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Different effects of transcriptional regulators MarA, SoxS and Rob on susceptibility of *Escherichia coli* to cationic antimicrobial peptides (CAMPs): Rob-dependent CAMP induction of the *marRAB* operon

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Cationic antimicrobial peptides (CAMPs), a component of the mammalian immune system, protect the host from bacterial infections. The roles of the *Escherichia coli* transcriptional regulators MarA, SoxS and Rob in susceptibility to these peptides were examined. Overexpression of *marA*, either in an antibiotic-resistant *marR* mutant or from a plasmid, decreased bacterial susceptibility to CAMPs. Overexpression of the *soxS* gene from a plasmid, which decreased susceptibility to antibiotics, unexpectedly caused no decrease in CAMP susceptibility; instead it produced increased susceptibility to different CAMPs. Deletion or overexpression of *rob* had little effect on CAMP susceptibility. The *marRAB* operon was upregulated when *E. coli* was incubated in sublethal amounts of CAMPs polymyxin B, LL-37 or human β-defensin-1; however, this upregulation required Rob. Deletion of *acrAB* increased bacterial susceptibility to polymyxin B, LL-37 and human β-defensin-1 peptides. Deletion of *tolC* yielded an even greater increase in susceptibility to these peptides and also led to increased susceptibility to human α-defensin-2. Inhibition of cellular proton-motive force increased peptide susceptibility for wild-type and *acrAB* deletion strains; however, it decreased susceptibility of *tolC* mutants. These findings demonstrate that CAMPs are both inducers of *marA*-mediated drug resistance through interaction with Rob and also substrates for efflux in *E. coli*. The three related transcriptional regulators show different effects on bacterial cell susceptibility to CAMPs.

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INTRODUCTION

The bacterium *Escherichia coli* occupies a variety of niches in the mammalian host, where it faces challenges from the innate immune system. One component of this system are cationic antimicrobial peptides (CAMPs), amphipathic proteins produced by a wide range of mammalian cells, including neutrophils and epithelial cells in the urogenital and gastrointestinal tracts. CAMPs utilize the negative charge of the bacterial cell membranes to collect on, and form hydrophilic channels through, the outer and inner membranes of the bacterial cells, causing osmotic damage to the bacterium (Oren *et al.*, 1999). CAMPs also affect bacterial cytoplasmic proteins (Jenssen *et al.*, 2006). One of the more potent CAMPs is LL-37, a cathelicidin that has demonstrated antimicrobial activity against many bacteria including *E. coli* (Chromek *et al.*, 2006). Other CAMPs found in the gastrointestinal tract include both α- and β-defensins, which are synthesized by mucosal cells and neutrophils at a basal level but show increased expression in response to bacterial infection (Ganz, 2003). The amphipathic structure and membrane-disrupting function of these peptides resemble the properties of similar antibacterial compounds made by bacteria, such as polymyxin B (Hancock, 2001), an important therapeutic for multidrug-resistant Gram-negative infections (Li *et al.*, 2006).

The first described mechanism for CAMP resistance in *E. coli* was an alteration of the outer-membrane charge by modification of the lipid A moiety of LPS (Guo *et al.*, 1998). Other work has shown that efflux pumps of the resistance-nodulation-division (RND) family decrease bacterial susceptibility to CAMPs in neisseriae (Shafer *et al.*, 1998; Tzeng *et al.*, 2005). In addition, polymyxin B has

Abbreviations: CAMP, cationic antimicrobial peptide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; PMF, proton-motive force; RND, resistance-nodulation-division.

been described as a substrate of homologous RND efflux pumps in *Campylobacter* (Akiba *et al.*, 2006), *Pseudomonas* (Masuda *et al.*, 2000), *Yersinia* (Bengoechea & Skurnik, 2000) and *Helicobacter* (Bina *et al.*, 2000). The AcrB and AcrA proteins respectively make up the inner membrane and periplasm-spanning regions of the tripartite *E. coli* RND efflux pump AcrAB-TolC, which acts to expel a wide variety of substrates including dyes, bile salts, organic solvents, and structurally dissimilar antibiotics (Nikaido & Zgurskaya, 2001). The TolC protein component is located in the bacterial outer membrane and also pairs with subunits of other membrane pumps (reviewed by Koronakis *et al.*, 2004). CAMPs have a molecular mass of 3–4 kDa, much larger than that of the chemicals and dyes that are known substrates of the AcrAB-TolC efflux pump. Still, TolC can act as a portal for such large substrates as haemolysin and colicins (Wandersman & Delepelaire, 1990).

