Salicylate Functions as an Efflux Pump Inducer and Promotes the Emergence of Fluoroquinolone-Resistant
Campylobacter jejuni Mutants

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Salicylate, a nonsteroidal anti-inflammatory compound, has been shown to increase the resistance of Campylobacter to antimicrobials. However, the molecular mechanism underlying salicylate-induced resistance has not yet been established. In this study, we determined how salicylate increases antibiotic resistance and evaluated its impact on the development of fluoroquinolone-resistant Campylobacter mutants. Transcriptional fusion assays, real-time quantitative reverse transcription-PCR (RT-PCR), and immunoblotting assays consistently demonstrated the induction of the CmeABC multidrug efflux pump by salicylate. Electrophoretic mobility shift assays further showed that salicylate inhibits the binding of CmeR (a transcriptional repressor of the TetR family) to the promoter DNA of cmeABC, suggesting that salicylate inhibits the function of CmeR. The presence of salicylate in the culture medium not only decreased the susceptibility of Campylobacter to ciprofloxacin but also resulted in an approximately 70-fold increase in the observed frequency of emergence of fluoroquinolone-resistant mutants under selection with ciprofloxacin. Together, these results indicate that in Campylobacter, salicylate inhibits the binding of CmeR to the promoter DNA and induces expression of cmeABC, resulting in decreased susceptibility to antibiotics and in increased emergence of fluoroquinolone-resistant mutants under selection pressure.

Sodium salicylates are commonly used as nonsteroidal anti-inflammatory drugs (NSAIDs). The acetyl form of salicylate, aspirin, is used widely in medicines and cosmetics. It has been estimated that around 40,000 metric tons of aspirin are consumed each year in the world (32). The main functions of aspirin are to relieve minor aches and pains and to reduce fever. Aspirin also has functions in decreasing the incidence of strokes and heart attacks (3). Salicylic acid and salicylate are the principal metabolites of aspirin (11). Sodium salicylate is also used as an antipyretic, antiphlogistic, and analgesic agent in livestock and poultry (12). In addition, salicylic acid is a common compound in plants and in numerous foods and beverages (13, 33). Therefore, salicylate is available to humans and food-producing animals via multiple sources.

In addition to its effects in mammalian cells, salicylate also alters the susceptibility of bacteria to antibiotics. Growth of several bacterial species in the presence of salicylate induces nonheritable resistance to multiple antibiotics (25). In Escherichia coli, the presence of salicylate increases resistance to multiple antibiotics, including quinolones, cephalosporins, ampicillin, nalidixic acid, tetracycline, and chloramphenicol (25, 27). Salicylate-induced multiple antibiotic resistance in E. coli is mediated by increased transcription of the marRAB operon. Salicylate inhibits the binding of the repressor protein MarR to marO, the operator region of the mar operon, which then leads to overexpression of the transcriptional activator protein MarA (4). MarA modulates the transcription of a number of genes, including decreased expression of OmpF (a porin) and increased expression of the multidrug efflux pump AcrAB-TolC, which results in multiple antibiotic resistance (2). Increased resistance to chloramphenicol and enoxacin in Salmonella enterica serovar Typhimurium is also due to induction of the mar regulon by salicylate (31). In Klebsiella pneumoniae, salicylate-induced antibiotic resistance is due to increased expression of a MarA homologue, RamA, and the reduced production of two porins (5, 7).

Campylobacter is recognized as a leading bacterial cause of food-borne diseases in the United States and other developed countries (30). According to a CDC report, campylobacteriosis is estimated to affect over 0.84 million people every year in the United States (29). Worldwide, Campylobacter infections account for 400 to 500 million cases of diarrhea each year (28). Antibiotic treatment is recommended when the infection by Campylobacter is severe or occurs in immunocompromised patients. However, Campylobacter has become increasingly resistant to antimicrobials (18, 24). Among the known antibiotic resistance mechanisms in Campylobacter, the CmeABC efflux pump is an important player and confers resistance to structurally diverse antibiotics and toxic compounds (17). It has been demonstrated that CmeABC belongs to the RND family of efflux transporters and is regulated by a transcriptional repressor, CmeR, which binds to a specific site in the promoter region of cmeABC (15, 17). Expression of CmeABC is inducible by bile compounds, which interact with the ligand-binding domain of CmeR and prevent binding of CmeR to the cmeABC promoter in Campylobacter jejuni (14, 16). Further-
more, it has been shown that overexpression of CmeABC in *Campylobacter* significantly increases the frequency of emergence of fluoroquinolone-resistant mutants (35).

