the MIC of DOC. However, a Δ*ybiS* mutant (SV7557) showed increased resistance to DOC.

(ii) The level of DOC resistance of a double mutant Δ*erfK* Δ*ycfS* was similar or identical to that of the wild type. Increased DOC resistance, however, was detected in Δ*ybiS* Δ*ycfS and* Δ*ybiS* Δ*erfK* strains.

(iii) A Δ*ybiS* Δ*ycfS* Δ*erfK* strain (SV7563) showed hiperesistance to DOC (>14% compared with 7% in the wild type).

These observations further support the existence of an inverse relationship between Braun protein anchorage and bile sensitivity. The increased resistance to DOC observed in strains carrying the Δ*ybiS* deletion, alone or combined with Δ*ycfS* Δ*erfK*, is consistent with the fact that deletion of the *E. coli ybiS* gene severely reduces covalent linkage of Braun lipoprotein to peptidoglycan [22]. However, the most compelling observations were made in the triple mutant Δ*ybiS* Δ*ycfS* Δ*erfK*, which does not contain detectable amounts of muropeptides carrying the fragment of the Braun lipoprotein (Table 4 and Fig. S4):

(i) The Δ*ybiS* Δ*ycfS* Δ*erfK* mutant (SV7563) was hyperesistant to DOC (Table 3).

(ii) Partial complementation of the Δ*ybiS* Δ*ycfS* Δ*erfK* mutant with a plasmid-borne *ybiS* gene (strain SV7756) increased covalent linkage of Braun lipoprotein to peptidoglycan (Fig. S4) and decreased resistance to DOC (Table 3).

Altogether, these observations may support the view that reduction in the amount of Braun lipoprotein anchored to the peptidoglycan increases bile resistance in *S. enterica*.

Table 3. Minimal inhibitory concentrations of DOC[a]

Strain	Genotype	MIC of DOC (%)
SL1344	Wt	7
SV7557	Δ*ybiS*	9
SV7558	Δ*ycfS*	7
SV7559	Δ*erfK*	7
SV7560	Δ*ybiS* Δ*ycfS*	10
SV7561	Δ*ybiS* Δ*erfK*	10
SV7562	Δ*ycfS* Δ*erfK*	7
SV7563	Δ*ybiS* Δ*ycfS* Δ*erfK*	>14
SV7564	Δ*ycbB*	7
SV7566	Δ*ynhG*	7
SV7567	Δ*ycbB* Δ*ynhG*	7
SV7756	Δ*ybiS* Δ*ycfS* Δ*erfK* p$_{BAD}$::*ybiS*	7

[a] Averages of 3 or more independent experiments

Table 4. Muropeptide composition of peptidoglycan in *S. typhimurium* SL1344 and in a Δ*ybiS* Δ*ycfS* Δ*erfK* triple mutant (SV7563)

Muropeptide group	Relative abundance (mol %)	
	wt	Δ*ybiS* Δ*ycfS* Δ*erfK*
Monomers	46.7	63.7
Dimers	49.3	34.0
Trimers	4.0	2.3
Lipoprotein	13.5	0.0
Anhydro	4.8	3.1
Dap-dap	9.9	8.0
Pentapeptide	0.0	0.5
Penta-glycine	0.0	0.0

Contribution of 3-3 crosslink reduction to bile resistance

Genetic analysis was also performed to investigate whether reduction of 3-3 crosslinking contributed to increase resistance to DOC in *S. enterica*. Neither the single mutants Δ*ycbB* and Δ*ynhG* nor the double mutant Δ*ycbB* Δ*ynhG* showed significant changes in DOC resistance (Table 3). However, these observations did not rule out the possibility that high levels of 3-3 crosslinking could cause bile sensitivity. To address this possibility, one of the *S. enterica* homologs of the *E. coli* gene that encodes an L,D-transpeptidase that catalyzes 3-3 cross-linkage (*ynhG*) was cloned on the pBAD18 vector under the control of the arabinose-inducible promoter p_{BAD}. The relative abundance of peptidoglycan 3-3 crosslinks was determined in the wild-type, in a mutant devoid of Braun lipoprotein anchored to the peptydoglycan (Δ*ybiS* Δ*ycfS* Δ*erfK*), and in a mutant lacking crosslink L,D-transpeptidases (Δ*ycbB* Δ*ynhG*) carrying the empty vector pBAD18 and in derivate strains carrying plasmid pIZ2019 (p_{BAD}::*ynhG*). All cultures for muropeptide analysis were grown in LB containing arabinose, and the results can be summarized as follows:

(i) The presence of plasmid pIZ2019 increased the amount of muropeptides containing 3-3 crosslinks in all strains (Table 4 and Figs. S5, S6, and S7), therefore confirming that the cloned *ynhG* was functional.

(ii) p_{BAD}-driven expression of *ynhG* decreased the MIC of DOC from 7% to 4% in both the wild type and in the Δ*ycbB* Δ*ynhG* mutant, and from >14% to 7% in the hyperesistant mutant devoid of Braun lipoprotein Table 5).

Table 5. Relative abundance of muropeptides cross-linked through a DAP-DAP peptide bridge (3-3 cross-link) of strains overexpressing (+) or not (-) the *ynhG* L,D-transpeptidase gene and MIC of DOC of these strains.

Strain	*ynhG* overexpression	Relative abundance of 3-3 cross-links (mol %)	MIC of DOC
Wild-type	-	4.00	7
	+	10.72	4
Δ*ybiS* Δ*ycfS* Δ*erfK*	-	5.24	>12
	+	8.32	7
Δ*ycbB* Δ*ynhG*	-	0.00	7
	+	11.70	4

A tentative interpretation of these observations is that either low levels of crosslinking or absence of crosslinking may be compatible with bile resistance while high levels of crosslinking may be associated with bile sensitivity.

Discussion

Genetic analysis has provided evidence that bacterial surface structures contribute to bile resistance. Mutants of *S. enterica* lacking O antigen chains in the lipopolysaccharide (LPS) are bile-sensitive, indicating that LPS serves as a barrier for bile salts [11]. LPS modifications under PhoPQ control also contribute to bile resistance [35]. As a consequence, *Salmonella phoP* constitutive mutants show increased bile resistance [35], while *phoP* mutants are bile-sensitive [12]. Absence of lipid A modifications sensitize *E. coli* to bile salts [36]. Bile sensitivity is likewise observed in *S. enterica* mutants lacking enterobacterial common antigen (ECA), an outer membrane glycolipid [14]. Furthermore, mutations that destabilize the outer membrane (e. g., in the *tolQRA* gene cluster) cause bile sensitivity in both *E. coli* and *Salmonella* [37].

Our analysis of peptidoglycan structure in the presence of bile was stimulated by previous evidence indicating that *S. enterica* remodels peptidoglycan in response to diverse environmental cues including oxidative stress [38]. Furthermore, bile salts are known to induce stress responses and, among other injuries, cause oxidative damage [5]

Growth of *S. enterica* in the presence of a sublethal concentration of DOC was accompanied by a reduction in the amount of Braun protein anchored to peptidoglycan (Table 1). Evidence that this reduction is associated with bile resistance was provided by two additional observations: (i) *S. enterica* cultures adapted to grow in the presence of a lethal concentration of DOC also showed reduced amounts of muropeptide-bound Braun lipoprotein (Table 2); a *S. enterica* mutant lacking Braun protein anchored to peptidoglycan (strain SV7563, Δ*ybiS* Δ*ycfS* Δ*erfK* strain) was found to be hyperesistant to bile (Tables 3 and 4). Because peptidoglycan-bound Braun lipoprotein provides covalent linkage between the outer membrane and the peptidoglycan, reduction or loss of this union could decrease rigidity of the cell envelope, perhaps altering outer membrane fluidity. In some cases, increase of membrane fluidity has been associated with sensitivity to envelope-damaging compounds [39, 40]. However, other studies have suggested that increase of outer membrane fluidity might permit resistance to stress conditions and antibacterial compounds [41, 42]. The latter examples may provide analogies to understand why reduction of peptidoglycan-bound lipoprotein increases bile resistance.

A reduction in 3-3 crosslinks was also detected in the presence of bile, suggesting that crosslinking may be reduced in the presence of bile (Tables 1 and 2). A Δ*ycbB* Δ*ynhG* mutant, which lacks Dap-Dap crosslinks, shows a level of DOC sensitivity identical to that of the wild type (Table 5), suggesting that crosslinking is not actually necessary for bile resistance. However, overproduction of YnhG transpeptidase causes a strong decrease in bile resistance (Table 5), indicating that high levels of crosslinking may cause bile sensitivity. We thus tentatively conclude that low crosslinking or absence of crosslinking may be compatible with bile resistance while high levels of crosslinking may be associated with bile sensitivity.

Experimental procedures

Bacterial strains, bacteriophages, and standard strain construction

All the strains of *Salmonella enterica* used in this study listed in Table 6 belong to serovar Typhimurium, and derive from the mouse-virulent strain SL1344. Transduction was performed with phage P22 HT 105/1 *int201 ([43]* and G. Roberts, unpublished data). The P22 HT transduction protocol was described elsewhere [44]. To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan et al. [45], except that methyl blue (Sigma Chemical Co., St. Louis, Missouri) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Oligonucleotides used in this study are listed in Table S1. Targeted gene disruption was achieved using plasmids pKD13 [46] and oligonucleotides FOR and REV. Oligonucleotides E1 and E2 were used for allele verification. Antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 [46]. Plasmids p_{BAD}-*ybiS* (pIZ2018) and p_{BAD}-*ynhG* (pIZ2019) were constructed by cloning the respective gene under the control of the arabinose-dependent p_{BAD} promoter of pBAD18 [47] using the pairs of oligonucleotides ybiS-pBAD-FOR and ybiS-pBAD-REV, and ynhG-pBAD-FOR and ynhG-pBAD-REV, respectively (Table S1).

Growth media and conditions

Luria–Bertani (LB) broth was used as liquid medium. Liquid cultures were grown with aeration by shaking in an orbital incubator. If necessary for plasmids maintenance or selection of recombinant strains appropriate antibiotics: kanamycin sulfate (Km), or ampicillin (Ap) were added to broth or agar plates at a final concentration of 50 μg ml^{-1} and 100 μg ml^{-1}, respectively. When specified, sodium deoxycholate (DOC) (Sigma Chemical Co.) was added. Cultures in the presence of DOC were started with an aliquot from an overnight grown culture in LB. Solid LB contained agar at 1.5% final concentration. To induce arabinose-promoter of the pBAD18 vector 0.2% of arabinose was added.

Table 6. Strains of Salmonella enterica serovar Typhimurium constructed for this study

Strain	Genotype
SV7490	*ybiS::km*
SV7491	*ycbB::km*
SV7492	*erfK::km*
SV7493	*ycfS::km*
SV7500	*ynhG::km*
SV7557	Δ*ybiS*
SV7558	Δ*ycfS*
SV7559	Δ*erfK*
SV7560	Δ*ybiS* Δ*ycfS*
SV7561	Δ*ybiS* Δ*erfK*
SV7562	Δ*ycfS* Δ*erfK*
SV7563	Δ*ybiS* Δ*ycfS* Δ*erfK*
SV7564	Δ*ycbB*
SV7566	Δ*ynhG*
SV7567	Δ*ycbB* Δ*ynhG*
SV7264	SL1344 / pBAD18
SV7752	Δ*ybiS* Δ*ycfS* Δ*erfK* / pBAD18
SV7753	Δ*ycbB* Δ*ynhG* / pBAD18
SV7756	Δ*ybiS* Δ*ycfS* Δ*erfK* / pIZ2018 (p_{BAD}::*ybiS*)
SV7758	wt p_{BAD}::*ynhG*
SV7760	Δ*ybiS* Δ*ycfS* Δ*erfK* / pIZ2019 (p_{BAD}::*ynhG*)
SV7761	Δ*ycbB* Δ*ynhG* / pIZ2019 (p_{BAD}::*ynhG*)

Determination of minimal inhibitory concentration

Exponential cultures in LB broth were prepared, and samples containing around 3×10^2 colony-forming-units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of DOC. After 12 h incubation at 37°C, growth was visually monitored. Assays were carried out in triplicate. Student's *t*-test was used to analyze every MIC. The null hypothesis was that MICs were not significantly different from the MIC for the wild-type. *P* values of 0.01 or less were considered significant.

Peptidoglycan purification and muramidase digestion

Peptidoglycan was prepared as described before [48]. Cells from exponential or stationary cultures (25ml) were harvested by centrifugation and resuspended in 1 volume of the culture medium used. The samples were slowly dropped into an equal volumen of boiling 10% (wt/vol) SDS and vigorously stirred for more than 4 h and left stirring overnight at 37ºC. The insoluble fraction (peptidoglycan) was recovered by centrifugation (300,000x g, 15 min, 30ºC) and washed until it was free of SDS by successive suspension in destilated water and high speed centrifugation. The last pellet of the washing procedure was suspended in 20mM Tris-HCl (pH 7.5) and digested first with 100 µg/ml α-amylase (EC 3.2.1.1; Sigma-Aldrich, Saint Louis, MO) for 1 h at 37°C and then with 100 µg/ml preactivated pronase E (EC 3.4.24.4; Merck, Darmstadt, Germany) at 60°C for 90 min. The enzymes were inactivated by boiling for 2 min in 1% (final concentration) SDS. Cell walls were collected by centrifugation as described above and washed with water. It was again centrifuged (Eppendorf centrifuge at maximum speed for 10 min) to remove insoluble debris. The pellet was digested in 50 mM phosphate buffer (pH 4.9) with Cellosyl (the muramidase, from Hoechst AG, Frankfurt, Germany) 100 µg/ml final concentration at 37°C overnight. The enzyme reaction was stopped by boiling the sample for 2 min in a water bath and centrifugating it (Eppendorf centrifuge at maximum speed for 10 min). The supernatant (muropeptides) was mixed with 1/3 volume of 0.75 M sodium borate buffer (pH 9.0) and reduced with excess sodium borohydride ($NaBH_4$) for 30 min at room temperature. The pH was tested with pH indicator strips (Acilit, Merck) and adjusted to 3 with orthophosphoric acid. The samples were stored at −20°C.

Separation and quantification of muropeptides

Muropeptide composition was determined by high-performance liquid chromatography (HPLC). Separation of the reduced muropeptides by HPLC (325 system; Kontron Instruments) was performed essentially by the method of Glauner et al. [31]. The eluted muropeptides were monitored by measuring absorbance at 204 nm (Jasco UV-1570 spectrophotometer). Individual muropeptides were quantified from their integrated areas using samples of known concentration as standards [49], and the muropeptides were grouped into classes according to structural similarities [31]. Chromatograms are presented as supplementary material (Figs. S1-S7).

Supplemental Material

Table S1. Oligonucleotides used in this study (5'→3')

Oligonucleotide	Sequence
erfK-E1	gcacaacaccagccatgtg
erfK-E2	aaggtctaccgggtttcttg
erfK-P1-REV	agcccatgaaaaccagcaatgcgacaggatatttaaaggctgtaggctggagctgcttcg
erfK-P4-FOR	cacctgccgcgacaaaggaagggatataatgcgtcgtatcattccggggatccgtcgacc
ybiS-E1	ctgtacaaagggctacacgt
ybiS-E2	actgatgccgtaagagaagc
ybiS-P1-REV	tatctatcgggcttcgcaggcagagtctgtcagttcagactgtaggctggagctgcttcg
ybiS-P4-FOR	ctagcctgcttggcacagaatctcgcataacatgaatatgattccggggatccgtcgacc
ycfS-E1	gctattgcataagcgtcagg
ycfS-E2	ccgtattacctctgttaccg
ycfS-P1-REV	gcgaggctttttgtcggtcagctattcacgcttacagcgtgtaggctggagctgcttcg
ycfS-P4-FOR	ataaccacataataatcatgggtttattataatgttcaacattccggggatccgtcgacc
ycbB-E1	gttaagcctgacgagtgag
ycbB-E2	ctggcacagacttctgaacg
ycbB-P1-REV	acattaggacaacatatttaccagaacttcttcatttacctgtaggctggagctgcttcg
ycbB-P4-FOR	tgcggagtatgataacgaaaacagggggcaagggatgttgattccggggatccgtcgacc
ynhG-E1	tgtacagacagcgttctacc
ynhG-E2	ccaacgtctggacaacaaag
ynhG-P4-FOR	tcgcagagggcatcaccaggaatacaggaggtttggtatgattccggggatccgtcgacc
ynhG-P1-REV	gccattttttgtccgtcgtttgctgcaaaggctactgcgtgtaggctggagctgcttcg
ybiS-pBAD-FOR	tcggaattcaggaggcagaatctcgcataacatgaatatg
ybiS-pBAD-REV	tcgagtcgaccttcgcaggcagagtctgtcagttcagacg
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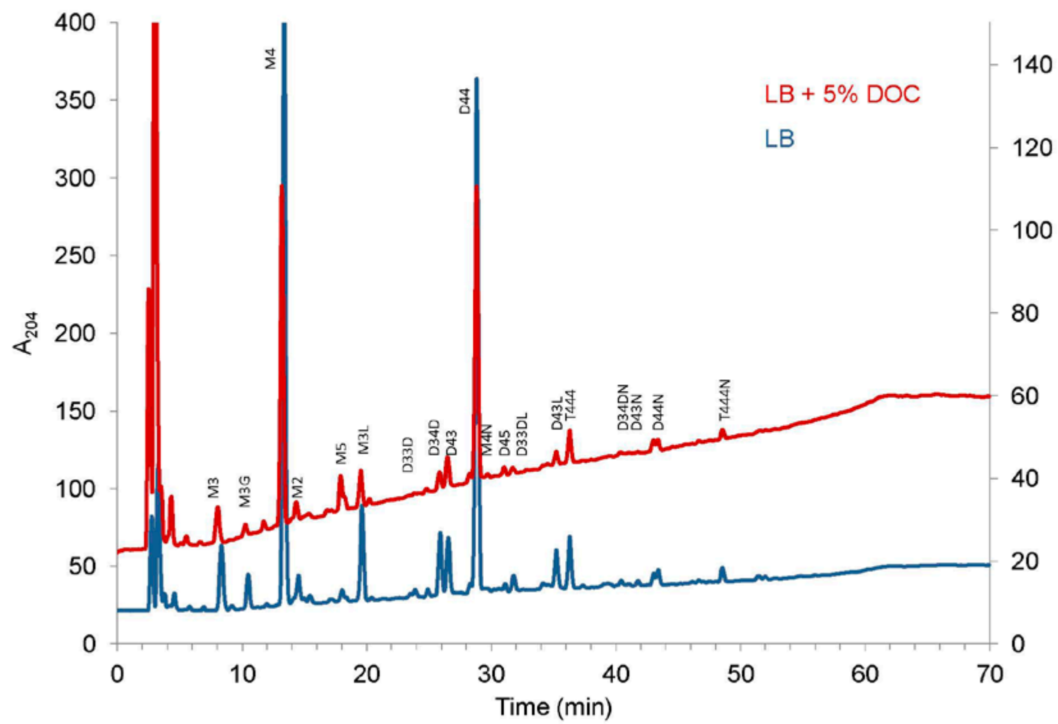

Figure S1. HPLC separation of muropeptides from exponential cultures of. *S. enterica* SL1344 grown in LB and LB supplemented with 5% DOC.

