Stress response and resistance of *Salmonella enterica* serotype Enteritidis to the efflux pump inhibitor neuroleptic drug thioridazine

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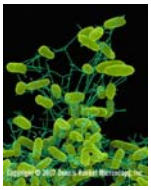
ABSTRACT

Introduction. The main reason for problematic therapy lies in the variety of responses that *Salmonella* activates when in a noxious environment, rendering the organism quite resistant to most antibiotics. Multidrug resistant (MDR) phenotype of most clinical bacterial isolates is due to the over-expression of multidrug efflux pumps. Compounds that are efflux pump inhibitors (EPIs) reduce or reverse resistance to antibiotics to which the bacterial strain is initially resistant.
Objectives. In the present study, thioridazine (TZ)-induced accumulation of the universal efflux pump substrate ethidium bromide and its subsequent efflux by *Salmonella enterica* serotype Enteritidis strains was investigated under different physiological conditions. **Methods.** Concentrations of TZ were evaluated for activity against over-expressed MDR efflux pumps of *Salmonella* strains with the aid of the automated ethidium bromide (EB) real-time fluorimetric method. The activity of genes that regulate and code for the AcrB transporter, was demonstrated by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). **Results.** *Salmonella enterica* serotype Enteritidis cultured in medium containing increasing concentrations of TZ does not grow during the first 6–8 h, after which time its growth is similar to unexposed controls. At the end of a 16-h exposure period, the organism is resistant to >250 mg/L TZ. The TZ promoted increase of accumulation of EB that is followed by efflux may be the reason for the resistance of *Salmonellae* to this phenothiazine.
The genetic response against TZ treatment was assessed by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) at periodic intervals. It is demonstrated that a sequence of activation beginning with the stress gene *soxS*, followed by the global regulator *ramA*, then by the local regulator *marA* and then by the transporter *acrB* which remains over-expressed by the end of the 16 h culturing period. **Conclusions.** TZ seems to present an environmental challenge to the organism, namely TZ induces resistance to the agent as a consequence of the activation of genes that regulate and code for the main efflux pump AcrAB. Furthermore, TZ also activates the two-component regulon PmrA/B and because the activation of *pmrA/B* also activates *acrB*, the development of high resistance to TZ during a 16-h culture period is in part due to activation of the two-component regulon.

INTRODUCTION



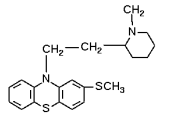
The ability of *Salmonella* to survive in the food chain is due, in part, to its ability to respond effectively to environmental changes.
Among these responses is activation of the PmrA/B two-component regulon, which is activated by the low pH of the phagolysosome of the neutrophil that temporarily imprisons the organism subsequent to its phagocytosis. Activation of the PmrA/B two-step regulon eventually leads to the synthesis of lipid A, which is rapidly introduced into the nascent lipopolysaccharide (LPS) layer of the outer cell membrane. The increase in LPS makes the organism resistant to practically everything, including most antibiotics and antimicrobial agents.

MATERIALS AND METHODS

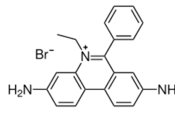
Materials
Mueller–Hinton (MH) powder (Sigma, Madrid, Spain) was used for preparation of MH agar and MH broth. Ethidium bromide (EB) and thioridazine (TZ) were also purchased from Sigma.
Bacteria
Salmonella Enteritidis NCTC 13349, *S.* Enteritidis 104 and 104_{CIP} and *S.* Enteritidis 5408 and 5408_{CIP} strains were studied. *Salmonella* Enteritidis 104_{CIP} and 5408_{CIP} strains were derived from their respective parental strains by gradual exposure to ciprofloxacin, achieving high-level resistance to this antibiotic.
Detection of efflux pump activity
Detection of efflux pump activity by the *Salmonella* strains was conducted by a semi-automated fluorometric method as described previously (1). The method follows the real-time accumulation of EtBr by a bacterial population using a Rotor-Gene 3000™ thermocycler (Corbett Research, Sydney, Australia).
Determination of growth of Salmonella strains in the absence and presence of thioridazine
Isolated colonies of the *Salmonella* strains were transferred to 10 mL tubes containing increasing concentrations of TZ in MH broth. Growth was followed spectrophotometrically at 600 nm for up to 24 h.
Assessment of the activity of genes that regulate and code for the AcrB transporter by real-time reverse transcriptase polymerase chain reaction
Isolated colonies of *S.* Enteritidis 104 were transferred to 10 mL tubes containing a subinhibitory concentration of TZ (100 mg/L) and at intervals of 0, 0,5, 1, 4, 8 and 16 h of culture at 37 °C the tubes were centrifuged at 1200 × *g* for 15 min. Pellets were suspended in PBS, centrifuged and re-suspended in PBS and the OD_{600} was adjusted to 0.6. From aliquots of 1.5 mL of each tube, total RNA was isolated in an RNase-free environment using an RNeasy Protect Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.
Real-time quantification of the RNA templates by real-time one-step RT-qPCR was performed in a Rotor-Gene 3000™ thermocycler (Corbett Research) strictly adhering to the manufacturer's recommendations of the QuantiTect® SYBR® Green RT-PCR Kit (QIAGEN).
The forward and reverse primers used for assessment of the activity of the global regulator *ramA*, the stress genes *soxS* and *rob*, the local regulator *marA*, the transporter *acrB* and the two-component regulon *pmrA* and *pmrB* are shown in Table 1.

Table 1. Primers used in this study.

