RESEARCH ARTICLE
INTERNATIONAL MICROBIOLOGY (2013) 16:87-92
doi: 10.2436/20.1501.01.183 ISSN 1139-6709 **www.im.microbios.org**
INTERNATIONAL MICROBIOLOGY OPEN ACCESS

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

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Received 29 May 2013 · Accepted 26 June 2013

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):87-92]

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.

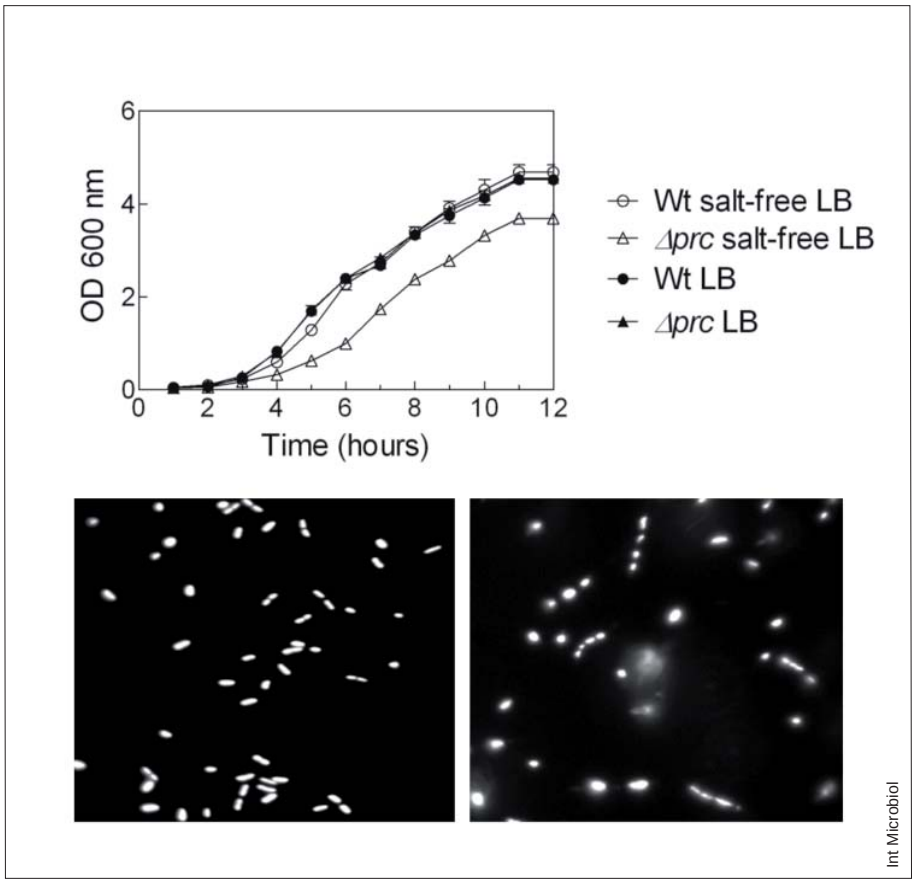

Fig. 1. Top: Growth of the wild type strain and its Prc⁻ derivative SV6278 in LB and salt-free LB. Bottom: Microscopic photographs of wild type (SL1344, left) and Prc⁻ (SV6278, right) *S. enterica* cells grown in salt-free LB.

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

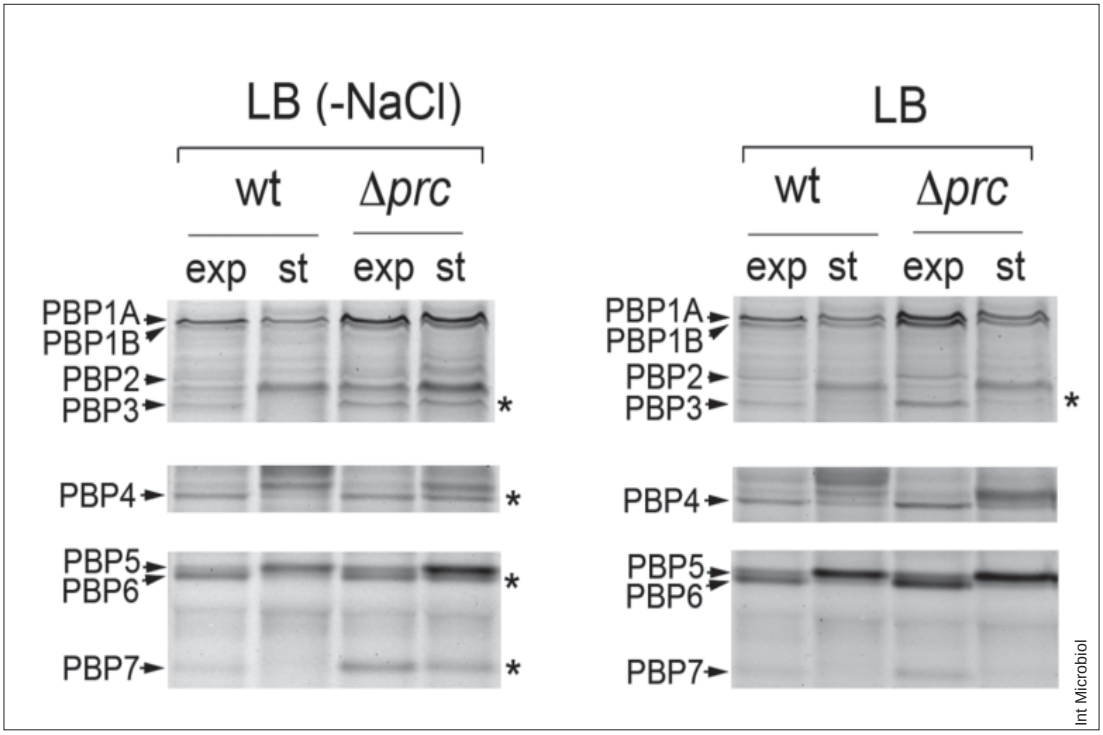

Fig. 2. Binding of bocillin FL to cell envelopes of stationary (st) and exponential (exp) cultures from wild-type *S. enterica* (wt) and from the Prc⁻ mutant SV6278 (Δ*prc*) grown in LB and salt-free LB (LB – NaCl).

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.

Acknowledgements. This study was supported by the Spanish Government and the European Regional Fund (grants BIO2010-15023, BIO2010-18885, and BFU2009-09200) and by the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (grant CVI-5879). G.R. is supported by a fellowship from the Programa de Formación de Personal Universitario (FPU) of the Spanish Ministry of Education and Culture.

Competing interests. None declared.

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