Expression of *acrAB* is negatively regulated by AcrR (Ma *et al.*, 1996), and both *acrAB* and *tolC* are positively regulated by the related MarA, Rob and SoxS transcriptional regulators (Barbosa & Levy, 2000; Jair *et al.*, 1996; Miller *et al.*, 1994; White *et al.*, 1997). MarA, Rob and SoxS act in different ways to control the expression of not only *acrAB* and *tolC*, but also more than 80 other genes (White *et al.*, 2005). Expression of the *marRAB* operon is itself controlled by the repressor *marR* (Cohen *et al.*, 1993a). Spontaneous inactivating mutations in *marR* are recovered *in vitro* and *in vivo*, resulting in resistance to a range of antibiotics and disinfectants via MarA (Maneewannakul & Levy, 1996; Oethinger *et al.*, 1998). The Rob protein increases expression of the *mar* operon (Jair *et al.*, 1996), is activated by bile salts (Rosenberg *et al.*, 2003), and is necessary for polymyxin B-induced upregulation of *micF* (Oh *et al.*, 2000). The *soxRS* response system is transcriptionally activated by reactive oxygen species to increase resistance to antibiotics and other agents via the AcrAB-TolC efflux pump (Amabile-Cuevas & Demple, 1991; Miller *et al.*, 1994). In consideration of the roles of homologous pumps of the RND family in CAMP resistance, and a previous study that showed a *mar*/*rob*/*sox* triple mutant did not persist in a mouse model of ascending pyelonephritis (Casaz *et al.*, 2006), we examined the roles of MarA, Rob and SoxS in susceptibility to CAMPs in *E. coli*, with particular attention to their effect on the AcrAB-TolC efflux pump.

METHODS

Bacterial strains and culture conditions. All bacterial strains (Table 1) were cultured on LB agar plates or in LB broth at 37 °C. Antibiotic concentrations were 100 μg ml^{-1} for ampicillin (Amp), and 50 μg ml^{-1} for kanamycin (Km) and chloramphenicol (Cm). For IPTG induction, strains were grown in the presence of 0.5 mM IPTG. Mutant strains created in this study were made by P1 transduction from the indicated donor to recipient (Table 1) as described by Nicoloff *et al.* (2006). Strain DMW1000 was created by the sequential transduction of *marA*::*kan*, *rob*::*kan* and *soxS*::*kan* mutated genes from strains from the Keio collection (Baba *et al.*, 2006; Table 1) into an AG100 background. Mutations in these strains had been made by partial deletion and insertion of a Km^R cassette flanked by FRT regions. Each Km^R transductant was transformed with the Amp^R pCP20 plasmid, which carries the FLP gene and has a temperature-sensitive origin of replication (Datsenko & Wanner, 2000). Plasmid-containing colonies were selected at 30 °C on LB agar containing Amp, and were then transferred to non-selective agar and grown at the non-permissive temperature of 43 °C, to simultaneously cure the strain of the plasmid and excise the FRT-flanked Km^R cassette. Strains were then screened for loss of both Amp^R and Km^R; deletion of the gene was confirmed by PCR. Plasmid pCP20 was also used to remove the Km^R cassette from strain AG100T before transduction of the *soxS*::*kan* mutation to form strain AG100TS. *E. coli* strains were transformed with plasmids by electroporation as described for the Bio-Rad Genepulser.

LL-37 survival assay. Bacteria were grown to mid-exponential phase in LB or Mueller–Hinton (MH) broth, before being diluted 1 : 10 into PBS in tubes with and without LL-37 at a final concentration of 35 μg ml^{-1} unless otherwise indicated. In addition, some strains were incubated for 45 min in the presence or absence of paraquat (250 μM) in order to induce transcription of *soxS* (Amabile-Cuevas & Demple, 1991). All mixtures were incubated for 60 min at 37 °C before being serially diluted and cultured on LB agar overnight at 37 °C. The percentage survival was determined by comparing titres between LL-37-treated and non-treated cultures. LL-37 was synthesized by the Core Facility at Tufts University-Boston Campus, from its known sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES (Shafer *et al.*, 1998; Gudmundsson *et al.*, 1996).

Defensin survival assays. Mid-exponential-phase cultures were centrifuged at 9000 r.p.m. for 5 min, washed twice with cold deionized water, and added in a 1 : 10 dilution to PBS with 10 % (v/v) LB broth and the listed amounts of α-defensin HNP-2 (Sigma), β-defensin HBD-1 (Phoenix Pharmaceuticals) or polymyxin B (Sigma). These mixtures were incubated at 37 °C for 2 h before serial dilution and plating on LB agar. Polymyxin B was stable in LB agar allowing for efficiency of plating (e.o.p.) assays, determined by culturing ~10^6 c.f.u. bacteria grown to early exponential phase on LB agar with and without polymyxin B (0.06 μg ml^{-1}) and/or IPTG (0.5 mM) overnight at 37 °C. Results shown represent the mean of three independent experiments performed in duplicate.