Previously, it was shown that growth of *Campylobacter* in the presence of salicylate resulted in a small but statistically significant increase in resistance to ciprofloxacin, tetracycline, and erythromycin (26). Later, Hannula and Hanninen confirmed a salicylate-induced increase in resistance to ciprofloxacin in almost all examined *Campylobacter* strains (10). These studies indicated that salicylate modulates *Campylobacter* resistance to antibiotics, but how salicylate influences antibiotic resistance and if it affects the emergence of antibiotic-resistant *Campylobacter* mutants are unknown. Based on previous findings on salicylate and *cmeABC* regulation, we hypothesized that salicylate modulates antibiotic resistance in *Campylobacter* by altering the expression of the *CmeABC* efflux pump. To examine this hypothesis, we sought to compare the expression levels of *cmeABC* with and without salicylate, to determine the interaction of salicylate with the CmeR regulator, and to assess the impact of salicylate on the emergence of fluoroquinolone-resistant *Campylobacter* mutants.

### MATERIALS AND METHODS

#### Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* strains were cultured on Mueller-Hinton (MH) agar or in MH broth at 42°C microaerobically (5% O₂, 10% CO₂, and 85% N₂) in a gas incubator. *C. jejuni* strains with antimicrobial resistance markers were grown on kanamycin (30 µg/ml) or chloramphenicol (4 µg/ml) when appropriate. All strains were preserved as 30% glycerol stocks at -80°C.

#### Antimicrobial susceptibility tests.

The MICs of antibiotics against *C. jejuni* NCTC 11168 were determined using either *Campylobacter* MIC plates (Trekb Diagnostic Systems) or a broth microdilution method as described previously (1). Since the MICs of other examined antibiotics were determined by Student's t-test.

#### Real-time qRT-PCR.

To verify if salicylate affects the antimicrobial susceptibility of *Campylobacter*, we measured the MICs of different antibiotics in the absence and presence of salicylate (100 µg/ml). Growth of *C. jejuni* NCTC 11168 with salicylate resulted in a moderate (2-fold) but reproducible increase in the MIC of ciprofloxacin in multiple experiments. This result is consistent with previous findings reported by others (10, 26). In this study, salicylate did not affect the MICs of other examined antimicrobials, including azithromycin, gentamicin, florfenicol, nalidixic acid, clindamycin, rifampin, cefotaxime, and streptomycin. However, the presence of salicylate increased the growth rate of strain 11168 in the presence of various antibiotics, including ciprofloxacin, erythromycin, novobiocin, and tetracy-
Salicylate, at their corresponding MICs (Fig. 1). Together, these results confirmed that salicylate decreased the susceptibility of Campylobacter to antibiotics. It should be pointed out that the enhanced resistance induced by salicylate was not inheritable. After removing salicylate from the medium, the MIC of ciprofloxacin for strain 11168 returned to the baseline level (data not shown).

Salicylate induces the expression of cmeABC in Campylobacter. To determine if salicylate induces the expression of cmeABC, we measured the β-galactosidase activity of strain 11168/pABC11 grown in the presence or absence of salicylate. Compared to the basal level of transcription in MH broth, addition of salicylate (100 μg/ml) to the culture resulted in a 3-fold increase in the expression of cmeABC (Fig. 2A). We further examined the levels of the cmeB transcript in Campylobacter cultures grown with different concentrations of salicylate (0, 100, and 200 μg/ml), using real-time qRT-PCR. As shown in Fig. 2B, salicylate induced the transcription of cmeB 2- to 3-fold, in a dose-dependent manner. An immunoblotting assay using anti-CmeABC antibodies further confirmed the induction of CmeABC by salicylate (Fig. 3). According to densitometric analysis (data not shown), the amounts of CmeABC proteins increased 1.5- to 3-fold in the presence of salicylate compared to the baseline control levels (in MH broth). The major outer membrane protein (MOMP) band, which was used as an internal control, did not show any changes among the samples (Fig. 3).

Salicylate interferes with CmeR binding to the cmeABC promoter. Since expression of CmeABC is controlled by CmeR (15), we further determined if salicylate affected the transcription of cmeB by using qRT-PCR. Results from three independent experiments did not reveal any significant changes in the transcription level of cmeB in the presence of salicylate (data not shown), indicating that salicylate did not alter the expression of cmeB. Thus, the enhanced expression of cmeABC by salicylate is unlikely to be due to a change in cmeR transcription.

