Multiple Antibiotic Resistance (*mar*) Locus in *Salmonella enterica* Serovar Typhimurium DT104

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In order to understand the role of the *mar* locus in *Salmonella* with regard to multiple antibiotic resistance, cyclohexane resistance, and outer membrane protein F (OmpF) regulation, a *marA*::*gfp* reporter mutant was constructed in an antibiotic-sensitive *Salmonella enterica* serovar Typhimurium DT104 background. Salicylate induced *marA*, whereas a number of antibiotics, disinfectants, and various growth conditions did not. Increased antibiotic resistance was observed upon salicylate induction, although this was shown to be by both *mar*-dependent and *mar*-independent pathways. Cyclohexane resistance, however, was induced by salicylate by a *mar*-dependent pathway. Complementation studies with a plasmid that constitutively expressed *marA* confirmed the involvement of *mar* in *Salmonella* with low-level antibiotic resistance and cyclohexane resistance, although the involvement of *mar* in down regulation of OmpF was unclear. However, *marA* overexpression did increase the expression of a ca. 50-kDa protein, but its identity remains to be elucidated. Passage of the *marA*::*gfp* reporter mutant with increasing levels of tetracycline, a method reported to select for *mar* mutants in *Escherichia coli*, led to both multiple-antibiotic and cyclohexane resistance. Collectively, these data indicate that low-level antibiotic resistance, cyclohexane resistance, and modulation of OMPs in *Salmonella*, as in *E. coli*, can occur in both a *mar*-dependent and *mar*-independent manner.

The multiple antibiotic resistance locus (*mar*) is reported to be present in *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter*, *Hafnia*, *Enterobacter*, and *Escherichia coli* (13). In recent years, much work has been carried out to elucidate the structure, function, and clinical significance of the *mar* locus, mainly in *E. coli* but also in *Salmonella* (1, 18, 29, 30, 42, 43). The *marRAB* operon, which is located at min 34.8 of the chromosomal map of *E. coli* K-12 (20), controls multiple antibiotic resistance in *E. coli* by production of MarA (12, 17, 27), a 127-amino-acid protein of the XylS/AraC family of transcriptional activator proteins, which alters the expression of several target genes (1). For example, in *E. coli* MarA positively regulates *acrAB* (25), which encodes a stress-induced efflux system, and *micF* (12, 38), which encodes an antisense RNA involved in the regulation of the porin outer membrane protein F (OmpF), through which hydrophilic substances enter the cell (35). The repressor MarR (43), encoded by *marR*, binds to the *marO* operator region (39) to negatively regulate expression of *marRAB*. Neither the function of MarB nor the natural inducer of the *marRAB* operon is known. A number of unrelated substances, including tetracycline, chloramphenicol, dinitrophenol, menadione, paraquat, plumbagin, benzoate, and sodium salicylate and related compounds, have been shown to induce the operon in *E. coli* (10, 38, 39), of which salicylate is the most potent inducer (10). In addition to the involvement of *mar* in multiple antibiotic resistance, it has also been shown to be involved in organic solvent tolerance (7, 47), resistance to disinfectants such as pine oil (31), and resistance to weak acids (5).

E. coli passaged on low levels of tetracycline or chloramphenicol produced *mar* mutants at a rate of about 10^{-8} per cell division, and these mutants had increased resistance to the unrelated antibiotics penicillin G, ampicillin, cephalothin, puromycin, rifampin, nalidixic acid, and fluoroquinolones (11, 19). Furthermore, *E. coli* strains resistant to pine oil, which is used in household disinfectants, showed resistance to multiple antibiotics (tetracycline, ampicillin, chloramphenicol, and nalidixic acid) that was associated with increased expression of *marA* (31). Continued passage of first-step *mar* mutants on media with increasing levels of tetracycline or chloramphenicol resulted in increased levels of antibiotic resistance (19). However, the genetic basis for second-step high-level resistant mutants was only partially attributed to *mar*, because transduction of the locus from high- or low-level *mar* mutants produced only a low level of multiple antibiotic resistance (1).

E. coli induced for *mar* has been demonstrated to have resistance to several unrelated antibiotics, which is in part associated with reduced levels of OmpF (11, 12). However, cyclohexane resistance in *E. coli* has been shown to be independent of OmpF but dependent on the *acrAB* efflux pump (6). For *Salmonella*, the involvement of reduced levels of OmpF in antibiotic and cyclohexane resistance is less well characterized. A *Salmonella enterica* serovar Typhi isolate resistant to chloramphenicol, carbenicillin, and ampicillin that lacked OmpF and did not encode a chloramphenicol acetyltransferase has been described (45). However, a lack of correlation between reduced levels of OmpF and quinolone resistance in clinical isolates of serovar Typhimurium from two patients that failed ciprofloxacin therapy has been shown (37).