Gene	Primer sequence (5'-3')	Amplicon Size (bp)	Reference
16SrRNA	Fw_GCGGCAGGCCTAACACAT Rv_GCAAGAGGCCCGAACGTC	182	O'Regan E *et al.*
ramA	Fw_CGTCATGCGGGGTATTCCAAGTG Rv_ CGCGCCGCCAGTTTTAGC	107	O'Regan E *et al.*
marA	Fw_ ATCCGCAGCCGTAAAATGAC Rv_ TGGTTCAGCGGCAGCATATA	180	O'Regan E *et al.*
soxS	Fw_ AAATCGGGCTACTCCAAGTG Rv_ CTACAGGCGGTGACGGTAAT	217	O'Regan E *et al.*
rob	Fw_ CATTACGGCTGGGCGAGTTTACC Rv_ CTGGCGGAATAGTTGGCGAATGAC	180	O'Regan E *et al.*
acrB	Fw_ TTTTGCAGGGCGCGGTCAGAATAC Rv_ TGCGGTGCCCAGCTCAACGAT	184	O'Regan E *et al.*
pmrA	Fw_CGCGCAAACCGAAGGCTATG Rv_GTATCGCGGGCGGTCAGAATG	192	This study
pmrB	Fw_GCGTGGGCCAGTCATTCTCTTCC Rv_ATCAATACCCGGCCCCTCGTCTT	299	This study



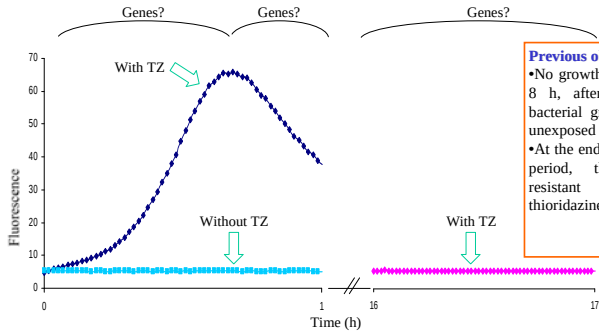
Thioridazine (TZ)



Ethidium bromide (EB)

OBJECTIVES

Figure 1. Efflux of the general efflux pump substrate EB by *Salmonella* Enteritidis 104 strain in the presence of 50 mg/L of TZ.

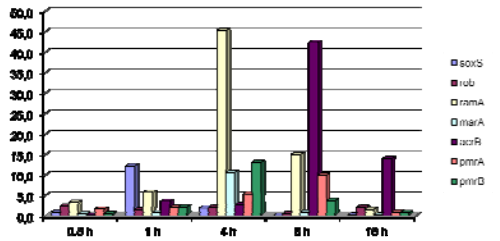


Previous observations:
•No growth during the first 6–8 h, after which time the bacterial growth is similar to unexposed controls.
•At the end of a 16-h exposure period, the organism is resistant to >250 mg/L thioridazine (TZ).

WHY?

RESULTS

Figure 2. Activities of genes during transient inhibition of growth from exposure to 100 mg/L thioridazine (TZ) on *Salmonella* Enteritidis 104. The values of the Y axis correspond to the comparison between the expression level compared to the control, using the comparative threshold cycle (C_T) method, using the formula $2^{-\Delta\Delta CT}$.



❖ During the first 8 h, the organism is not growing, however the genes that regulate and code for the AcrB transporter are sequentially activated.
❖ By the end of the 16-h culture period, only *acrB* remains elevated in activity.
❖ It is demonstrated that a sequence of activation beginning with the **stress gene *soxS***, followed by the **global regulator *ramA***, then by the **local regulator *marA*** and then by **the transporter *acrB*** which remains over-expressed by the end of the 16 h culturing period.

sequence of activation:
stress gene *soxS* → global regulator *ramA* → local regulator *marA* → transporter *acrB*

DISCUSSION

➲Because activation of the PmrA/B regulon takes place under a very acidic pH such as that present in an activated lysosome, TZ appears to present another environmental challenge to the organism that is independent of pH.

➲The exposure of *Salmonella* to the phenothiazine TZ induces resistance to the agent as a consequence of activation of genes that regulate and code for the main efflux pump AcrAB.

➲Because activation of the PmrA/B regulon results in the synthesis of lipid A that is introduced into the nascent LPS layer of the outer membrane, and this makes the organism resistant to practically everything, the eventual resistance to the phenothiazine (MIC > 200 mg/L) must in part be due to activation of the PmrA/B regulon.

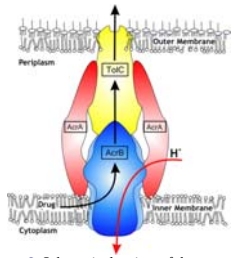


Figure 3. Schematic drawing of the AcrAB-TolC efflux pump from *E. coli.* *(http://www.pnas.org/content/106/17/6893/F1.large.jpg)*

CONCLUSIONS

❖ TZ seems to present an environmental challenge to the organism, namely TZ induces resistance to the agent as a consequence of the activation of genes that regulate and code for the main efflux pump AcrAB. Furthermore, TZ also activates the two-component regulon PmrA/B and because the activation of *pmrA/B* also activates *acrB*, causing the development of high resistance to TZ during a 16-h culture period.

❖ The transition from susceptibility to TZ to eventual high resistance to this phenothiazine, in our opinion, mimics what takes place in the patient who is initially treated with an antibiotic that proves ineffective: the adjunct use of TZ or perhaps any other similar phenothiazine may actually contribute to additional resistance.

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