Disruption of proton-motive force (PMF). The PMF was disrupted by incubating mid-exponential-phase bacteria without shaking in carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 100 μM in PBS for 20 min at 37 °C prior to incubation with CAMPs. The system was re-energized by addition of glucose (30 mM) and the culture was incubated for an additional 10 min before addition of CAMPs. All strains tested displayed no difference in viability between CCCP-treated and non-treated samples in the absence of CAMPs. Of note, while mutants AG100A and AG100T showed increased susceptibility to CAMPs compared to the parent strain (see Tables 2 and 5), the increase was somewhat different when cells were incubated without shaking in PBS (as a control for CCCP or glucose) (Table 5) from that found under shaking conditions (Table 2).

β-Galactosidase assays. All β-galactosidase assays were performed with derivatives of strain SPC105, which contains both the *marRAB* operon and a *lacZ* gene fused downstream of the promoter of the *marRAB* operon inserted at the λ *att* site on the *E. coli* chromosome (Cohen *et al.*, 1993b). Strains grown to mid-exponential phase in LB broth were diluted 1 : 50 into 1 ml PBS or water, with or without the appropriate antimicrobial peptide, and were incubated for 1 h at 37 °C. The OD_{600} was measured, followed by lysis and a standard Miller assay protocol (Miller, 1972). OD_{420} values were converted to

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
***E. coli* strains**		
AG100	Wild-type K12 strain	George & Levy (1983)
AG100A	*acrAB*::*kan*	Okusu *et al.* (1996)
AG100B	*acrR* deletion	Okusu *et al.* (1996)
AG100T	*tolC*::*kan* from JW5503-1	This study
AG112	*marR* point mutant of AG100; inactivated MarR	Oethinger *et al.* (2000)
AG112A	*marR acrAB*::*kan*	Okusu *et al.* (1996)
AG112T	*marR tolC*::*kan* from JW5503-1	This study
JW5503-1	Source of *tolC*::*kan*	Baba *et al.* (2006)
JW5249-1	Source of *marA*::*kan*	Baba *et al.* (2006)
AG100M	*marA*::*kan* from JW5249-1	This study
AG100R	*rob*::*kan*	White *et al.* (1997)
AG100S	*soxS*::*kan*	Greenberg *et al.* (1990)
AG100soxR105	*soxR105* sjc-2204::Tn*10*Kan from strain JTG1078	This study
AG100TS	Δ*tolC soxS*::*kan*	This study
DMW1000	Δ*marA* Δ*rob* Δ*soxS*	This study
JTG1078	*soxR105 zjc-2204*::Tn*10*Kan, overexpression of *soxS*	Greenberg *et al.* (1991)
SPC105	*marRAB* promoter fused to *lacZ* on phage λ inserted at chromosomal λ *att* site; MC4100 host	Cohen *et al.* (1993b)
SPC105R	*rob*::*kan* from AG100R	This study
Plasmids		
pSMarAB	IPTG-inducible *marA* plasmid (Amp^R)*	White *et al.* (1997)
pSRob	IPTG-inducible *rob* plasmid (Amp^R)	White *et al.* (1997)
pSXS	IPTG-inducible *soxS* plasmid (Amp^R)	Amabile-Cuevas & Demple (1991)
pCP20	Low-copy FLP-encoding temperature-sensitive plasmid (Amp^R)	Datsenko & Wanner (2000)

*The *marB* gene has no effect on antibiotic resistance (Martin *et al.*, 1995).

Table 2. Susceptibility to cationic peptides

Assays were performed with a starting bacterial concentration of 10^8 c.f.u. ml^{-1} diluted 1 : 10 in PBS ± CAMP. The mixtures were then incubated for 45 min at 37 °C before serial dilution and plating on LB agar plates for overnight growth to determine percentage of bacteria that survived the CAMP challenge.

Strain	Survival (%)				MIC PXB (μg ml^{-1})‡
	LL-37* (35 μg ml^{-1})	HNP-2* (25 μg ml^{-1})	HBD-1* (10 μg ml^{-1})	PXB† (0.015 μg ml^{-1})	
AG100	0.6 ± 0.4	26 ± 2.3	5.1 ± 0.8	0.87 ± 0.08	0.03
AG112 (*marR*)	16 ± 3.0§	58 ± 3.6§	11.8 ± 1.8§	0.92 ± 0.14	0.06
AG112A (*marR acrAB*)	0.02 ± 0.01‖	53 ± 2.8	1.4 ± 0.1‖	0.13 ± 0.11‖	0.03
AG112T (*marR tolC*)	0.005 ± 0.002‖	7 ± 2.0‖	0.02 ± 0.005‖	0.027 ± 0.02‖	0.03
AG100A (*acrAB*)	0.03 ± 0.02§	25 ± 2.1	1.1 ± 0.1§	0.11 ± 0.08§	0.015
AG100T (*tolC*)	0.003 ± 0.002§	15 ± 1.8§	0.7 ± 0.2§	0.00012 ± 0.00001§	0.015
AG100B (*acrR*)	31 ± 2.0§	25 ± 2.0	50.0 ± 5.0§	0.91 ± 0.045	0.06
AG100soxR105	0.9 ± 0.3	30 ± 3.2	7.5 ± 2.3	0.56 ± 0.23	0.03

*Data are expressed as mean percentage survival at the given peptide concentration compared to non-treated controls in at least three separate experiments (± SEM).

