Salicylate reduces the antimicrobial activity of ciprofloxacin against extracellular *Salmonella enterica* serovar Typhimurium, but not against *Salmonella* in macrophages

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**Objectives:** Salicylate, a potent inducer of the MarA activator in *Salmonella enterica*, is the principal metabolite of aspirin, which is often consumed for medicinal and cosmetic uses. Our research was aimed at testing if salicylate activates the mar regulon in macrophage-associated *Salmonella* (intracellular bacteria), and investigating its effects on bacterial susceptibility to ciprofloxacin extracellularly and intracellularly.

**Methods:** J774 macrophages were infected with *S. enterica* serovar Typhimurium (wild-type and marA null mutant), treated with ciprofloxacin with and without pre-exposure to salicylate, and the surviving bacteria were counted. Similar experiments were conducted with bacteria in broth (extracellular bacteria). Phe-Arg-b-naphthylamide (PABN) was added to investigate the role of efflux pumps in resistance. The transcriptional regulation of marRAB, acrAB and micF in extracellular and intracellular *Salmonella* Typhimurium with and without salicylate and ciprofloxacin was investigated using green fluorescent protein as a marker protein and quantitative real time PCR.

**Results:** Pre-exposure of *Salmonella* to salicylate increased the resistance of extracellular but not intracellular bacteria to ciprofloxacin, although salicylate stimulated the expression of mar genes in intracellular and extracellular bacteria. Using marA mutants and the inhibitor PABN, we showed that the improved resistance in extracellular bacteria is derived from the induction of acrAB by salicylate, which is mediated by MarA.

**Conclusions:** In intracellular bacteria, the expression of acrAB is already higher when compared with extracellular cells; therefore, salicylate does not result in significant acrAB induction intracellularly and subsequent resistance enhancement. Results show that conclusions raised from extracellular studies cannot be applied to intracellular bacteria, although the systems have similar functions.

**Keywords:** antimicrobial agents, antibiotic resistance, microbial adaptation, infected macrophages

**Introduction**

Salicylic acid (or sodium salicylate) is a non-steroidal anti-inflammatory compound. It has been used for medicinal purposes since the ancient Egyptian period. The acetylation of salicylate to form aspirin extended its usage in medicines and cosmetics, and today >16,000 tons of aspirin are consumed annually in the USA. It is often used to reduce fever, to relieve pain and as an anti-inflammatory drug. It also has an ‘anti-clotting’ effect; thus, it is used in low doses to prevent heart attacks, strokes and blood clot formation (reviewed in Price et al.). Salicylic acid and salicylate, which are the principal metabolites of aspirin, are also key ingredients in many skincare products and shampoos. In addition to its activity in mammals, salicylate induces morphological and physiological alterations in bacteria. For instance, in *Staphylococcus aureus* and *Escherichia coli* it modulates the transcription of dozens of genes. Salicylate is also associated with the production of factors involved with bacterial virulence and with reduction in the resistance to a variety of antimicrobials.

In *E. coli* and *Salmonella enterica*, the global regulators MarA and MarR are induced by salicylate. MarR binds the mar operator that flanks the promoter of marRAB and represses the transcription of these genes. Expression of MarA and MarR is induced by the prevention of MarR binding to the operator, and this may occur by the binding of MarR to salicylate. Salicylate is, in fact, the most potent known inducer of marRAB. The expression of MarA is further activated when MarA binds marbox in the operator of marRAB. MarA not only regulates its own expression, but also the expression of many genes involved in adaptation to...
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Materials and methods

**Eukaryotic cell line, bacterial strains and construction of mutants**

Eukaryotic cells used in this study are the mouse macrophage-like cell line J774.A1. *S. enterica* serovar Typhimurium ATCC 14028 was used for bacterial infection of the macrophages. Two mutants, ΔmarA and ΔacrAB, were constructed essentially according to Datsenko and Wanner21 using the primer pairs marFor5′-TCA TAG CAT TTG GGA CTG GAT CGA GGA TAA CCT GGA GTC Gtt gag cga tgt tgt agg ctg-3′ and marRev 5′-CGG GGT CAA TGT TTG CTG TGA CTC AAA GCC ATA GCG TTC Cat ggg aat tag cca tgg tcc-3′ for inactivating the marA gene, as well as acrBFor5′-TGC CAG AAG TTG GGG TTG TCA CAC TAA AAA CGG AAC CAC Ttt gag cga tgt tgt agg ctg-3′ and acrBRev 5′-TAA ACG TCG TTA GTC AGT CGG AAG GTC GCA GCC AGC Aat ggg aat tag cca tgg tcc-3′ for inactivating acrAB. Capital letters represent the homology region for site-specific recombination with the target gene and lowercase letters represent the primer part for PCR with the plasmid DNA pKD3.