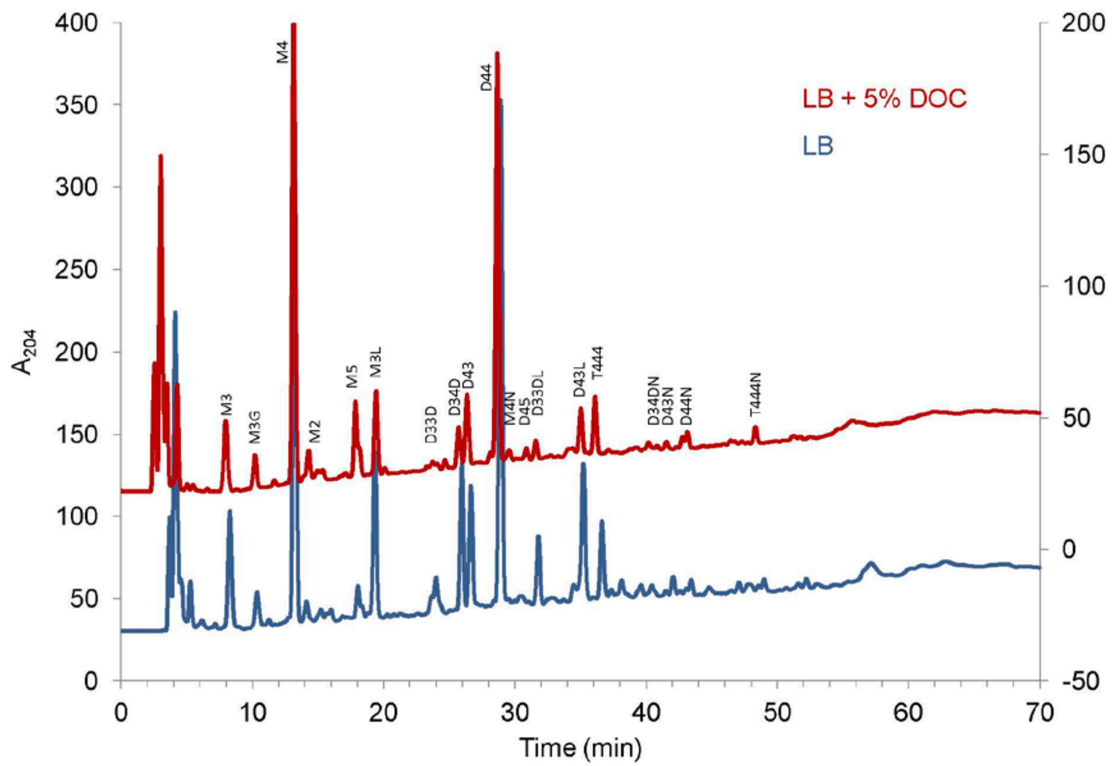

Figure S2. HPLC separation of muropeptides from stationary cultures of. *S. enterica* SL1344 grown in LB and LB supplemented with 5% DOC.

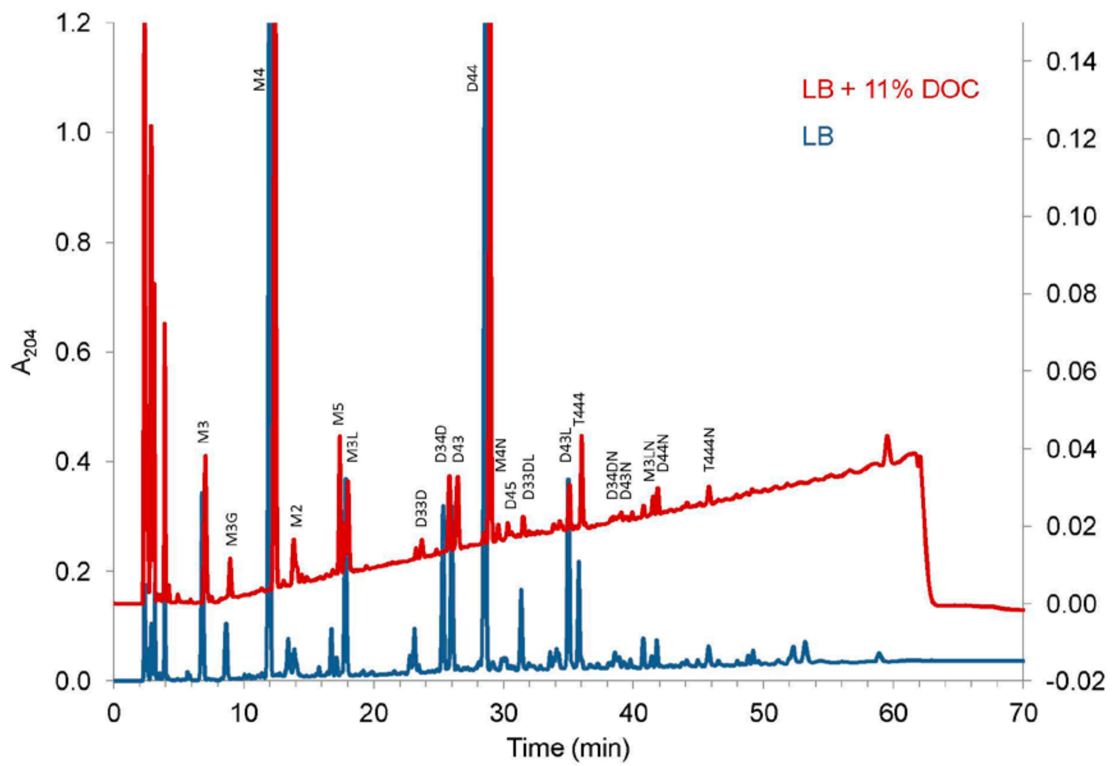

Figure S3. HPLC separation of muropeptides from stationary cultures of. *S. enterica* SL1344 grown in LB and LB supplemented with 11% DOC.

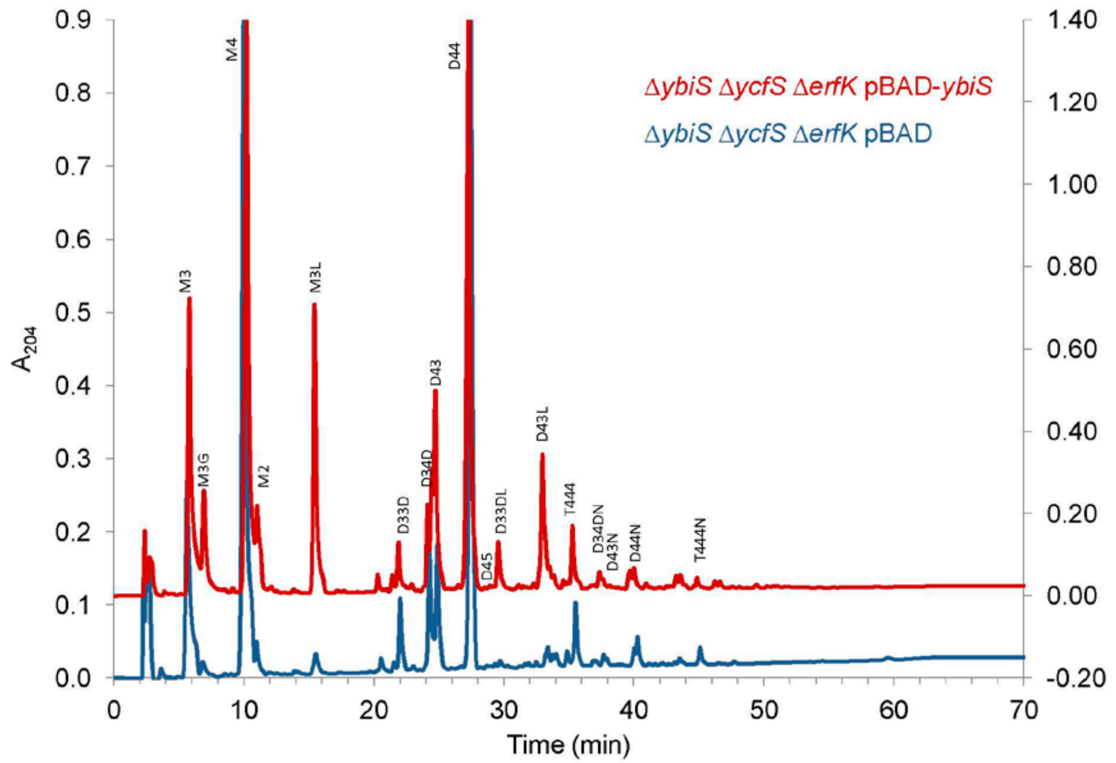

Figure S4. HPLC separation of muropeptides from stationary cultures of *S. enterica* strains SV7756 (Δ*ybiS* Δ*ycfS* Δ*erfK* / p_{BAD}::*ybiS*) and SV7753 ((Δ*ybiS* Δ*ycfS* Δ*erfK* / pBAD) grown in LB.

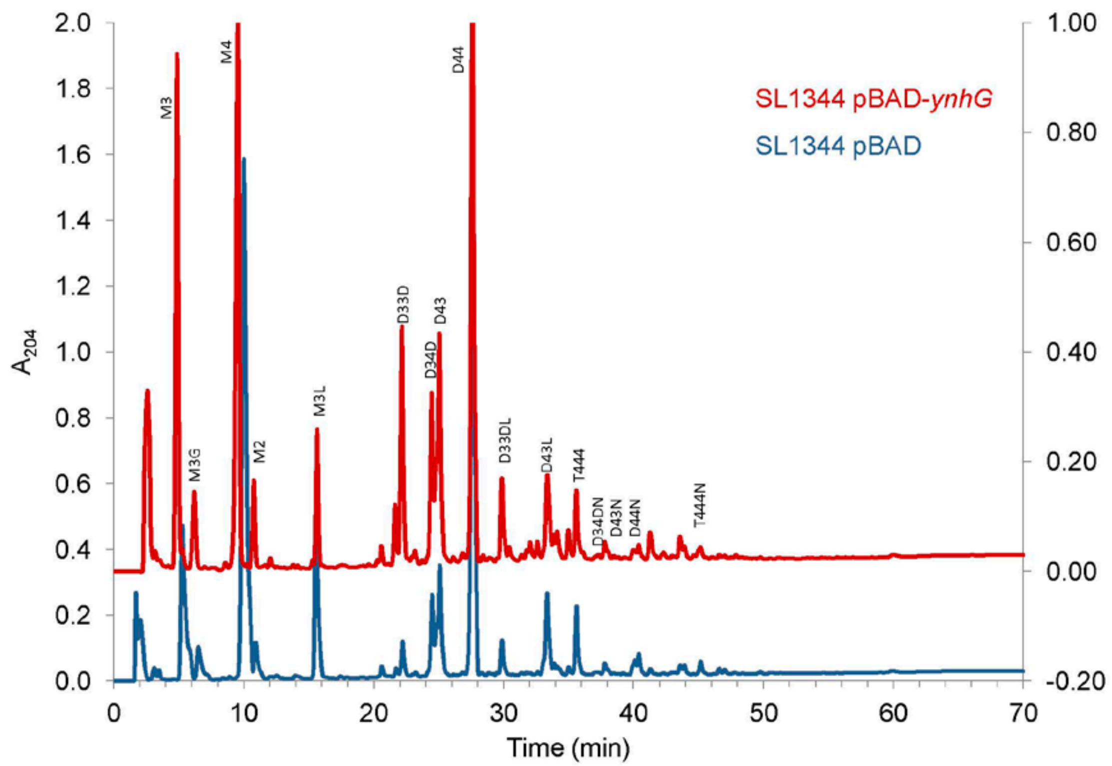

Figure S4. HPLC separation of muropeptides from stationary cultures of *S. enterica* strains SV7755 (SL1344 / pBAD::*ynhG*) and SV7464 (SL1344 / pBAD) grown in LB.

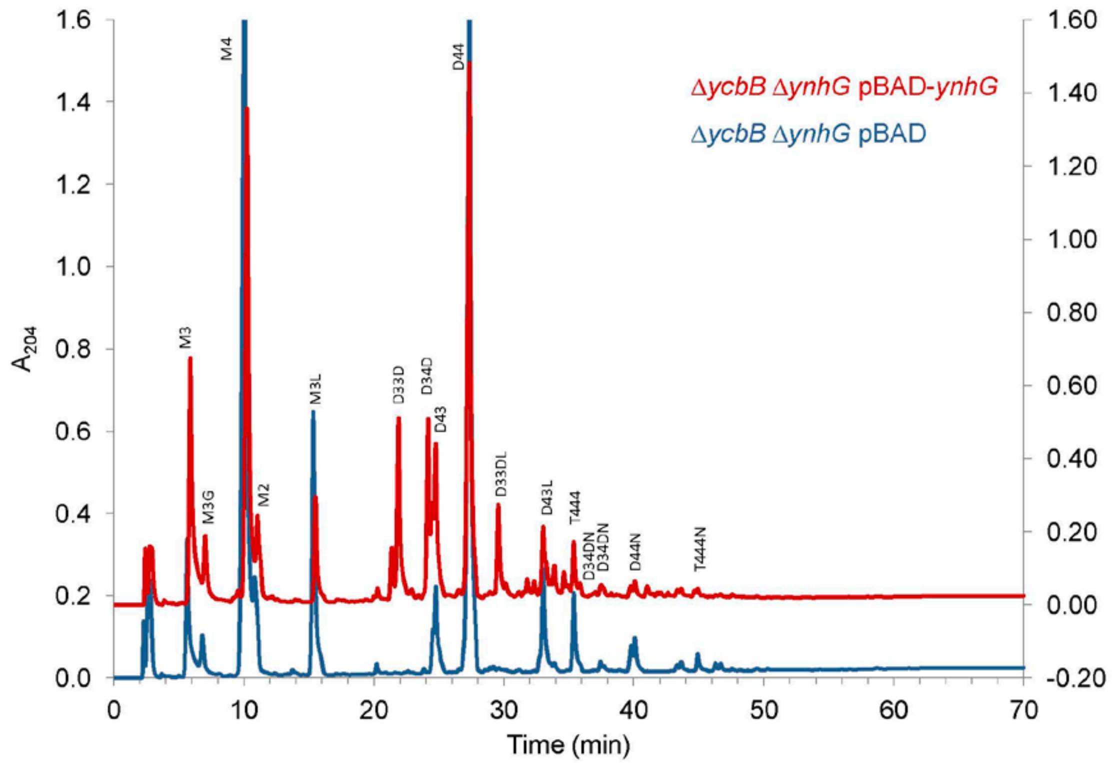

Figure S6. HPLC separation of muropeptides from stationary cultures of. *S. enterica* strains SV7757 (Δ*ycbB* Δ*ynhG* / pBAD::*ynhG*) and SV7753 Δ*ycbB* Δ*ynhG* / pBAD)

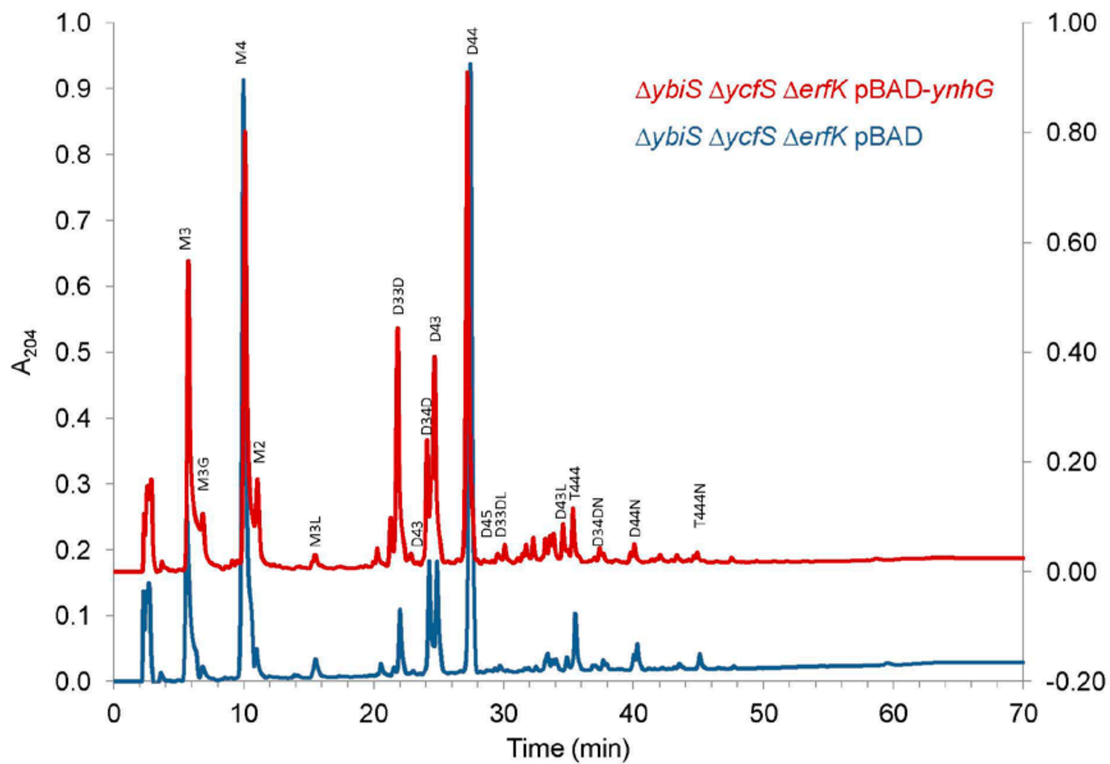

Figure S7. HPLC separation of muropeptides from stationary cultures of. *S. enterica* strains SV7760 (Δ*ybiS* Δ*ycfS* Δ*erfK* / pBAD::*ynhG*) and SV7761 (Δ*ybiS* Δ*ycfS* Δ*erfK* / pBAD) grown in LB.