There is an increasing concern regarding the veterinary use of antibiotics, which prompts a closer examination of the

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TABLE 1. Strains used

Strain	Properties or use	Reference
E. coli DH5α	F′*endA1 hsdR17*(r$_K$ m$_K^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1Δ* (*lacIZYA-argF*) U169 *deoR* (φ80 [d*lac*Δ(*lacZ*) *M15*]; general-purpose cloning strain	48
E. coli K-12 S-17(λ*pir*)	*pro res$^+$ mod$^+$* RP4 2-Tc::Mu-Km::*Tn*7 λ*pir*; recipient strain for suicide plasmid construct	40
E. coli AG100	Wild-type K-12 used as cyclohexane-sensitive control	19
E. coli AG102	*marR1* mutant of AG100 used as cyclohexane-resistant control	19
Serovar Typhimurium DT104 S3530/96	DNA template for construction of *marA*::*gfp*::*cam*	This study
Serovar Typhimurium DT104 S3992/96	Recipient strain for *marA*::*gfp*::*cam* construct (referred to as parent strain)	This study
LR1 to LR5	*marA*::*gfp*::*cam* reporter mutant clones of S3992/96	This study
LR6	S3992/96 + *marA* plasmid pLR1	This study
LR7	LR1 + *marA* plasmid pLR1	This study

mechanisms of resistance in zoonotic pathogens. While there has been considerable work done in relation to the role of the *mar* locus of *E. coli*, the role of the *mar* locus in the biology of *Salmonella* has not been investigated in such depth. It cannot be assumed that the *mar* locus will be isofunctional in *E. coli* and *Salmonella*. Thus, the aim of this work was to investigate the role of *mar* in *Salmonella* in antibiotic resistance, cyclohexane resistance, and modulation of OmpF.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Tables 1 and 2, respectively. Additionally, 44 serotypes of *Salmonella* were used. These are not listed in Table 1. All *Salmonella* strains were obtained from the collection of strains at the Veterinary Laboratories Agency, Weybridge, United Kingdom, and were originally isolated from animals or their environment. As negative controls for *marA* DNA hybridization studies, the following strains (not listed in Table 1) were used: *E. coli* NCTC 10418, *Enterobacter aerogenes* NCTC 10006, *Klebsiella aerogenes* NCTC 418, *Pseudomonas diminuta* NCTC 8545, *Proteus vulgaris* NCTC 4175, *Streptococcus mutans* NCTC 10920, *Streptococcus agalactiae* NCTC 8181, and *Staphylococcus aureus* NCTC 1803.

Antibiotics, chemicals, bacterial growth, and media. Sodium ampicillin, erythromycin, streptomycin sulphate, sodium cefoperazone, sodium sulfadiazine, chloramphenicol, sodium nalidixic acid, and tetracycline hydrochloride were obtained from Sigma-Aldrich Company, Ltd., as was hexane, cyclohexane, and salicylic acid. Zeocin was obtained from Invitrogen. Co-amoxiclav (amoxicillin and clavulanate, 2:1) and ciprofloxacin were obtained from SmithKline Beecham and Bayer, respectively.

All bacteria were grown in Luria-Bertani (LB) broth or agar except where stated. For MIC determinations, strains grown overnight at 37°C and diluted 1:10 in saline were inoculated onto Iso-Sensitest agar (Oxoid CM471) supplemented with antibiotics as required and also with 2.5 or 10 mM salicylate when measuring the effect of salicylate on antibiotic resistance. When measuring the effect of salicylate on MICs, strains were also grown in broth containing salicylate. For production of antibiotic-resistant (*mar*-like) mutants, LR1 and its isogenic parent (Table 1) were grown at 30°C for 48 to 72 h on Trypticase soy agar (Difco) supplemented with tetracycline, according to the method of George and Levy (19).

For OMP extraction, bacteria were grown for 48 h at 37°C on M9 minimal agar

supplemented with glucose (0.2% [wt/vol]), thiamine (4 mg/liter), and nicotinamide (0.6 mg/liter). For acid tolerance assays, strains were grown overnight in buffered peptone water (BDH). Acid tolerance was determined in prewarmed buffered peptone water adjusted to specific pH values with hydrochloric acid.

To assess induction of *marA*, the serovar Typhimurium *marA*::*gfp*::*cam* reporter mutant and isogenic parent were grown at 30 and 37°C in the presence of salicylate ranging from 2.5 to 20 mM. Additionally, induction of *marA* at 37°C was monitored when strains were grown in the presence of the antibiotics and disinfectants at one-fourth or one-half of the MICs for those strains. The disinfectants tested were a synthetic phenol-xylenol mixture, a quaternary ammonium compound, a peroxygen compound (acetic acid in equilibrium with hydrogen peroxide), and a compound product of formaldehyde, glutaraldehyde, and the quaternary ammonium compound. To monitor the effects of some environmental stresses on induction of *marA*, strains were grown in LB broth supplemented with 15 and 30% (wt/vol) sucrose (high osmolarity), LB without NaCl (low osmolarity), or in M9 minimal medium supplemented with 0.025% (wt/vol) glucose (carbon limitation). Overnight LB cultures were held at 46°C for 2 h (heat shock).

PCR amplification. The oligonucleotide primers used are listed in Table 3 and were designed from the published *mar* sequence for serovar Typhimurium (42) using the DNASTAR software LaserGene. PCR was performed as described previously (2), and serovar Typhimurium DT104 S3530/96 DNA was used as a template.