**Plasmids used in this study**

Reporter plasmids pmarAB:gfp, pacrAB:gfp and pmicF:gfp used in this research were described previously.20 In these plasmids, the green fluorescent protein (GFP) gene was placed under the control of the marRAB, acrAB and micF promoters, respectively, in the low-copy vector pCS21. The reporter plasmid pChv1 was described previously.22 It carries the Vibrio fischeri luciferase system, luxABCDE and luxIR.

**Expression experiments in broth with GFP as a reporter**

Cultures harbouring each one of the GFP plasmids were grown in Luria-Bertani (LB) broth containing 30 mg/L kanamycin. Overnight cultures were diluted (1:100) in a fresh medium and 150 μL was distributed into each well of flat-bottomed 96-well plates. For induction, 0.1–20 mM sodium salicylate (Sigma Chemical) was added. Incubation was performed in a Victor2 multimwell fluorimeter (Wallac), set at 37°C with continuous shaking (normal speed), and assayed every 20 min. Conditions of incubation and measurements of fluorescence were conducted as described previously.20,21 The minimum concentration of salicylate that stimulated the highest induction of marA without inhibition of the bacterial growth was 5 mM and this concentration was used in further experiments. To analyse induction with ciprofloxacin, the antibiotic was added at a concentration of 0–0.3 mg/L (MIC = 0.125 mg/L). Experiments were repeated three times and each experiment contained triplicate assays. The fluorescence intensity (Flu) was calculated by subtracting the fluorescence of the control cultures (bacteria with pCS21 without insert). The fluorescence intensity at each point of the growth curve was normalized by dividing the fluorescence by the optical density of the bacteria (at 600 nm).

**Effect of salicylate on the antibacterial activity of ciprofloxacin against bacteria in broth**

Overnight cultures were diluted (1:100) in fresh medium and grown for 1.5 h (to an approximate OD<sub>600</sub> of 0.3). Aliquots of the culture (25 μL) were distributed into wells of 24-well plates and bacteria were harvested by centrifugation. The supernatant was removed and the bacteria were resuspended in fresh medium (500 μL) supplemented with 0 or 5 mM salicylate. Plates were incubated at 37°C for 1 h with shaking and the medium was replaced with fresh LB supplemented with 0 or 0.1 mg/L ciprofloxacin. Bacteria were incubated with the antibiotic for 1 h, harvested by centrifugation, and the pellet was resuspended in saline, serially diluted (1:10) and plated on LB agar for viable counts of the survivors. To study the role of AcrAB in salicylate-dependent tolerance to ciprofloxacin, the same experiments were conducted with 20 mg/L Phe-Arg-β-naphthylamide (PAβN), which inhibits efflux pumps.24

**Bacterial infection of macrophages**

The J774 macrophages were grown in 24-well plates in Roswell Park Memorial Institute (RPMI) medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum, 40 μM β-mercaptoethanol, 5000 U/L penicillin and 50 mg/mL streptomycin, as described previously.12 Overnight cultures of *Salmonella* carrying one of the GFP genes in

stress conditions, such as oxidative stress, heavy metals or antimicrobials.10

A well-known example of an adaptive phenotype controlled by MarA is increased tolerance to antibiotics such as tetracycline, chloramphenicol and fluoroquinolones,11,12 and to other antimicrobial compounds such as triclosan13 and pine oil.14 This is achieved mainly due to control of efflux by up-regulation of the AcrAB-ToLC efflux pump15 and control of influx by the induction of micF,13,16 which encodes an antisense RNA involved in the regulation of outer membrane protein F (OmpF), through which hydrophilic substances enter the cell. However, micF and acrAB are also induced by stress signals in additional ways that are not dependent on the marRAB system.17 Interestingly, antibiotics that serve as substrates of the AcrAB efflux pump are very weak inducers of the marRAB operon in *E. coli* and do not induce these genes in *Salmonella*.5,8 Based on that, it was suggested that exposure of *E. coli* or *S. enterica* to salicylate activates MarA through binding to its repressor, MarR. This induction increases efflux and decreases influx of multiple antimicrobials, resulting in increased tolerance to the antimicrobials.2,6 While this effect has been extensively shown in vitro, very little is known about the effect of salicylate on pathogens in the host.