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DISCUSSION

A small number of *Salmonella* serovars are specialists that establish systemic infections in immunocompetent hosts. Perhaps the most infamous of these pathogens is *S.* Typhi, the causative agent of typhoid fever, which causes systemic infection in humans and can also establish chronic carriage in the gall bladder. Human chronic carriers of *S.* Typhi are important reservoirs for transmission through an intermittent release of the pathogen from the gall bladder into the intestine. The bacterial lifestyle in the gall bladder is one of the less known aspects of *Salmonella* biology. The high concentration of bile present in the gall bladder makes it a harsh environment for bacteria because bile salts are bactericidal.

Resistance to bile can be studied under reductionist laboratory conditions by adding ox bile or individual bile salts to culture media. Biochemical analysis of transport and/or efflux of bile salts are likewise feasible in the laboratory.

Under laboratory conditions, growth of *Salmonella* in the presence of sublethal concentrations of either bile or bile salts 'adapts´ the bacterial population to survive lethal concentrations. Adaptation can be distinguished from mutation because adaptive bile resistance is reversible, and cultivation in the absence of bile renders the population bile-sensitive. This phenomenon is likely to occur also *in vivo*: a fraction of *S. enterica* isolates recovered from the gall bladder of BALB/c mice lose bile resistance upon cultivation in LB.

Although adaptation of *S. enterica* to bile was a well known phenomenon, the underlying mechanisms had not been investigated previously. This study has investigated adaptation using a combination of classical genetics, molecular biology, and single cells analysis, and the main findings are as follows:

Sublethal concentrations of bile trigger changes of gene expression that may facilitate survival. Transcriptomic analysis in the presence of 5% DOC reveals multiple changes in gene expression, some of which provide tentative explanations for the observed increase in bile resistance. Especially relevant is the observation that exposure to DOC activates the RpoS-dependent general stress response. This response facilitates survival of *E. coli* and other gamma-proteobacteria under conditions that do not support active growth, and is also known to be activated by multiple stress conditions. Hence it is

not surprising that bile salts, which denature proteins and cause DNA damage, can trigger the general stress response. The conclusion that the RpoS-dependent general stress response plays a crucial role in bile resistance is supported by additional observations: (i) Lack of RpoS causes bile sensitivity; (ii) *S. enterica* stationary cultures, in which the RpoS response is physiologically active, are more resistant to both DOC and ox bile. The occurrence of extreme levels of resistance (≥14% DOC) in adapted cultures admits several explanations (not mutually exclusive). Activation of the RpoS regulon by sublethal concentrations of bile may be stronger than physiological activation in stationary cells. It is also possible that high levels of bile resistance result from simultaneous activation of more than one bile-resistance response. This latter possibility is supported by the observation that stress-inducible genes that do not belong to the RpoS regulon are also activated by exposure to 5% DOC. Furthermore, exposure to bile upregulates the expression of genes of unknown function, some of which might be part of stress response networks. An example is *yiiU*, which is activated by DOC and is essential for bile resistance (see below). High throughput analysis of gene expression also indicates that adaptation to bile may involve downregulation of porin genes and other genes encoding envelope structures, as well as upregulation of efflux pumps. All these gene expression changes fit well in the literature: porins provide passage to bile salts, envelope structures are major barriers for bile salt uptake, and efflux pumps can transport bile salts outside the cell.

Mutational preadaptation to bile. Preadaptation of *S. enterica* to bile is a different phenomenon, which does not pertain to *Salmonella* populations but to individual bacterial cells. When an aliquot of a *S. enterica* batch culture is plated on LB agar containing a lethal concentration of ox bile, bile-resistant colonies appear at frequencies ranging from 10^{-6} to 10^{-7} per cell and generation. These numbers fall in the known range of bacterial mutation frequencies. Not surprisingly, Luria-Delbrück fluctuation analysis confirms that such colonies arise from bile-resistant cells found in the culture aliquot used for inoculation. Full genome sequencing of 6 bile-resistant mutants revealed that 3 mutants carried mutations in *yrbK* and one in *rlpB*. The high frequency of mutations in lipopolysaccharide transport genes leaves little doubt that altered LPS transport can cause bile resistance. However, the identification of such mutants has intriguing aspects. One comes from the

fact that LPS transport genes are known to be essential in *E. coli*. If such is also the case in *Salmonella*, the mutations found (two nucleotide substitutions, one in-frame deletion and one premature stop codon relatively close to the 3' end of the coding sequence) must all be leaky. Another intriguing question is related to the fact that LPS is a major barrier against bile salts. However, it is conceivable that transport of LPS components across the envelope might sensitize the cell to bile salts, thus explaining why altered transport may confer bile resistance. An alternative, speculative explanation is that the LPS transport proteins altered in bile-resistant mutants might be also involved in LPS assembly and/or modification, and that specific mutations might boost bile resistance. This view may be tentatively supported by the observation that some of the bile-resistant mutants described in this study have altered LPS profiles.

Non mutational preadaptation to bile. Although lethal selection in the presence of bile yields mutants, a fraction of bile-resistant isolates obtained under such conditions are unstable, and lose bile resistance if grown overnight in LB without bile. Hence, preadaptive bile resistance seems to involve also non mutational preadaptation, at first sight an intriguing phenomenon. How can a *Salmonella* batch culture contain cells that are bile-resistant without previous adaptation by growth at sublethal concentrations? However, it is well known that bacterial cultures, albeit genetically clonal, can contain subpopulations of cells with distinct patterns of gene expression, either as a consequence of epigenetic control or as the result of stochastic fluctuations in gene expression. We thus hypothesize that non mutational preadaptation may be caused by activation of bile-resistance responses in the absence of bile. This hypothesis was tested by analyzing expression of *osmY*, an RpoS-dependent gene, and *cspD*, a stress response gene that does not belong to the RpoS regulon, in individual *Salmonella* cells grown in the presence and in the absence of a sublethal concentration of DOC. Microscopic microfluidics confirmed that exposure to DOC activates *osmY* and *cspD* expression in most *Salmonella* cells. However, upregulation of *osmY* and *cspD* expression was also observed in subpopulations of *Salmonella* cells in the absence of DOC. Non mutational preadaptation may thus result from activation of the RpoS-dependent general stress response and/or other stress responses in a cell subpopulation. Stress response activation may either be triggered by a stress situation encountered by individual cells (e. g., increased

concentrations of harmful metabolic products) or be accidental. Repression of specific loci, which has not been addressed in this study, may also contribute to preadaptation.

Role for the cell division factor ZapB in bile resistance. Among the *S. enterica* loci that showed increased expression in the presence of a sublethal concentration of bile a locus of unknown function, annotated as *yiiU*, was found. *In silico* analysis indicated that yiiU was a *S. enterica* homolog of the *zapB* gene of *E. coli*, which encodes a non-essential cell division factor, and complementation analysis proved that the ZapB products of *E. coli* and *Salmonella* are interchangeable. Furthermore, the *Salmonella* ZapB protein was found to be localized at the septum like its *E. coli* counterpart. Disruption of the *Salmonella zapB* locus causes sensitivity to DOC, indicating that *yiiU* is not merely a bile-induced locus but also a gene necessary for bile resistance.

Upregulation of *S. enterica zapB* expression in the presence of DOC is still observed when *zapB* transcription is driven by a heterologous promoter, suggesting the involvement of a postranscriptional mechanism. Comparison of *zapB* mRNA decay in LB and LB + 5% DOC reveals that *zapB* mRNA is more stable in the presence of DOC, thus explaining the higher mRNA level detected both in the initial transcriptomic analysis and in Northern blots. Stabilization of the *zapB* transcript in the presence of DOC requires the Hfq RNA chaperone, an effect that admits two alternative explanations: (i) Hfq binding might protect *zapB* mRNA from degradation; (ii) Hfq might catalyze the interaction of *zapB* mRNA with a small regulatory RNA, and the mRNA:sRNA interaction might increase *zapB* mRNA stability. The latter hypothesis seems more likely considering that Hfq typically catalyzes mRNA:sRNA interactions. *In silico* search for sRNAs containing regions that might interact with *zapB* mRNA by base pairing have identified two relevant candidates, OxyS and RprA, two sRNAS which are known to be produced at higher levels in the presence of bile (J. Hinton, pers, comm.).

A paradox is that the increased *zapB* mRNA level found in the presence of bile does not result in higher amounts of ZapB protein. Actually, the ZapB protein level decreases in the presence of DOC, and ZapB protein analysis in a Lon$^-$ background provides evidence that degradation of ZapB in presence of bile is mediated by the Lon protease. In *E. coli* Lon protease has been shown to play a main role in degradation of abnormally

folded proteins. Because bile salts are known to cause misfolding and denaturation of proteins, we tentatively propose that DOC may cause ZapB misfolding, and that misfolding may trigger degradation by the Lon protease. If this view is correct, increased *zapB* mRNA stability in the presence of bile might provide a mechanism to compensate Lon-mediated degradation and to maintain an intracellular amount of ZapB needed for survive in the presence of bile.

The cause of bile sensitivity in the absence of ZapB remains unknown. However, it is remarkable that another non-essential cell division factor, DamX, is also necessary for bile resistance in both *S. enterica* and *E. coli*. It is thus conceivable that perturbations in Z-ring assembly might render the *Salmonella* cell bile-sensitive. This view is consistent with the major role played by the bacterial envelope as a protective barrier against bile salts.

Role for the cell wall remodeling in bile resistance. Characterization of a bile-resistant mutant obtained in a genetic screen indicated that lack of the periplasmic protease Prc increased bile resistance. Because Prc is known to be involved in processing of peptidoglycan-binding protein 3 (PBP3), we analyzed PBP activity in a Prc^{-} background, and detected multiple PBP alterations. This observation suggested that peptidoglycan remodeling might contribute to bile resistance. Our analysis of peptidoglycan structure in the presence of bile was further stimulated by previous evidence indicating that *S. enterica* remodels peptidoglycan in response to diverse environmental cues including oxidative stress (and bile salts are known to cause oxidative damage).

Growth of *S. enterica* in the presence of a sublethal concentration of DOC was accompanied by a reduction in the amount of Braun protein anchored to peptidoglycan. Evidence that this reduction is associated with bile resistance was provided by two observations: (i) *S. enterica* cultures adapted to grow in the presence of a lethal concentration of DOC also showed reduced amounts of muropeptide-bound Braun lipoprotein; (ii) a *S. enterica* mutant lacking Braun protein anchored to peptidoglycan was found to be hyperesistant to bile. Because peptidoglycan-bound Braun lipoprotein provides covalent linkage between the outer membrane and the peptidoglycan, reduction or loss of this union could decrease rigidity of the cell envelope, perhaps altering outer

membrane fluidity. In some cases, increase of membrane fluidity has been associated with sensitivity to envelope-damaging compounds. However, other studies have suggested that increase of outer membrane fluidity might permit resistance to stress conditions and antibacterial compounds. The latter examples may provide analogies to understand why reduction of peptidoglycan-bound lipoprotein increases bile resistance.

A reduction in 3-3 crosslinks was also detected in the presence of bile, suggesting that crosslinking may be reduced in the presence of bile. In support of this view, overproduction of YnhG transpeptidase causes a strong decrease in bile resistance, indicating that high levels of crosslinking may cause bile sensitivity. We thus tentatively conclude that low crosslinking or absence of crosslinking may be compatible with bile resistance while high levels of crosslinking may be associated with bile sensitivity.

CONCLUSIONS

1. *Salmonella enterica* can survive in the presence of lethal concentrations of bile by three distinct mechanisms: adaptation, mutational preadaptation and non mutational preadaptation. Non mutational preadaptation to bile is described for the fist time in this study.

2. Adaptation of *Salmonella enterica* to bile is associated with multiple changes in gene expression, which include upregulation of the RpoS-dependent general stress response and other stress responses.

3. Individual *Salmonella* cells can become bile-resistant without adaptation. A fraction of such isolates are stable, indicating that bile resistance can be acquired by mutation. Full genome sequencing of bile-resistant mutants shows that alteration of lipopolysaccharide transport is a frequent cause of mutational bile resistance.

4. Selection in the presence of a lethal concentration of provides bile-resistant isolates of *S. enterica* that are not mutants. We propose that such isolates derive from rare cells whose physiological state permitted survival upon encountering bile. Single cell analysis of gene expression confirms that batch cultures of *Salmonella* contain cells that activate stress response genes in the absence of DOC. Hence, phenotypic heterogeneity in clonal bacterial populations of *Salmonella* may explain the origin of non-mutational preadapted isolates.

5. The cell division factor ZapB is essential for bile resistance.

6. Bile induces remodeling of the peptidoglycan layer in the *Salmonella enterica* cell wall. Reduced amounts of Braun lipoprotein covalently anchored to peptidoglycan and decreased levels of 3-3 cross-links may contribute to bile resistance.

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June 26, 2013

Dear Dr. Casadesús,

We have the pleasure to inform you that your manuscript ref. 13-048 **Increased bile resistance in *Salmonella enterica* mutants lacking Prc periplasmic protease,** authored by Sara B. Hernández, Juan A. Ayala, Gadea Rico-Pérez, Francisco García-del Portillo, Josep Casadesús has been accepted for publication.

The manuscript is scheduled to be included in the next issue of our journal, INTERNATIONAL MICROBIOLOGY.

We thank you for your interest in INTERNATIONAL MICROBIOLOGY.

.

Sincerely yours,
Ricardo Guerrero & José Berenguer
Coeditors-in-Chief

Adaptation and Preadaptation of *Salmonella enterica* to Bile

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Abstract

Bile possesses antibacterial activity because bile salts disrupt membranes, denature proteins, and damage DNA. This study describes mechanisms employed by the bacterium *Salmonella enterica* to survive bile. Sublethal concentrations of the bile salt sodium deoxycholate (DOC) adapt *Salmonella* to survive lethal concentrations of bile. Adaptation seems to be associated to multiple changes in gene expression, which include upregulation of the RpoS-dependent general stress response and other stress responses. The crucial role of the general stress response in adaptation to bile is supported by the observation that $RpoS^{-}$ mutants are bile-sensitive. While adaptation to bile involves a response by the bacterial population, individual cells can become bile-resistant without adaptation: plating of a non-adapted *S. enterica* culture on medium containing a lethal concentration of bile yields bile-resistant colonies at frequencies between 10^{-6} and 10^{-7} per cell and generation. Fluctuation analysis indicates that such colonies derive from bile-resistant cells present in the previous culture. A fraction of such isolates are stable, indicating that bile resistance can be acquired by mutation. Full genome sequencing of bile-resistant mutants shows that alteration of the lipopolysaccharide transport machinery is a frequent cause of mutational bile resistance. However, selection on lethal concentrations of bile also provides bile-resistant isolates that are not mutants. We propose that such isolates derive from rare cells whose physiological state permitted survival upon encountering bile. This view is supported by single cell analysis of gene expression using a microscope fluidic system: batch cultures of *Salmonella* contain cells that activate stress response genes in the absence of DOC. This phenomenon underscores the existence of phenotypic heterogeneity in clonal populations of bacteria and may illustrate the adaptive value of gene expression fluctuations.

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Introduction

Bile is a fluid containing bile salts, cholesterol, and a variety of proteins and electrolytes [1]. Bile is synthesized by parenchymal cells (hepatocytes) in the liver. In mammals with a gall bladder, a fraction of bile is stored in the gall bladder while another fraction flows directly into the small intestine [1]. When food passes by the small intestine, gall bladder contraction releases bile into the duodenum. Bile aids in the digestion of fats, facilitates absorption of fat-soluble vitamins in the intestine, and contributes to the elimination of excess cholesterol and waste metabolic products produced in the liver [1].

About two thirds of bile (dry weight) are made of bile salts, a family of molecules with steroid structure which derive from cholesterol [2]. Bile salts dissolve membrane lipids and cause dissociation of integral membrane proteins. Inside the cell, the detergent activity of bile salts causes misfolding and denaturation of proteins [3,4]. Chelation of calcium and iron by bile salts is also a source of physiological perturbations [3,4]. Furthermore, bile salts have DNA damaging capacity, stimulate DNA rearrangements, and induce plasmid curing [4,5,6,7]. However, certain bacterial species are resistant to the antibacterial activities of bile salts [3,8]. This trait has been exploited for the design of selective microbiological media such as the one-century-old MacConkey agar used in the identification of genera of the family Enterobacteriaceae. On the other hand, bile salts regulate the expression of specific bacterial genes, some of them necessary for bile resistance and others involved in pathogenesis [3,8]. Bile salts may thus be viewed both as environmental signals used by bacteria to identify bile-containing animal environments and as antibacterial compounds [8].

An extreme example of bile-resistant pathogen is *Salmonella enterica*, which colonizes the hepatobiliary tract during systemic infection and persists in the gall bladder during chronic infection [9,10]. *Salmonella* survival in the mammalian gall bladder seems to involve several strategies. Invasion of the gall bladder epithelium may permit escape from the extremely high concentrations of bile salts present in the gall bladder lumen [11]. Formation of biofilms on gallstones may also protect *Salmonella* from the bactericidal activities of bile salts [12,13]. However, planktonic *Salmonella* cells are also found at high numbers in the bile-laden gall bladder lumen, and the mechanisms employed to thrive in such a harsh environment remain to be identified.

Bile resistance can be studied under laboratory conditions by adding ox bile or individual bile salts to microbiological media

Author Summary

This study describes mechanisms employed by the bacterium *Salmonella enterica* to survive bile: adaptation, mutation, and non-mutational preadaptation. Adaptation is easily observed in the laboratory: when a *Salmonella* culture is grown in the presence of a sublethal concentration of the bile salt sodium deoxycholate (DOC), the minimal inhibitory concentration of DOC increases. Adaptation appears to be associated to multiple changes in gene expression induced by DOC. Mutational bile resistance is also a common phenomenon: plating on agar containing a lethal concentration of bile yields bile-resistant colonies. Fluctuation analysis indicates that such colonies derive from bile-resistant cells present in the previous culture. However, selection on lethal concentrations of bile also provides bile-resistant isolates that are not mutants. Non-mutational preadaptation, a non-canonical phenomenon *a priori*, suggests that batch cultures contain rare *Salmonella* cells whose physiological state permits survival upon encountering bile. The view that non-mutational preadaptation may be a consequence of phenotypic heterogeneity is supported by the observation that *Salmonella* cultures contain cells that activate stress response genes in the absence of DOC.