DNA hybridization and Southern blotting. Colony dot blot DNA hybridization studies were performed to test if *marA* sequence homology was present in the 44 *Salmonella* serotypes. The serotypes tested were Agama, Agona, Anatum, Arizonae, Binza, Bovis morbificans, Bradenburg, Bredeney, Cubana, Derby, Dublin, Ealing, Enteritidis, Give, Gold Coast, Hadar, Harford, Heidelberg, Idikan, Indiana, Infantis, Kedougou, Kentucky, Lexington, London, Manhattan, Mbandaka, Montevideo, Muenster, Newington, Newport, Ohio, Oranienberg, Orion, Panama, Poona, Pullorum, Senftenberg, Taksony, Tennessee, Thompson, Typhimurium, Virchow, and Wangata. At least two isolates of each serotype were tested. Other strains were also tested as negative controls.

Colony dot blotting was performed using the *marA* 387-kb probe (from this study) and Southern blotting was performed using the *marRAB* 1.3-kb probe (from this study). Additionally, Southern blotting was performed using linearized suicide plasmid pLR4 without *marRAB* or *cam*. The probes were prepared by PCR. Probe amplicons were labeled using a Rediprime random primer labeling kit (Amersham Life Sciences) according to the manufacturer's instructions for dot blotting or were labeled using a nonradioactive labeling kit (Gene-Images

TABLE 2. Plasmids used

Plasmid	Description	Reference or source
pCR-SCRIPT Am$^+$	Direct cloning vector	Commercially available from Stratagene (9)
pERFORM-Z	pERFORM-C with *cam* resistance gene replaced by zeocin resistance gene	2
pLR1	pCR-SCRIPT with *marRAB* 1.3 kb in MCS^a	This study
pLR2	As pLR1 with *cam* cloned into *Xcm*I site of *marA*	This study
pLR3	As pLR2 with *gfp* cloned into *Age*I site of *marA*	This study
pLR4	pERFORM-Z suicide plasmid with *marA*::*gfp*::*cam* cloned into MCS	This study

^{a} MCS, multiple cloning site.

TABLE 3. Primer sequences for PCR

Primer name	Direction[a]	5′-to-3′ DNA sequence of primer	Position of primer (bp)[b]
marA 387	F	ATGACGATGTCCAGACGCAACACT	1433 to 1456
marA 387	R	CTAGTAGTTGCCATGGTTCAGCGG	1799 to 1822
marRAB 1.3	F	ATTCCGCTGGGTCGCTTGA	1018 to 1036
marRAB 1.3	R	AGCGCCGCGGTGTTACTC	1996 to 2313
mar gfp seq[c]	F	TCATCAACGACCAGGGCAAGAC	1317 to 1338
mar gfp seq	R	ATCCGCAGCCGTAAAATGAC	1610 to 1629

[a] F, forward; R, reverse.
[b] bp, positions as related to positions in published sequence of serovar Typhimurium (42).
[c] seq, sequencing primer to check insertion of *gfp* into *marA*.

AlkPhos Direct; Amersham Life Sciences) according to the manufacturer's instructions for Southern blotting. Colony dot blot and Southern blot analyses were performed as described previously (26). For Southern blots, DNA was fixed to Hybond N+ membranes (Amersham Life Sciences) by incubation at 80°C for 2 h. For nonradioactive probing, posthybridization washes were performed according to the manufacturer's instructions. For radioactive probing, three washes were performed at 62°C. Wash 1 comprised 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS), and washes 2 and 3 comprised 1× SSC and 0.1% SDS.

Construction of serovar Typhimurium DT104 *marA::gfp::cam* mutants. The serovar Typhimurium DT104 *marA::gfp::cam* reporter mutants (LR1 to LR5) were produced from strain S3992/96 by allelic exchange (Fig. 1). Briefly, the *marRAB* region amplified with the *marRAB* 1.3-kb primers (Table 3) was cloned according to the manufacturer's instructions into pCR-script plasmid (Stratagene) to give plasmid pLR1 (Table 2). A 1.1-kb chloramphenicol resistance cassette (Stratagene) was cloned into the *Xcm*I site in the distal part of *marA* to give plasmid pLR2 (Table 2). The promoterless green fluorescent protein (*gfp*) gene (14) was obtained by restriction endonuclease digestion of its plasmid vector using *Pst*I and *Xba*I. The promoterless *gfp* gene was cloned blunt ended into the *Age*I site in the proximal region of *marA* to give plasmid pLR3 (Table 2). The *marA::gfp::cam* construct was amplified by *marRAB* 1.3-kb primers (Table 3) and ligated into the zeocin-resistant pERFORM-Z suicide plasmid (2) to give plasmid pLR4 (Table 2). Plasmid pLR4 was transformed into electrocompetent permissive host *E. coli* S17(λpir) cells by electroporation. Conjugation with *E. coli* S17pLR4 as donor and serovar Typhimurium DT104 S3992/96 (Table 1) as recipient was performed by filter mating, with selection made for chloramphenicol. Five putative recombinant clones designated LR1 to LR5 that were zeocin sensitive and chloramphenicol resistant were retained for further analysis.