The foodborne pathogen *S. enterica* serovar Typhimurium is able to survive and replicate in host macrophages and other types of cells. Studies of the role of AcrAB or MarA in virulence or resistance in the host cell environment are few and contradictory. Inactivation of marA in *Salmonella* Typhimurium was shown not to affect the virulence of this pathogen in mice,12 but it significantly lowered the adherence to human gut cells and survival within the macrophages.2 AcrAB was shown to play a role in the colonization of chickens by *Salmonella* Typhimurium, in invasion into macrophages in vitro and in the expression of proteins involved in pathogenesis.18,19 Moreover, although it was shown that an active mar operon slightly enhances the resistance of *Salmonella* to bile salts and to different types of antibiotics in the laboratory and in the environment, very little is known about the importance of this system for the success of antibiotic treatment within the host. Recently, we have shown that in *S. enterica*, but not in *E. coli*, marA and acrAB are better expressed at 37°C (in comparison with 30°C) and are induced by salicylate to higher levels at 37°C.20 This revealed the hypothesis that pre-exposure of intracellular *Salmonella* to salicylate would increase its tolerance to antibiotics. The primary goal of this study was to better understand the roles that salicylate plays in the tolerance to antibiotics in macrophages. For this purpose, we determined the effect of salicylate on the susceptibility to ciprofloxacin, and compared the transcriptional regulation of marRAB, acrAB and micF in macrophages and in extracellular bacteria.

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reporter plasmids, which had been grown with 0 or 20 mg/L PAbN, were diluted (1:100) and grown in LB supplemented with/without the inhibitor for 1.5 h (until they reached mid-log phase), harvested by centrifugation, resuspended in fresh RPMI, and added to the wells with the macrophages to reach a multiplicity of infection of 1:50 to 1:100 macrophages to bacteria. After incubation for 45 min (37°C, 5% CO₂), the infected macrophages were washed three times with PBS and incubated for 1 h in fresh RPMI supplemented with 100 mg/L gentamicin to kill the extracellular bacteria.

**The effect of salicylate on the transcription of acrAB, marRAB and micF in intracellular Salmonella**

After an additional three washing steps, salicylate at concentrations of 0, 1, 3 or 5 mM was added to the RPMI medium and the infected macrophages were incubated for 1 h. To estimate the bacterial GFP expression in each infected macrophage, the macrophages were analysed by the mean of flow cytometry with FACS calibur (Becton Dickinson) using WinMDI version 2.8 software. Experiments were repeated three times and each experiment contained triplicate assays. In each assay, 10,000 cells were analysed. Since 5 mM gave the highest induction without affecting macrophage viability or morphology, this concentration was used in further experiments.

**Permeability of macrophages to ciprofloxacin**

The time over which ciprofloxacin permeates macrophages and influences intracellular bacteria was analysed using the bioluminescence assay, as described previously. Briefly, the bacteria were transformed with the pCh1 plasmid that carries the entire *Vibrio fischeri* luciferase system. J774 macrophages were infected with the pCh1-carrying bacteria, as described above, and 500 μg/L homoserine lactone was added to the medium for induction of the luciferase system. Following incubation for 20 min, ciprofloxacin was added at concentrations of 0–10 mg/L. The plates were incubated in the luminometer and every 5 min orbital shaking was performed for 5 s, followed by a luminescence measurement. In each plate, three wells contained a ciprofloxacin-treated culture (of a single concentration) and three wells contained the untreated control culture.

**Activity of ciprofloxacin against intracellular bacteria with and without induction with salicylate**

Macrophages were infected with *Salmonella* as described above, treated with 0 or 5 mM salicylate for 1 h, washed and then treated with ciprofloxacin. *Salmonella* in macrophages was not affected by ciprofloxacin at the concentration used in broth (0.1 mg/L) and we had to increase the ciprofloxacin concentration to 20 mg/L in order to obtain a 2-log reduction. In half of the samples, 20 mg/L PAbN was added. Following incubation for 1 h, one of the two following options was performed: (i) flow-cytometric analysis to estimate the bacterial GFP expression in each infected macrophage; or (ii) viable counts of intracellular bacteria. For viable counts, macrophages were washed with PBS and incubated for 15 min with a lysis buffer, as described previously. Lysates were diluted (1:10 in PBS) and plated on LB agar.

