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# Adaptation of *Salmonella enterica* to bile: essential role of AcrAB-mediated efflux

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## Summary

**Adaptation to bile is the ability to endure the lethal effects of bile salts after growth on sublethal concentrations. Surveys of adaptation to bile in *Salmonella enterica* ser. Tyhimurium reveal that active efflux is essential for adaptation while other bacterial functions involved in bile resistance are not. Among *S. enterica* mutants lacking one or more efflux systems, only strains lacking AcrAB are unable to adapt, thus revealing an essential role for AcrAB. Transcription of the *acrAB* operon is upregulated in the presence of a sublethal concentration of sodium deoxycholate (DOC) while other efflux loci are either weakly upregulated or irresponsive. Upregulation of *acrAB* transcription is strong during exponential growth, and weak in stationary cultures. Single cell analysis of ethidium bromide accumulation indicates that DOC-induced AcrAB-mediated efflux occurs in both exponential and stationary cultures. Upregulation of *acrAB* expression may thus be crucial at early stages of adaptation, while sustained AcrAB activity may be sufficient to confer bile resistance in nondividing cells.**

## Introduction

Bile is a fluid synthesized by parenchymal cells in the liver. 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 products of hepatic metabolism (Hofmann, 1998). About two-thirds of bile (dry weight) are made of bile salts, a family of molecules with steroid structure which derive from cholesterol (Hofmann and Hagey, 2008). Aside from their role in digestion, bile salts are physiological signals for transcriptional regulation of both mammalian and bacterial genes (Forman *et al.*, 1995; Gupta and Chowdhury, 1997; Makishima *et al.*, 1999; Schuhmacher and Klose, 1999; Van Velkinburgh and Gunn, 1999; Makishima *et al.*, 2002; Prouty *et al.*, 2004a; Malik-Kale *et al.*, 2008; D'Aldebert *et al.*, 2009; Eade *et al.*, 2016). In a sort of Dr. Jekyll and Mr. Hyde dichotomy, however, bile salts are also antibacterial compounds that disrupt membranes, denature proteins and cause oxidative DNA damage. At high concentrations, bile salts are bactericidal (Coleman *et al.*, 1980; Kandell and Bernstein, 1991; Gómez Zavaglia *et al.*, 2002; Leverrier *et al.*, 2003; Prieto *et al.*, 2004; 2006; Merritt and Donaldson, 2009).

Bacterial species that colonize the mammalian intestine are resistant to bile salts, and a relevant example is *Salmonella enterica*, whose ability to endure bile permits not only intestinal survival but also colonization of the hepatobiliary tract including the gall bladder (Sinnott and Teall, 1987; Andrews-Polymenis *et al.*, 2010; Crawford *et al.*, 2010; Gonzalez-Escobedo and Gunn, 2013). In fact, the gall bladder is the major niche for the human-adapted serovar *Salmonella* Typhi in chronic carriers of typhoid (Gonzalez-Escobedo *et al.*, 2011). *Salmonella* survival in the mammalian gall bladder appears to involve several strategies. One is invasion of the gall bladder epithelium (Menendez *et al.*, 2009; Gonzalez-Escobedo *et al.*, 2013), which may permit escape from the high concentrations of bile salts present in the gall bladder lumen. Formation of biofilms on gallstones may also protect *Salmonella* from bile salts (Prouty *et al.*, 2002; Crawford *et al.*, 2008; 2010). In addition, planktonic *Salmonella* cells are found in the gall bladder lumen, and the physiological responses that permit survival remain unknown.

Bile resistance can be studied under laboratory conditions by supplementing microbiological culture media with either ox bile or individual bile salts (Pace *et al.*, 1997; Van Velkinburgh and Gunn, 1999; Raphael *et al.*, 2005; Froelich *et al.*, 2006; Malik-Kale *et al.*, 2008; Casadesús *et al.*, 2010; Lertpiriyapong *et al.*, 2012). Using this reductionist approach, genetic and biochemical analysis has permitted the identification of cell components and mechanisms involved in bacterial resistance to bile (Gunn, 2000; Casadesús *et al.*, 2010; Hernández *et al.*, 2012; 2013; 2015). Examples include envelope structures like the

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lipopolysaccharide (Picken and Beacham, 1977; Van Velkinburgh and Gunn, 1999; Murata *et al*., 2007; Crawford *et al*., 2012; Hernández *et al*., 2012; May and Groisman, 2013) and the enterobacterial common antigen (Ramos-Morales *et al*., 2003), which serve as barriers that reduce intake of bile salts. Downregulation of genes encoding porins (Hernández *et al*., 2012) may also reduce intake of bile salts. In turn, peptidoglycan remodelling may permit bile-insensitive cell wall synthesis (Hernández *et al*., 2015). However, envelope barriers are insufficient to prevent bile intake, and the activity of efflux pumps is necessary to prevent intracellular accumulation of bile salts (Thanassi *et al*., 1997; Piddock, 2006).

Efflux systems are responsible for resistance to numerous compounds (Gunn, 2000). Nine efflux systems have been characterized in *Salmonella enterica*, belonging to four families: resistance nodulation division (RND); major facilitator superfamily (MF); multidrug and toxic-compound extrusion (MATE) and ATP-binding cassette superfamily (ABC). AcrAB-TolC, an RND efflux transporter, is the best characterized efflux system in enteric bacteria (Ma *et al*., 1993; 1995; Zgurskaya and Nikaido, 1999; Nikaido *et al*., 2008). RND efflux transporters (in this case, AcrB) are embedded in the inner membrane, and form a complex with two other proteins, a periplasmic adaptor protein (AcrA) and an outer membrane channel (TolC) (Dinh *et al*., 1994; Paulsen *et al*., 1997; Pos, 2009). RND efflux systems transport a wide range of substances outside the cell, including bile salts (Nikaido, 1996; 2001; Murakami *et al*., 2002).

The concentrations of bile or individual bile salts that inhibit *Salmonella* growth are not absolute, and 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 either bile or individual bile salts (Gunn, 2000; Prouty *et al*., 2004a; Prieto *et al*., 2009; Hernández *et al*., 2012). This phenomenon, known as adaptation, is accompanied by dramatic changes in gene expression (Prouty *et al*., 2004a; Hernández *et al*., 2012). Physiological adjustments that permit adaptation to bile include upregulation of stress responses and DNA repair mechanisms (Prieto *et al*., 2006; Hernández *et al*., 2012), remodelling of the cell envelope (Hernández *et al*., 2015) and activation of efflux systems (Gunn, 2000; Prouty *et al*., 2004a; Nishino *et al*., 2006; Nikaido *et al*., 2008; Baucheron *et al*., 2014). Many other gene expression changes occur (Hernández *et al*., 2012), and remain to be interpreted.