Complementation of strains. Plasmid pLR1 (Table 2) was electroporated into S3992/96 and LR1 (Table 1), with selection made for ampicillin resistance to give LR6 and LR7, respectively (Table 1).

Induction of *marA* over time. The fluorescence and cell optical density of LR1 and its isogenic parent strain were determined at various time points up to 24 h when grown at 30 or 37°C with test substances (see "Antibiotics, chemicals, bacterial growth, and media" above). After washing and resuspension in half the original volume of phosphate-buffered saline were carried out, fluorescence (200-μl volumes in a 96-well plate) was measured using a Millipore CytoFluor 2350 set to an excitation wavelength of 485 nm and emission wavelength of 530 nm. As a negative control, the isogenic parent strain was grown under identical conditions. All experiments were performed in triplicate, and the relative fluo-

rescence was calculated as fluorescence divided by the optical density at 630 nm. A paired *t* test was used to determine if differences in fluorescence between LR1 and its isogenic parent were statistically significant.

MIC determinations. MICs of antibiotics were determined by agar doubling dilution using the National Committee for Clinical Laboratory Standards method (34), except that Iso-Sensitest agar was used instead of Mueller-Hinton agar. Inoculated plates were incubated at 37°C overnight before the MIC was recorded. All antibiotic MICs were determined using multiple clones, and MIC experiments were repeated.

For experiments to determine induction of *marA* in the reporter mutant LR1, growth in broth was determined. As such, for antibiotics and disinfectants used in induction experiments, MICs in broth were determined by a method similar to the doubling dilution method used in agar.

Cyclohexane resistance. Cyclohexane resistance was determined by the method of Asako et. al. (7). Briefly, bacteria grown overnight at 30°C were inoculated (using a multipoint inoculator) onto duplicate solid agar media in glass petri dishes. Each plate was flooded to a depth of 3 mm (6 ml) with hexane or cyclohexane, sealed with Nescofilm, and incubated overnight at 30°C. Strains that grew in the presence of cyclohexane were deemed cyclohexane resistant.

Preparation and separation of OMPs. OMP extracts of strains were prepared by a method similar to that of Inokuchi et al. (23). The outer membranes were recovered in 50 μl of 10 mM sodium phosphate buffer (pH 7), and the protein levels were determined and adjusted to 2,000 μg/ml in the final sample buffer. Samples (20 μl) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 300 V for 7 h in a 12.5% (wt/vol) gel without urea (36). Marker standards used were carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and egg albumin (45 kDa). After being run, gels were stained overnight in 0.1% Coomassie blue (Sigma). Strains with plasmid pLR1 (LR6 and LR7) were grown with 50 μg of ampicillin/ml at all stages prior to OMP extraction.

N′-terminal sequencing of proteins. The identity of OmpC, OmpF, and OmpA bands from SDS-PAGE gels (see Fig. 3) were determined previously (unpublished observations). To identify the unknown protein (see Fig. 3) from SDS-PAGE, the band was transferred by Western blotting (28) onto a polyvinylidene fluoride membrane (Sequi-blot; Bio-Rad). N′-terminal sequencing of the first few amino acids of the unknown protein was carried out with an Applied Biosystems 477A sequencer.

Comparisons of protein sequences. Comparisons of N′-terminal protein sequences were made using the Jalview multiple alignment editor (http://www
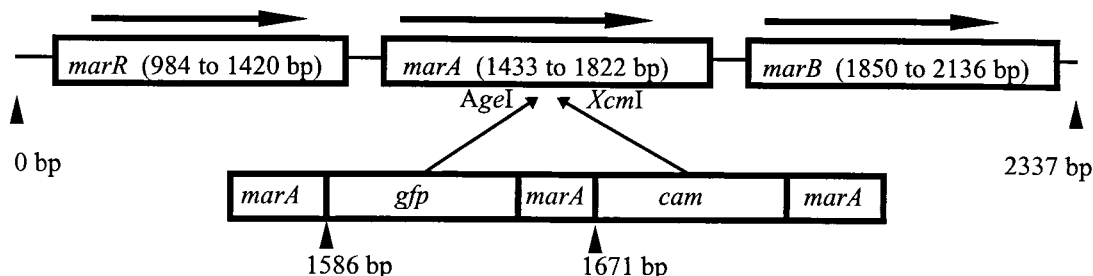

FIG 1. Construction of *marA::gfp::cam*. Base pair positions refer to positions in the published sequence of serovar Typhimurium (42). The *gfp* gene was inserted into the *Age*I site of *marA* at bp 1586, and the *cam* gene was inserted into the *Xcm*I site of *marA* at bp 1671.

.ebi.ac.uk/~michele/jalview/). Comparisons were to OMPs of specific interest, such as OmpC, OmpF, OmpA, and TolC.

The N′-terminal sequences were also compared using BLAST search programs (BLASTp and BLASTn) (3), which are available online at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The unknown N′-terminal protein sequence was compared to sequences within the *E. coli* database only.