†Data shown are the mean efficiency of plating on LB agar plates supplemented with polymyxin B (0.015 μg ml^{-1}) as compared to control plates in three separate experiments (± SEM).

‡Data shown represent the MIC of the strains on LB agar plates supplemented with polymyxin B.

§$P \leqslant 0.05$ when compared to strain AG100.

‖$P \leqslant 0.05$ when compared to strain AG112.

Miller units and normalized to the non-treated strain. Data are expressed as fold increase with peptide compared to the untreated strain, and represent the mean ± 95 % confidence interval of three independent experiments performed in triplicate. The indicated concentrations of inducers had no effect on the growth of strains SPC105 or SPC105R.

Statistical analysis. The statistical significance of differences between strains was tested by using a Student's *t*-test assuming two samples of equal variance.

RESULTS AND DISCUSSION

Role of *marRAB* in CAMP susceptibility

Transcriptional regulators are an important component of intrinsic antibiotic resistance and are frequently the sites of spontaneous mutation in antibiotic-resistant strains isolated from the clinic or environment (Maneewannakul & Levy, 1996). Antimicrobial peptides are found in locations and cells that interact with *E. coli* during colonization of the human host. We compared the CAMP susceptibilities of the antibiotic-resistant *marR* point mutant AG112, in which the MarR repressor is inactive and *marA* is therefore overexpressed, with the parent AG100 strain. AG112 showed a tenfold decrease in LL-37 susceptibility, a twofold decrease in susceptibility to HNP-2 and HBD-1 and a twofold increase in polymyxin B MIC (Table 2). These effects can be attributed to MarA activation of the expression of the efflux pump AcrAB-TolC, since loss of either *acrAB* or *tolC* (AG112A and AG112T) negated the decreased susceptibility (Table 2). The polymyxin B susceptibility levels for *tolC* strain AG100T compared to AG112T (also *marR*) showed lower susceptibility for AG112T, which suggests that additional genes under the control of MarA may be contributing to lower polymyxin B susceptibility. Inversely, higher susceptibility was observed for AG112T compared to AG100T for HNP-2 and HBP-1, but not for the other CAMPs, suggesting a resistance disadvantage to α- and β-defensins conveyed by overexpression of *marA* in the absence of *tolC*. These TolC-independent opposing susceptibility changes may be due to a MarA-based downregulation of other membrane transport proteins, or a change in the outer-membrane profile of the *marR* mutant leading to changes in the attraction of CAMPs to the membrane of the bacterium. We attribute these phenotypes to the *marR*-dependent increase of *marA* transcription because similar findings were seen with overexpression of *marA* from a plasmid (Table 3). While deletion of the *marA* gene had only a slight impact on CAMP susceptibility, overexpression of MarA from plasmid pSMarAB produced an antibiotic-resistant *E. coli* strain that was also sevenfold more resistant to host CAMPs (Table 3).

Table 3. Effects of deletion and overexpression of regulatory genes *marA*, *rob* and *soxS* on CAMP susceptibility

Strain	Survival (%)		MIC PXB (μg ml^{-1})†
	LL-37 (35 μg ml^{-1})*	PXB‡ (0.015 μg ml^{-1})	
AG100	0.63 ± 0.4	0.87 ± 0.08	0.03
AG100M (Δ*marA*)	0.31 ± 0.2	0.53 ± 0.13	0.03
AG100R (Δ*rob*)	0.22 ± 0.2	0.44 ± 0.17	0.03
AG100S (Δ*soxS*)	0.37 ± 0.2	0.39 ± 0.13	0.03
DMW1000 (Δ*marA* Δ*rob* Δ*soxS*)	0.30 ± 0.2	0.40 ± 0.09	0.03
AG100 with plasmid:§			
–	0.62 ± 0.015	0.65 ± 0.02	0.03
pSMarAB	4.4 ± 0.025‖	0.65 ± 0.14	0.06
pSRob	0.63 ± 0.33	0.74 ± 0.09	0.03
pSXS	0.19 ± 0.011‖	0.63 ± 0.05	0.03
AG100T with plasmid:§			
–	0.006 ± 0.001	0.00018 ± 0.00008	0.015
pSMarAB	0.007 ± 0.005	0.00016 ± 0.00005	0.015
pSRob	0.006 ± 0.002	0.00020 ± 0.00002	0.015
pSXS	0.004 ± 0.001	0.00010 ± 0.00008	0.0075

*Data expressed as mean percentage survival at the given peptide concentration compared to non-treated controls in at least three separate experiments (± SEM).

†Data shown represent the MIC of the strains on polymyxin B (PXB) plates.

