

## Antibiotic Resistance in *Salmonella enterica* Serovar Typhimurium Exposed to Microcin-Producing *Escherichia coli*

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**Microcin 24 is an antimicrobial peptide secreted by uropathogenic *Escherichia coli*. Secretion of microcin 24 provides an antibacterial defense mechanism for *E. coli*. In a plasmid-based system using transformed *Salmonella enterica*, we found that resistance to microcin 24 could be seen in concert with a multiple-antibiotic resistance phenotype. This multidrug-resistant phenotype appeared when *Salmonella* was exposed to an *E. coli* strain expressing microcin 24. Therefore, it appears that multidrug-resistant *Salmonella* can arise as a result of an insult from other pathogenic bacteria.**

Microcins are antimicrobial peptides secreted by bacteria as a means of disabling neighboring bacteria (1). Microcin 24 (Mcc24) is a colicin secreted by a strain of uropathogenic *Escherichia coli* (18). A previous study demonstrated that Mcc24 has activity against *Salmonella enterica* and most *E. coli* strains but not against *Campylobacter* or *Listeria* strains (18). We also found that Mcc24 does not have activity against multiple-antibiotic-resistant *Klebsiella pneumoniae* (ATCC MCV37) (data not shown). In the present study, we evaluated the ability of *S. enterica* serotype Typhimurium to develop resistance to Mcc24 and how the development of Mcc24 resistance related to resistance to antibiotics. This study was undertaken to determine if the use of Mcc24 in probiotic bacteria, as described previously (23), could lead to antibiotic resistance in *Salmonella*.

We pursued the multiple-antibiotic resistance (*mar*) operon as a potential component of Mcc24 resistance since this operon regulates resistance to a variety of foreign substances in *Salmonella* (7). The *mar* operon regulates an efflux system that facilitates the expulsion of antibiotics and organic solvents (6). The *mar* operon is regulated by MarR, a repressor protein that prevents the transcription of *marAB* (15). Transcription of *marAB* occurs when MarR is absent due to an inhibition of translation of *marR* (20), altered as a result of mutagenesis of *marR* (20) or sequestration by salicylate (5). As part of this study we used a plasmid-based system to attenuate the Mar phenotype in a manner similar to that of White et al. (22), who used antisense DNA inhibition to block the expression of *marA*, a regulator of an efflux system (16). *S. enterica* serotype Typhimurium strain SL1344 (24) was transformed with pBAD, a high-copy-number plasmid with a pBM1 replicon (Invitrogen) containing the PCR-derived *marR* gene. The *marR* gene was cloned from SL1344 DNA using PCR conditions described previously (3) with 5'-ATGAAAAGCACCAGTGATCTGTT C-3' and 5'-CCTACGGCAGATT-TTTCTTGAGCAA-3' as

forward and reverse primers, respectively. The expression of *marR* in pBAD is under the control of arabinose via the *araBAD* promoter (12, 14).

SL1344 was also transformed with another plasmid containing the CFP (cyan derivative of green fluorescent protein [8]; Clontech) gene cloned into pCRII Blunt, a high-copy-number plasmid with a pBMI replicon and the Lac promoter for transcription of the cloned gene (Invitrogen), as previously described (10). This plasmid was included to visually distinguish *S. enterica* serotype Typhimurium from an Mcc24-producing *E. coli* strain (ampicillin-resistant strain MC4100pGOB18 [23]). SL1344 cotransformants, designated SL1344/CFP/*marR*, were propagated in Lennox L broth (GIBCO-BRL) containing 100 µg of ampicillin per ml and 64 µg of kanamycin per ml. Ampicillin resistance is conferred by a beta-lactamase encoded by pBAD, while kanamycin resistance is conferred by a phosphotransferase encoded by pCRII Blunt.