This study addresses the question of whether adaptation of *Salmonella* to bile involves multiple physiological adjustments or can be ascribed to a discrete set of bile resistance functions. The issue has been investigated by genetic analysis, using assays that distinguish sensitivity to bile from adaptation to bile. To make interpretation possible, the mutants chosen for the study lacked cell functions whose role in bile resistance is more or less understood: the enterobacterial common antigen (*wec*) (Ramos-Morales *et al*., 2003), DNA methylation (*dam*) (Pucciarelli *et al*., 2002; Prieto *et al*., 2004), virulence regulation (*phoP*) (Van Velkinburgh and Gunn, 1999), cell division factors (*damX* and *zapB*) (López-Garrido *et al*., 2010; Hernández *et al*., 2012), the general stress response (*rpoS*) (Hernández *et al*., 2012) and active efflux (*acrA, acrB* and *tolC*) (Thanassi *et al*., 1997; Prouty *et al*., 2004a; Nishino *et al*., 2006). Only a subset of the bile resistance functions included in the study appear to be required for adaptation: Dam methylation, the PhoPQ signal transduction system and active efflux. Among them, efflux mediated by the AcrAB pump stands out as essential. The need of AcrAB for adaptation is supported by gene expression assays and flow cytometry analyses that reveal a correlation between the presence of DOC and AcrAB-mediated efflux. We thus propose that AcrAB-mediated efflux is essential for adaptation to bile, while other functions may have complementary roles.

## Results

### *Surveys of bile adaptation in bile-sensitive mutants of* S. enterica

The ability of *S. enterica* bile-sensitive mutants to adapt to bile was tested in two types of trials, outlined in Supporting Information Fig. S1: (i) tests of the minimal concentration (MIC) of DOC after incubation in the presence of a sublethal concentration of DOC; (ii) plate assays of resistance to ox bile after incubation in the presence of a sublethal concentration of DOC. In the latter assay, we used LB agar containing gradients of ox bile, a classical technique in studies of antibiotic resistance (Szybalski and Bryson, 1952). Gradient plates permit visual assessment of adaptation in the presence of ox bile, and sort out the problem posed by the dark colour of bile, which makes MIC determination difficult and unreliable. In both kinds of assays, adaptation was confirmed when the increased resistance level was found to be reversible (Supporting Information Fig. S1). This criterion excluded the possibility that bile-resistant mutants had been selected. The mutants used in these trials carried loss-of-function mutations in pleiotropic regulatory loci (*dam, phoP, rpoS*), in genes encoding components of the bacterial envelope (*wec*, *zapB*), and in genes encoding efflux pump components (*acrA, acrB*, *tolC*). The adaptation trials were performed in two steps:

(i) The maximal concentration of DOC that permitted survival was determined for each strain (Table 1). Sensitivity to DOC was found to be extreme in *acrA*, *acrB* and *tolC*. This conclusion is in agreement with previous studies showing that AcrAB-mediated efflux

**Table 1.** Maximal concentrations of sodium deoxycholate (DOC) that permitted survival of *S. enterica* mutants with a bile-sensitive phenotype.[a]

| Strain | Genotype | Maximal survival concentration (%) |
|---|---|---|
| SV5015 | *SL 1344* (WT) | 6 |
| SV5367 | Δ*dam* | 1 |
| SV6947 | *damX*::Km | 4 |
| SV6934 | *phoP*::MudJ | 5 |
| SV4227 | *rpoS*::Ap | 4 |
| SV4586 | *wecD*::Km | 4 |
| SV6268 | *zapB*::Km | 4 |
| SV6629 | *tolC*::Km | 1 |
| SV7248 | *acrA*::Km | 1 |
| SV7458 | *acrB*::Km | 1 |

**a.** Averages from ≥3 independent experiments.

is essential for bile resistance in *Escherichia coli* and *Salmonella* (Thanassi *et al*., 1997; Prouty *et al*., 2004a; Nishino *et al*., 2006).

(ii) Aliquots from exponential cultures grown in LB containing a sublethal concentration of DOC were either used to monitor the MIC of DOC in microtiter plates or spread on LB agar containing a gradient of ox bile (0%–30%). Bacterial growth was monitored after overnight incubation at 37°C. As controls, aliquots from exponential cultures in LB (in other words, not pre-exposed to DOC) were also subjected to MIC determination and plated on bile gradient agar. An additional control was SV6544, a strain carrying a mutant allele of *yrbK* that confers hyper-resistance to bile (Hernández *et al*., 2012). To test whether adaptation had occurred or not, the cultures were diluted and grown overnight in LB broth. The following day, aliquots were either used for MIC determination or plated on ox bile gradient agar. A decrease in the level of resistance to bile confirmed the occurrence of adaptation, and ruled out the selection of bile-resistant mutants.

The main observations made in these trials were as follows:

(i) Exposure to a sublethal concentration of DOC increased the level of bile resistance of certain bile-sensitive mutants (*rpoS, wec, zapB, damX*) in a fashion similar or identical to that of the wild type, indicating that full adaptation had occurred (Table 2 and Fig. 1).

(ii) Other mutants (*dam* and *phoP*) increased their resistance to bile but did not reach the wild type level, suggesting that DNA methylation and the PhoPQ regulon are involved in adaptation (Table 2 and Fig. 1).

(iii) The *acrA, acrB* and *tolC* mutants were unable to adapt to bile (Table 2 and Fig. 1).

### *Surveys of adaptation to bile in* S. enterica *mutants lacking efflux systems*

The observation that only mutants lacking efflux pump components (*acrA, acrB* and *tolC*) were unable to adapt to bile led us to investigate whether the additional efflux systems described in *S. enterica* had also a role in adaptation. For this purpose, single efflux pump mutants were constructed and the minimal inhibitory concentrations (MICs) of sodium deoxycholate (DOC) were determined for each strain. Lack of efflux pumps other than AcrAB did not severely impair bile resistance (Table 3), as described previously in strain ATCC 14028 (Nishino *et al*., 2006). Furthermore, mutants lacking individual efflux pumps other than AcrAB were able to adapt after overnight growth in LB + 5% DOC (Table 4 and Fig. 2).