‡Data shown as the mean efficiency of plating on PXB plates (0.015 μg ml^{-1}) as compared to control plates in three separate experiments (± SEM).

§Plates and broth supplemented with IPTG (0.5 mM).

‖$P \leqslant 0.05$ when compared to strain AG100.

Role of *soxS* in CAMP susceptibility

The *soxS* gene product of the *soxRS* two-component system also acts as an activator of efflux and has been implicated in antibiotic resistance (Pomposiello *et al.*, 2001). Surprisingly, deletion of *soxS* or overexpression of *soxS* by mutation of *soxR* (*soxR105*) did not significantly affect CAMP susceptibility of the wild-type strain (Tables 2 and 3). The *soxR105* mutation is a naturally occurring single nucleotide mutation which causes a change in SoxR that greatly increases its transcriptional activation of *soxS*, leading to increased antibiotic resistance (data not shown). Overexpression of *soxS* from a plasmid (pSXS) in strain AG100, however, increased susceptibility to LL-37 (three-fold) but not polymyxin B (Table 3). Surprisingly, deletion of *soxS* or overexpression of *soxS* by a mutation in *soxR* did not significantly affect CAMP susceptibility in the wild-type strain (Tables 2 and 3), although these mutations do affect efflux and antibiotic resistance. These findings suggested that SoxS-regulated genes may be causing increases in CAMP susceptibility, which counteract the expected decrease in susceptibility that should be conferred by increased efflux. To test this theory, we utilized lower concentrations of LL-37 to allow for closer study of the effects of *soxS* expression in a *tolC* background. Susceptibility to lower concentrations of LL-37 was measured in *tolC* strain AG100T containing pSXS, and the low levels of viability were normalized to those of the plasmid-less *tolC* parent. Here overexpression of *soxS* on plasmid pSXS caused a tenfold drop in survival of the *tolC* mutant in the presence of LL-37 and a small decrease in polymyxin B MIC (Table 4). Similarly, paraquat induction of the *soxS* gene led to an 18-fold drop in survival in the *tolC* strain (Table 4). These data indicate that SoxS-controlled genes other than *tolC* can increase LL-37 susceptibility; this may explain why overexpression of *soxS* had no effect on CAMP susceptibility in the wild-type background (AG100).

Table 4. TolC-independent effects of *soxS* expression on LL-37 and polymyxin B susceptibility

Strain	Survival ratio*		MIC polymyxin B (μg ml^{-1})†
	LL-37 (20 μg ml^{-1})	LL-37 (10 μg ml^{-1})	
AG100T	1.0	1.0	0.015
AG100T + pSXS	0.09 ± 0.1‡	0.16 ± 0.09‡	0.0075
AG100T (PQ)§	0.06 ± 0.04‡	ND	ND

*Values represent the mean ratio of survival compared to AG100T (± SEM). ND, Not determined.

†Polymyxin B MIC values as determined by serial dilution on plates containing IPTG (0.5 mM).

§*soxS* was induced by 250 μM paraquat before incubation with LL-37.

‡$P \leqslant 0.05$ when compared to strain AG100T.

Role of *rob* in CAMP susceptibility

The absence of the Rob protein slightly increased susceptibility to LL-37 and polymyxin B; however, over-expression of *rob* by use of an IPTG-inducible plasmid caused no change in CAMP susceptibility (Table 3). These results suggest that the Rob protein is not important in CAMP susceptibility at lethal concentrations; however, Rob may act as a signal of osmotic stress caused by membrane disruption by CAMPs or as a recognition protein of the CAMPs, as it does for bile salts and fatty acids (Rosenberg *et al.*, 2003).

The above three transcriptional activators act differently in CAMP susceptibility: MarA leads to decreased susceptibility via increased efflux, SoxS leads to increased susceptibility via some unknown mechanism, and Rob has minimal effects on CAMP susceptibility. Previous work showed that deletion of all three transcription factors *marA*, *rob* and *soxS* led to a marked decrease in bacterial persistence in infected mouse kidneys (Casaz *et al.*, 2006). We therefore tested a *mar/rob/sox* triple mutant (DMW1000) to see if there was a greater increase in CAMP susceptibility compared to the nominal increases found for *marA*, *rob* or *soxS* single deletion mutants. Susceptibility to LL-37 and polymyxin B did not differ between the single mutants and the triple mutant strain (Table 3). While these *in vitro* data show that the loss of all three of the transcriptional factors is not cumulative, they do identify a possible contributor to the findings in mice, namely AcrAB-TolC-mediated tolerance to host antimicrobial peptides.