To evaluate Mcc24 resistance in *S. enterica* serotype Typhimurium in a system that mimics the in vivo commingling of *Salmonella* and *E. coli*, we flooded SL1344/CFP/*marR* (approximately  $2 \times 10^8$  bacteria) on Lennox L agar. Next we soaked a filter disk (Bacto concentration disks, sterile blanks; Difco) in Lennox L broth containing Mcc24-producing *E. coli* MC4100pGOB18 ( $10^9$  organisms/ml) and placed this disk in the middle of the SL1344/CFP/*marR*-laden agar plate. Bacteria were then grown together on Lennox L agar plates containing ampicillin at 37°C overnight.

As shown in Fig. 1, a zone of inhibition was observed around the disk soaked in *E. coli* strain MC4100pGOB18. Table 1 reveals that this zone was due to a secreted protein since the inhibition was present if trichloroacetic acid (TCA)-precipitated supernatants from *E. coli* strain MC4100pGOB18 were used instead of broth containing the bacteria. The zone also disappeared for SL1344 transformed with pCRXL, a high-copy-number plasmid with a ColE1 replicon, the Lac promoter for transcription of the cloned gene (Invitrogen), and the Mcc24 immunity gene (*mtfI*; GenBank accession number U47048). The *mtfI* gene was amplified by PCR from the pGOB18 plasmid. Additionally, the zone of inhibition disappeared if TCA-precipitated supernatants from *E. coli* strain MC4100pGOB18

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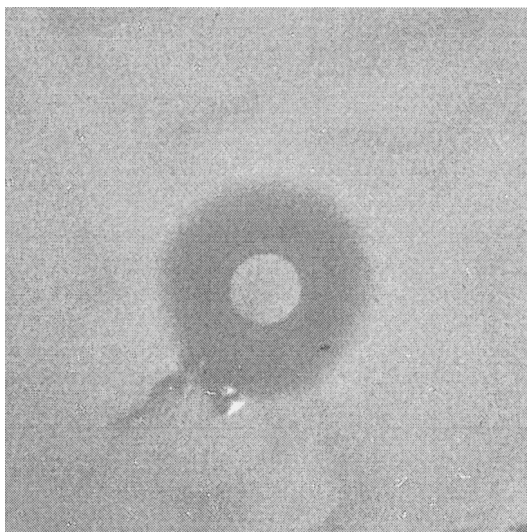


FIG. 1. Mcc24-mediated zone of inhibition of SL1344/CFP/*marR*. SL1344/CFP/*marR* and *E. coli* strain MC4100pGOB18 (center of plate) were incubated together as described in the text.

were treated with 1 mg of pronase E (Sigma) per ml. As shown in Fig. 2, the zone was also absent if SL1344/CFP/*marR* or SL1344 was grown in Lennox L broth and incubated on Lennox L agar, both containing 3.5 mM salicylate (Sigma). Salicylate can activate the Mar phenotype by physically binding to MarR (5), thus competitively eliminating the repressor effect of MarR on the *mar* operon (15). Therefore, it appears that resistance to Mcc24 can occur through the *mar* response. This is also apparent since the zone was absent for *S. enterica* serotype Typhimurium strain 8431 (Table 1), a mutant exhibiting a Mar-like phenotype (4). Additionally, the salicylate-mediated induction of Mcc24 resistance was prevented by 0.2%-arabinose-induced episomal expression of MarR from pBAD. That is, repression of *marA* transcription occurred as a result of



FIG. 2. Induction of the Mar phenotype in SL1344/CFP/*marR*. SL1344/CFP/*marR* and *E. coli* strain MC4100pGOB18 (center of plate) were incubated together in the presence of 3.5 mM salicylate.

competitive antagonism of salicylate-mediated derepression by episomally derived MarR. Chromosomal and episomal *marR* DNA sequences were not changed by the salicylate or arabinose treatment. Sequencing was performed using pBAD-specific primers for episomal *marR*, open reading frame 221, and *marA* flanking sequences for chromosomal *marR* (GenBank accession number U54468; data not shown).