RESULTS

The *marA* region is present in *Salmonella*. It is widely assumed that the *marRAB* region is conserved among *Salmonella* serotypes. Colony dot blot DNA hybridization studies confirmed the presence of sequences that shared homology with serovar Typhimurium *marA* for all 44 *Salmonella* serotypes tested (data not shown). Two *E. coli* strains hybridized with this probe, but other non-*Salmonella* strains tested did not hybridize (results not shown). Also, Southern hybridizations were performed to test if *marRAB* in *Salmonella* was present as a single copy and in a conserved region of the *Salmonella* genome. Chromosomal DNA from *Salmonella* serotypes Arizonae ($n = 1$), Dublin ($n = 3$), Enteritidis ($n = 3$), Hadar ($n = 1$), Infantis ($n = 1$), Kedougou ($n = 1$), Newport ($n = 1$), Typhimurium ($n = 5$), and Virchow ($n = 1$) was digested to completion with *Hin*dIII and probed. All serotypes hybridized a single fragment of approximately 3.5 kb except for serotype Arizonae strain S170/97, for which a single fragment of approximately 4.5 kb hybridized (results not shown).

Construction of *marA*::*gfp*::*cam* mutants. In order to study the regulation of the *marRAB* region and the phenotype of a *mar* null mutant, *marA*::*gfp*::*cam* reporter mutants LR1 to LR5 were constructed by allelic exchange in serovar Typhimurium DT104 S3992/96. The correct orientation of the *gfp* gene in the *marRAB*::*gfp*::*cam* construct was confirmed by sequencing (results not shown), using custom-made primers (Table 3). To confirm successful allelic exchange in colonies that were chloramphenicol resistant but zeocin sensitive, Southern blotting was performed (results not shown). Chromosomal DNA digested to completion with *Hin*dIII and *Pvu*II was hybridized with the *marRAB* 1.3-kb probe and the *marRAB*::*gfp*::*cam* probe. *Hin*dIII did not cut within *gfp, cam,* or the *marRAB* 1.3-kb region, and a single, larger DNA fragment was seen for the recombinants (5.2 kb) than for that of the parent (3.5 kb). *Pvu*II did not cut in the *marRAB* 1.3-kb region but cut in both *gfp* and *cam*, and so a single product was seen for the parent and three products were seen for the recombinants. The combined lengths of *gfp* and *cam* were approximately 1.8 kb, so the predicted increases in the sizes of the hybridizing region were observed (results not shown). Plasmid pLR4 without the *marA*::*gfp*::*cam* construct did not hybridize Southern blots of putative reporter mutants that were chloramphenicol resistant but zeocin sensitive (results not shown), thus confirming the absence of suicide plasmid DNA in the reporter mutants.

marA was induced by salicylate but not by antibiotics. Tests were done to assess what compounds or conditions induced *marA*. Salicylate (2.5 to 20 mM) increased fluorescence of the *marA*::*gfp* reporter mutant LR1 two- to fourfold ($P < 0.05$ to $P < 0.01$) (Table 4 and Fig. 2), whereas other substances, including nine antibiotics or simulated environmental stresses such as disinfectants (see Materials and Methods), did not. For these, the mean fluorescence of LR1 ranged from 0.6 to 1.7

TABLE 4. Induction of fluorescence in reporter mutant LR1

Salicylate concn (mM)	Growth condition (°C)	Relative increase[a] in fluorescence (SEM)	P value[b]
2.5	37	1.56 (0.16)	0.031
5	30	2.30 (0.16)	0.004
5	37	2.40 (0.16)	0.006
10	30	4.00 (0.47)	0.001
10	37	2.50 (0.12)	0.005
20	30	4.00 (0.16)	0.006
20	37	4.55 (0.41)	0.008

[a] Mean increase in relative fluorescence of LR1 compared to parent strain under identical growth conditions. SEM, standard error of the mean.
[b] P value for comparison of relative fluorescence of LR1 and parent.

times that of the parent strain, and these differences were not significant at $P < 0.05$ (results not shown).

With regard to the antibiotics tested, LR1 was more sensitive than the parent by 1 dilution to nalidixic acid and ciprofloxacin when grown without salicylate (Table 5). However, growth with salicylate induced decreased sensitivity to most antibiotics tested, more so for the parent than for LR1 (Table 5). Here was clear evidence that salicylate, the inducer of *marA*, was associated with the Mar-like phenotype.