Salicylate induces the expression of Cj0561c. In addition to the control of cmeABC expression, CmeR also functions as a repressor of Cj0561c (a putative periplasmic protein) in Cam-
Salicylate by specifically binding to the cj0561c promoter (9). Since salicylate inhibited the function of CmeR, we suspected that it might also induce the expression of Cj0561 in Campylobacter. To examine this possibility, we evaluated the promoter activity of cj0561c by using a transcriptional fusion construct (pMW561) (Table 1). The β-galactosidase activity of strain W7/pMW561 grown in MH broth supplemented with 100 μg/ml of salicylate increased 2.5-fold compared with that for growth in MH broth without salicylate (Fig. 5). An immunoblotting assay using anti-Cj0561c antibodies further confirmed the induction of Cj0561c by salicylate (Fig. 3). This induction result for Cj0561c is consistent with the result that salicylate interferes with the function of CmeR in Campylobacter.

Salicylate increases the frequency of emergence of fluoroquinolone-resistant Campylobacter mutants. Since salicylate induced the expression of CmeABC, we further examined if the presence of salicylate modulates the emergence of fluoroquinolone-resistant Campylobacter. The results are shown in Table 2. When 0.625 or 1.25 μg/ml ciprofloxacin was used in the selection plates for enumeration of mutants, C. jejuni 11168 exhibited similar frequencies of emergence of fluoroquinolone-resistant mutants, regardless of the presence of salicylate in the growth medium (~10⁻⁶; P ≥ 0.05). However, at a ciprofloxacin concentration of 4 μg/ml, incubation of strain 11168 with salicylate resulted in a 70-fold increase in the frequency of emergence of ciprofloxacin-resistant mutants, and the difference was statistically significant (P < 0.05). These findings indicate that salicylate increases the frequency of emergence of fluoroquinolone-resistant mutants on plates with a high concentration of ciprofloxacin.

**DISCUSSION**

The results from this study revealed that salicylate-mediated decreases in the susceptibility of Campylobacter to antibiotics are due at least partially to induction of expression of the CmeABC efflux pump. This conclusion is based on multiple pieces of experimental evidence derived from transcriptional fusion assays (Fig. 2A), real-time qPCRs (Fig. 2B), and immunoblotting assays (Fig. 3). We further showed that salicylate inhibits the binding of CmeR to its target promoters (Fig. 4), leading to enhanced expression of CmeABC and Cj0561c in the presence of salicylate (Fig. 2, 3, and 5). Together, these results provide a molecular basis for salicylate-induced resistance to antibiotics in Campylobacter.

The CmeABC efflux system plays an important role in resistance to antibiotics and toxic compounds in Campylobacter (17), and the expression of this efflux pump is modulated by CmeR (15). Previous studies demonstrated that overexpression of CmeABC results in modest increases in the MICs of antibiotics, including ciprofloxacin, erythromycin, novobiocin, tetracycline, cefotaxime, and fusidic acid (15, 16). In this study, we found that salicylate caused a small but reproducible increase in the MIC of ciprofloxacin and facilitated the growth of Campylobacter in the presence of inhibitory concentrations of antibiotics (Fig. 1). This finding is consistent with the results from previous studies using salicylate (10, 26).

CmeR is a pleiotropic regulator and functions as a transcriptional repressor of cmeABC and cj0561c (9, 15). Cj0561c is a periplasmic protein and is tightly controlled by CmeR due to the presence of two CmeR-binding sites in the promoter sequence of Cj0561c (9). Although the exact function of Cj0561c is unknown, a previous study showed that it contributes to the in vivo fitness of Campylobacter in chickens (9). In this study, we found that salicylate induced the expression of both CmeABC and Cj0561c. This induction can be explained by the fact that salicylate inhibited the binding of CmeR to promoter DNA, as shown by EMSA (Fig. 4). Compared with that by bile salts, which are strong inhibitors of CmeR binding (16), the
inhibition by salicylate was relatively weak but was visually apparent (Fig. 4). This finding suggests that salicylate releases the repression of CmeR on \textit{cmeABC} and \textit{cj0561c}, leading to increased expression of the two genes. Salicylate inhibits the function of CmeR, not the expression level of this regulatory protein. This conclusion is based on the fact that qRT-PCR did not detect altered transcription of \textit{cmeR} in the presence of salicylate (data not shown). How salicylate modulates the function of CmeR is unclear at present, but it is known that CmeR has a DNA-binding motif in the N-terminal region and a flexible ligand-binding pocket in the C-terminal region (8). It is possible that salicylate interacts with the ligand-binding pocket and triggers a conformational change in the DNA-binding domain, preventing CmeR binding to promoter DNA. This possibility remains to be examined in future studies. Based on results from this study and previously known information on CmeR regulation, we present a model that depicts the induction mechanisms of salicylate in \textit{Campylobacter} (Fig. 6). The binding of salicylate to CmeR appears to be reversible, since removal of salicylate from the culture medium restored \textit{cmeABC} expression to the basal level (data not shown).