[14]. Genetic and biochemical analysis in *E. coli* and *S. enterica* in the laboratory has permitted the identification of cell functions and mechanisms involved in bile resistance [3,8,14]. The relevance of these reductionist studies is supported by the fact that mutations that cause bile sensitivity *in vitro* often result in virulence attenuation in the mouse model of *S. enterica* infection [14]. The list of bile resistance factors in *Salmonella* and other enteric species includes envelope barriers such as the lipopolysaccharide [15,16] and the enterobacterial common antigen [17], the outer membrane [18,19], the cytoplasmic membrane [20], efflux pump systems [21], genes of the multiple antibiotic resistence (*mar*) and PhoPQ regulons [22,23], and DNA repair functions [5,6]. Genetic analysis has also identified cell functions whose loss increases bile resistance, probably by activating cell defense responses [20].

The bile resistance level of wild type *Salmonella* can be increased over the customary minimal inhibitory concentration by growth in the presence of sublethal concentrations of bile [8,24]. This phenomenon, henceforth called "adaptation", is easily observed in the laboratory and may be relevant during *Salmonella* colonization of the hepatobiliary tract. Below we describe studies of *Salmonella* adaptation to bile *in vitro*, and show that growth of *S. enterica* on sublethal concentrations of bile is accompanied by dramatic changes in gene expression. We also report that batch *Salmonella* cultures contain cells that show high levels of bile resistance without previous adaptation. This phenomenon, henceforth called "preadaptation", seems to involve two unrelated processes. One is mutation in specific loci, often related to lipopolysaccharide transport; another is activation of bile resistance responses in a subpopulation of bacterial cells. The latter phenomenon fits well in current views indicating that bacterial populations are heterogeneous, and that fluctuations in gene expression can have adaptive value [25,26].

Results

Viability of *S. enterica* SL1344 in the presence of sodium deoxycholate

The minimal inhibitory concentration (MIC) of sodium deoxycholate for *S. enterica* strain SL1344 grown in LB is 7%, and the MIC of ox bile is 12% under the same conditions. These MICs are similar to those previously reported for strain ATCC 14028 [5,6]. To ascertain whether inhibition of bacterial growth by bile salts involves bacterial death or merely growth arrest, we performed viability tests to distinguish live and dead *Salmonella* cells in the presence of DOC. Aliquots from *Salmonella* exponential cultures grown in LB were treated with various concentrations of DOC (1%, 3%, 5%, 7%, and 9%) for 30 min. Examination under the microscope using a commercial live/dead color-based kit was then performed, and the numbers of live/dead cells were counted. Cell counting was randomly performed, and the minimal number of bacterial cells counted was 1,700. A representative experiment is shown in Figure 1, top panel. A direct correlation was found between the percentage of dead *Salmonella* cells and the concentration of DOC.

The bactericidal capacity of bile was also monitored by plaque counts of colony-forming-units. Aliquots from *Salmonella* exponential cultures grown in LB were treated with various concentrations of DOC (1%, 3%, 5%, 7%, and 9%) for 30 min. The cultures were then diluted, plated on LB, and incubated overnight at 37°C. Colony counts confirmed that exposure to DOC renders *S. enterica* cells inviable in a dose-dependent maner (Figure 1, bottom panel).

Adaptation of *S. enterica* to lethal concentrations of sodium deoxycholate

Although the level of resistance to bile is fairly constant under given conditions, *Salmonella* can be adapted to grow at higher concentrations of bile by previous growth in the presence of sublethal concentrations [8]. To determine the concentration(s) of DOC that permit adaptation in strain SL1344, *S. enterica* cultures were grown in LB containing different concentrations of DOC (from 1% to 7%). Aliquots from the cultures were then transferred to microtiter plates containing DOC at concentrations ranging from 1% to 14%. As shown in the diagram of Figure 2, growth at concentrations slightly lower than the MIC (4% and 5%) increased the MIC of DOC to ≥14%. A smaller increase of the MIC was likewise observed after growth in 3% DOC.

The bacterial cells that had survived bile in the microtiter plate were transferred to LB and cultured overnight. Aliquots from these cultures were then used to determine the MIC of DOC in microtiter plates. As shown in Figure 2, a MIC of 7% DOC was determined for all cultures, indicating that resistance had decreased back to the level characteristic of strain SL1344. Hence, adaptation to DOC by growth at concentrations of 3–5% is reversible, and does not involve selection of bile-resistant mutants, at least under the conditions tested.

Transcriptomic analysis of *S. enterica* gene expression during growth on sublethal concentrations of sodium deoxycholate

The capacity of bile salts to induce changes in gene expression is well known in *S. enterica* [22,27] and in other bacteria [3], as well as in eukaryotes [28]. On these grounds, we hypothesized that the reversible increase of MIC observed when *S. enterica* is grown in the presence of sublethal concentrations of DOC might involve changes in gene expression. This hypothesis was tested by transcriptomic analysis using the Salgenomics microarray [29].

RNA extraction was performed in exponential and stationary cultures grown in LB with and without 5% DOC ($O.D._{600} = 0.4$ and $O.D._{600} \geq 1$, respectively). *S. enterica* grew at slower rates in LB+DOC than in LB, and the concentration of bacterial cells in LB+DOC reached a plateau well below the LB control. However, growth did occur, thus indicating that 5% is a sublethal

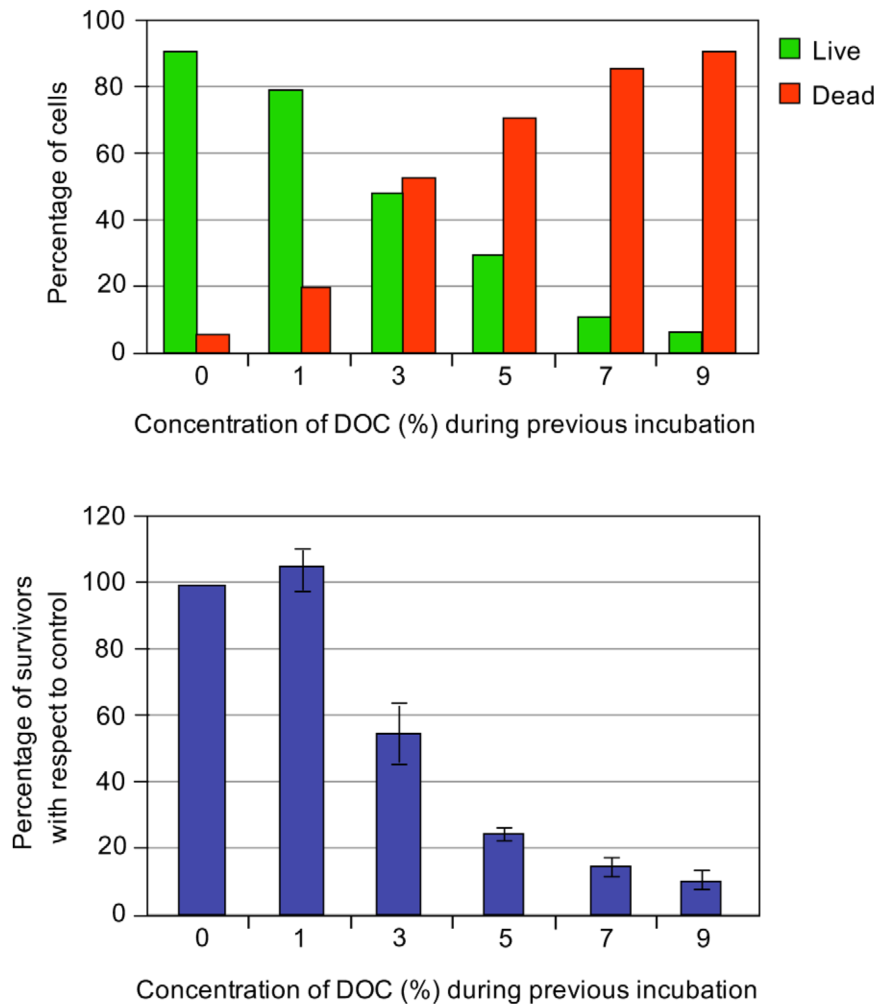

Figure 1. Percentages of live and dead bacteria and relative numbers of colony-forming units. Top panel: Percentages of live and dead bacteria (green and red histograms, respectively) found in 1 ml aliquots of an exponential culture of *S. enterica* SL1344 incubated in the presence of different concentrations of sodium deoxycholate (1%, 3%, 5%, 7% and 9%) during 30 minutes at 37°C. Bottom panel: Relative numbers of colony forming-units (CFU) after incubation of *S. enterica* SL1344 in the presence of different concentrations of sodium deoxycholate (1%, 3%, 5%, 7% and 9%) during 30 minutes at 37°C. The number of CFU in the absence of DOC is shown as 100%.
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concentration of DOC under such conditions (LB medium, 37°C). Note that these conditions are different from those used in the viability tests described above. This does not exclude, of course, that a fraction of bacterial cells may have been killed, and that the culture derives from the surviving subpopulation. Under these conditions, a large number of *Salmonella* loci showed differences in their RNA levels depending on whether DOC was present or absent. Raw data from transcriptomic analysis in the presence of 5% DOC have been deposited at the Array Express database (http://www.ebi.ac.uk/miamexpress) with accession number E-MTAB-637. Relevant data are summarized in Table 1 and Table 2. The main conclusions from these experiments can be summarized as follows:

(i) The RpoS-dependent genes *osmY*, *dps*, *uspB*, and *ecnB* [30] were found to be strongly upregulated by DOC in exponential cultures. The poorly characterized RpoS-dependent gene *ybiF*, located next to *dps* on the *Salmonella* chromosome, showed also >3 fold upregulation. In stationary cultures, *dps* and *osmY* were also upregulated by DOC. Their lower upregulation under such conditions is consistent with the fact that the RpoS-dependent general stress response is activated in non dividing cells [31]. These observations suggested that activation of the RpoS-dependent general stress response might play a role in adaptation to bile. A previous transcriptomic study in strain ATCC 14028 did not detect RpoS activation upon exposure to DOC [22]. However, the concentration of DOC used by Prouty et al. (3% ox bile) [22] may have been insufficient to trigger RpoS activation. In fact, under the conditions used in our study, concentrations of DOC much higher than those expected to be present in 3% ox bile (around 1% bile salts [1]) did not adapt *S. enterica* to bile (Figure 2).

(ii) The general stress response is not the only stress response activated by sublethal concentrations of bile salts: the stress-inducible *cspD* gene [32,33] was found to be upregulated by DOC in both exponential and stationary cultures, and the *uspA* gene [34] in stationary cultures only.

(iii) The outer membrane protein (OMP) genes *ompC* and *ompD* [35] were downregulated by DOC during exponential

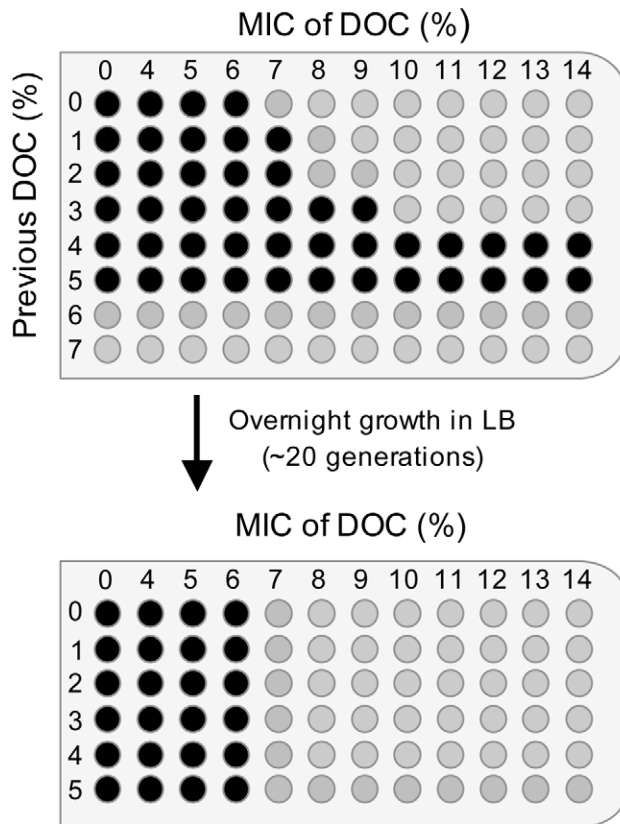

Figure 2. Minimal inhibitory concentrations (MICs) of sodium deoxycholate (DOC) for *Salmonella* cultures pre-exposed to various concentrations of DOC, and MICs for the same cultures after overnight growth in LB.
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growth. Because porins provide passage to bile salts [8], downregulation of *ompC* and *ompD* may be tentatively interpreted as a defensive modification of the outer membrane to decrease uptake of bile salts.

(iv) Modification of the cell envelope may not be limited to outer membrane remodeling. Downregulation of *mltB*, a gene involved in peptidoglycan recycling [36], may provide evidence for cell wall changes produced in response to DOC. The crucial role of the cell envelope in bile resistance is well established in the literature [15,17,18,19,37].

(v) A sublethal concentration of DOC upregulated *acrD*, which encodes a component of a multidrug resistance efflux pump [38]. Because efflux systems are known to transport bile salts outside the cell [21], upregulation of *acrD* may be viewed as another defensive response. Upregulation of other transport genes (*ugpB* and *pnuC*) was also observed but it is difficult to interpret.

(vi) *Salmonella* pathogenicity islands SPI-1 and SPI-2 were strongly downregulated by DOC, as previously described by other authors [27,39]. Downregulation was observed in stationary cultures only, a result consistent with the fact that neither SPI-1 nor SPI-2 are expressed in exponential cultures [40].

(vii) Miscellaneous gene expression changes of difficult interpretation are also presented in Table 1 and Table 2. Changes in the synthesis of cytochrome components may suggest alterations in electron transport. The possibility that chemotaxis is altered in the presence of DOC may be also considered, as previously proposed [22]. Altered expression of numerous metabolic genes is also observed.

Table 1. *S. enterica* loci showing altered expression (>3 fold) in the presence of 5% DOC during exponential growth.

Locus	Function of product	Fold change
aroG	Phenylalanine biosynthesis	+9.94
osmY	RpoS-dependent general stress response	+7.10
dps	RpoS-dependent general stress response	+7.04
ecnB	Entericidin synthesis	+6.28
ugpB	ABC transporter	+5.02
yiiU	Unkown function	+4.58
cspD	Stress response	+3.55
cyoA	Cytochrome oxidase	+3.39
cfa	Fatty acid synthesis	+3.22
pnuC	Nucleoside transport	+3.11
acrD	Efflux pump	+3.00
sseA	SPI-2 virulence effector	−12.01
malZ	Maltose catabolism	−5.28
ssaS	Component of the SPI-2 secretion apparatus	−4.48
ompC	Outer membrane	−3.42
ompF	Outer membrane	−3.39
ibpA	Heat shock	−3.02
cheM-cheW	Chemotaxis	−3.01
mltB	Peptidoglycan synthesis	−3.00

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Validation of microarray analysis using *lac* fusions

Data provided by transcriptomic analysis were validated by monitoring the effect of DOC on the expression of transcriptional *lac* fusions in 14 *S. enterica* genes. As a general rule, we chose genes which had shown strong expression changes in the presence of DOC. The sample included genes of the RpoS regulon (*osmY*, *dps*, and *ecnB*), a transport gene (*ugpB*), a metabolic gene (*aroG*), and two SPI-1 genes (*hilA* and *prgH*). Genes of unknown function, present in all enterics (*yiiU*, *yceK*, *ytfK*, *ybjM*, and *yajI*) or *Salmonella*-specific (*STM1441* and *STM1672*), were also included. The selection of these loci was based on the bile-sensitive phenotype of their mutants (e. g., *yiiU*), their DNA sequence relatedness to known bile resistance genes (e. g., *STM1441*, encoding a putative efflux pump) or the cellular location of their products (e. g., *yceK*, *yajI* and *STM1642*, which may encode outer membrane proteins, and *ybjM*, which may encode a cytoplasmic membrane protein). ß-galactosidase activities were measured in LB and in LB containing 5% DOC. Raw data are shown in Tables S1 and S2. Figure 3 is an elaboration that compares the ß-galactosidase activities of the *lac* fusions and the expression levels of the corresponding mRNAs detected by microarray analysis. Although differences in expression levels are observed, a correlation between mRNA content and ß-galactosidase activity is found in all cases.

Activation of the RpoS-dependent general stress response in the presence of sublethal concentrations of DOC was further analyzed by monitoring expression of RpoS-dependent genes other than *dps* and *osmY*. An additional goal of these experiments was to confirm

Table 2. *S. enterica* loci showing altered expression (>3 fold) in the presence of 5% DOC during stationary phase.

Locus	Function of product	Fold change
cyoA	Cytochrome oxidase	+26.33
mdh	Central metabolism	+12.02
tsr	Chemotaxis	+10.38
shdB	Central metabolism	+8.23
sucA	Central metabolism	+8.23
ugpB	ABC transporter	+7.40
ompF	Outer membrane	+7.34
uspA	Stress response	+6.50
dps	RpoS-dependent general stress response	+5.42
filE-fliF	Flagellum	+5.08
narG-narK	Nitrate reduction	+4.63
ahpC-ahpF	Stress response	+4.62
nagB-nagE	PTS system	+4.56
uspB	RpoS-dependent general stress response	+4.06
cspD	Stress response	+3.70
osmY	RpoS-dependent general stress response	+3.35
nirD-nirC	Nitrite reduction	+3.26
phdR	Central metabolism	+3.24
ppa	Central metabolism	+3.19
fpb	Central metabolism	+3.18
rrmA	Ribosomal RNA modification	+3.17
cydA	Cytochrome oxidase	+3.17
pipA-pibB	SPI-1 virulence effectors	−119.08
sseA	SPI-2 virulence effector	−68.34
sopE	SPI-1 virulence effector	−63.96
sifB	SPI-2 virulence effector	−54.29
ssaS-ssaT	SPI-2 secretion apparatus	−39.73
ssaB	SPI-2 secretion apparatus	−17.48
hilC	SPI-1 regulatory protein	−12.85
prgH	SPI-1 regulatory protein	−10.51
hilD	SPI-1 regulatory protein	−10.50
pagC	Intracellular survival in macrophages	−9.94
iroN	Siderophore	−9.23
sseJ	SPI-2 virulence effector	−7.91
valW	tRNA	−6.41
potC	Spermidine/putrescine transporter	−6.08
sifA	SPI-2 virulence effector	−6.08
sifB	SPI-2 virulence effector	−5.17
invH	SPI-1-encoded outer membrane lipoprotein	−5.10
cheM-cheW	Chemotaxis	−4.33
hilA	SPI-1 regulatory protein	−4.14
tnpA	IS*200* transposase	−4.09
traX	Conjugal transfer of the virulence plasmid	−3.39
pagD	Resistance to antimicrobial peptides	−3.18
pyrI	Central metabolism	−3.15

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that DOC upregulated the RpoS regulon in both exponential and stationary cultures. For these experiments, *lac* fusions in *katE*, *xthA*, *ots*, *dps*, and *osmY* were used. ß-galactosidase activities were

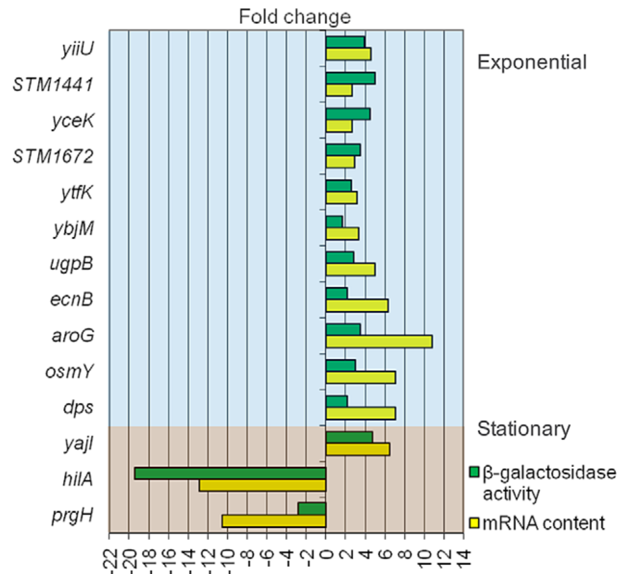

Figure 3. Validation of transcriptomic analysis: comparison of gene expression differences between LB and LB+5% deoxycholate as measured by RNA content (microarray analysis) and activity of *lac* fusions.
doi:10.1371/journal.pgen.1002459.g003

measured in LB and in LB containing 5% DOC. Expression in exponential cultures was tested at an $O.D._{600} = 0.4$. Stationary cultures were tested at an $O.D._{600} \geq 1$. Data shown in Table 3 and Table 4 confirmed that the RpoS regulon is upregulated by DOC. Upregulation occurs in both exponential and stationary cultures, and the induction ratios vary depending on the gene under study.