Even though single mutants lacking efflux pumps other than AcrAB were still able to adapt, we considered the possibility that these pumps might play an ancillary role in bile resistance. To investigate this possibility, mutants that

**Table 2.** Minimal inhibitory concentrations of sodium deoxycholate in non-adapted and adapted cultures of *S. enterica* mutants.[a]

| Strain | Genotype | MIC (%), day 1 (after growth in LB) | MIC (%), day 2 (after growth in LB + DOC) | MIC (%), day 3 (after growth in LB) |
|---|---|---|---|---|
| SV5015 | *SL 1344* (WT) | 7 | > 11 | 8 |
| SV5367 | Δ*dam* | 2 | > 11 | 3 |
| SV6947 | *damX*::Km | 5 | > 11 | 7 |
| SV6934 | *phoP*::MudJ | 6 | > 11 | 7 |
| SV4227 | *rpoS*::Ap | 5 | > 11 | 5 |
| SV4586 | *wecD*::Km | 5 | > 11 | 8 |
| SV6268 | *zapB*::Km | 5 | > 11 | 5 |
| SV6629 | *tolC*::Km | 2 | 2 | 2 |
| SV7248 | *acrA*::Km | 2 | 2 | 2 |
| SV7458 | *acrB*::Km | 2 | 2 | 2 |
| SV6544 | *yrbK* (Δ30nt) | > 11 | > 11 | > 11 |

**a.** Averages from ≥ 3 independent experiments.


**Fig. 1.** Adaptation assays of bile sensitive mutants on 0%–30% ox bile gradient plates. The plates on the left side of each column show the bile resistance level of each strain cultured overnight in LB broth. The middle column shows the resistance levels of the same strains after overnight growth in LB containing a strain-specific sublethal concentration of sodium deoxycholate (DOC). The left column corresponds to cultures of the adapted strains after overnight incubation in a medium free of bile or bile salts. The percentage of DOC used for adaptation is indicated on the right side of the plates (middle column). Arrows indicate the boundary of the growth area. [Colour figure can be viewed at wileyonlinelibrary.com]

lacked from two to eight efflux systems were constructed by sequential disruption of each efflux pump gene (leaving the *acrAB* genes intact). When these strains were used to determine MICs of DOC and to monitor growth on ox bile gradient plates, their resistance levels were found to be similar or slightly lower than that of the wild type (Table 4) as previously described in strain ATCC 14028 (Nishino *et al.*, 2006). Adaptation trials were then carried out, using the customary 5% DOC as the adaptive concentration. All mutants, including the strain lacking 8 efflux systems (all but AcrAB), were able to adapt (Fig. 3). A noteworthy observation is that an *acrD* mutant was able to adapt,

**Table 3.** Minimal inhibitory concentrations (MIC) of sodium deoxycholate (DOC) for *S. enterica* strains lacking efflux systems.[a]

| Strain | Genotype | Minimal inhibitory concentration (%)[a] |
|---|---|---|
| SV7248 | *acrA*::Km | 2 |
| SV6745 | *acrD*::Km | 7 |
| SV7249 | *acrF*::Km | 8 |
| SV7250 | *emrA*::Km | 7 |
| SV7251 | *macA*::Km | 8 |
| SV7252 | *mdfA*::Km | 8 |
| SV7457 | *mdsA*::Cm | 8 |
| SV7253 | *mdtA*::Km | 7 |
| SV7254 | *mdtK*::Km | 8 |
| SV7617 | Δ*acrD mdtK*::Km$^R$ | 7 |
| SV7620 | Δ*acrD* Δ*mdtK mdfA*::Km$^R$ | 7 |
| SV7511 | Δ*acrD* Δ*mdtK* Δ*mdfA emrA*::Km$^R$ | 7 |
| SV7626 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA acrF*::Km$^R$ | 7 |
| SV7631 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF mdtA*::Km$^R$ | 7 |
| SV7634 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA, macA*::Km$^R$ | 7 |
| SV7636 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA, macA*::Km$^R$, *mdsA*::Cm$^R$ | 7 |

**a.** Averages from ≥ 3 independent experiments.

indicating that the AcrAD pump is dispensable. Altogether, these observations support the conclusion that adaptation to bile specifically requires AcrAB-mediated efflux, and that the additional efflux systems of *S. enterica* ser. Typhimurium are dispensable.

### *Activation of* acrAB *expression by sodium deoxycholate*

The relevance of AcrAB-mediated efflux in adaptation to bile led us to study the expression pattern of the *acrA* and a*crB* genes during the adaptation process. For this purpose, the levels of *acrA* and *acrB* mRNAs were determined by quantitative real-time PCR (qRT-PCR) in cultures grown with 5% DOC. The strains under study were the wild type and SV7636 (*acrD mdtK mdfA emrA acrF mdtA macA mdsA*). In the wild type, the expression levels of efflux pump genes other than *acrAB* were also determined. Results shown in Fig. 4 can be summarized as follows:

(i) Expression of the *acrA* and *acrB* genes increased 10-fold or more during exponential growth in the presence of a sublethal concentration of DOC, an observation in agreement with previous studies (Lacroix *et al.*, 1996; Prouty *et al.*, 2004b; Nikaido *et al.*, 2008; Baucheron *et al.*, 2014). Increased *acrAB* expression in the presence of DOC was likewise observed in

**Table 4.** Minimal inhibitory concentrations (MICs) of sodium deoxycholate in non-adapted and adapted cultures of *S. enterica* mutants lacking efflux systems.[a]

| Strain | Genotype | MIC (%) day 1 (after growth in LB) | MIC (%) day 2 (after growth in LB + DOC) | MIC (%) day 3 (after growth in LB) |
|---|---|---|---|---|
| SV7248 | *acrA*::Km | 2 | 2 | 2 |
| SV6745 | *acrD*::Km | 7 | > 11 | 9 |
| SV7249 | *acrF*::Km | 8 | > 11 | 9 |
| SV7250 | *emrA*::Km | 7 | > 11 | 8 |
| SV7251 | *macA*::Km | 8 | > 11 | 9 |
| SV7252 | *mdfA*::Km | 8 | > 11 | 10 |
| SV7457 | *mdsA*::Cm | 8 | > 11 | 10 |
| SV7253 | *mdtA*::Km | 7 | > 11 | 7 |
| SV7254 | *mdtK*::Km | 8 | > 11 | 10 |
| SV7617 | Δ*acrD mdtK*::Km$^R$ | 7 | > 11 | 9 |
| SV7620 | Δ*acrD* Δ*mdtK mdfA*::Km$^R$ | 7 | > 11 | 8 |
| SV7511 | Δ*acrD* Δ*mdtK* Δ*mdfA emrA*::Km$^R$ | 7 | > 11 | 7 |
| SV7626 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA acrF*::Km$^R$ | 7 | > 11 | 8 |
| SV7631 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF mdtA*::Km$^R$ | 7 | > 11 | 9 |
| SV7634 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA, macA*::Km$^R$ | 7 | > 11 | 8 |
| SV7636 | Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA, macA*::Km$^R$, *mdsA*::Cm$^R$ | 7 | > 11 | 8 |