To evaluate the potential relationship between resistance to Mcc24 and multiple-antibiotic resistance, we determined the MICs of ciprofloxacin, chloramphenicol, tetracycline, and rifampin. These four antibiotics were chosen since they represent part of the Mar phenotype (6). MICs were determined by inoculating  $10^6$  bacteria into 1-ml aliquots of Mueller-Hinton broth (Difco) containing serial dilutions of antibiotics per NCCLS guidelines. Bacteria were grown aerobically, and MICs were ascribed based on the lowest concentration of antibiotic that inhibited growth. Table 1 reveals that salicylate-mediated

TABLE 1. Changes in MICs in relationship to Mcc24 resistance

Treatment	Strain	MIC ( $\mu\text{g/ml}$ ) of <sup>a</sup> :				Zone of inhibition around MC4100pGOB18 disk
		CIP	TET	CHL	RIF	
Exposure to disk soaked in MC4100pGOB18	SL1344	0.125	4	2	12.5	Yes
Exposure to TCA-precipitated supernatants from MC4100pGOB18	SL1344	ND <sup>b</sup>	ND	ND	ND	Yes
Exposure to pronase-treated TCA-precipitated supernatants from MC4100pGOB18	SL1344	ND	ND	ND	ND	No
3.5 mM salicylate	SL1344	4	16	32	50	No
Exposure to disk soaked in MC4100pGOB18	SL1344/ <i>mtfI</i> -pCRXL	ND	ND	ND	ND	No
None	8431	4	32	64	50	ND
Exposure to disk soaked in MC4100pGOB18	8431	4	32	64	50	No
None	SL1344/CFP/ <i>marR</i>	0.125	4	2	12.5	ND
Exposure to disk soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i>	0.125	4	2	12.5	Yes
Exposure to disk soaked in MC4100pGOB18 in the presence of 3.5 mM salicylate	SL1344/CFP/ <i>marR</i>	4	16	32	50	No
Exposure to disk soaked in MC4100pGOB18 in the presence of 3.5 mM salicylate plus 0.2% arabinose	SL1344/CFP/ <i>marR</i>	0.5	8	8	12.5	Yes
Exposure to disk soaked in MC4100pGOB18 in the presence of 3.5 mM salicylate plus two exposures to 0.2% arabinose	SL1344/CFP/ <i>marR</i>	0.25	4	4	12.5	Yes

<sup>a</sup> Concentrations of antibiotics were as follows: 8, 4, 2, 1, 0.5, 0.25, and 0.125  $\mu\text{g/ml}$  for ciprofloxacin (CIP); 64, 32, 16, 8, 4, 2, and 1  $\mu\text{g/ml}$  for tetracycline (TET) and for chloramphenicol (CHL); and 100, 50, 25, 12.5, 6.3, 3.2, and 1.6  $\mu\text{g/ml}$  for rifampin (RIF).

<sup>b</sup> ND, not determined.

TABLE 2. Changes in MICs in relationship to Mcc24 exposure in a representative experiment

Treatment	Strain <sup>a</sup>	MIC ( $\mu\text{g/ml}$ ) of <sup>b</sup> :				Zone of inhibition around MC4100pGOB18 disk
		CIP	TET	CHL	RIF	
Two exposures to disks soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i> border colonies	0.125	4	2	12.5	Yes
17 exposures to disks soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i> border colonies	0.25	ND	ND	ND	Yes
18 exposures to disks soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i> border colonies	2	16	32	50	No
17 exposures to disks soaked in MC4100pGOB18 followed by another exposure in the presence of 0.2% arabinose	SL1344/CFP/ <i>marR</i> border colonies	0.5	ND	ND	ND	Yes
17 exposures to disks soaked in MC4100pGOB18 and then two more exposures in the presence of 0.2% arabinose	SL1344/CFP/ <i>marR</i> border colonies	0.25	ND	ND	ND	Yes
17 exposures to disks soaked in MC4100pGOB18 one exposure to 0.2% arabinose, and then 2 to 3 additional exposures to disks soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i> border colonies	2	16	32	50	No
18 expansions without exposure to disks soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i> edge colonies	0.125	4	2	12.5	Yes
50 expansions without exposure to disks soaked in MC4100pGOB18	SL1344/CFP/ <i>marR</i> edge colonies	0.125	4	2	12.5	Yes

<sup>a</sup> "Border" indicates that colonies were selected from the border of the zone of inhibition, and "edge" indicates that colonies were selected from the edge of the agar plate.