Identification of bile-responsive genes necessary for bile resistance

The identification of genes whose expression was altered in the presence of sublethal concentrations of DOC raised the possibility that such loci might be necessary for bile resistance. We thus tested the MIC of sodium deoxycholate for mutants carrying loss-of-function mutations in 16 genes identified above as upregulated by bile: *acrD*, *yiiU*, *STM1441*, *yceK*, *STM1672*, *ytfK*, *ybjM*, *ugpB*, *ecnB*, *aroG*, *osmY*, *dps*, *yajI*, *katE*, *ots*, and *xthA*. Results from these trials are shown in Table S3, and can be summarized as follows:

Table 3. ß-galactosidase activities of *lac* fusions in RpoS-regulated genes in the presence and in the absence of 5% sodium deoxycholate during exponential growth.

Strain	Gene fusion	LB	LB+DOC
SV6065	*katE::lac*	104±33	758±121
SV6888	*katE::lac rpoS*	10±3	18±3
SV6066	*ots::lac*	66±5	267±34
SV6067	*xthA::lac*	5±1	13±5
SV6068	*osmY::lac*	17±4	95±9
SV6069	*dps::lac*	5±2	12±2

ß-galactosidase activities are shown in Miller units. Data are averages and standard deviations from 3–5 independent experiments.
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Table 4. ß-galactosidase activities of *lac* fusions in RpoS-regulated genes in the presence and in the absence of sodium deoxycholate in stationary phase.

Strain	Gene fusion	LB	LB+DOC
SV6065	*katE::lac*	512±88	1204±104
SV6888	*katE::lac rpoS*	14±6	22±5
SV6066	*ots::lac*	200±74	1575±220
SV6067	*xthA::lac*	32±4	63±11
SV6068	*osmY::lac*	105±27	370±49
SV6069	*dps::lac*	30±10	71±14

ß-galactosidase activities are shown in Miller units. Data are averages and standard deviations from 3–5 independent experiments.
doi:10.1371/journal.pgen.1002459.t004

(i) The only bile-sensitive mutant was YiiU$^-$ (MIC of DOC≅1.5%, compared with 7% in the wild type). Because this locus is virtually unknown, no explanation can be offered for its role in bile resistance.

(ii) The observation that the AcrD$^-$ mutant was not bile-sensitive is in agreement with previous observations made in strain ATCC 14028 [41], and can be explained by redundancy: *S. enterica* possesses multiple efflux systems, many of them versatile and with overlapping substrate specificity. Hence, bile sensitivity occurs only if multiple efflux systems are eliminated [41]. In agreement with this view, a TolC$^-$ mutant (strain SV6629), which lacks an outer membrane protein of all RND efflux pumps [38,41], showed extreme sensitivity to DOC (MIC≅0.02%, 350 fold lower than the MIC for the wild type). Extreme bile sensitivity of a TolC$^-$ mutant has been likewise described in strain ATCC 14028 [41].

(iii) Redundancy may also explain why mutants lacking individual RpoS-dependent genes (*osmY*, *dps*, *xthA*, *katE*, and *ots*) are not bile-sensitive. However, an RpoS$^-$ derivative of SL1344 (strain SV5561) showed a MIC of DOC≅3%. Hence, an active RpoS regulon appears to be necessary for bile resistance but the individual RpoS-dependent functions tested in this study (OsmY, Dps, XthA, KatE, and Ots) are dispensable.

Isolation of bile-resistant derivatives of *S. enterica* SL1344

Non adapted *Salmonella* populations (e. g., laboratory cultures in LB) are unable to grow on lethal concentrations of bile. However, bile resistance can be acquired by mutation, and bile-resistant mutants can be easily isolated upon plating on lethal concentrations of bile [14,37]. However, previous descriptions of bile-resistant mutants had involved transposon insertions, which usually cause loss of function and are lethal if they occur in essential genes. To avoid these constraints, we isolated bile-resistant mutants of *S. enterica* of spontaneous origin. Aliquots from a *S. enterica* culture grown in LB were plated on LB supplemented with a lethal concentration of ox bile (180 g/l). Use of ox bile instead of DOC was justified by the fact that high concentrations of DOC prevent agar solidification. Bile-resistant colonies appeared at frequencies ranging between 10^{-6} and 10^{-7} per cell and generation. Because high concentrations of bile salts are bactericidal (Figure 1), we expected that bile-resistant colonies would derive from bile-resistant cells present in the previous culture. This hypothesis was supported by Luria-Delbrück fluctuation analysis [42]: the averages of bile-resistant colonies obtained from independent cultures showed a variance much higher than the averages from a single culture (Table S4).

Bile-resistant colonies were purified in LB and plated again on LB+bile to confirm bile resistance. During these routine purification procedures, we made the unexpected observation that a relatively large number of bile-resistant isolates had become bile-sensitive. A systematic analysis of the phenomenon was then carried out. Bile resistant isolates were obtained by plating strain SL1344 on LB+ox bile. Colonies were transferred to LB and grown overnight. The MIC of DOC for each isolate was then determined in microtiter plates. These trials confirmed that bile-resistant isolates were of two types: (i) stable, putatively carrying mutations that confer bile resistance; (ii) unstable isolates that lose bile resistance, either partially or completely, upon nonselective growth in LB. The frequencies of mutants and unstable isolates varied from one trial to another (data not shown). A representative experiment involving 59 independent bile-resistant isolates is shown in Figure 4. In this case, 10 isolates turned out to be mutants while the other 49 were unstable bile-resistant isolates.

Characterization of bile-resistant mutant derivatives of *S. enterica* SL1344

Six spontaneous bile-resistant mutants (MIC of DOC ≥14%) of independent origin were chosen for full genome sequencing with the SOLiD platform [43]. Genome sequencing was followed by alignment with the *S. enterica* SL1344 genome sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs) to identify DNA sequence differences. The genome of the laboratory stock of *S. enterica* SL1344 was also sequenced as a control. The mutations found are described in Table 5. Relevant observations are as follows:

(i) Four bile-resistant strains were single mutants. Mutants #1 and #6 harbored a nucleotide substitution and an in-frame deletion, respectively, in the *S. enterica yrbK* gene. YrbK (recently renamed LptC) is a lipopolysaccharide transport protein in *E. coli* [44]. Mutant #3 harbored a nucleotide substitution in the *rlpB* gene (also known as *lptE*), which encodes the *Salmonella* homolog of *E. coli* RlpB, a lipopolysaccharide assembly protein [45]. Mutant #4 harbored a nucleotide substitution in the poorly known *deaD* gene, which in *E. coli* encodes a putative ATP-dependent RNA helicase [46].

(ii) Mutants #2 and #5 were double mutants. Interestingly, one of the mutations found in strain #2 mapped in the lipopolysaccharide transport gene *yrbK*. Strain #2 carried

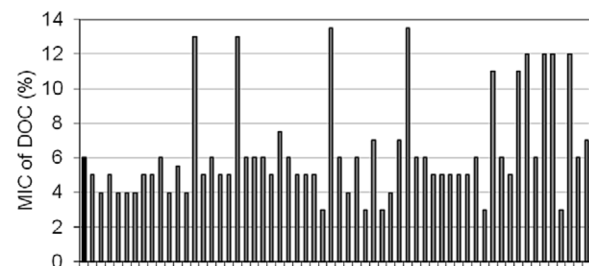

Figure 4. Minimal inhibitory concentration of sodium deoxycholate for bile resistant isolates after non-selective growth in LB. The isolates had been originally obtained on plates containing 18% ox bile.
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Table 5. Mutations present in the genomes of bile-resistant derivatives of *S. enterica* SL1344.

Mutant	Locus affected	Mutation[a]	Location of the mutation[b]	Predicted mutational change	Cellular function affected
1	*yrbK*	G→C substitution	Nucleotide 182	Arg→Pro	Lipopolysaccharide transport
2	*yrbK*	+1 frameshift	After nucleotide 399	Premature stop codon after amino acid 134	Lipopolysaccharide transport
2	Putative intergenic region on plasmid 2	A→G substitution	Base pair 13926	Unknown	Unknown
3	*rlpB*	C→A substitution	Nucleotide 287	Ala→Glu	Lipopolysaccharide transport
4	*deaD*	C→G substitution	Nucleotide 923	Ala→Gly	Putative ATP-dependent RNA helicase
5	*strA* locus on plasmid 3	+1 frameshift	Base pair 7949	Unknown	Unknown
5	*sul2* locus on plasmid 3	+1 frameshift	Base pair 38	Unknown	Unknown
6	*yrbK*	Deletion of 30 nucleotides	Base pairs 415–444	Loss of 10 amino acids	Lipopolysaccharide transport

[a]Nucleotide change is indicated for the coding sequence, when known.
[b]Base pair numbers are those of the annotated genome of *S. enterica* strain SL1344 (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs).
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an additional mutation in a putative intergenic region of plasmid 2. In turn, mutant #5 carried frameshifts in two putative loci of unknown function (annotated as *strA* and *sul2*) on plasmid 3. These plasmids are specific of strain SL1344 and have not been described in other strains of serovar Typhimurium. A potential role of these plasmids in adaptation to bile seems unlikely, because strain ATCC 14028, which lacks the plasmids, shows MICs of DOC and bile virtually identical to those of SL1344 [5,17,19]. The occurrence of plasmid-borne mutations that cause bile resistance is thus interesting but difficult to interpret.

Bile-resistant mutants carrying mutations in known genes (*yrbK*, *rlpB*, and *deaD*) were reconstructed (see Materials and Methods for reconstruction procedures). All reconstructed mutants showed a MIC of DOC ≥14%, thus confirming that single mutations in *yrbK*, *rlpB*, and *deaD* caused the bile-resistant phenotype of these isolates. The cause of bile resistance in the DeaD$^-$ mutant was not further investigated since *deaD* is a poorly known gene [46]. In contrast, the high frequency of mutations found in lipopolysaccharide transport genes (3 in *yrbK* and 1 in *rlpB*) provided evidence that alterations in lipopolysaccharide transport can cause bile resistance. LPS transport genes are known to be essential in *E. coli* [47,48]. If such is also the case in *S. enterica*, the mutations detected must be leaky. Leakiness may seem normal for the G→C (*yrbK*) and C→A (*rlpB*) substitutions detected in mutants #1 and #3, and even for the *yrbK* in-frame deletion detected in mutant #6. The mutation detected in strain #2 (*yrbK*), however, is a frameshift, a mutation type that often causes loss of function. In fact, the frameshift consists of a C insertion, and results in the formation of a premature stop codon (TAA) eight nucleotides downstream. However, these changes map near the C-terminal region, suggesting that a truncated YrbK protein may be leaky. The view that the C-terminal region of YrbK is dispensable is further supported by the observation that the in-frame deletion found in mutant #6 maps in the same region (Table 5).

Analysis of lipopolysaccharide in bile-resistant mutants

Because the *rlpB* and *yrbK* genes have been described in *E. coli* as involved in LPS transport across the periplasm and LPS assembly at the outer membrane [49], we examined whether the bile-resistant mutants under study showed LPS alterations. The LPS of the DeaD$^-$ mutant was also examined. Migration of the LPS in polyacrylamide gel is known to be affected by the number and size of repeating oligosaccharide units in long-chain LPS, such that bands in the profile represent progressively larger concatemers of the repeating oligosaccharide units [45]. Comparison of the LPS profiles of the mutants and the wild type shows that the *rlpB*, *yrbK*, *and deaD* mutations under study do not visibly alter the amount of LPS. However, structural differences are clearly observed between the wild type and mutants #1, #2, and #6, which carry *yrbK* mutant alleles (Figure 5). Reconstructed YrbK$^-$ mutants showed LPS profiles identical to those of their parental mutants (Figure S1). The profiles found in these mutants may indicate differences in the oligosaccharide units that form long-chain LPS [48].

Single cell analysis of gene expression in the presence and in the absence of sodium deoxycholate

The observation that preadaptation of *S. enterica* to bile can occur by reversible, non mutational mechanisms raised the possibility that the bacterial population might contain cells which activate bile resistance responses in the absence of bile. This hypothesis was initially tested by examining the expression level of *osmY*, a gene of the RpoS regulon, in single *Salmonella* cells grown in the presence and in the absence of a sublethal concentration of DOC (5%, the same concentration used for transcriptomic analysis). To monitor *osmY* expression, a green fluorescent protein (GFP) fusion was constructed at the 3′ end of the *osmY* coding sequence (strain SV6562). Expression of the *osmY*::GFP fusion in individual *Salmonella* cells was monitored using a microscope automated fluidic system [50]. These experiments were of two kinds:

(i) Single point experiments: *S. enterica* cultures grown in LB and LB+5% DOC were diluted and transferred to agar pads. Ten fields, each containing ≥30 cells, were manually defined, and the fluorescence level of individual cells was measured [50]. Two representative, independent experiments are shown in Figure 6A and 6B. Exposure to 5% DOC increased *osmY*::GFP expression in an heterogeneous but consistent manner (red histograms). Cells grown in the absence of DOC (grey histograms) showed lower and more homogeneous levels of *osmY*::GFP expression. However, a significant degree of heterogeneity was observed, indicating that some cells activate the RpoS general stress response in the absence of DOC. Comparison of panels A and B reveals that the

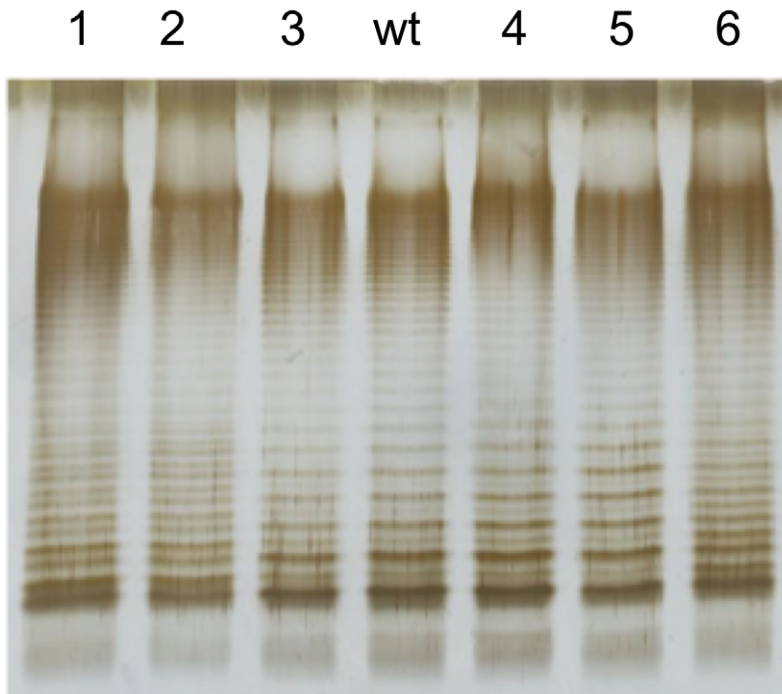

Figure 5. Lipopolysaccharide profiles of bile-resistant derivatives of *S. enterica* SL1344, as observed by electrophoresis and silver staining. The lane marked "wt" shows the LPS profile of the wild type strain. Lanes 1–6 show the LPS profiles of bile-resistant mutants #1, #2, #3, #4, #5, and #6.
doi:10.1371/journal.pgen.1002459.g005

number of cells that activate the RpoS general response in the absence of DOC varies from one experiment to another, thus providing further evidence that phenotypic heterogeneity occurs in batch cultures. As a control, we analyzed *osmY*::GFP expression in an RpoS^{-} background (strain SV6780). As expected, the level of expression of *osmY* was significantly lower in the absence of RpoS, and *osmY*::GFP induction by DOC was very modest in the RpoS^{-} mutant (Figure 6C).

(ii) Time lapse experiments: *S. enterica* cultures grown in LB were diluted, transferred to microscope slides, and covered with agar pads containing either LB or LB+5% DOC. The fluorescence of individual cells was then monitored during 90 minutes. A representative experiment involving 14 *Salmonella* cells is shown in Figure 7. The presence of DOC increased the level of fluorescence, albeit at different levels in different cells (perhaps reflecting differences in the initial level of *osmY*::GFP induction). Lysis of some cells was observed in the presence of DOC. In the absence of DOC, the fluorescence levels were lower. However, heterogeneous expression of *osmY*::GFP was observed in the absence of DOC, as in the single point experiments shown in Figure 6.

These experiments suggest that *S. enterica* cultures grown in LB contain cells with elevated expression of the RpoS-dependent general stress response. This observation offers a tentative explanation for non mutational preadaptation to bile: when an aliquot from an LB culture is plated on a lethal concentration of bile, sustainment and/or amplification of the general stress response in certain cells may permit the formation of bile-resistant colonies.