LL-37, polymyxin B and defensin HBD-1 are substrates of the AcrAB-TolC efflux pump

Previous work with AcrAB-TolC homologues in other bacterial systems displayed a role for efflux in bacterial susceptibility to the human antimicrobial peptide LL-37 (Shafer *et al.*, 1998). Those results and our findings above led us to investigate the apparent role of the AcrAB-TolC efflux pump in CAMP susceptibility. Using *acrAB* and *tolC* deletion mutants of *E. coli*, we showed increased LL-37 sensitivity compared to the wild-type *E. coli* strain (Table 2): AG100, 0.6 % survival; AG100A, 0.03 %; and AG100T, 0.003 %. The 20-fold survival difference between the wild-type and *acrAB* deletion mutant (AG100A) implicates the AcrAB efflux pump, and the 200-fold difference between wild-type and *tolC* mutant strain AG100T suggests the role of additional *tolC*-dependent efflux pumps in CAMP susceptibility. Deletion of *acrR* (AG100B), the repressor of *acrAB*, led to a nearly 50-fold decrease in bacterial LL-37 susceptibility, also consistent with an AcrAB-based CAMP resistance mechanism (Table 2). These findings are in line with similar observations of efflux pump repressor mutants in other bacteria (Hagman & Shafer, 1995).

It appeared that AcrAB and other RND efflux pumps that use TolC were mediating efflux of CAMPs. To further test this hypothesis, we utilized the energy uncoupler CCCP to

Table 5. Effect of PMF on peptide susceptibility

Bacterial inocula of 10^8 c.f.u. ml^{-1} were incubated in PBS ± CCCP (100 μM) for 20 min at 37 °C, followed by the addition of PBS or glucose (30 mM) and an additional 10 min at 37 °C.

Strain	−CCCP	+CCCP	+CCCP+glucose
		LL-37 survival after treatment with CCCP*	
AG100	0.97 ± 0.27	0.11 ± 0.02	1.34 ± 0.14
AG100A (*acrAB*)	0.40 ± 0.06	0.070 ± 0.0035	0.28 ± 0.003
AG100T (*tolC*)	0.0009 ± 0.0001‡	0.072 ± 0.004	0.0009 ± 0.0003‡
		PXB survival after treatment with CCCP†	
AG100	24.1 ± 4.2	18.5 ± 1.8	26.1 ± 3.2
AG100A	16.1 ± 1.3	15.8 ± 1.2	16.8 ± 1.4
AG100T	4.6 ± 0.51‡	15.2 ± 2.5	4.1 ± 0.35‡

*Values represent the mean percentage survival of strains incubated in LL-37 (35 μg ml^{-1}) for 45 min at 37 °C compared to non-LL-37-treated controls (± SEM).
†Data represent the mean percentage survival of strains incubated for 2 h in polymyxin B (1.5 μg ml^{-1}) compared to non-treated controls (± SEM).
‡$P \leqslant 0.05$ when compared to strain AG100 in the same treatment column.

establish the role of active transport in the observed CAMP survival phenotypes. The loss of PMF increased the levels of LL-37 susceptibility, equalizing values for the wild-type and the *acrAB* deletion strains (Table 5). Surprisingly, loss of PMF in the *tolC* deletion strain AG100T, which should have no RND active efflux, caused a 70-fold decrease in susceptibility compared to the non-CCCP-treated control, bringing the *tolC* mutant susceptibility levels to that of CCCP-treated wild-type and *acrAB* deletion mutant (Table 5). The CCCP-dependent decrease in susceptibility for strain AG100T is in contrast to increases in susceptibility for CCCP-treated wild-type and *acrAB* strains, and suggests the presence of a PMF-dependent active CAMP uptake system in *E. coli*, which may normally be overshadowed by basal levels of TolC efflux pumps. Alternatively, the loss of PMF may alter the charge of the outer membrane of the *tolC* mutant, causing CAMPs to be less attracted to the bacterium. When the cells were treated with glucose after CCCP to re-energize the PMF, CAMP susceptibility was subsequently restored to non-CCCP-treated control levels (Table 5).

Forms of α- and β-defensins are found throughout the human body where *E. coli* may encounter any or all of these CAMPs. While these peptides are similar in size to LL-37, they differ by the presence of disulfide bonds, which may interfere with the ability of the peptides to be effluxed through the AcrAB-TolC complex. Susceptibility to both α- (HNP-2) and β- (HBD-1) peptides was increased in a *tolC* mutant (two- and tenfold respectively compared to the wild-type strain), while an *acrAB* deletion mutant only showed an increase in susceptibility to β-defensin, HBD-1 (fivefold) (Table 2). These findings also hold true in the presence of a *marR* mutation and show that a MarR-repressed, TolC-dependent mechanism is responsible for bacterial susceptibility to α-defensin HNP-2. Efficiency of plating assays using polymyxin B showed susceptibility increases for *acrAB* mutants (eightfold) and *tolC* mutants (10 000-fold) (Table 2). Treatment of the strains with the energy uncoupler CCCP produced increases in polymyxin susceptibility for the wild-type strain, implicating active efflux in polymyxin susceptibility, similar to the effects described for LL-37 (Table 3).