<sup>b</sup> Concentrations of antibiotics were as described in Table 1, note a. ND, not determined.

Mcc24 resistance occurred in concert with an increase in ciprofloxacin MICs. Additionally, SL1344/CFP/*marR* exhibited increases in the MICs of chloramphenicol, tetracycline, and rifampin. Simultaneous exposure to arabinose prevented the salicylate-mediated change in ciprofloxacin MICs.

To evaluate the *mar* response in relationship to Mcc24 resistance in *Salmonella*, we repeatedly selected SL1344/CFP/*marR* colonies that bordered the zone of inhibition (Table 2). *Salmonella* colonies were expanded by vigorous shaking for 4 to 6 h at 37°C in Lennox L broth containing ampicillin and kanamycin. Broth cultures (approximately  $2 \times 10^8$  bacteria) were then replated with the disk soaked in *E. coli* strain MC4100pGOB18 on Lennox L agar containing ampicillin. The zone disappeared after 9, 11, or 18 expansions and replatings in three separate experiments. As a control, SL1344/CFP/*marR* colonies from the edge of the plate were also expanded and replated. SL1344/CFP/*marR* colonies from the edge of the plate remained sensitive to Mcc24. We have been unable to isolate Mcc24-resistant SL1344/CFP/*marR* colonies from the edge of the plate. In a representative experiment in which 18 expansion-replating procedures were required to induce Mcc24 resistance, we found that resistance to Mcc24 and increased MICs of ciprofloxacin, tetracycline, chloramphenicol, and rifampin could be prevented by episomal expression of MarR. Mcc24-sensitive microbes became Mcc24 resistant, however, after removal of the arabinose. Chromosomal and episomal *marR* DNA sequences were not changed by the expansion processes or by the salicylate or arabinose treatment. Thus, Mcc24 exposure-mediated resistance was apparently due to activation of the *mar* system. While a previous study suggests that resistance to microcin MccB17 can be due to an export of the peptide in *E. coli* (11), this is the first study to document *mar*-mediated resistance to a microcin.

Although the mechanism of action for Mcc24 is currently unknown, other microcins provide antimicrobial action by inhibiting DNA gyrase, by inhibiting protein expression (17, 21), or by forming pores in target bacteria (9). Since *marR* DNA sequences were not altered and since ancillary MarR may pre-

vent derepression, it is feasible that Mcc24 can inhibit translation. The Mcc24-mediated inhibition of translation may decrease MarR levels below a threshold that normally maintains repression of *mar*. Derepression then ensues when this threshold is breached. That is, Mcc24 indirectly mediates resistance to itself.

In this study, we found that *Salmonella* can develop resistance to an *E. coli*-derived microcin. Extrapolation of our results indicates that exposure of *Salmonella* to Mcc24 can result in multidrug-resistant *Salmonella*. Therefore, the ongoing problem with multidrug-resistant *Salmonella* may be due to the activation of innate pathways and the acquisition of genetic determinants of antibiotic resistance such as integrons (2, 13, 19). Since ciprofloxacin resistance in *Salmonella* is uncommon, the events described in this study are probably a rare situation in vivo. Nonetheless, it unfortunately appears that microcin production gives an advantage to pathogenic *E. coli* while also potentially selecting for multidrug-resistant *Salmonella*.

We thank Ruth Willson for technical assistance, Sandy Johnson for secretarial assistance, Chuck Greiner for photography, and Kristi Anderson, Kim Brogden, and Bonita Glatz for reviewing the manuscript. S.A.C. and T.S.F. contributed equally to this work.

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