**a.** Averages from ≥ 3 independent experiments.

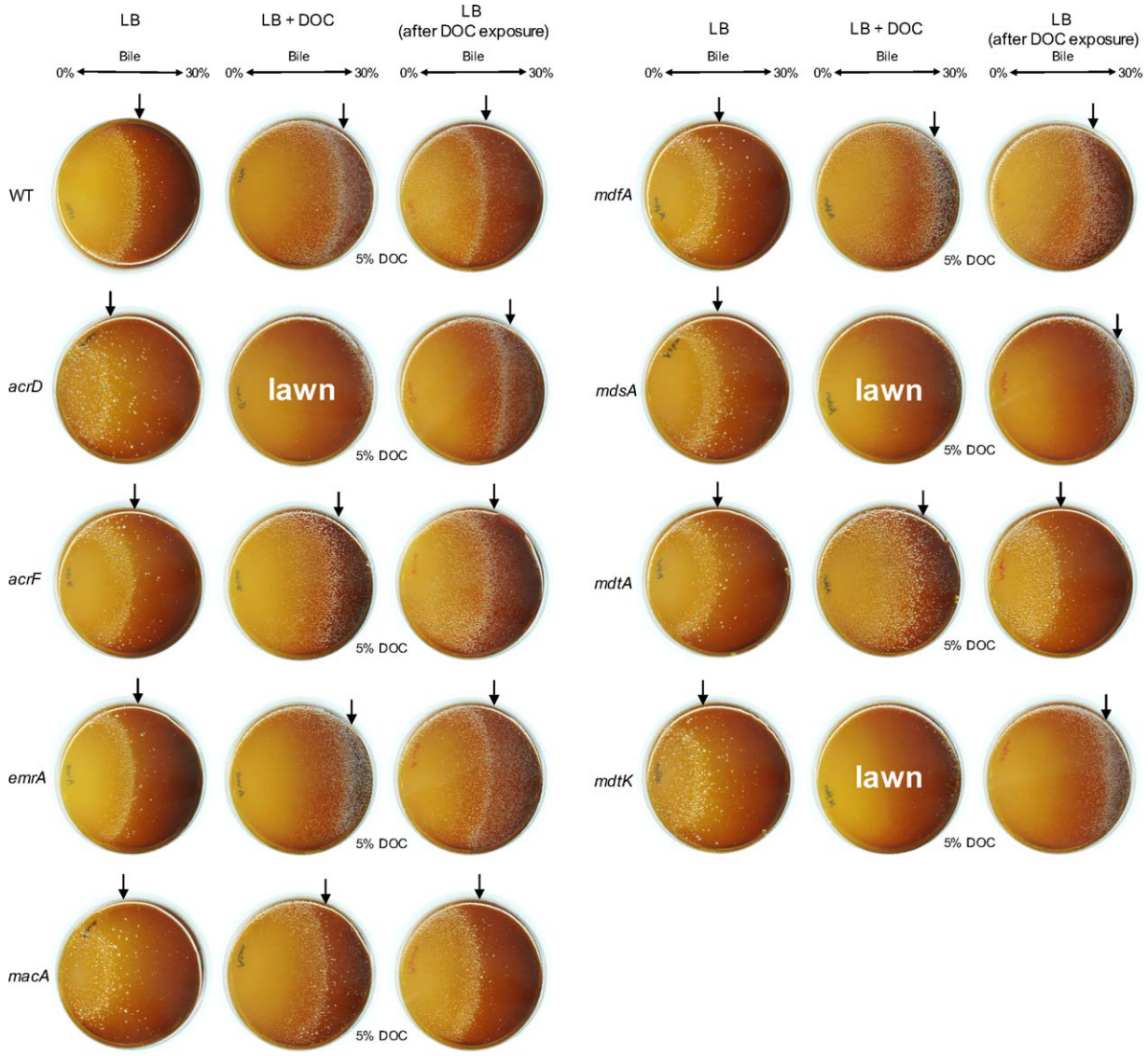


**Fig. 2.** Adaptation assays of mutants lacking individual efflux systems on 0%–30% ox bile gradient plates. The plates on the left side of each column show the bile resistance level of each strain cultured overnight in LB broth. The middle column shows the resistance levels of the same strains after overnight growth in LB containing a strain-specific sublethal concentration of sodium deoxycholate (DOC). The left column corresponds to cultures of the adapted strains after overnight incubation in a medium free of bile or bile salts. The percentage of DOC used for adaptation is indicated on the right side of the plates (middle column). Arrows indicate the boundary of the growth area. [Colour figure can be viewed at wileyonlinelibrary.com]

SV7636, the strain that lacks 8 efflux pumps. The increase was minor in stationary cultures.

(ii) Besides *acrAB*, other efflux genes increased their expression during exponential growth in the presence of DOC albeit at moderate levels. The list includes *acrD, mdtA* and *emrA*, which encode components of efflux pumps reported to export bile and sodium deoxycholate, as well as *macA*, *mdsA*, *mdfA* and *mdtK*, whose products are not known to be involved in bile transport (Nishino *et al.*, 2006; Horiyama *et al.*, 2010). Expression of these efflux genes increased to a lesser extent in stationary cultures, with the exception of *acrF* and *mdfA*.

### *Single cell analysis of AcrAB-mediated efflux*

Efflux activity was monitored in individual cells by flow cytometry using ethidium bromide (EtBr) as substrate. The strains analyzed were the wild type and SV7636 (*acrD mdtK mdfA emrA acrF mdtA macA mdsA*), grown in LB and under bile adaptation conditions (LB + 5% DOC). Relevant observations were as follows:

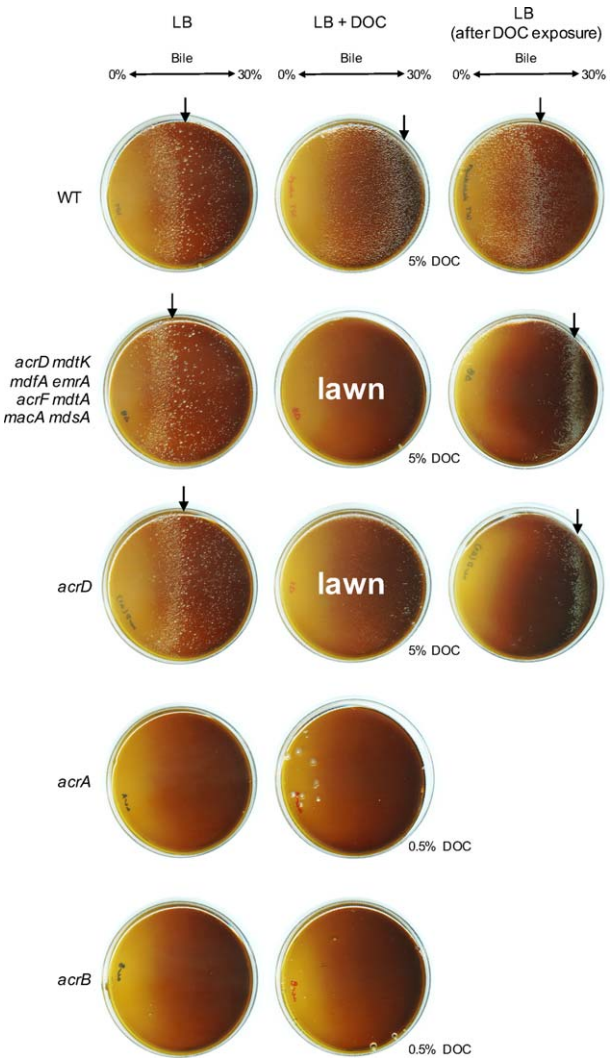


**Fig. 3.** Adaptation assay of a mutant lacking eight efflux systems, SV7636 (Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA macA::Km mdsA::Cm*) on 0%–30% ox bile gradient plates. The plates on the left side of each column show the bile resistance level of each strain cultured overnight in LB broth. The middle column shows the resistance levels of the same strains after overnight growth in LB containing a strain-specific sublethal concentration of sodium deoxycholate (DOC). The left column corresponds to cultures of the adapted strains after overnight incubation in a medium free of bile or bile salts. The percentage of DOC used for adaptation is indicated on the right side of the plates (middle column). The strains used as controls were SL1344 (wild type), SV6745 (*acrD*), SV7248 (*acrA*) and SV7458 (*acrB*). Arrows indicate the boundary of the growth area. [Colour figure can be viewed at wileyonlinelibrary.com]

(i) A decrease in EtBr fluorescence in the presence of DOC was detected in exponential cultures of both the wild type and the SV7636 strain, indicating increased efflux (Fig. 5A). The fact that the behaviour of SV7636 (which lacks all efflux systems except AcrAB) was similar to that of the wild type suggests that efflux pumps other than AcrAB do not play a major role in bile and/or sodium deoxycholate elimination in the first stages of adaptation to bile.

(ii) Four hours after the addition of sodium deoxycholate to the cultures, the ethidium bromide fluorescence within the cells started to increase both in the wild type and in SV7636, the strain devoid of efflux pumps other than AcrAB. However, the level of EtBr fluorescence was lower in adapted cultures (LB + 5% DOC) than in non-adapted cultures (LB), indicating that efflux remained active (Fig. 5A). Hence, DOC appears to keep efflux active independently of the growth phase but its intensity decreases as cells enter the stationary phase. A different representation of the same flow cytometry data (Fig. 5B) shows more clearly the occurrence of sustained efflux in the presence of DOC in stationary cultures.

## Discussion

This study distinguishes *Salmonella* cell functions involved in resistance to bile from functions involved in adaptation to bile. The distinction is simple: bile-sensitive mutants that are able to increase their bile resistance level identify functions that are dispensable for adaptation, while mutants that increase bile resistance to levels below the wild type identify cell functions involved in adaptation.

Adaptation to bile is not hampered in *damX, wecD*, *rpoS* and *zapB* mutants despite the fact that they are bile-sensitive (Table 1). A tentative interpretation is that adaptation may be a multitask, and that the existence of redundant or alternative adaptation mechanisms may permit that certain functions compensate for the absence of others. Especially remarkable is the dispensability of the general stress response, given that the RpoS regulon is strongly upregulated by bile and *rpoS* mutants are bile-sensitive (Hernández *et al.*, 2012) (Tables 1 and 2, Fig. 1).

Adaptation was found to be suboptimal in other bile-sensitive mutants, thereby identifying bile-resistant functions that play a role in adaptation. Lack of Dam methylation permitted adaptation to concentrations of bile lower than the wild type, a defect that may be tentatively attributed to lack of DNA strand discrimination upon mismatch repair and/or to an altered pattern of gene expression (Prieto *et al.*, 2004; Balbontín *et al.*, 2006). Suboptimal adaptation was also observed in a *phoP* mutant, suggesting that functions under PhoPQ control (Van Velkinburgh and Gunn, 1999; Murata *et al.*, 2007) are necessary for full adaptation. An interesting observation was that a *dam* mutant adapted better to bile than a *phoP* mutant despite the fact that its more sensitive to bile (Tables 1 and 2, Fig. 1). This difference may illustrate the complexity of adaptation, in agreement with the occurrence of multiple gene expression changes in the presence of a sublethal