**RNA extraction, real-time PCR (RT-PCR) experiments and quantification of mRNA**

Bacteria were grown as described above for infection of macrophages. For extraction of RNA from extracellular bacteria, samples (2.5 mL) were collected before the infection (at mid-log phase), added to 2.5 mL of RNAProtect Bacteria Reagent (Qiagen) and harvested, as instructed by the manufacturer. For extraction of RNA from intracellular bacteria, infected macrophages were washed three times with PBS and incubated for 15 min with a lysis buffer. Lysates (2.5 mL) were added to 2.5 mL of RNAProtect Bacteria Reagent (Qiagen) and harvested by centrifugation, as instructed by the manufacturer. RNA was extracted from extracellular and intracellular bacteria by using the RNaseasy Mini Kit (Qiagen). The extracted RNA was purified from DNA contaminants by on-column digestion using the RNase-free DNase Set (Qiagen) and cDNA was synthesized using the Reverse-IT 1st strand synthesis Kit (ABgene), as described previously.

RT-PCR for quantification of the specific mRNA molecules (marA, acrA and micF) was performed in a Rotor-Gene 3000 (Corbett Research) using the ABsoluteQPCR SYBR Green Mix (ABgene), as described previously. To determine if samples were contaminated with DNA, the total RNA was used as a template in PCR reactions using the same primers. Concentrations of the experimental transcripts were calculated from the linear regression of a standard curve of a PCR product and normalized by referring to the 16S rRNA calculated concentrations. For each culture, the normalized concentration of the untreated sample (without induction) was set at 1 and the other normalized concentrations were calculated proportionally.

**Data analysis**

Experiments were repeated three times and each experiment contained triplicate assays, so, in all, nine replicates were performed. Data were analysed with Microsoft Excel version 7 and statistically processed using the one-way analysis of variance method. P values ≤ 0.05 were regarded as significant.

**Results**

**The effect of salicylate on the activity of ciprofloxacin in extracellular and intracellular Salmonella**

We initially determined if pre-exposure of *Salmonella* to salicylate modulates its susceptibility to ciprofloxacin in broth and in macrophages. As can be seen in Table 1, induction with salicylate prior to exposure to ciprofloxacin in broth significantly improved the sensitivity of extracellular and intracellular *Salmonella* to ciprofloxacin, while in macrophages, no significant difference in sensitivity between the untreated sample (without induction) and the sample pre-exposed to salicylate prior to exposure to ciprofloxacin was observed.

| Strains and conditions | No induction | SAL | CIP | SAL+CIP |
|------------------------|--------------|-----|-----|---------|
| WT—extracellular       | 7.5          | 7.7 | 4.3 | 6.1<sup>a</sup> |
| WT—extracellular + PAbN | 7.9          | 7.4 | 2.4 | 3.9<sup>b</sup> |
| ΔmarA—extracellular    | 7.1          | 7.4 | 2.4 | 2.7<sup>c</sup> |
| WT—intracellular       | 6.2          | 6.2 | 4.2 | 3.8<sup>c</sup> |
| WT—intracellular + PAbN | 4.8          | 4.8 | 3.6 | 3.3<sup>c</sup> |
| ΔmarA—intracellular    | 6.1          | 6.6 | 4.9 | 4.8<sup>c</sup> |

WT, wild-type.  
<sup>a</sup>SAL concentration was 5 mM; CIP concentration was 0.1 mg/L in extracellular cultures and 20 mg/L in intracellular cultures.  
<sup>b</sup>The AcrAB inhibitor PAbN (20 mg/L) was added.  
<sup>c</sup>Statistically significant difference between treated and non-treated cells (P < 0.001).  
<sup>d</sup>Statistically significant difference between CIP treatment and SAL+CIP treatment (P < 0.001).
the tolerance of extracellular Salmonella to ciprofloxacin. On the other hand, exposure of intracellular Salmonella to salicylate prior to its exposure to the antibiotic did not affect significantly the susceptibility of intracellular Salmonella to ciprofloxacin (Table 1).