LR1 could be trained to Mar-like resistance. Passage of *E. coli* with tetracycline is reported to select for *mar* constitutive mutants that have increased multiple antibiotic resistance (19, 20). We hypothesized that LR1 would not yield such derivatives, due to insertional inactivation of *marA*, if the *marRAB* was the only operon involved in low-level antibiotic resistance in *Salmonella*. To test this, LR1 and its parent were passaged eight times with tetracycline, and both showed increased resistance up to 16 times higher to tetracycline, ampicillin, nalidixic acid, ciprofloxacin, and erythromycin, as compared to the strains before passage with tetracycline (Table 5). However,
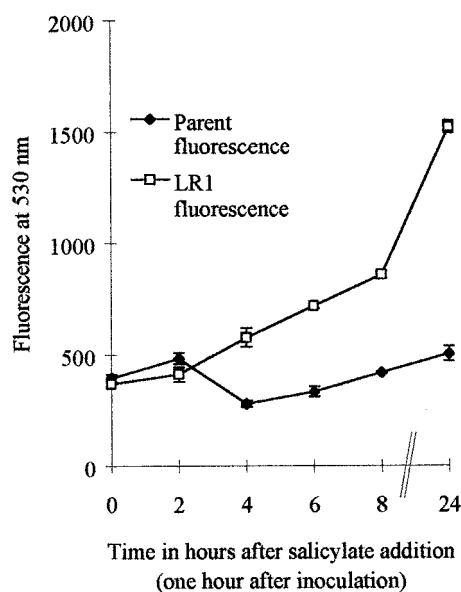

FIG. 2. Induction of *marA* in reporter mutant LR1 grown with 10 mM salicylate.

TABLE 5. MICs for LR1 and its parent before and after passage with salicylate or tetracycline and with and without plasmid expressing *marA*

Strain	Growth[a]	Result type[b]	MIC (μg/ml)[c]									
			TET	CHL	AMP	AMC	CFP	STR	NAL	CIP	ERY	SUL
Parent	LB broth	MIC	1.25	5	1.25	1.25	0.63	20	2.5	0.02	80	160
	2.5 mM Sal	MIC	2.5	10	2.5	NT	1.25	20	5	0.04	NT	NT
	10 mM Sal	MIC	5	10	2.5	NT	1.25	20	20	0.16	NT	NT
	Ex TET 50	MIC	20	NT	10	5	5	10	20	0.63	320	640
LR6	AMP 50 μg/ml	MIC	5	NT	NT	NT	NT	NT	10	NT	NT	NT
LR1	LB broth	MIC min	1.25	NT	1.25	1.25	0.31	10	2.5	0.02	80	160
		MIC max	1.25	NT	1.25	1.25	0.63	20	2.5	0.02	160	320
	2.5 mM Sal	MIC	1.25	80[d]	1.25	NT	0.31	10	2.5	0.02	NT	NT
	10 mM Sal	MIC	2.5	80[d]	1.25	NT	0.63	20	5	0.04	NT	NT
	Ex TET 50	MIC min	10	NT	1.25	1.25	0.63	5	10	0.08	320	160
		MIC max	10	NT	5	1.25	0.63	5	20	0.16	320	320
LR7	AMP 50 μg/ml	MIC	5	NT	NT	NT	NT	NT	10	NT	NT	NT

[a] Strains were normally grown in LB broth prior to MIC determinations. LB broths were supplemented as shown with 2.5 and 10 mM salicylate (Sal) or with 50 μg of ampicillin/ml (to maintain plasmid pLR1). Ex Tet 50, strains grown for eight passages with increasing concentrations of tetracycline (TET) up to 50 μg/ml.
[b] MIC min, lowest recorded MIC; MIC max, highest recorded MIC.
[c] TET, tetracycline; CHL, chloramphenicol; AMP, ampicillin; AMC, Co-amoxiclav (amoxicillin:clavulanic acid, 2:1); CFP, cefoperazone; STR, streptomycin; NAL, nalidixic acid; CIP, ciprofloxacin, ERY, erythromycin; SUL, sulfadiazine. NT, not tested. Experiments were performed with multiple clones and/or repeated with identical or comparable results.
[d] Resistant to chloramphenicol due to insertion of *cam* in LR1.

LR1 showed a lower increase in resistance than its parent against these antibiotics (Table 5). The parent strain also showed some increase in resistance to cefoperazone. Little or no increase in resistance after antibiotic passage was observed against the antibiotics streptomycin and sulfadiazine for either LR1 or its parent. Complementation of the parent and LR1 with pLR1 gave the Mar phenotype with respect to low-level antibiotic resistance (Table 5). This level of resistance was not as high as that seen for strains passaged eight times with tetracycline. This further suggests that non-*mar* mechanisms were involved in the acquisition of resistance after multiple passage with tetracycline.

Cyclohexane resistance is induced by salicylate. Organic solvent tolerance in *E. coli* has been shown to be linked to mutations in *marR*, to overexpression of *marA, soxS, and rob*, to upregulation of *acrAB*, and to increased resistance to numerous antibiotics (6, 7, 47). We hypothesized that LR1 would remain cyclohexane sensitive even under inducing conditions, due to insertional inactivation of *marA* if the *marRAB* was involved in cyclohexane resistance in *Salmonella*. Both LR1 and its parent were sensitive to cyclohexane when grown on LB agar medium (results not shown). However, the parent strain but not LR1 became cyclohexane resistant when grown on LB agar medium supplemented with salicylate. Complementation of the parent and LR1 with pLR1 led to cyclohexane resistance in both strains (data not shown).

Both LR1 and its parent became resistant to cyclohexane after multiple (eight) passage with tetracycline (results not shown), which suggested that cyclohexane resistance in *Salmonella* was not controlled by *marRAB* exclusively.