Curiously, our findings differ sharply from those of a recent study that found no difference in susceptibility to CAMPs LL-37 and polymyxin B between an *E. coli acrAB* deletion mutant and an antibiotic-resistant *acrR* mutant grown in MH broth (Rieg *et al.*, 2009). We performed parallel experiments with both media comparing wild-type, *acrAB*, *tolC* and *acrR* mutants. With MH, as described by Rieg *et al.* (2009), we found no effect of *acrAB* and *acrR* deletions on susceptibility to LL-37 or polymyxin B. However, in LB broth these deletions affected susceptibilities to these and other CAMPs as described above (Table 2). The different results with MH and LB may reflect differences in ion concentrations between the two media that could affect the activity of the CAMPs and/or the ability of the AcrAB complex to effectively expel these substrates. Other studies have described ion-dependent differences in both CAMP stability and efflux pump function (D'Amato *et al.*, 1975; Dorschner *et al.*, 2006). Also, differences in the media could affect the membrane structure of the bacterium, increasing attraction of CAMPs to the membrane and/or increasing membrane permeability. Of note, the *tolC* deletion mutant (not tested by Rieg *et al.*, 2009) displayed an increase in susceptibility to LL-37 and polymyxin B in both LB (Table 2) and MH media (Table 6).

The AcrAB-TolC pump in *E. coli* can now be categorized with efflux pumps NorM and MtrCDE of *Neisseria* spp. (Shafer *et al.*, 1998; Tzeng *et al.*, 2005) and YejABEF of *Salmonella* (Eswarappa *et al.*, 2008) as a CAMP resistance

Table 6. CAMP susceptibilities in MH agar

Strain	LL-37*	PXB†
AG100	7.2 ± 0.2	0.1 ± 0.007
AG100T (*tolC*)	0.2 ± 0.01‡	0.005 ± 0.001‡

*Data expressed as mean percentage survival in LL-37 (35 μg ml^{-1}) compared to non-treated controls in at least three separate experiments (± SEM).
†Data shown as the mean efficiency of plating on polymyxin B (PXB) plates (0.015 μg ml^{-1}) as compared to control plates in three separate experiments (± SEM).
‡$P \leqslant 0.05$ when compared to strain AG100 in the same treatment column.

factor. We also demonstrate human α- and β-defensins as substrates of an *E. coli* RND efflux pump. Defensins have been shown as substrates for a *Salmonella* ABC efflux pump (Eswarappa *et al.*, 2008), and active efflux in general was implicated as a defensin susceptibility factor for the oral pathogen *Treponema denticolum* (Brissette & Lukehart, 2007). The results for LL-37, HNP-2 and HBD-1 susceptibility are made more noteworthy by the fact that these defensins are chiefly found in the granules of neutrophils or secreted by mucosal epithelial cells in response to bacterial infection (Ganz, 2003).

Exposure to sublethal levels of CAMPs increases expression of the *marRAB* operon in a Rob-dependent manner

We hypothesized that the *mar* operon may be induced by CAMPs at sublethal concentrations in order to decrease bacterial susceptibility at higher CAMP concentrations, as would be found with induction of inflammation when bacteria colonize a host niche. Using the *mar-lacZ* reporter strain SPC105, we found that exposure to a sublethal amount of LL-37, polymyxin B or HBD-1 reproducibly increased transcription of the *mar* operon approximately 1.5-fold compared to non-exposed cultures (Fig. 1). These levels, however, are less than the three- to sixfold increases reported with the classic MarR inhibitor salicylate (Alekshun & Levy, 1999). Previous workers have shown that the Rob protein is activated by bile salts, leading to upregulation of Rob targets including the *mar* operon (Rosenberg *et al.*, 2003). In addition, the Rob protein has been shown to play a role in the polymyxin B-dependent upregulation of *micF* (Oh *et al.*, 2000). Therefore, we transduced a *rob*::*kan* mutation into reporter strain SPC105 and found that the increases in *marRAB* expression from exposure to sublethal concentrations of CAMPs were eliminated (Fig. 1). Therefore it is likely that upregulation of *marRAB* by CAMPs occurs indirectly through or by association with Rob, and this activity reveals a new phenotype for *rob* as a virulence gene.

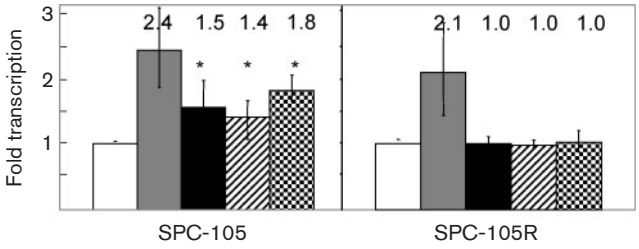

Fig. 1. Peptide-induced activation of the *marRAB* operon is *rob*-dependent. The fold levels of *marRAB* transcription are shown for strain SPC105 (*mar-lacZ*) and its *rob*::*kan* derivative, SPC105R, using a β-galactosidase assay. Strains were grown in the absence (white bars) or presence of salicylate (0.5 mM) (grey), polymyxin B (0.001 μg ml^{-1}) (black), LL-37 (1.0 μg ml^{-1}) (hatch) or HBD-1 (1.0 μg ml^{-1}) (check), and compared to non-treated controls. Data are expressed as fold transcription compared to parallel control samples, and represent the mean of three separate independent experiments. Error bars represent the 95 % confidence intervals. Mean values are shown above the bars. *$P \leqslant 0.05$ when compared to the non-induced control.