**Penetration of ciprofloxacin to intracellular Salmonella**

The low activity of ciprofloxacin against intracellular Salmonella raised concerns about the ability of ciprofloxacin to permeate the macrophages and affect the intracellular bacteria, so we initially determined the time over which ciprofloxacin affects the intracellular bacteria. For this purpose, the macrophages were infected with Salmonella Typhimurium carrying the luciferase reporter plasmid pChv1. The infected macrophages were treated with different concentrations of ciprofloxacin for up to 1 h and the emitted light produced by the intracellular bacteria was measured. Bacteria were also treated in broth for comparison. The bacterial light emission reported changes in the general bacterial physiology, since the luciferase activity declined when the bacteria were killed or subjected to stress.22 In extracellular Salmonella, the light emission declined within <5 min after exposure to 0.1 mg/L ciprofloxacin. In intracellular bacteria, ciprofloxacin at concentrations of <1 mg/L had a very low effect on the light emission, but the intensity of the light produced by intracellular Salmonella declined a few minutes after exposure to 10 mg/L ciprofloxacin, indicating that the bacteria were under stress caused by ciprofloxacin. These results reveal that ciprofloxacin at a concentration above 10 mg/L (i.e., 20 mg/L) permeates macrophages and influences the intracellular bacteria within minutes.

**The effect of salicylate and ciprofloxacin on the transcription of marRAB, micF and acrAB**

The induction of marRAB, acrAB and micF by salicylate and/or ciprofloxacin was investigated. Induction levels are dependent on exposure time. However, experiments of long duration with infected macrophages are complicated, because the macrophages and the bacteria undergo different changes during the infection, increasing the heterogeneity of the cultures. Therefore, we measured the effect of salicylate and ciprofloxacin on the transcription of the investigated genes after 1 h. As can be seen in Table 2, salicylate induced the expression of GFP under the marRAB, acrAB and micF promoters in broth, even after 1 h. On the other hand, ciprofloxacin did not change the GFP expression in any of the reporter plasmids used and we did not observe significant differences between the effect of salicylate alone or salicylate with ciprofloxacin. Since the induction ratios were very low after 1 h, we also looked at the induction of the genes after longer incubation to determine if ciprofloxacin has any effect on the expression. After 6 h of exposure to salicylate in broth, the expression of GFP under the marRAB, acrAB and micF promoters was stimulated with salicylate by 30.7-, 3.6- and 9.6-fold, respectively, and again ciprofloxacin did not change the GFP expression (Figure 1). Very similar results were observed when mRNA levels of the respective genes were measured directly using quantitative RT-PCR (data not shown).

To study the induction of the investigated genes in intracellular Salmonella, the fluorescence of each macrophage was measured. Two parameters were calculated in the infected macrophages: the average intensity of the fluorescence in the macrophages; and the percentage of macrophages with fluorescence levels above that of the control. Figure 2 shows representative flow cytometry histograms of macrophages infected with GFP-expressing Salmonella Typhimurium in which gfp is under the control of each promoter. It also illustrates the method used to determine the percentages of macrophages infected with Salmonella that express detectable amounts of GFP and the mean relative fluorescence intensity with and without salicylate. Table 3 summarizes the average changes of three experiments. The percentage of fluorescent macrophages

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Table 2. Changes (fold) in the fluorescence of extracellular Salmonella Typhimurium harbouring the pCS21, pmarRAB:gfp, pmicF:gfp and pacrAB:gfp plasmids after exposure to sodium salicylate (SAL, 5 mM), ciprofloxacin (CIP, 0.1 mg/L) or salicylate followed by ciprofloxacin for 1 h each.  

| Plasmid                  | SAL       | CIP       | SAL+CIP   |
|--------------------------|-----------|-----------|-----------|
| pCS21                    | 1.0       | 1.0       | 1.0       |
| pmarRAB:gfp              | 1.4*      | 1.0       | 1.4*      |
| pmicF:gfp                | 1.9*      | 1.0       | 1.7*      |
| pacrAB:gfp               | 1.2*      | 1.0       | 1.2*      |
| pCS21+PA6N               | 1.0       | 1.0       | 1.0       |
| pmarRAB:gfp+PA6N         | 1.5*      | 0.8       | 1.6*      |
| pmicF:gfp+PA6N           | 1.7*      | 1.1       | 1.5*      |
| pacrAB:gfp+PA6N          | 1.0       | 0.9       | 0.9       |

* Asterisks represent statistically significant differences between the induced and uninduced cultures (P < 0.001).

b The AcrAB inhibitor PA6N (20 mg/L) was added.