OMP profiles were not altered by the *marA::gfp::cam* mutation. Induction of *mar* in *E. coli* has been reported to lead to down regulation of OmpF (10, 12). To investigate this in *Salmonella*, OMP profiling was performed for the parent and LR1 when grown in normal media or media supplemented with 5 mM salicylate and when complemented with the plasmid

pLR1 (media supplemented with 50 μg of ampicillin/ml to maintain the plasmid). The OMP profiles of the parent and LR1 when grown in normal media were similar (Fig. 3, lanes 2 and 3), and previous work has confirmed the major bands shown to be OmpC, OmpF, and OmpA (unpublished observations). Growth of the parent and LR1 with salicylate did
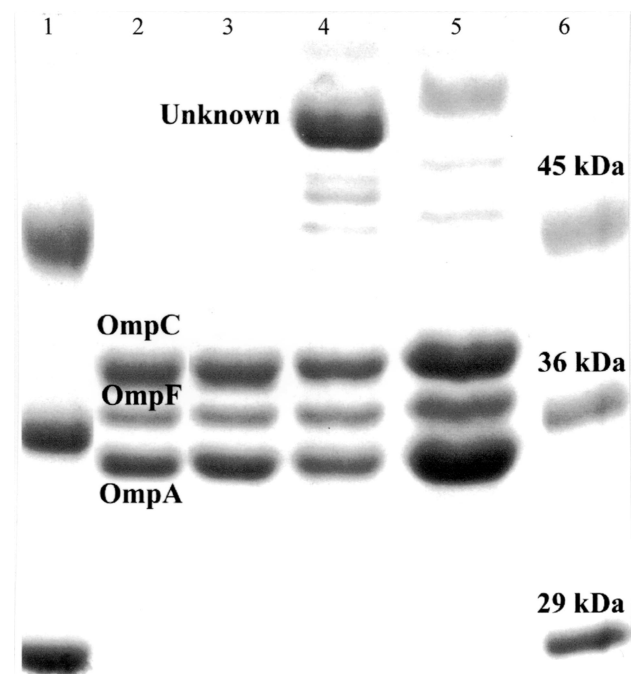

FIG. 3. OMPs of parent and LR1 complemented with plasmid pLR1. Lane 1 and 6, standards (sizes as shown); 2, parent; 3, LR1; 4, parent complemented with plasmid pLR1; 5, LR1 complemented with plasmid pLR1.

cause down regulation of OmpF (results not shown). These data showed that the mutation of *marA* was not correlated with altered OmpF levels.

Complementation of the parent and LR1 strains with plasmid pLR1 did not affect the levels of OmpC, OmpF, and OmpA (Fig. 3, lanes 4 and 5), but an unknown protein of ca. 50 kDa was up regulated in the parent. N′-terminal sequencing of this novel protein yielded the sequence APCDNTIFA. When this sequence was compared with *Salmonella* OmpF, OmpC, OmpA, and TolC proteins using an alignment program (http://www.ebi.ac.uk/~michele/jalview/), it showed the most similarity to OmpA. The sequence APCDNTIFA was identical to six of the nine amino acids of the OmpA sequence (amino acids 22 to 30). The OmpA sequence to which the N′-terminal sequence showed some homology immediately followed a classic type 1 cleavage sequence (AQA).

The APCDNTIFA sequence showed similarity to 9 *E. coli* proteins using the BLASTP search programs and to 19 proteins using the TBLASTN search programs (3; http://www.ncbi.nlm.nih.gov). However, the results of these searches were generally inconclusive, although it was interesting that some homology was shown to the *E. coli* multiple drug resistance proteins EmrY and EmrK (44).

It was considered possible that changes to the bacterial surface induced by mutation, salicylate induction, or overexpression of the novel 50-kDa protein alter other observable phenotypes. No alterations to cell surface hydrophobicity or acid tolerance between LR1 and its isogenic parent were observed under the conditions tested (results not shown).

DISCUSSION

All serotypes of *Salmonella* tested possessed *marA*, and Southern blot analysis showed one copy of a *marRAB*-like sequence in a conserved region of the chromosome. *Salmonella marA* is reported to share 86% sequence homology with the same region of *E. coli* (42), and this would explain the hybridization of the serovar Typhimurium *marA* probe with *E. coli* under the stringency conditions used.

Southern blot analysis and sequencing confirmed the insertion of *gfp* and *cam* into *marA* in the reporter mutant LR1. Induction of *marA* in the reporter mutant showed that background fluorescence increased in a linear fashion with increased growth over time. However, when LR1 and the isogenic parent strain were grown with salicylate, the reporter mutant clearly showed a greater increase in fluorescence, which was most noticeable 24 h after inoculation. Salicylate was both an inhibitor of growth and an inducer of *marA*, and interpretation of induction was dependent upon a comparison of relative fluorescence of the parent and LR1 under identical conditions. Only salicylate enhanced the transcription of *marA* significantly. Previous studies with *E. coli* showed that chloramphenicol and tetracycline, but not ampicillin, nalidixic acid, or norfloxacin, caused an increase in *marA* hybridizable mRNA (22). None of these antibiotics caused a significant increase in *marA* transcription in this study; however, Northern blotting may be needed to detect low levels. Alternatively, the inducers of *marA* in *Salmonella* differ from those for *E. coli*. For *E. coli*, salicylate has been shown to bind to MarR, inhibiting the formation of the MarR-*marO* complex (27). It is

assumed that salicylate induces *mar* in *Salmonella* in a similar way.