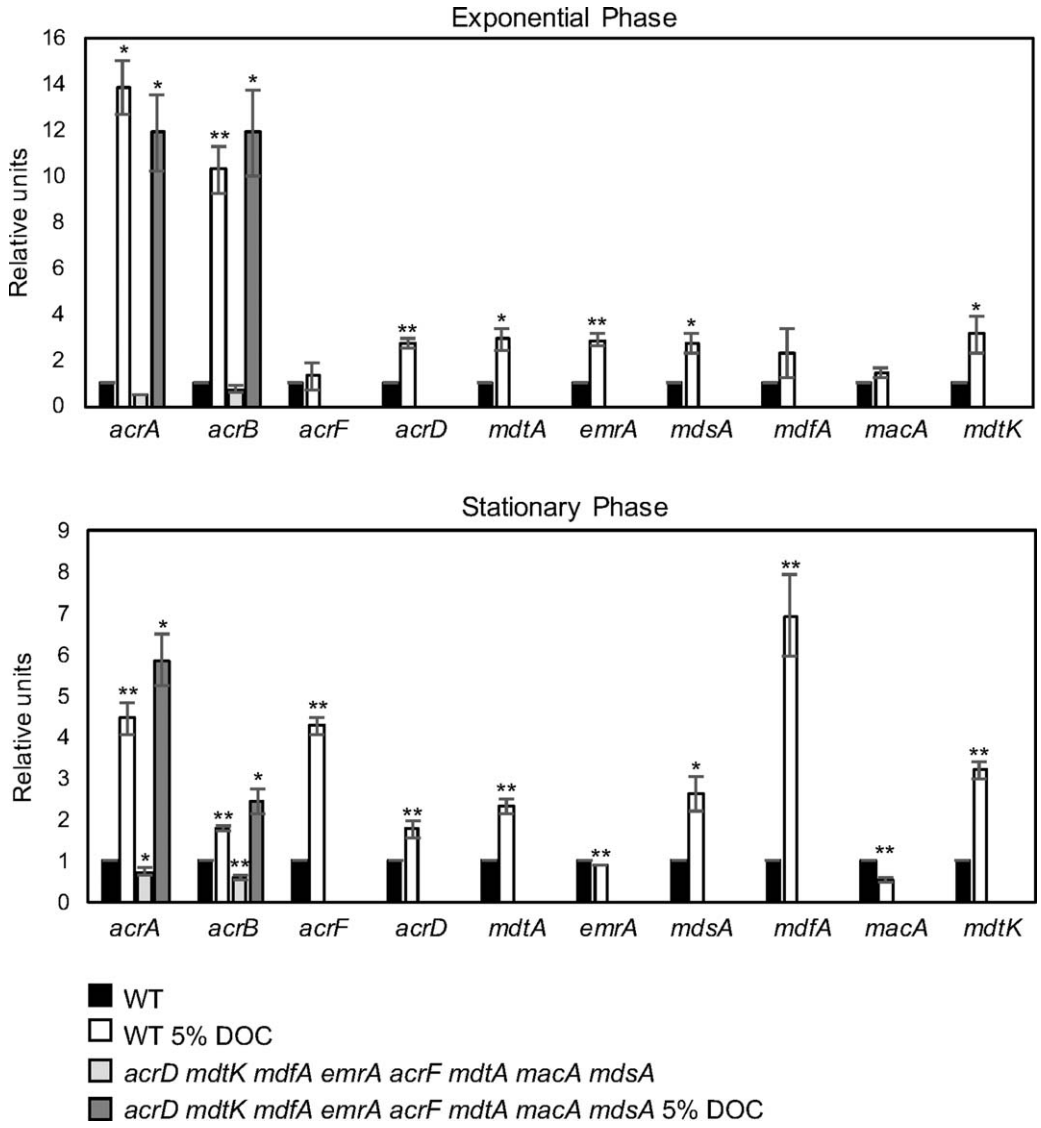


**Fig. 4.** Relative expression levels of *S. enterica* efflux pump genes in the wild type and in SV7636 (Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA macA::Km mdsA::Cm*). The strains were grown in LB and LB + 5% DOC and mRNA content was monitored in exponential (panel A) and stationary phase (panel B). Each number is the fold change in expression compared with the wild type strain grown in LB. Data are normalized to the RNA levels obtained in the wild type strain cultured in LB (value for the wild type = 1). Error bars denote standard errors; bars with asterisks are significantly different according to the two-tailed *t* test (* $P < 0.05$; ** $P < 0.01$).

concentration of DOC (Prouty *et al*., 2004a; Hernández *et al*., 2012).

Unlike other gene losses, mutations in *acrA, acrB* and *tolC* did not permit adaptation (Tables 1 and 2, Fig. 1). Hence, adaptation to bile may be a multitask but the need of AcrAB-mediated efflux appears to be absolute. The possibility that lack of adaptation might be caused by use of a low concentration of DOC seems unlikely as the concentration of DOC used for *acrA, acrB* and *tolC* mutants did permit adaptation of a *dam* mutant (Table 2, Fig. 1). Expression of the *acrAB* and *tolC* genes is upregulated by bile (Prouty *et al*., 2004a; Nikaido *et al*., 2008; Baucheron *et al*., 2014), and the AcrAB-TolC efflux system is required for bile resistance (Lacroix *et al*., 1996; Prouty *et al*., 2004a; Begley *et al*., 2005). Not surprisingly, *S. enterica* mutants lacking AcrAB-TolC are avirulent upon infection of immunodeficient mice, which involves colonization of the hepatobiliary tract (Stone and Miller, 1995; Lacroix *et al*., 1996; Nishino *et al*., 2006). However, sensitivity to bile is not only cause of attenuation as the AcrAB-TolC system plays multiple roles in bacterial physiology and virulence (Piddock, 2006; Sun *et al*., 2014; Buckner *et al*., 2016; Wang-Kan *et al*., 2017).

The unique role of AcrAB in adaptation to bile is emphasized by the fact that other *Salmonella* efflux