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![Figure 1. The effect of sodium salicylate and ciprofloxacin on the activity of the marRAB, acrAB and micF promoters in LB broth. Data show the induction (fold of normalized GFP fluorescence intensity) of Salmonella Typhimurium cultures harbouring the promoter–gfp fusions pmarRAB:gfp, pmicF:gfp and pacrAB:gfp after exposure to 5 mM salicylate or 0.1 mg/L ciprofloxacin for 6 h at 37°C in LB. Columns are an average of at least three different experiments; each experiment was conducted in triplicate. Standard errors of the means of nine assays are shown. In all genes induction with salicylate significantly increased transcription.](https://academic.oup.com/jac/article-abstract/65/5/888/826304/5mM-salicylate-0.1mgL-ciprofloxacin)
and the mean fluorescence intensity of the positive cells significantly increased after induction with salicylate when GFP was under the control of the marRAB promoter, but were not affected in macrophages infected with non GFP-expressing Salmonella. As in broth, the expression of GFP under the micF promoter was very strong even without induction and both parameters were induced with salicylate. Interestingly, when GFP was under the control of the acrAB promoter exposure to salicylate increased the number of fluorescent macrophages, but the mean fluorescence intensity of the positive cells did not change significantly after induction. Thus, even without induction, a portion of the bacteria produced the highest fluorescence. Salicylate mainly induced the expression in non-producing bacteria, but did not further induce it in the GFP-producing cells. Ciprofloxacin did not affect the fluorescence in macrophages. Combined treatment with salicylate and ciprofloxacin also did not show any

Figure 2. Fluorescence of intracellular Salmonella within J774A.1 macrophages with (b, d, f, h, j) and without (a, c, e, g, i) 5 mM salicylate, as measured by flow cytometry. The x-axis of each histogram represents the intensity of GFP fluorescence (log scale); the y-axis represents the cell number. S. enterica serovar Typhimurium harboured the following promoter–gfp fusions: pmarRAB:gfp (e, f); pmicF:gfp (g, h); and pacrAB:gfp (i, j). Controls were cultures of macrophages without infection (a, b) and macrophages infected with Salmonella harbouring the pCS21 plasmid (c, d). Events, the percentage of cells (out of 10000) within the positive region (M1); Flu, the mean fluorescence intensity for positive cells.
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Table 3. Changes (fold) in the percentage of fluorescent macrophages and their mean of fluorescence with and without salicylate (SAL) or ciprofloxacin (CIP) after infection with Salmonella harbouring the pCS21, pmarRAB:gfp, pmicF:gfp and pacrAB:gfp plasmids.

| Plasmid          | With 5 mM SAL | With 20 mg/L CIP | With 5 mM SAL + 20 mg/L CIP |
|------------------|---------------|-----------------|----------------------------|
|                  | events^c      | Flu^d           | events^c                   | Flu^d |
| Control          | 0.9           | 1.0             | 1.1                       | 1.0   |
| pmarRAB:gfp      | 10.6*         | 1.3^*           | 0.9                       | 1.0   |
| pmicF:gfp        | 6.2^*         | 1.3^*           | 0.8                       | 1.0   |
| pacrAB:gfp       | 2.9^*         | 1.0             | 1.3                       | 1.0   |
| Control + PAβN   | 1.0           | 1.0             | 1.1                       | 1.0   |
| pmarRAB:gfp + PAβN^b | 0.7^*       | 1.0             | 0.1^*                     | 0.9   |
| pmicF:gfp + PAβN^b | 7.3^*       | 1.3^*           | 2.3^*                     | 1.2   |
| pacrAB:gfp + PAβN^b | 0.5^*       | 0.7^*           | 0.2^*                     | 0.6^* |

^a Asterisks represent statistically significant differences between the induced and uninduced cultures (P<0.001).
^b The AcrAB inhibitor PAβN (20 mg/L) was added.
^c Events, change in % macrophages within the positive region (fold).
^d Flu, change in mean fluorescence intensity (fold).

significant difference compared with induction with salicylate only (Tables 2 and 3).

**Transcription in intracellular cells compared with extracellular cells**

Presuming that the methods used were sensitive enough to detect changes in the transcription of acrAB, it seems that the acrAB transcription was already high in many (but not all) intracellular Salmonella. Hence, salicylate did not induce the fluorescence in these cells. To confirm that transcription of acrAB is indeed higher in intracellular Salmonella, the acrA mRNA levels in intracellular bacteria and in bacteria grown in LB broth were compared. We found that not only are the normalized acrA mRNA levels higher in intracellular bacteria, so are the marA mRNA levels. On the other hand, micF transcription was lower in the intracellular bacteria (Figure 3).