Conclusions

Our investigation of the transcriptional regulators of antibiotic resistance allows us to conclude that overexpression of *marA* decreases susceptibility to CAMPs via upregulation of the AcrAB-TolC efflux pump and possibly other TolC-dependent RND efflux pumps. We show that CAMPs themselves act to upregulate the *mar* operon, but require Rob for this activity. Unexpectedly, overexpression of SoxS, another transcriptional activator of *marRAB* and the *acrAB* and *tolC* efflux pump genes, did not lead to a change in CAMP susceptibility in a wild-type strain. However, overexpression of SoxS in a *tolC* mutant increased CAMP susceptibility, which suggests that other genes under the control of SoxS are detrimental to bacterial survival in the presence of CAMPs. In addition, we propose that AcrAB and other TolC-dependent efflux pumps actively excrete CAMPs, thereby decreasing the susceptibility of *E. coli* to CAMPs (Fig. 2).

The prominent role of CAMPs in innate immunity provides a selective pressure on *E. coli* to retain the transcriptional regulators MarA and Rob, and the efflux pump AcrAB-TolC, which play major roles in bacterial resistance to therapeutic antibiotics such as chloramphenicol, tetracyclines and fluoroquinolones, as well as triclosan, and now polymyxin B. Also, this evolutionary pressure could contribute to the retention of such regulators and efflux pumps in natural flora, which act as sources of horizontal gene transfer of antibiotic-resistance factors (Salyers *et al.*, 2004). The past decade has unveiled a wealth of information linking the RND family of efflux pumps to resistance to natural host-derived substrates (Piddock, 2006). Further understanding of the CAMP recognition and possible uptake mechanisms may lead to the

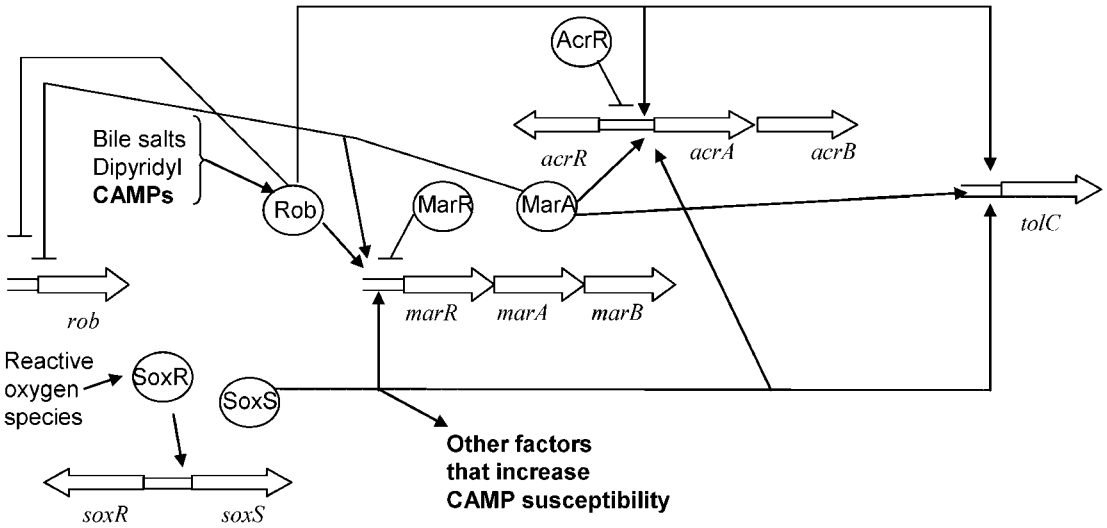

Fig. 2. Transcriptional circuitry of the *mar* operon with regard to CAMP susceptibility. Activation of the *mar* operon and how this affects transcription of the *acrAB* and *tolC* operons are shown. We propose that CAMPs act like bile salts and dipyridyl to activate the Rob protein and thus increase transcription of the *mar* operon. We also show that certain CAMP sensitivity factors are induced by activation of the SoxR protein, and this increased sensitivity offsets the decrease in susceptibility that should accompany increased levels of AcrAB and TolC. Arrows mark positive interactions and horizonal lines represent repression. CAMP activity is highlighted.

development of therapeutics that not only decrease intrinsic drug resistance, but also increase the potency of the host immune system against these pathogens.

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