To investigate whether heterogeneous gene expression occurred also in bile-responsive loci that do not belong to the RpoS regulon, we monitored expression of *cspD* [32,33], a stress response gene which is upregulated by exposure to a sublethal concentration of DOC (Table 1 and Table 2). For this purpose, a GFP fusion was constructed at the 3′ end of the *cspD* coding sequence (strain SV6802). Single point experiments in LB and LB+5% DOC were carried out as above, and the fluorescence level of individual cells was measured [50]. Exposure to 5% DOC increased *cspD*::GFP expression in an heterogeneous but consistent manner (Figure 8). However, heterogeneity was also observed in LB (Figure 8), indicating that some cells activate *cspD* expression in the absence of DOC. Hence, phenotypic heterogeneity in gene expression is not restricted to RpoS regulon. This observation suggests that non mutational preadaptation to bile may require specific gene expression patterns, perhaps involving multiple loci.

Discussion

Bacteria live in a changing environment, devoid of the homeostatic mechanisms that create stable conditions in the tissues of multicellular eukaryotes. Except for obligate parasites that have adapted to stable environments, survival of bacteria depends on ceaseless adaptation. This study describes mechanisms employed by *Salmonella enterica* to survive bile, a fluid with antibacterial capacity due to the presence of bile salts [3,4,8]. The adaptation mechanisms have been investigated using either ox bile, which contains a mixture of bile salts [1], or sodium deoxycholate, the most abundant and well known bile salt [2].

The concentrations of bile and DOC that inhibit *S. enterica* growth depend on the conditions used. For instance, it is well known that dividing cells are more sensitive to bile salts than non dividing cultures [19]. Furthermore, even under specific conditions, the minimal inhibitory concentration of bile increases if the *S. enterica* culture is previously grown on sublethal concentrations

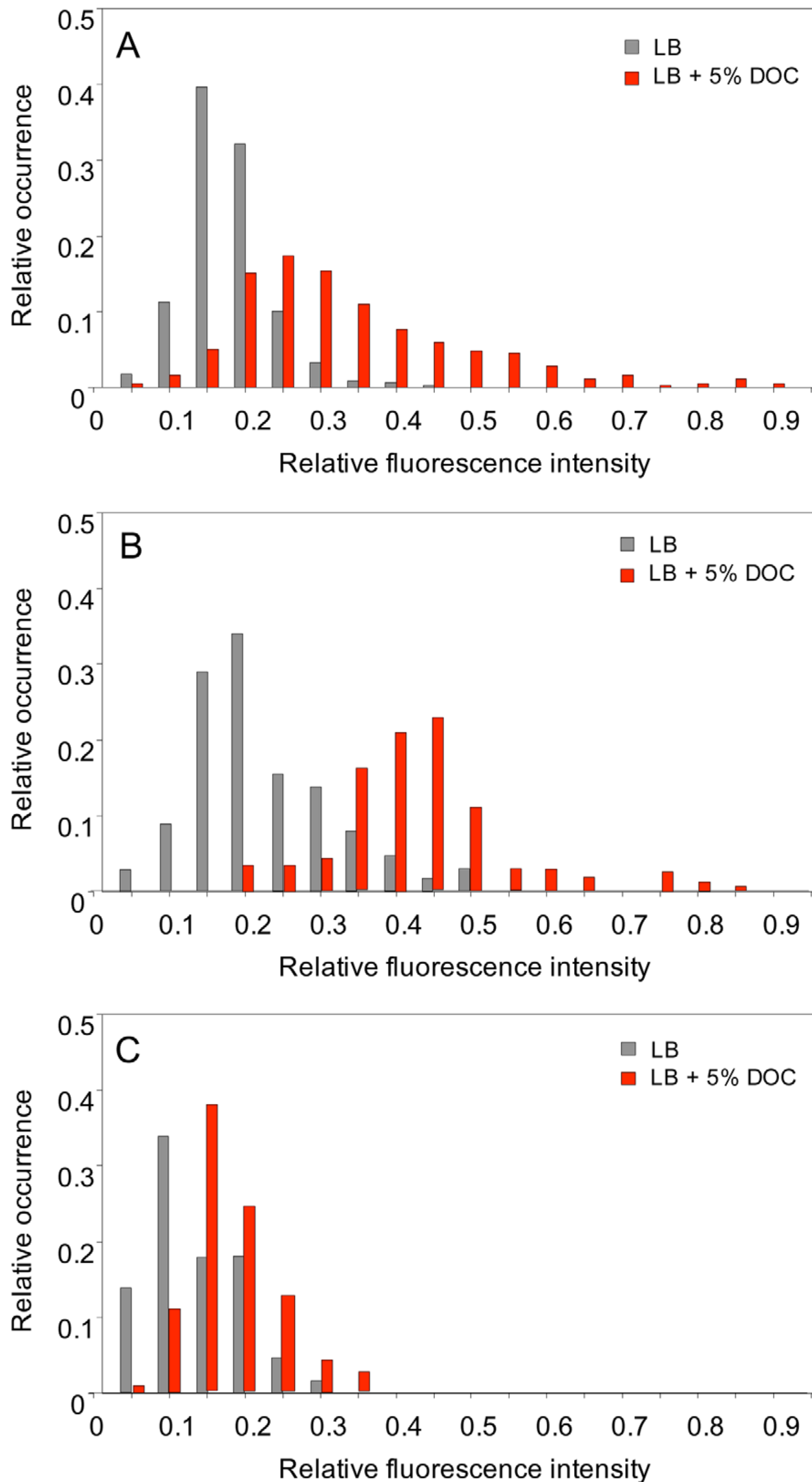

Figure 6. Levels of *osmY* gene expression in individual bacterial cells. Panels A and B show the distribution of fluorescence intensity in individual cells (*N*>300) of *S. enterica* SV6562 (*osmY*::GFP) in two independent experiments. In both cases, strain SV6562 was grown during 5 h in LB with or without 5% sodium deoxycholate. Histograms represent the proportion of bacterial cells showing distinct fluorescence levels in LB (grey) and LB+DOC (red). Fluorescence intensities are shown in an arbitrary scale (0–1). Panel C shows the distribution of fluorescence intensity in individual cells (*N*>300) of *S. enterica* SV6780 (*osmY*::GFP RpoS^{-}) under conditions identical to those of experiments A and B.
doi:10.1371/journal.pgen.1002459.g006

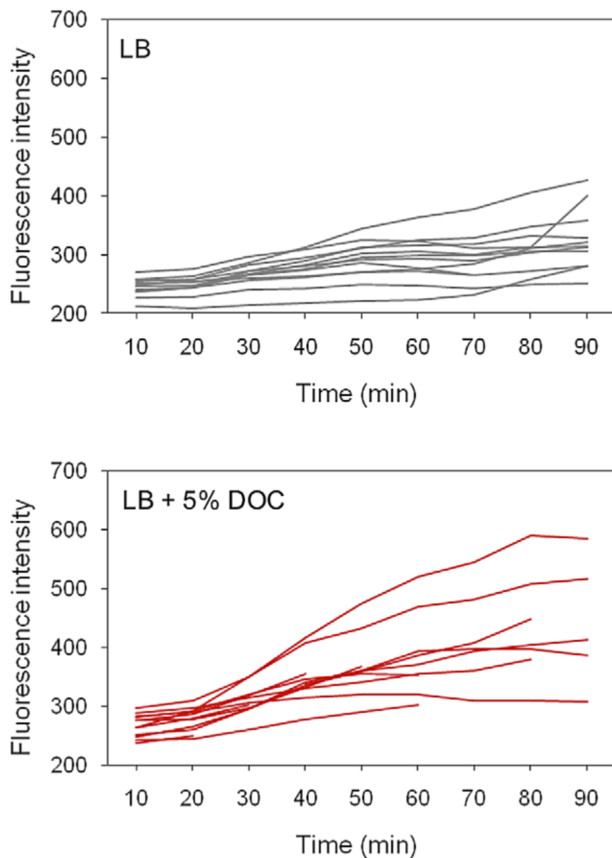

Figure 7. Time course of *osmY*::GFP expression in individual cells in the presence and in the absence of DOC. Aliquots from an exponential culture (O.D.$_{600}$ = 0.5) of *S. enterica* SV6562 (*osmY*::GFP) grown in LB were transferred to agar pads containing or not 5% sodium deoxycholate. Bacterial cells were fixed *in situ*, and GFP fluorescence intensity was measured at 10 min intervals during 90 min.
doi:10.1371/journal.pgen.1002459.g007

[8]. For instance, minimal inhibitory concentration analyses shown in Figure 2 indicate that growth on 4–5% DOC, a concentration which does not inhibit *Salmonella* growth, increases the MIC to ≥14%. Growth at DOC concentrations below 3% does not increase the MIC, and concentrations higher than 5% are inhibitory (Figure 2). Hence, sublethal (but relatively high) concentrations of DOC are necessary to increase the MIC above the standard inhibitory concentration.

A conceivable explanation for *Salmonella* adaptation to bile was that sublethal concentrations of bile might trigger changes of gene expression that could facilitate survival. Transcriptomic analysis in the presence of 5% DOC revealed indeed multiple changes in gene expression, some of which provided tentative explanations for the observed increase in bile resistance. Especially relevant was the observation that exposure to DOC activates the RpoS-dependent general stress response (Table 1, Table 2, Table 3, Table 4, and Figure 3). This response facilitates survival of *E. coli* and other gamma-proteobacteria under conditions that do not support active growth, and is also known to be activated by multiple stress conditions [31]. Hence it is not surprising that bile salts, which denature proteins and cause DNA damage [3,4], can trigger the general stress response. The conclusion that the RpoS-dependent general stress response plays a crucial role in bile resistance is supported by two additional observations: (i) Lack of RpoS causes bile sensitivity (Table S3); (ii) *S. enterica* stationary cultures, in which the RpoS response is physiologically active, are more resistant to both DOC and ox bile [19]. The occurrence of extreme levels of resistance (≥14% DOC) in adapted cultures admits several explanations (not mutually exclusive). Activation of the RpoS regulon by sublethal concentrations of bile may be stronger than physiological activation in stationary cells. It is also possible that high levels of bile resistance result from simultaneous activation of more than one bile-resistance response. This latter possibility is supported by the observation that stress-inducible genes (*cspD*, *uspA*, *aphC*, etc.) that do not belong to the RpoS regulon are also activated by exposure to 5% DOC (Table 1 and Table 2). Furthermore, exposure to bile upregulates the expression of genes of unknown function, some of which might be part of stress

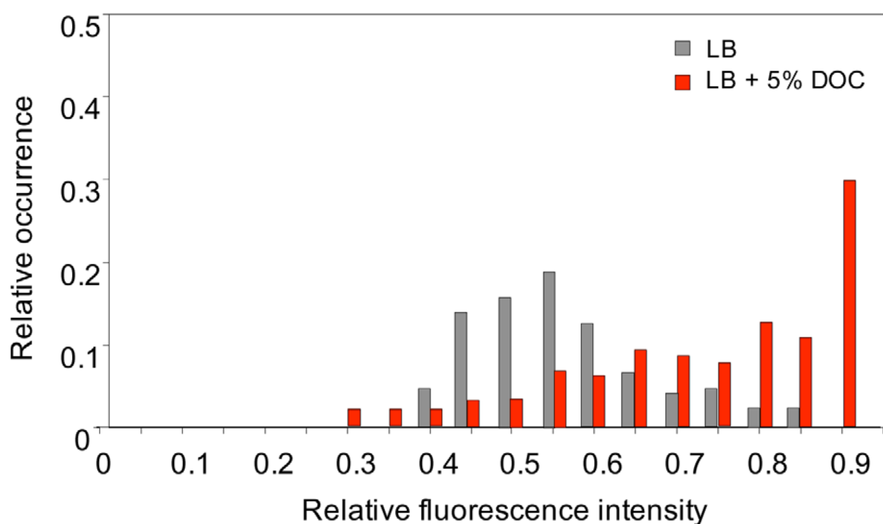

Figure 8. Levels of *cspD* gene expression in individual bacterial cells. The distribution of fluorescence intensity was measured in individual cells (*N*>300) of *S. enterica* SV6802 (*cspD*::GFP) after growth during 5 h in LB with or without 5% sodium deoxycholate. Histograms represent the proportion of bacterial cells showing distinct fluorescence levels in LB (grey) and LB+DOC (red). Fluorescence intensities are shown in an arbitrary scale (0–1).
doi:10.1371/journal.pgen.1002459.g008

response networks. An example is *yiiU*, which is activated by DOC (Table 1 and Figure 3) and is essential for bile resistance (Table S3).

High throughput analyses of gene expression also indicated that adaptation to bile may involve downregulation of porin genes and other genes encoding envelope structures, as well as upregulation of efflux pumps (Table 1 and Table 2). All these gene expression changes fit well in the literature: porins provide passage to bile salts [8], envelope structures are major barriers for bile salt uptake [8], and efflux pumps can transport bile salts outside the cell [21]. Hence, growth on sublethal concentrations of bile may permit *Salmonella* adaptation to lethal concentrations by triggering multiple changes in gene expression. Downregulation of pathogenicity island 1 by sublethal concentrations of DOC (Table 2), a phenomenon previously described [27], may be viewed as a signalling system used by *Salmonella* to identify environments that are not appropriate for epithelial cell invasion (e. g., the duodenum). In turn, downregulation of pathogenicity island 2 in the presence of bile (Table 2) may be viewed as a response that prevents activation of *Salmonella* functions involved in intracellular survival.

Preadaptation of *S. enterica* to bile is a completely different phenomenon, which does not pertain to *Salmonella* populations but to individual bacterial cells. Preadaptation is easily observed under laboratory conditions: when an aliquot of a *S. enterica* batch culture is plated on LB agar containing a lethal concentration of ox bile, bile-resistant colonies appear at frequencies ranging from 10^{-6} to 10^{-7} per cell and generation. These numbers fall in the known range of bacterial mutation frequencies [51]. Not surprisingly, Luria-Delbrück fluctuation analysis confirms that such colonies arise from bile-resistant cells found in the culture aliquot used for inoculation (Table S4). However, we were surprised to find that the majority of bile-resistant isolates obtained under such conditions were unstable, and lost bile resistance if grown overnight in LB without bile. Hence, preadaptive bile resistance seems to involve two distinct phenomena: mutation and non mutational preadaptation.

Full genome sequencing of 6 bile-resistant mutants revealed that 3 mutants carried mutations in *yrbK* and one in *rlpB.* The high frequency of mutations in lipopolysaccharide transport genes leaves little doubt that altered LPS transport can cause bile resistance. However, the identification of such mutants has intriguing aspects. One comes from the fact that LPS transport genes are known to be essential in *E. coli* [47,48]. If such is also the case in *Salmonella*, the mutations found (two nucleotide substitutions, one in-frame deletion and one premature stop codon relatively close to the 3′ end of the coding sequence) must all be leaky. Another intriguing question is related to the fact that LPS is a major barrier against bile salts [15]. However, it is conceivable that transport of LPS components across the envelope might sensitize the cell to bile salts, thus explaining why altered transport may confer bile resistance. An alternative, speculative explanation is that the LPS transport proteins altered in bile-resistant mutants might be also involved in LPS assembly and/or modification, and that specific mutations might boost bile resistance. This view may be tentatively supported by the observation that some of the bile-resistant mutants described in this study have altered LPS profiles (Figure 5 and Figure S1).

Non mutational preadaptation to bile was at first sight an intriguing phenomenon. How can a *Salmonella* batch culture contain cells that are bile-resistant without previous adaptation by growth at sublethal concentrations? However, it is well known that bacterial cultures, albeit genetically clonal, can contain subpopulations of cells with distinct patterns of gene expression [25], either as a consequence of epigenetic control [52] or as the result of stochastic fluctuations in gene expression [25,53]. We thus hypothesized that non mutational preadaptation might be caused by activation of bile-resistance responses in the absence of bile. This hypothesis was tested by analyzing expression of *osmY*, an RpoS-dependent gene, and *cspD*, a stress response gene that does not belong to the RpoS regulon [32,33], in individual *Salmonella* cells grown in the presence and in the absence of a sublethal concentration of DOC. Microscopic microfluidics [50] confirmed that exposure to DOC activates *osmY* and *cspD* expression in most *Salmonella* cells (Figure 6 and Figure 7). However, upregulation of *osmY* and *cspD* expression was also observed in subpopulations of *Salmonella* cells in the absence of DOC (Figure 6, Figure 7, and Figure 8). Non mutational preadaptation may thus result from activation of the RpoS-dependent general stress response and/or other stress responses in a cell subpopulation. Stress response activation may either be triggered by a stress situation encountered by individual cells (e. g., increased concentrations of harmful metabolic products) or be accidental. Repression of specific loci, which has not been addressed in this study, may also contribute to preadaptation. It is possible that non mutational preadaptation to environmental challenges is a common phenomenon, and that the acquisition of bile resistance described in this study is merely one example among many others. The widespread occurrence of phenotypic polymorphism in clonal populations of bacteria [25,52,53] may support this possibility.

At this stage, it is impossible to ascertain whether the three distinct modes of *Salmonella* adaptation to bile described in this study may occur or not upon infection of animals. Current evidence obtained in the mouse model of typhoid fever indicates that *Salmonella* cells can escape the high concentrations of bile salts found in the gall bladder lumen by invading the gall bladder epithelium [11] and by forming biofilms on the surface of gallstones [12,13]. However, planktonic *Salmonella* cells are also found in the gall bladder lumen, and little is known about the mechanisms that permit their survival and multiplication. Our model envisions that planktonic *Salmonella* cells may adapt to the gall bladder lumen by changing their gene expression pattern. Passage by the small intestine and the liver, which contain bile concentrations much lower than those found in the gall bladder [1,2], might facilitate adaptation in an analogous manner as growth on sublethal concentrations of DOC in the laboratory. Phenotypic heterogeneity and subpopulation formation may additionally contribute to adaptation by activating bile resistance responses prior to colonization of the hepatobiliary tract. Lastly, appearance of bile-resistant mutants may provide an alternative mechanism for *Salmonella* adaptation to the gall bladder. Because bile salts are mutagenic [5,6] and the dose of a mutagen is the product of its concentration by the time of exposure [54], mutational adaptation of *Salmonella* to bile might be speeded up by bile itself, especially during long term infection of the bile-laden gall bladder. This phenomenon might be relevant during persistent and chronic infections, as found for instance in human carriers of *Salmonella* Typhi [10].