**The role of the AcrAB efflux pump in the tolerance of Salmonella to ciprofloxacin**

To examine if the AcrAB efflux pumps have a role in the tolerance of intracellular bacteria to ciprofloxacin and in the salicylate-dependent tolerance to ciprofloxacin in broth, we constructed a mutant deleted in the acrAB genes. However, this mutant grew very slowly in LB and its ability to infect the macrophages was significantly lower than that of the wild-type (data not shown). As a result, comparison between the susceptibility of both strains to ciprofloxacin in broth and in macrophages was not possible, so we chose a different strategy to inactivate the AcrAB efflux pumps, using the PAβN inhibitor. PAβN affected the susceptibility to ciprofloxacin of both intracellular and extracellular Salmonella (Table 1), and it also diminished the salicylate-dependent tolerance of the bacteria to ciprofloxacin in broth. It should be noted that treatment with the inhibitor reduced the bacterial counts in the macrophages by 1.5-log. Looking at the expression of the genes after exposure to salicylate, PAβN did not influence the expression of GFP under the marAB and micF promoters in extracellular bacteria and micF in intracellular bacteria, but unexpectedly inhibited the expression under the marAB and acrAB promoters in intracellular bacteria. A decrease in the transcription under the marAB and acrAB promoters in parallel to the induction under the micF promoter was also observed in intracellular bacteria after exposure to ciprofloxacin (Tables 2 and 3).

**The role of MarA in the tolerance of intracellular and extracellular bacteria to ciprofloxacin**

To determine if the activation of the AcrAB efflux pump is mediated by MarA, we performed the described experiments with ΔmarA mutant (Table 1). We found that in contrast to the...
Discussion

Salicylic acid and its derivatives are frequently used in medical applications, but their effects on the development of tolerance to antibiotics have not been sufficiently documented to date, either in host cells or in vivo. The main conclusion of the present study is that exposure to salicylate improves the tolerance of Salmonella Typhimurium in broth to ciprofloxacin, but does not affect the tolerance of Salmonella Typhimurium in macrophages. Our results with extracellular Salmonella confirm previous in vitro studies that showed induction of low levels of resistance to multiple antibiotics in various bacterial species by salicylate, including resistance to fluoroquinolones, the drugs of choice for treating bacteraemic enteric fevers due to Salmonella.2,6,26,27 To validate that the salicylate-dependent increase in tolerance in broth is mediated through MarA, which activates the expression of AcrAB, we investigated the effect of salicylate on the activity of ciprofloxacin against marA null mutant. Results clearly showed that deletion of the marA gene increased the susceptibility of Salmonella in broth to ciprofloxacin, although ciprofloxacin itself was not a marA inducer. Moreover, salicylate did not have any effect on the susceptibility of the mutant to ciprofloxacin. Inhibition of AcrAB decreased the tolerance to ciprofloxacin and this tolerance was not affected by salicylate in the presence of the inhibitor. These results, together with the results of the induction of marA, micF and acrAB by salicylate in broth at 37°C, and the lack of the induction of acrAB by salicylate in the ΔmarA mutant strongly support the hypothesis that the salicylate-dependent increase in tolerance to ciprofloxacin in broth at 37°C is mediated by MarA, which activates the expression of the AcrAB efflux pumps, leading to a reduction in ciprofloxacin accumulation. The effect of salicylate on increased fluoroquinolone resistance in vitro and its mechanism of action through the Mar homologues have already been studied by diverse methods in different strains such as S. enterica, Serratia marcescens and S. aureus.6,28 For instance, in Salmonella Typhimurium DT104, 10 mM salicylate increased the MIC of ciprofloxacin 8-fold and only 2-fold in a marA null mutant.6 Based on this and other observations, there is a need to understand that salicylate used therapeutically could give rise to ciprofloxacin tolerance in vivo.6,28 Thus, this study was aimed to determine the effect of salicylate on the ciprofloxacin tolerance of Salmonella in macrophages. Salmonella in macrophages was less susceptible to ciprofloxacin. Much higher concentrations of antibiotics were needed to obtain a 2-log reduction, although the uptake of ciprofloxacin was rapid. Previous studies also demonstrated that ciprofloxacin rapidly penetrates J774 macrophages29 and accumulates in the cells, but we further demonstrated that ciprofloxacin not only penetrates the macrophages within minutes, but also affects intracellular bacteria. Ciprofloxacin also impaired DNA and protein synthesis in J774 macrophages at high concentrations (>30 mg/L).30 Nevertheless, these concentrations are above the concentrations used in this study. Similar to our results, ciprofloxacin showed reduced activity against intracellular bacteria like Listeria monocytogenes and S. aureus. It was suggested that several potential mechanisms may reduce the activity of quinolones intracellularly: (i) intracellular bacteria only poorly respond to the antibiotic due to the low growth rate; (ii) lower uptake by the intracellular bacteria; and (iii) the activity is defeated by the local environment in the host cells, such as low pH.29