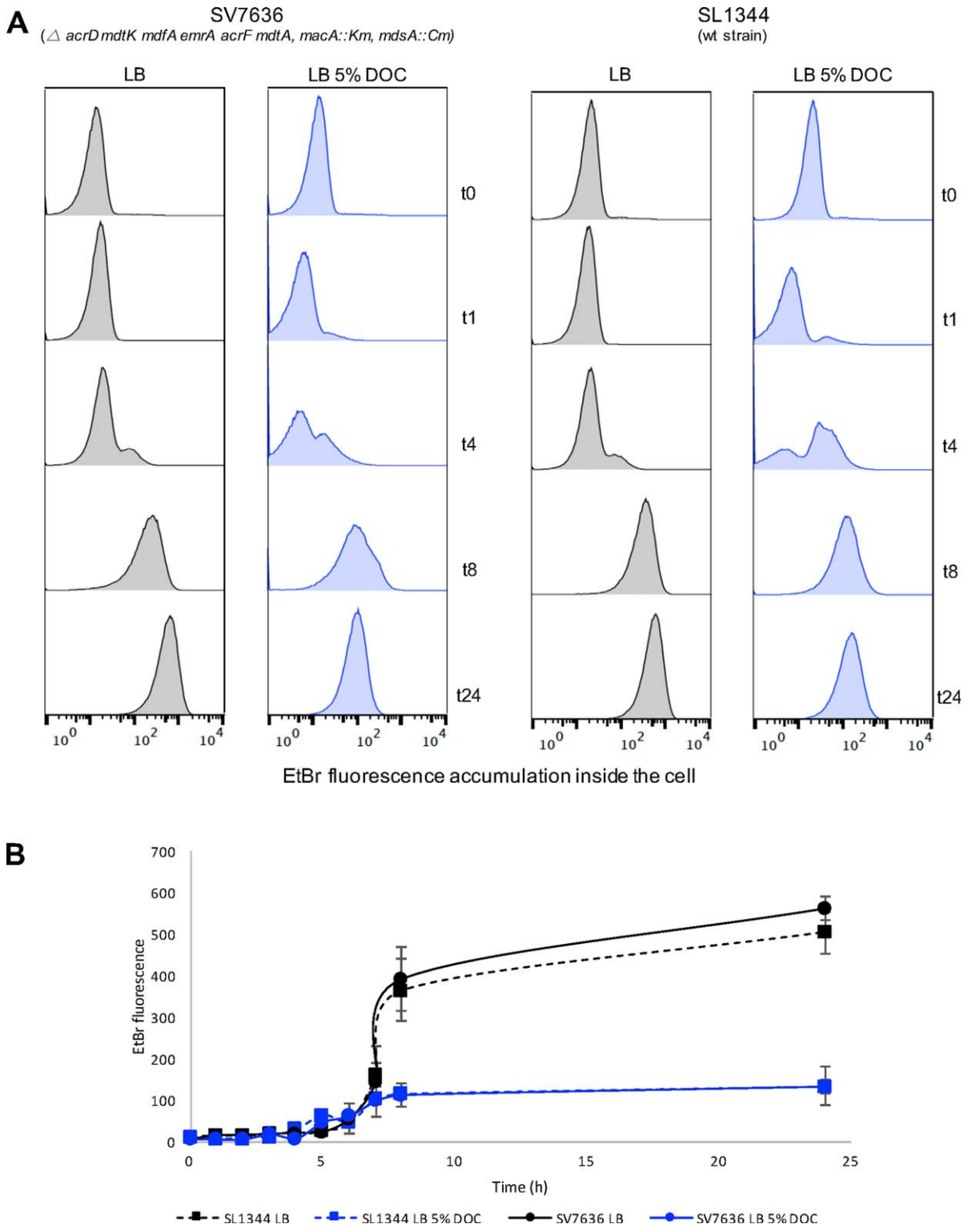


**Fig. 5.** Single cell analysis of AcrAB-mediated efflux during a 24-h period.
**A**. Histograms of ethidium bromide fluorescence of SV7636 and the wild type cultured in LB + 1 μg ml$^{-1}$ ethidium bromide and LB + 1 μg ml$^{-1}$ ethidium bromide + 5% DOC. $t_0$ corresponds to early exponential cultures before adding ethidium bromide; $t_1$, $t_4$, $t_8$ and $t_{24}$ indicate 1, 4, 8 and 24 h after EtBr addition, respectively.
**B.** Curves of ethidium bromide fluorescence for SV7636 and for the wild type strain, plotted using the same flow cytometry data represented in the histograms. Averages and standard deviations from > 3 independent experiments are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

systems appear to be dispensable for adaptation (Tables 3 and 4, Figs. 2 and 3). A tentative interpretation is that the contribution of these efflux systems, some of them known to transport bile salts, may be minor in the presence of a functional AcrAB efflux system. In support of this view, a mutant lacking all efflux pumps except AcrAB remained able to adapt (Table 4, Fig. 3).

Analysis of transcript abundance by quantitative real-time PCR revealed that sublethal concentrations of DOC upregulate expression of the *acrAB* operon (Fig. 4). In turn, higher *acrAB* expression can be correlated with the increased efflux activity detected by single cell analysis of EtBr accumulation in the presence of DOC (Fig. 5). Hence, *acrAB* upregulation may be a key event for adaptation, and may occur immediately or shortly after the encounter with bile. However, a steady state may be reached when a sufficient amount of AcrAB is present in the cell envelope, thereby explaining that upregulation of *acrAB* expression is lower in stationary cultures.

DOC-induced efflux decreases when the cultures approach the stationary phase and remains low thereafter (Fig. 5). One tentative explanation may be that physiological responses involved in adaptation are active in stationary cultures, thus reducing bile intake and bile-induced intracellular damage. This possibility is supported by two observations: (i) stationary cultures are more resistant to bile than exponentially growing cultures (Pucciarelli *et al*., 2002); (ii) stress responses induced by sublethal concentrations of bile are active in nondividing cells (Prieto *et al*., 2006; Merritt and Donaldson, 2009; Hernández *et al*., 2012). Furthermore, bile influx may be lower in adapted cultures as a consequence of cellular envelope remodelling, active stress responses and DNA repair mechanisms (Prieto *et al*., 2006; Hernández *et al*., 2012; 2015). These responses may reduce the need of AcrAB-mediated efflux.

The list of bile adaptation functions identified in this study is unlikely to be complete. However, our observations support a tentative model in which AcrAB-mediated efflux is essential to adapt, while other functions may have complementary roles. If one considers the multiple types of damage caused by bile salts (Coleman *et al*., 1980; Kandell and Bernstein, 1991; Gómez Zavaglia *et al*., 2002; Leverrier *et al*., 2003; Prieto *et al*., 2004; 2006; Merritt and Donaldson, 2009), it is not surprising that lowering their concentration inside the cell by active efflux may be a *sine qua non* condition to survive.

The fundamental role of AcrAB-mediated efflux in adaptation to bile is reminiscent of other adaptive strategies involving active efflux. The list of compounds extruded by bacterial efflux pumps includes antibiotics, antiseptics, heavy metals, solvents, detergents, animal hormones and host-defense molecules (Piddock, 2006; Martínez *et al*., 2009; Blair *et al*., 2014). Furthermore, certain efflux pumps can export virulence factors (Piddock, 2006). The observation that lack of AcrB function impairs *Salmonella enterica* virulence *in vivo* and in cell cultures (Wang-Kan *et al*. 2017) therefore suggests that AcrAB-mediated activity may be necessary inside the animal host not only for efflux of bile and other obnoxious compounds but also for the secretion of virulence effectors.

## Experimental procedures

### *Bacterial strains, media, culture conditions and chemicals*

Strains of *Salmonella enterica* serovar Typhimurium (often abbreviated as *S. enterica*) derive from SV5015, a $His^+$ derivative of the mouse-virulent strain SL1344 (Supporting Information Table S1). Strains SV7249 and SV7254 were constructed by transducing alleles from ATCC14028 to SL1344. Transduction was performed with phage P22 HT 105/1 int201 (Schmieger, 1972 and G. Roberts, unpublished data). The P22 HT transduction protocol was described elsewhere (Garzón *et al*., 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan *et al*. (1972), except that methyl blue (Sigma Aldrich) was substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Lysogeny Broth (LB) was used as liquid medium. LB plates contained agar at 15 g $L^{-1}$ final concentration. Cultures were grown at 37°C with shaking at 250 rpm in a New Brunswick Innova 3100 waterbath. Deoxycholic acid (sodium salt) and sodium choleate (ox bile extract) were both from Sigma Aldrich. Antibiotics were used at the final concentrations described previously (Prieto *et al*., 2004). Ethidium bromide was purchased from Sigma Aldrich.