Salicylate not only increases antibiotic resistance but also promotes the emergence of spontaneous fluoroquinolone-resistant \textit{Campylobacter} mutants under selection pressure. In \textit{Campylobacter}, fluoroquinolone resistance is mediated by target modification (GyrA mutations) and by the efflux function of CmeABC (6, 20, 23). A single point mutation in the quinolone resistance-determining region of \textit{gyrA} DNA is sufficient to significantly increase the resistance of \textit{Campylobacter} to fluoroquinolone antimicrobials (6, 18, 19, 24). A T86I substitution in GyrA confers high-level resistance to fluoroquinolones, while T86K, A70T, and D90N substitutions are associated with moderate resistance to fluoroquinolones (18, 24). It is important that CmeABC functions synergistically with GyrA mutations in conferring fluoroquinolone resistance and that without CmeABC, GyrA mutants are unable to maintain the resistance phenotype (18, 35). Thus, the expression level of CmeABC affects the frequency of emergence of fluoroquinolone-resistant mutants in \textit{Campylobacter} (35). In this study, we showed that addition of salicylate to the culture medium resulted in a 70-fold increase in the frequency of emergence of ciprofloxacin-resistant mutants at a higher concentration of the antibiotic (4 \mu g/ml) (Table 2). This result is consistent with our previous finding for a \textit{cmeR} mutant in which CmeABC was overexpressed, for which the frequency of emergence of fluoroquinolone-resistant mutants increased significantly (35).

For \textit{Campylobacter}, it is known that different GyrA mutations confer different levels of resistance, and the measured frequencies of emergence of resistant mutants vary with the concentration of ciprofloxacin in the plates (35). The presence of salicylate in the medium did not alter the frequencies of emergence of fluoroquinolone-resistant mutants (~10^{-6}) when the concentrations of ciprofloxacin in the selection plates were 5 times (0.625 \mu g/ml) and 10 times (1.25 \mu g/ml) higher than the MIC (Table 2). This result can be explained by the facts that the basal expression of CmeABC is sufficient and that overexpression of this efflux pump is not required for GyrA mutants to survive at low selection pressure (0.625 and 1.25 \mu g/ml) (35). In contrast, with 4 \mu g/ml of ciprofloxacin, those GyrA mutants with lower MICs would require overexpression of \textit{cmeABC} to survive the selection pressure, resulting in a difference in the numbers of detected mutants with and without salicylate. These results indicate that salicylate does not affect the spontaneous mutation rate but facilitates the emergence of fluoroquinolone-resistant mutants under anti-

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**FIG. 6.** Diagram depicting the molecular basis of salicylate-mediated induction of CmeABC and \textit{cj0561c}. (A) Baseline expression of the genes in the absence of salicylate. Transcription of \textit{cmeABC} and \textit{cj0561c} is at a low level due to inhibition by CmeR. (B) Induction of the genes by salicylate. When salicylate is present, it inhibits the binding of CmeR and ameliorates the repression on \textit{cmeABC} and \textit{cj0561c}, leading to overexpression of CmeABC and \textit{cj0561c}.
otic selection by inducing the expression of cmeABC. Similar findings were obtained in a previous study in which mutation of cmeR (overexpressed CmeABC) led to increased emergence of ciprofloxacin-resistant mutants for selection with ciprofloxacin at 4 µg/ml but not at 0.625 and 1.25 µg/ml (35).

In summary, this study identified the molecular mechanism underlying salicylate-induced antibiotic resistance in Campylobacter and revealed that salicylate promotes the emergence of fluoroquinolone-resistant Campylobacter mutants under selection pressure. Although these findings were made under laboratory conditions, they might be applicable to the ecological niches occupied by C. jejuni under natural conditions. Considering the common presence of salicylate in plant and food as well as its widespread use in veterinary and human medicine, it is possible that C. jejuni is exposed to salicylate in animal reservoirs and in the human host. Such exposure could conceivably influence the development of antibiotic resistance in this pathogenic organism. Those treating Campylobacter infections with fluoroquinolones should consider this possibility and avoid the simultaneous use of salicylate-containing medicine or salicylate-rich nutrients.

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