The results of this study demonstrate that exposure of Salmonella in macrophages to salicylate induces the expression of MarA, but does not affect the resistance to ciprofloxacin. Moreover, deletion of MarA also does not decrease the resistance to ciprofloxacin. We suggest that the differences between intracellular and extracellular Salmonella reflect the fact that bacteria in macrophages already express high levels of the AcrAB efflux pumps in a MarA-independent pathway. In the macrophages, bacteria are faced with oxidative stress, nitrosative stress, nutrient-poor conditions, an acidic environment and the presence of defensins,31,32 and these special conditions affect the transcriptional profiles of the genes expressed by Salmonella. Indeed, results of the RT-PCR analysis show that marA and acrA are up-regulated in the macrophages. Hautefort et al. performed a microarray analysis of Salmonella Typhimurium and showed that the transcription level of marA in intracellular Salmonella was 4.4-fold higher compared with extracellular bacteria.32 The authors suggested that weak acids accumulating in the Salmonella-containing vacuole induce marAB.32 Surprisingly, the authors showed that acrA transcription was not significantly higher in intracellular compared with extracellular bacteria.32 The differences between the results of the microarray and the RT-PCR probably rely on the ability to detect lower levels of up-regulation with RT-PCR. The induction of marRAB and acrAB by salicylate in intracellular bacteria was diminished by the efflux pump inhibitor PAβN, while micF was not affected. This is surprising, because we would anticipate the opposite response: higher expression when the pumps are inhibited. It is possible that PAβN also inhibits pumps of macrophages and changes the environment in the host cell.

Three observations in the present study support the importance of AcrAB or other efflux pumps during infection of macrophages. First, acrAB null mutants had a lower infection dose compared with the wild-type strain. Second, exposure of intracellular Salmonella to an efflux pump inhibitor caused a significant reduction in bacterial numbers in the macrophages. Third, intracellular Salmonella are much more resistant to ciprofloxacin compared with extracellular bacteria. Weiber et al. have demonstrated that the AcrAB efflux pump is required for efficient adhesion, invasion and persistence in epithelial cells and macrophages by Salmonella.18,19 All these demonstrate that AcrAB and, perhaps, additional efflux pumps (that are inhibited by PAβN) are activated in intracellular Salmonella and might also be required to be efficient. The question that still requires an answer is why Salmonella is not functional during infection with Salmonella alone, without other bacteria that produce the SdiA inducer.25 SoxS is activated through SoxR by superoxide ions,

salicylate-mediated tolerance in the wild-type strain, in the ΔmarA strain salicylate did not influence the tolerance in broth. Deletion of marA did not significantly affect the bacterial counts of intracellular bacteria with and without salicylate (P<0.05).
H₂O₂ and paraquat. Since Salmonella is faced with oxidative stress in the macrophages, it is not surprising that intracellular bacteria levels of saxS mRNA are 3.7-fold higher compared with those in extracellular bacteria. Bile salts were shown to induce the expression of AcrAB by activation of RamA; however, it is possible that RamA contributes to the induction of AcrAB in macrophages by the detection of other signals in the host.

We can conclude that intracellular bacteria resist ciprofloxacin due to local environmental conditions, such as low pH, which reduce the activity of ciprofloxacin, and due to bacterial expression of systems aimed to stimulate adaptation to stress signals in the macrophage environment. For that reason, compounds such as salicylate do not have the same effect on the expression of bacterial genes in the macrophage. Salicylate up-regulates MarA in macrophages, but since AcrAB efflux pumps are already up-regulated, probably through SoxS or RamA, salicylate does not activate the efflux pumps significantly enough to affect the tolerance to ciprofloxacin. Our study points to the importance of AcrAB in both pathogenicity and resistance, and demonstrates that conclusions arising from experiments with extracellular bacteria cannot always be applied to intracellular bacteria, although the systems function in a similar way. The significance of these findings for salicylate and antibiotic resistance needs to be further evaluated in other host cells, such as epithelial cells, which lack oxidative or nitrosative stress and thus do not up-regulate SoxS. It will be interesting to further investigate the role of efflux systems in protecting against compounds that occur in the environment that the bacterium inhabits, in order to reveal the evolutionary role of these systems.

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None to declare.

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