### *Gene disruption*

Targeted gene disruption was achieved using plasmids pKD3, pKD4 and pKD13 (Datsenko and Wanner, 2000). To construct multiple efflux pump mutants (e.g., SV7636), antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 (Datsenko and Wanner, 2000) followed by transductions with mutant lysates of P22 HT phage (Schmieger, 1972). The oligonucleotides used for disruption (labelled 'FOR' and 'REV') are listed in Supporting Information Table S2 together with the oligonucleotides used for allele verification (labelled 'E') by the polymerase chain reaction.

### *Adaptation assay on bile gradient plates*

Stationary cultures in LB broth were diluted 25 times (100 μl of bacterial suspension in 2.5 ml LB) and incubated at 37°C with shaking until they reached a concentration of approximately $1.2 \times 10^8$ cells $ml^{-1}$. From these cultures, 100 μl were plated on 0%–30% ox bile agar gradient plates, and another 100 μl were diluted in 2.5 ml of LB with a sublethal concentration of sodium deoxycholate (DOC) and incubated o/n at 37°C with shaking. The following day, the cultures were diluted like the

day before in LB with DOC and incubated until there where roughly $1.2 \times 10^8$ cells $ml^{-1}$; 100 μl of the culture were plated on the ox bile gradient plates while other 100 μl were cultured o/n in LB. The overnight cultures where diluted in LB like the previous days, and plated again on ox bile gradient plates. All the plates were incubated at 37°C for 2 days, growth was visually monitored.

### *Determination of minimal inhibitory concentrations (MIC) of sodium deoxycholate*

Exponential cultures in LB broth were prepared, and samples containing around $3 \times 10^2$ colony-forming-units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab) containing known amounts of DOC. After 12 h incubation at 37°C, growth was visually monitored.

### *RNA isolation, quantitative reverse transcriptase PCR (qRT-PCR) and data analysis*

To prepare cells for RNA extraction, 5 ml of fresh LB ± 5% DOC was inoculated with 100 μl from an overnight bacterial culture, and incubated with shaking at 37°C. Three biological replicates were performed for each strain. In exponential cultures (O.D.600 ≈ 0.6), a 2 ml aliquot from cultures in LB was extracted after 1.5 h, and after 3 h in LB + 5% DOC; in stationary phase cultures (OD 600 ≈ 2), 2 ml aliquots were extracted from o/n cultures. The samples were centrifuged at 13 000 rpm at 4°C for 2 min in a 5415 R Eppendorf centrifuge. The pellet was washed three times with NaCl 0.9% and resuspended in 100 μl of a solution of lysozyme (3 mg $ml^{-1}$). Cell lysis was facilitated by a freeze–thaw cycle. After lysis, RNA was extracted using 1 ml of TRIsure reagent following manufacturer's instructions (Bioline). Total RNA was then resuspended in 30 μl of RNase-free water. The quantity and quality of the RNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies). An aliquot of 1 μg of RNA was used for cDNA synthesis using QuantiTect® Reverse Transcription Kit (Qiagen) following manufacturer's instructions. Quantitative RT-PCR reactions were performed in a Light Cycler 480 II apparatus (Roche). Each reaction was carried out in a total volume of 10 μl on a 480-well optical reaction plate (Roche) containing 5 μl SYBR, 0.2 μl DYE II (Takara), 4 μl cDNA (1/10 dilution) and two gene-specific primers at a final concentration of 0.2 mM each. Real-time cycling conditions were as follows: (i) 95°C for 10 min and (ii) 40 cycles at 95°C for 15 s, 60°C for 1 min. A non-RT control was included for each primer set. Triplicates were run for each reaction, and the $C_t$ value is averaged from them. Absence of primer dimers was corroborated by running a dissociation curve at the end of each experiment to determine the melting temperature of the amplicon. Melting curve analysis verified that each reaction contained a single PCR product. Gene-specific primers were designed with Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), and are listed in Supporting Information Table S2.

For quantification, the efficiency of each primer pair was determined to be between 90%–110%, following the instructions for efficiency determination described in the 'Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR' (Applied Biosystems, 2014). These efficiencies indicate that the amount of DNA is doubled in each PCR cycle, and allows for direct comparison between different genes. Relative RNA levels were determined using the $\Delta\Delta C_t$ method as described in the Applied Biosystems manual. Briefly, each gene $C_t$ value is normalized to the $C_t$ value for the internal control (*hdfR*), which provides the $\Delta C_t$ value. This value is then related to a given gene in the reference strain (SL1344, in this case), providing the $\Delta\Delta C_t$ value. Since the amount of DNA doubles in each PCR cycle, the relative amount of input cDNA can be determined by using the formula $2\text{-}\Delta\Delta C_t$. Each $\Delta\Delta C_t$ determination was performed at least in three different RNA samples (three biological replicates), and the results presented here are representative examples of such determinations. The Student's *t* test was used to determine whether the differences in RNA content were significant.

### *Assessment of efflux pump activity by flow cytometry*

Flow cytometry was used to monitor accumulation and efflux of ethidium bromide (EtBr) on a real-time basis, a method previously described (Martins *et al.*, 2006; Viveiros *et al.*, 2008). Data acquisition and analysis were performed using a Cytomics FC500-MPL cytometer (Beckman Coulter). EtBr (Sigma Chemical Co.) was excited at 488 nm, and fluorescence was detected using a 585 nm filter. To study AcrAB activity with DOC, exponential cultures of strains SV7636 (Δ*acrD* Δ*mdtK* Δ*mdfA* Δ*emrA* Δ*acrF* Δ*mdtA macA::Km mdsA::Cm*) and SL1344 were diluted in LB and LB + 5% DOC and incubated for 2 h (for exponential phase) or overnight (for stationary phase). EtBr was then added (1 μg $ml^{-1}$) and the cultures were incubated for 60 additional minutes at 37°C in the dark to permit EtBr accumulation. Finally, 0.5 ml of each culture was centrifuged for 3 min at 13 000 rpm and the pellet was resuspended in phosphate saline buffer for fluorescence measurement. Data were collected for 100 000 events per sample, and were analyzed with CXP and FlowJo 10 Software.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Procedures employed to assay adaptation to bile: MIC determinations in 96-well microtiter plates and growth on agar containing a gradient of ox bile. In both types of trials, adaptation was detected as a transient increase in bile resistance after growth in the presence of a sublethal concentration of DOC.

**Table S1.** Strains of *Salmonella enterica* serovar Typhimurium.

**Table S2.** Oligonucleotides used in this study.