Materials and Methods

Bacterial strains, plasmids, bacteriophages, media, and culture conditions

Strains of *Salmonella enterica* serovar Typhimurium (often abbreviated as *S. enterica*) used in this study (Table 1) derive from the mouse-virulent strain SL1344. Strains SV6065, SV6066, SV6067, SV6068, SV6069, SV6629, and SV6745 were constructed by transducing alleles from ATCC 14028 or LT2 to SL1344.

Transduction was performed with phage P22 HT 105/1 *int201* ([55] and G. Roberts, unpublished data). The P22 HT transduction protocol was described elsewhere [56]. To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan et al. [57], except that methyl blue (Sigma Chemical Co., St. Louis, Missouri) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Luria-Bertani broth (LB) was used as standard rich medium and E medium [58] as minimal medium. Solid media contained agar at 1.5% final concentration. Cultures were grown at 37°C. Aeration of liquid cultures was obtained by shaking in an orbital incubator. Deoxycholic acid (sodium salt) and sodium choleate (ox bile extract) were both from Sigma. Antibiotics were used at the final concentrations described previously [5]. A strain list is provided as Table 6.

Gene disruption and directed construction of *lac* fusions

Targeted gene disruption was achieved using plasmids pKD3, pKD4, and pKD13 [59]. Antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 [59]. The oligonucleotides used for disruption (labeled "FOR" and "REV") are listed in Table S5 together with the oligonucleotides (labeled "E") used for allele verification by the polymerase chain reaction. For the construction of transcriptional and translational *lac* fusions in the *Salmonella* chromosome, FRT sites generated by excision of Km^r cassettes were used to integrate either plasmid pCE36 or pCE40 [60].

Table 6. Strain list.

Strain	Genotype
SV5561	*rpoS*::Ap^r
SV6065	*katE*::MudK (Km^r)
SV6066	*ots*::MudJ (Km^r)
SV6067	*xthA*::*lacZ*
SV6068	*osmY*::*lacZ*
SV6069	*dps*::*lacZ*
SV6088	*hilA*::*lacZ*
SV6090	*prgH*::*lacZ*
SV6109	*STM1441*::*lacZ*
SV6112	*ybjM*::*lacZ*
SV6115	*ecnB*::*lacZ*
SV6118	*STM1672*::*lacZ*
SV6124	*yajI*::*lacZ*
SV6127	*ugpB*::*lacZ*
SV6261	*aroG*::*lacZ*
SV6267	*ytfK*::*lacZ*
SV6270	*yiiU*::*lacZ*
SV6292	*yceK*::*lacZ*
SV6435	*rlpB* (287 C→A)
SV6562	*osmY*::GFP (Cm^r)
SV6629	*tolC*::Cm^r
SV6745	*acrD*:: Km^r
SV6780	*osmY*::GFP (Cm^r) *rpoS*::Ap^r
SV6802	*cspD*::GFP (Cm^r)
SV6880	*yrbK* (182 G→C) *yrbL*–Km^r–*mtgA*
SV6883	*yrbK* (399+) *yrbL*–Km^r–*mtgA*
SV6884	*yrbL*–Km^r–*mtgA*
SV6888	*katE*::MudK *rpoS*::Ap^r
SV6889	*deaD* (923 G→C)

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Construction of *osmY*::GFP and *cspD*::GFP fusions

An *osmY*::*gfp* fusion was constructed as described by Hautefort et al. [61]. A fragment containing the promoterless green fluorescent protein (*gfp*) gene and the chloramphenicol resistance cassette was amplified from the pZEP07 plasmid with primers 5′ AAG CCG TTG ATG GCG TAA AAA GTG TTA AAA ACG ATC TGA AAG TTC AGT AAT AAG AAG GAG ATA TAC ATA TGA G 3′, and 5′ GGT GCA CAT TAC GCC TCC CGA CAA ACG TCG GGA GGA CGA ATT ACG ACG AAT TAT CAC TTA TTC AGG CGT A 3′. Primers used for *cspD* amplification were 5′ GCA ATC ACG CCA GCG TCA TCG TGC CCA TCG AAG CAG AGG CCG TTG CAT AGT AAG AAG GAG ATA TAC ATA TGA G 3′, and 5′ CGA TCG GGC TGG CAT TTT GCC TCC TGG ATG TAC ACA ATG AGA CAG AGG AGT TAT CAC TTA TTC AGG CGT A 3′. The 5′ regions of these primers are homologous to the 3′ end of the *osmY* and *cspD* coding sequences, so that the fusion is formed immediately after the *osmY* and *cspD* stop codons. The constructs were integrated into the chromosome of *S. enterica* using the Lambda Red recombination system [59].

Directed construction of *rlpB* and *deaD* point mutations

The *rlpB* allele from mutant #3 and the *deaD* allele from mutant #4 were PCR-amplified using pairs of 30-nucleotide primers that contained XbaI and SacI targets. The primers for *rlpB* amplification were 5′ TTT TGA GCT CGA AGG TGA TAT CGA CAA CGC 3′, and 5′ TTT TTC TAG ACT CAT TCA TTG CCG CGT TAG 3′; for *deaD* amplification, 5′ TTT TGA GCT CCG TCT GCT TGA TCA CTT AAA 3′, and 5′ TTT TTC TAG AAC GAC GTT CAC GAC GCG GAC 3′. The resulting fragments were digested with XbaI and SacI, cloned onto pDMS197 [61] and propagated in *E. coli* CC118 lambda *pir* [62]. Plasmids derived from pMDS197 were transformed into *E. coli* S17-1 lambda *pir* [63]. The resulting strains were used as donors in matings with *S. enterica* SL1344 as recipients, selecting Tc^r transconjugants on E plates supplemented with tetracycline and histidine. Several Tc^r transconjugants were grown in nutrient broth (without NaCl) containing 5% sucrose. Individual tetracycline-sensitive segregants were then examined for the incorporation of the mutant allele by DNA sequencing.

Reconstruction of bile-resistant mutants by P22-mediated transduction of a linked marker

The kanamycin-resistant cassette of plasmid pKD4 was inserted at a region close to the mutation under study, using lambda Red recombination [59]. The oligonucleotides employed for gene targeting are listed in Table S5. To reconstruct *yrbK* mutations, the Km^r cassette was inserted at an intergenic region between *yrbL* and *mtgA*, 37 bp downstream the *yrbL* stop codon and 28 bp downstream the *mtgA* stop codon (note that *yrbL* and *mtgA* undergo divergent transcription). The distance from the Km^r cassette and the *yrbK* mutations under study is 6,135 bp for mutant #1, and 5,918 bp for mutant #2. To reconstruct the *rlpB* mutation of mutant #3, the Km^r cassette was introduced at an intergenic region between the putative ORFs *ybeL* and *ybeQ* (21 bp downstream the putative *ybeL* stop codon, and 8,253 bp away from the *rlpB* mutation). DNA sequence analysis employed the *S.*

Typhimurium SL1344 genome database (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs).

The presence of the original mutation in the reconstructed mutants was verified by PCR amplification using primers designed *ad hoc* (Table S5), followed by DNA sequencing. In all constructions, the distance between the Km^r cassette and the mutation under study permitted >90% cotransduction of the Km^r cassette and the point mutation under study, fulfilling calculations made with the formula of Wu [62].

Determination of minimal inhibitory concentrations of sodium deoxycholate

Exponential cultures in LB broth were prepared, and samples containing around 3×10^2 colony-forming-units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of sodium deoxycholate (DOC). After 12 h incubation at 37°C, growth was visually monitored. Assays were carried out in triplicate. Student's *t*-test was used to analyze every MIC. The null hypothesis was that MICs were not significantly different from the MIC for the wild-type. *P* values of 0.01 or less were considered significant.

Assessment of bacterial viability using a cell staining kit

One ml aliquots of an exponential culture of *S. enterica* SL1344 grown in LB were incubated in the presence of different concentrations of sodium deoxycholate (1%, 3%, 5%, 7% and 9%) during 30 minutes at 37°C. The cells were then washed three times with 0.85% NaCl and stained using the Viability/Cytotoxicity Assay Kit for Bacteria (Biotium Inc., Hayward, California). Control suspensions of live and dead cells were prepared as described in the kit protocol. Live and dead cells were distinguished using a Leica DMR 020-525.024 fluorescence microscope (Leica Camera AG, Solms, Germany). Live and dead bacteria were counted as the green and red cells (respectively) found in randomly selected 5×5 mm squares painted on a micro cover glass.

Assessment of bacterial viability by plate counts

Aliquots of *Salmonella* exponential cultures grown in LB, each containing 2×10^6 cells, were treated with various concentrations of DOC (1%, 3%, 5%, 7%, and 9%) for 30 min. The cultures were then diluted, plated on LB and incubated at 37°C. Counts of colony-forming-units were performed after overnight growth.

Isolation of bile-resistant mutants and Luria-Delbrück fluctuation assays

Bile-resistant derivatives of *S. enterica* SL1344 were isolated by plating 0.1 ml aliquots (approximately, 2×10^8 cells) from an overnight LB culture onto LB plates containing 18% ox bile (Sigma-Aldrich, St. Louis, Missouri). Fluctuation analysis was performed as described by Luria and Delbrück [42], and the number of independent cultures was 40.

DNA isolation and full-genome sequencing

Whole genome DNA samples from bile-resistant mutants and from the parent strain SL1344 were prepared by phenol extraction and ethanol precipitation. Whole genome sequencing was performed using the oligonucleotide ligation and detection (SOLiD, v2) platform [43] at the facilities of Sistemas Genómicos S.L., Parque Tecnológico de Valencia, Paterna, Spain, using mate-pair libraries and reads of 25 nucleotides [63]. DNA sequences were aligned with the genome sequence of *Salmonella* Typhimurium SL1344 available at the Wellcome Trust Sanger Institute, Hinxton, England (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs). Two mismatches or mispairs per reading were permitted.

RNA isolation, microarray procedures, and data analysis

To prepare cells for RNA extraction, 25 ml of fresh LB and LB+DOC 5% in a 250 ml flask was inoculated with a 1:100 dilution from an overnight bacterial culture, and incubated with shaking at 250 rpm in a New Brunswick Innova 3100 waterbath at 37°C. Three biological replicates were performed for each strain, and RNA was extracted at an optical density (OD_{600})~0.4 (exponential phase) and >1 (stationary phase). RNA extractions were performed as described by Mangan et al. [64], and their quality was assessed on an Agilent 2100 Bioanalyzer. Transcriptomic analyses were performed with the Salgenomics microarray [29]. Hybridization and microarray scanning were performed at the Genomics Service of the Centro Nacional de Biotecnología, C.S.I.C., Cantoblanco, Madrid, Spain (http://www.cnb.uam.es/content/services/genomics). For normalization of the two-color microarray data, LiMMA software [65] was used. Further bioinformatic analysis was carried out with the FIESTA programme (http://bioinfogp.cnb.csic.es/tools/FIESTA/index.php). Raw transcriptomic data were deposited at the Array Express database (http://www.ebi.ac.uk/miamexpress) under accession number E-MTAB-637.

ß-galactosidase assays

Levels of ß-galactosidase activity were assayed as described by Miller [66], using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

Electrophoretic visualization of lipopolysaccharide profiles

To investigate lipopolysaccharide (LPS) profiles, bacterial cultures were grown in LB. Bacterial cells were harvested and washed three times with 0.9% NaCl. The $O.D._{600}$ of the washed bacterial suspension was measured to calculate cell concentration. A bacterial mass containing about 3.2×10^8 cells was pelleted by centrifugation. Treatments applied to the bacterial pellet, electrophoresis of crude bacterial extracts, and silver staining procedures were performed as described by Buendía-Clavería *et al.* [67]. Three replicates per strain were performed.

Microscopy

Cells were inoculated onto a microscope cover slip and covered with a thin (2 mm thick) semisolid LB agar (1.5%) matrix with or without DOC. In the DOC-containing samples, the final concentration of DOC was 5%. The cover slip and the agar pad were then mounted in a seal flow chamber allowing constant aeration and reduced desiccation. Flow chambers were incubated in a temperature-controlled automated microscope (Nikon TE2000-E-PFS, Nikon, Champigny-sur-Marne, France) at 37°C. For single point experiments, 10 fields, each containing at least 30 cells treated or not with DOC, were manually defined. For 90 min time lapse experiments, a single field was examined, and time points were taken every 10 min under agar pads with or without DOC. Images were recorded using a CoolSNAP HQ2 high resolution camera (Roper Scientific, Evry, France) and a 100x/1.4 DLL objective. Digital analysis and image treatment were performed with Metamorph software 7.5 (Molecular Devices, Sunnyvale, California) as previously described [50].

Supporting Information

Figure S1 LPS profiles of bile resistant mutants #1 and #2 (both carrying *yrbK* mutations), reconstructed by P22-mediated transduction of a linked Km^r marker. Lanes are as follows: 1, $YrbK^+$ Km^r transductant obtained with a P22 HT lysate grown on SV6880 (*yrbK* G→C Km^r); 2, $YrbK^-$ Km^r transductant obtained with a P22 HT lysate grown on SV6880; wt, wild type; 3, $YrbK^-$ Km^r transductant obtained with a P22 HT lysate grown on SV6883 (*yrbK* +1 frameshift Km^r); $YrbK^+$ Km^r transductant obtained with a P22 HT lysate grown on SV6883. Transductants 1 and 4 were bile-sensitive, while transductants 2 and 3 were bile-resistant. The *yrbK* mutations carried by transductants 2 and 3 were confirmed by PCR amplification and DNA sequencing of the amplified fragments.
(PDF)

Table S1 ß-galactosidase activities of *lac* fusions in bile-responsive genes during exponential growth in the presence and in the absence of DOC.
(DOC)

Table S2 ß-galactosidase activities of *lac* fusions in bile-responsive genes in stationary cultures grown in the presence and in the absence of DOC.
(DOC)

Table S3 Minimal inhibitory concentrations (g/100 ml) of sodium deoxycholate for *S. enterica* strains mentioned in this study, all derived from SL1344.
(DOC)

Table S4 Fluctuation in the frequencies of bile-resistant mutants obtained upon plating of *S. enterica* SL1344 on LB+18% ox bile.
(DOC)

Table S5 Oligonucleotides.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JC LA. Performed the experiments: SBH IC AD LA. Analyzed the data: SBH IC AD LA JC. Contributed reagents/materials/analysis tools: SBH IC AD LA JC. Wrote the paper: SBH IC AD LA JC.

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Increased bile resistance in *Salmonella enterica* mutants lacking Prc periplasmic protease

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Summary. Prc is a periplasmic protease involved in processing of penicillin-binding protein 3 (PBP3). Lack of Prc suppresses bile sensitivity in Dam$^-$, Wec$^-$, PhoP$^-$, DamX$^-$, and SeqA$^-$ mutants of *Salmonella enterica*, and increases bile resistance in the wild type. Changes in the activity of penicillin binding proteins PBP3, PBP4, PBP5/6 and PBP7 are detected in a Prc$^-$ background, suggesting that peptidoglycan remodeling might contribute to bile resistance. [**Int Microbiol** 2013; 16(2):xx-xx]

Keywords: *Salmonella* · bile · Prc protease · peptidoglycan · penicillin-binding proteins

Introduction

Salmonella enterica is a bacterial pathogen that infects humans and livestock animals causing intestinal, systemic, and chronic infections [9]. In the intestine and in the hepatobiliary tract, *Salmonella* is exposed to bile, a fluid containing cholesterol, bile salts, phospholipids, proteins, bilirubin, and electrolytes [15]. About two thirds of bile (dry weight) are made of bile salts, a family of amphipathic molecules derived from cholesterol [16]. The relationship between intestinal bacteria and bile salts is complex. On one hand, bile salts control the expression of certain genes, and can be considered environmental signals used by the bacterium to identify the intestinal milieu [3]. On the other hand, bile salts are antibacterial compounds that disrupt membranes, denature proteins, and damage DNA [3,10]. Enteric bacteria are able to resist the antibacterial activities of bile salts, and an extreme example is *Salmonella enterica* which colonizes the bile-laden gall bladder during systemic and chronic infections [7,10]. In asymptomatic human carriers of *Salmonella* Typhi, persistence in the gall bladder can last for decades or even for a lifetime [7].

The mechanisms that permit *Salmonella* survival in the presence of bile are partially understood. Envelope structures such as the lipopolysaccharide and the enterobacterial common antigen serve as barriers that reduce intake of bile salts [3]. However, the protection provided by these barriers is incomplete, making other mechanisms necessary. Intake of bile salts induces the RpoS general stress response and other stress responses that facilitate survival [14]. In turn, activation of the

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SOS system helps to cope with bile-induced DNA injuries [26]. In addition, the intracellular concentration of bile salts is reduced by active transport of bile from the cytoplasm, especially by the AcrAB efflux pump [22].

Genetic analysis has proven useful for the identification of bile resistance functions. Isolation of bile-sensitive mutants has permitted the identification of cellular functions necessary for bile resistance, and searches for suppressors of bile sensitivity have helped to outline the responses or "pathways" involved. Especially productive has been the use of *Salmonella* Dam$^-$ mutants, which are extremely sensitive to bile [28]. Certain suppressors of bile sensitivity in the Dam$^-$ background have been found to suppress bile sensitivity caused by mutations other than *dam* [24,26,27]. Broad suppressor capacity usually indicates that a cellular defense response has been activated by the suppressor mutation, thus permitting the identification of bile defense responses [24].

Below, we describe a novel class of suppressors of bile sensitivity in *Salmonella* Dam$^-$ mutants. Loss of function in the *S. enterica prc* gene restores bile resistance in Dam$^-$ mutants and in other bile-sensitive mutants, and increases bile resistance in the wild type. Penicillin-binding proteins PBP3, PBP4, PBP5/6, and PBP7 show altered activity in *S. enterica* Prc$^-$ mutants, suggesting that changes in PBP activity can modulate bile resistance, perhaps by modification of peptidoglycan structure.

Materials and methods

Bacterial strains, bacteriophages, media and growth conditions. The strains of *Salmonella enterica* used in this study belong to serovar Typhimurium, and derive from the mouse-virulent strain SL1344 (His$^+$). An exception is TH3468 (*proAB47*/F'128 [*pro-lac*] *zzf-3834*::Tn10dTc[*del-20 del-25*] [T-POP3]), an LT2 derivative provided by K.T. Hughes, University of Utah, Salt Lake City. *Escherichia coli* DH5α [11] was used as the host of plasmids. Transductional crosses using phage P22 HT 105/1 *int201* [33] were used for strain construction in *S. enterica*. The P22 HT transduction protocol was described elsewhere [6]. To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan et al. [4] except that methyl blue (Sigma, St. Louis, MO, USA) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Luria-Bertani broth (LB) was used as standard rich medium. Liquid cultures were grown with aeration by shaking in an orbital incubator. Solid LB contained agar 1.5 % final concentration. When specified, sodium deoxycholate (DOC) (Sigma) was added.