Despite numerous papers on the function of *mar*, the natural inducer of the *mar* locus is still not known (1). It has been postulated that *mar* originated from primal chromosomal genes involved in responding to the hostile environment of antibiotic-producing soil microbes (24). This would explain partially the reported ability of antibiotics such as tetracycline and chloramphenicol to induce *mar* in *E. coli* (22). Other workers (29) have postulated that naturally occurring plant breakdown products such as naphthoquinones are the true substrates for intrinsic resistance systems such as *mar, emrRAB, sox*, and *acrAB*. While salicylate is the most potent inducer of *mar* in *E. coli* (10), naphthoquinones such as plumbagin and menadione are effective inducers at lower concentrations (10, 39).

The increased induction of *marA* transcription when LR1 was grown with 10 mM rather than 2.5 mM salicylate correlated with slight increases in the MICs of MAR-type antibiotics (i.e., antibiotics to which expression of *mar* confers some resistance, such as β-lactams, chloramphenicol, quinolones, and tetracyclines) when LR1 was grown with 10 mM rather than 2.5 mM salicylate. The lack of (or reduced) increase in MICs for LR1 when grown with salicylate compared to those for its parent implicates the involvement of *mar* in low-level antibiotic resistance in *Salmonella*. This was further endorsed by complementation studies with plasmid pLR1, which constitutively expresses *marA*, as demonstrated by constitutive expression of *gfp* from pLR3. In LR1 and its parent, pLR1 increased tetracycline and nalidixic acid MICs fourfold. For *E. coli*, salicylate has been shown to induce antibiotic resistance by a *mar*-dependent and a *mar*-independent pathway (10), and our results suggest that salicylate functions in *Salmonella* in a similar way. The *mar*-independent pathway was evident in that growth with salicylate caused increased antibiotic resistance for both LR1 and its parent. The *mar*-dependent pathway was suggested in that the plasmid expressing *marA* caused an 8-fold increase in resistance to nalidixic acid, whereas growth with 10 mM salicylate caused a 16-fold increase in resistance to nalidixic acid.

The effect of growing strains with salicylate on increased fluoroquinolone resistance in *Staphylococcus aureus* has been reported (21), and fears have been expressed that salicylate used therapeutically could give rise to sufficient levels in vivo (5 mM) to induce antibiotic resistance (8, 21). This could apply to *Salmonella* also. Fluoroquinolone antibiotics are the agents of choice for treating bacteremic enteric fevers due to *Salmonella* (15, 41, 46). For ciprofloxacin, MICs following growth with 2.5 and 10 mM salicylate increased by about 2 to 4 times and up to 8 times (MIC maximum, 0.31 μg/ml), respectively. Salicylate and acetylsalicylate (aspirin) are chemically similar, and growth of *E. coli* with both salicylate and acetylsalicylate has been shown to cause increased resistance to ampicillin, tetracycline, and nalidixic acid (38). Whether aspirin treatment of fever accompanying systemic *Salmonella* infection could make the difference between antibiotics curing and failing to cure a life-threatening infection is a matter of conjecture.

Passage of the parent and LR1 with tetracycline gave rise to increased antibiotic resistance for specific antibiotics, as has been observed for *E. coli* following passage with tetracycline (19). There was little increase in resistance for streptomycin,

but resistance was seen primarily against tetracycline, chloramphenicol, ampicillin, cefoperazone, and quinolones, a pattern of resistance also reported for *mar* mutants of *E. coli* (7). Therefore, multiple-antibiotic-resistant mutants of *Salmonella* with a similar phenotype to the classical *mar* mutants can arise from passage with antibiotics such as tetracycline but independently of *marA*.

Salicylate induced cyclohexane resistance in the parent strain but not the reporter mutant LR1. Plasmid pLR1, which constitutively expressed *marA*, made LR1 and its parent cyclohexane resistant in the absence of salicylate. These results clearly implicate *mar* in inducible cyclohexane resistance. However, results also showed that cyclohexane resistance could occur independently of *mar*, since the reporter mutant LR1 became cyclohexane resistant after passage with tetracycline.

Salicylate down regulated OmpF through *mar*-independent mechanisms as have been observed for *E. coli* (10), and complementation studies, using plasmid pLR1 expressing *marA*, gave expected results with respect to low-level multiple antibiotic resistance and cyclohexane resistance. However, the OMP profiles of complemented strains were unexpected, because a ca. 50-kDa protein was up regulated. In *E. coli*, TolC is an OMP that has a molecular mass of 51 kDa (4, 32, 33, 35) and is thought to link the *acrAB* efflux pump to the outside of the membrane (16). As such, one would expect TolC to be up regulated when *mar* is up regulated (16, 18, 33, 47). Homology searches were equivocal and this protein requires more detailed analysis.

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