Mutagenesis with T-POP and characterization of T-POP insertions. Pools of random T-POP3 [30] insertion mutants were generated using a P22 lysate grown on TH3468. The pools were then used to transduce strain SV6100 (Δ*dam-231*). Transductants were selected on LB plates supplemented with 20 μg/ml Tc and 2.5 % DOC. Putative suppressor-carrying isolates were lysed with P22 HT, and the lysates were used to transduce SV6100, selecting Tc^r transductants. A 100 % linkage between tetracycline resistance and DOC resistance confirmed the existence of a suppressor mutation generated by T-POP insertion. Chromosomal DNA from bile resistant mutants carrying T-POP insertions was digested with *Pst*I, which does not cleave within the T-POP element, and ligated to the *Pst*I site of plasmid pBlueScript II. Ligation mixtures were electroporated into *E. coli* DH5α, and transformants were selected on LB plates supplemented with 100 μg/ml Ap. Upon plasmid DNA purification, T-POP boundaries were sequenced with primers 5´ GAT CAC CAA GGT GCA GAG CC 3´, and 5´ TCT TGA TAA CCC AAG AGG GC 3´.

Construction of a Prc$^-$ mutant by site-directed mutagenesis. The *S. enterica prc* gene was disrupted by lambda Red recombination using plasmid pKD4 [5] and oligonucleotides 5´ CAC CTG GTG TTC TGA AAC GGA GGC CAG GCC TGG CAT GAA CTG TAG GCT GGA GCT GCT TCG 3´ and 5´ CCT GTT TAG CGT TAC TTA TTG GCT GCC GCC TGC TCC GCT GCA TAT GAA TAT CCT CCT TAG 3´. The external primers 5´ GTA GCG CGT CGT AAA GAA GG 3´and 5´ CCA TGA TCA GCA AGC CTT GC 3´ were used for allele verification. The antibiotic resistance cassette introduced during strain construction was excised by recombination with plasmid pCP20 [5].

Determination of the minimal inhibitory concentration [MIC]. An aliquot from an exponential culture, containing approx. 3×10^3 colony-forming-units/ml, was transferred to a polypropylene microtiter plate (Greiner, Frickenhausen, Germany) containing increasing concentrations of the antibacterial substance to be tested (DOC, antibiotic). After overnight incubation at 37°C, the MIC was determined by visual inspection.

Growth curves. To monitor growth rate, 200 μl from an overnight culture grown in LB was diluted in 20 ml of salt-free LB (0 % NaCl) or LB (0.5 % NaCl), and grown at 30 ºC or 37 ºC with aeration by shaking. Growth was monitored by measuring the OD_{600} at 1 hour intervals. Experiments were performed in triplicate.

Microscopic observation of cells. Cultures were grown at 37 °C to exponential phase. For DNA staining, samples suspended in 100 μl of phosphate-buffer saline (PBS) were mixed with 2 μl of Hoechst 33342 (500 μg μg/ml), incubated 20–30 min at 37°C, and washed with PBS. About 2-3 μl of the culture samples were placed on a microscope slide. Images were acquired with a Leica DMR fluorescence microscope using the 100× oil-objective lens, and were analyzed with the Leica IM50 software.

Preparation of cell envelopes. Envelopes were prepared as described elsewhere [28]. Briefly, *ca.* 10^{10} cells were rapidly cooled in an ice-salt mix and harvested by low-speed centrifugation (15 min,15,000 ×*g,* 4 ºC). Bacterial pellets were resuspended in 1 ml of PBS pH 8.0. Cell suspensions (0.5 ml, approx.) were subjected to three bursts of sonication (30 s pulses) with a Branson sonifier, mod. 250 (Branson Ultrasonics Co., Danbury, CT, USA). Unbroken cells were discarded by centrifugation at 5000 ×*g,* 10 min, 4 ºC. Cell envelopes were recovered by high speed centrifugation (200,000 ×*g*, 20 min, 4ºC) and resuspended in 100 μl of PBS pH 8.0.

In vitro assays of PBP activity. The assays were performed upon modification of previously described procedures [8]. Envelope fractions were prepared from exponential and stationary cultures grown in LB and LB without NaCl. The protein concentration was determined with a D-C protein assay kit (Bio-Rad, Hercules, California) and adjusted to 6 mg/ml in PBS, pH 8.0. Samples for binding assay were diluted 1/10 with PBS, pH 8.0, and 3 μl of bocillin FL (Molecular Probes, Eugene, Oregon) was added (10 μM final

concentration) in a final volume of 75 µl. The mixtures were incubated for 30 min at 37°C. Twenty five µl of NUPAGE sample buffer 4X (Life Technologies, Alcobendas, Spain) was added and samples were boiled for 10 min. Insoluble materials were removed by centrifugation at an Eppendorf centrifuge (14,500 rpm, 10 min, 20°C). Proteins in the sample (50 µl) were separated by SDS-PAGE in a NUPAGE 10 % BIS-TRIS acrylamide gel run in MOPS 1X buffer at a constant voltage of 75 V. The gel was washed in distilled water and fluorescence was detected directly on the gels using a Thyphon 9410 variable-mode imager (General Electric, Madrid, Spain) with an excitation wavelength of 588 nm and a 520BP40 emission filter.

Results

Increased bile resistance in *Salmonella enterica* Prc$^-$ mutants. A genetic screen with the T-POP3 transposon [30] was used to search for supressors of bile sensitivity in a *S. enterica* Dam$^-$ mutant. Cloning and sequencing of T-POP3 boundaries provided eight independent candidates in which T-POP3 had inserted at the *S. enterica prc* gene [2]. In *E. coli*, *prc* encodes a periplasmic protease also known as tail-specific protease [13,35].

Additional confirmation that loss of Prc function suppressed bile sensitivity in a Dam$^-$ mutant was obtained by disrupting the *prc* gene with the lambda Red recombination procedure, and introducing the mutant allele into the Dam$^-$ background. MIC analysis confirmed that bile sensitivity was suppressed by the *prc* mutation (data not shown). Further work was carried out with the *prc* deletion allele constructed by site-directed mutagenesis (strain SV6278).

To ascertain whether the ability of a *prc* mutation to suppress bile sensitivity was specific for Dam$^-$ mutants or broader, Prc$^-$ derivatives were constructed in other bile-sensitive mutants of *S. enterica* such as PhoP$^-$ [36], WecD$^-$ [29], DamX$^-$ [20] and SeqA$^-$ [25]. MIC determinations unambiguously showed that a *prc* mutation suppressed bile sensitivity in all genetic backgrounds under study (Table 1). We interpret broad suppression capacity as an indication that the *prc* mutation causes some structural or physiological change that increases bile resistance. This view is supported by an additional observation: introduction of a null *prc* allele in the wild type increased the MIC of DOC from 7 % to 12 % (Table 1).

Other phenotypes of *Salmonella enterica* Prc$^-$ mutants. Growth curves of the *S. enterica* wild type strain and a Prc$^-$ derivative (SV6278) were monitored under various osmolarity and temperature conditions. The Prc$^-$ mutant showed a growth defect at low osmolarity, irrespective of the incubation temperature (Fig. 1). Similar observations were made when a Prc$^-$ mutant was streaked for single colonies on a salt-free LB plate (data not shown). Unlike their *E. coli* counterparts [13], *S. enterica* Prc$^-$ mutants appeared to be sensitive to low osmolarity in a temperature-independent fashion. Growth in low osmolarity medium results in the formation of cell filaments, a morphological alteration previously described in *E. coli* [13]. Filament formation was observed in a fraction of cells only, and typically produced filaments of 3–6 cells (Fig. 1). Like sensitivity to low osmolarity,

Table 1. MICs of sodium deoxycholate

Strain	Genotype	MIC of DOC (%)*
SL1344	Wild type	7
SV6100	Δ*dam*	0.2
SV6278	Δ*prc*	12
SV6946	Δ*prc* Δ*dam*::*Km*^{r}	6
SV6934	Δ*phoP*::*MudJ*	0.5
SV6940	Δ*prc* Δ*phoP*::*MudJ*	6
SV6947	Δ*damX*::*Km*^{r}	0.5
SV6953	Δ*prc* Δ*damX*::*Km*^{r}	6
SV6954	Δ*wecD*::*MudJ*	2
SV6960	Δ*prc* Δ*wecD*::*MudJ*	6
SV6961	Δ*seqA*::*Tn10*	0.5
SV6967	Δ*prc* Δ*seqA*::*Tn10*	7

*Median of >5 independent experiments.

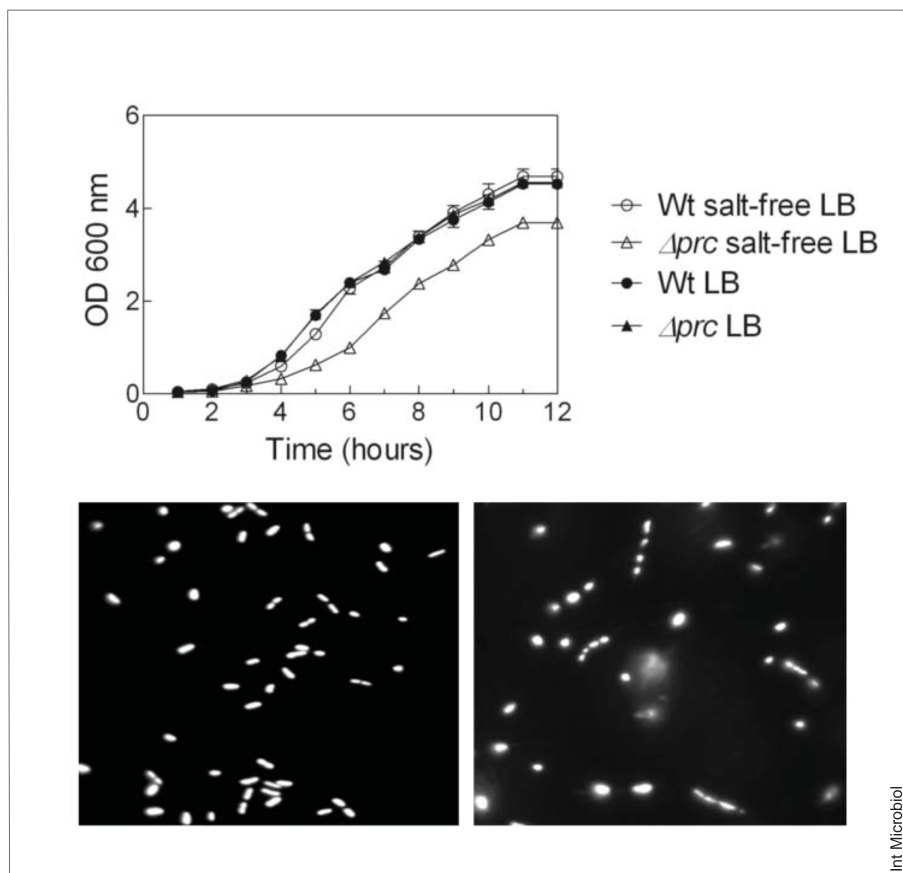

Fig. 1. Top: Growth of the wild type strain and its Prc⁻ derivative SV6278 in LB and salt-free LB. Bottom: Microscopic photographs of wild type (SL1344, left) and Prc⁻ (SV6278, right) *S. enterica* cells grown in salt-free LB.

filament formation turned out to be temperature-independent in *S. enterica.*

To investigate whether inactivation of the *prc* gene altered the susceptibility of *S. enterica* to antibiotics, the MICs of selected drugs were determined for the wild type and for SV6278 (Prc⁻). The Prc⁻ mutant showed increased sensitivity to nalidixic acid and chloramphenicol, as previously described in *E. coli* [34]. Increased sensitivity to malachite green and polymixin B (not tested in the *E. coli* study) was also detected. However, unlike *E. coli*, the levels of resistance to ampicillin and kanamycin remained unaltered in the *S. enterica* Prc⁻ mutant (data not shown).

Analysis of penicillin-binding proteins in Prc⁻ strains. Cell envelopes were prepared from the wild type and from a Prc⁻ mutant (SV6278). Bacteria were grown in LB and salt-free LB. The activity of PBPs was analyzed by detecting their capacity to bind bocillin FL [8]. A representative experiment is shown in Fig. 2. Under low osmolarity, differences were found between the wild type and the Prc⁻ mutant: (i) Bocillin binding bands corresponding to PBP3, PBP4, and PBP7 were detected in stationary cultures of Prc⁻ mutant but not in the wild type when grown in low osmolarity media; (ii) subtle differences in the PBP5/PB6 levels were also observed, and PBP5 was found to increase in stationary cultures of the Prc⁻ strain under low osmolarity conditions; and (iii) PBP7 activity increased in the Prc⁻ mutant in both exponential and stationary cultures.

Discussion

Mutations that increase the wild type level of bile resistance in *Salmonella enterica* have been described previously [14,24], and this study adds *prc* to the list. In *E. coli*, the *prc* gene encodes a periplasmic protease (also known as Tsp protease, for tail-specific protease) [35]. Prc/Tsp is involved in C-terminal processing of PBP 3 [13], in the degradation of abnormal proteins [17,18], and perhaps in fatty acid transport [1]. It seems likely that Prc may play similar roles in *S. enterica*, as the predicted gene product shows a 94 % identity in amino acid sequence with its *E. coli* counterpart. Furthermore, the phenotypes of the *S. enterica* Prc⁻ mutants described

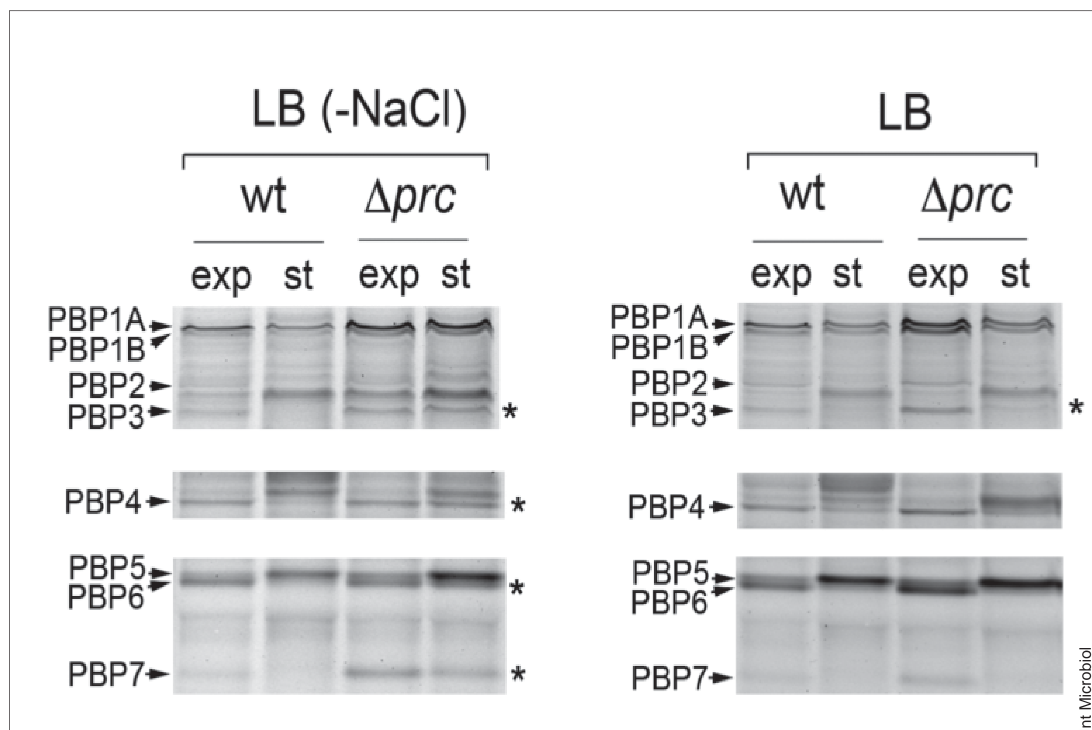

Fig. 2. Binding of bocillin FL to cell envelopes of stationary (st) and exponential (exp) cultures from wild-type *S. enterica* (wt) and from the Prc⁻ mutant SV6278 (Δ*prc*) grown in LB and salt-free LB (LB – NaCl).

in this study resemble those previously described in *E. coli* [13], with minor variations (Fig. 1).

The capacity of *prc* mutations to act as enhancers of bile resistance in the wild type and as general suppressors of bile sensitivity in a variety of mutant backgrounds (Table 1) suggests that bile resistance may result from a response triggered by Prc absence. One possibility is that enhanced bile resistance may be the consequence of the changes in activity of several PBPs such as PBP3, PBP4 and PBP7 that were detected in Prc⁻ mutants (Fig. 2). PBPs are membrane enzymes involved in polymerization and restructuring of peptidoglycan in the final steps of peptidoglycan biosynthesis [32]. PBP3 is an essential transpeptidase that catalyzes crosslink of the peptidoglycan strands during formation of the cell division septum [21]. PBP3 processing by Prc is not required for cell viability [12]. In turn, PBP7 and PBP4 are DD-endopeptidases that break the peptide cross-bridges between glycan chains in high-molecular-mass murein sacculi [31]. This study does not prove that the bile resistance phenotype of Prc⁻ mutants is a consequence of peptidoglycan remodeling. However, the increase in PBP7 and PBP4 activities observed in Prc⁻ mutants fits well in the view that these PBPs may produce a peptidoglycan structure necessary for cell survival under certain adverse conditions such as starving or exposure to oxidative damaging agents [19]. In fact, one of the antibacterial actions of bile salts is DNA oxidative damage [27].

Prc⁻ mutants are unlikely to be found in nature: during *Salmonella* infection, the potential advantage of acquiring a *prc* mutation would be compensated by its negative consequences, which include sensitivity to low osmolarity and impaired cell division. In fact, *S. enterica* Prc⁻ mutants show reduced survival within macrophages [2]. A parallel case is found in *S. enterica* AsmA⁻ mutants, which are hyperesistant to bile but show impaired capacity to invade epithelial cells [24]. However, hyperesistant mutants should not be merely viewed as laboratory curiosities as their physiological defects can unveil mechanisms that operate in the wild type. In the case of Prc⁻ mutants, their defects raise the possibility that alterations in the machinery for peptidoglycan synthesis may contribute to bile resistance. In support of this hypothesis, other components of the cell envelope are known to play roles in bile resistance [3,10]. A reason to overlook the cell wall in previous studies may have been the essential nature of most functions involved in cell wall biogenesis, which makes classical genetic analysis difficult.

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