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A Combination of *sbmA* and *tolC* Mutations in *Escherichia coli* K-12 Tn*10*-Carrying Strains Results in Hypersusceptibility to Tetracycline▽

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Previously, we demonstrated that *Escherichia coli tolC* mutations reduce the high-level resistance to tetracycline afforded by the transposon Tn*10*-encoded TetA pump from resistance at 200 μg/ml to resistance at 40 μg/ml. In this study, we found that the addition of an *sbmA* mutation to a *tolC*::Tn*10* mutant exacerbates this phenotype: the double mutant did not form colonies, even in the presence of tetracycline at a concentration as low as 5 μg/ml. Inactivation of *sbmA* alone partially inhibited high-level tetracycline resistance, from resistance at 200 μg/ml to resistance at 120 μg/ml. There thus appears to be an additive effect of the mutations, resulting in almost complete suppression of the phenotypic expression of Tn*10* tetracycline resistance.

Escherichia coli sbmA mutants show complete resistance to microcins B17 (13) and J25 (23) and increased resistance to bleomycin (25) and proline-rich antimicrobial peptides (17). Analysis of the 406-amino-acid sequence of SbmA, deduced from the gene, suggests that it is an inner membrane protein and predicts seven membrane-spanning domains (10). It was therefore inferred that SbmA transports these peptide antibiotics into the cell cytoplasm. The finding that mutations in *sbmA*, even deletions of the entire gene, result in no apparent growth phenotype (showing that the SbmA protein is dispensable for cell viability) raises the question of the biological role of SbmA. Homologs of the *sbmA* gene are found in a variety of bacteria (10). The *Rhizobium meliloti* homolog of SbmA, BacA, is essential for the symbiosis between *R. meliloti* and alfalfa (10), whereas in *Brucella abortus* a homolog of the *bacA* gene is critical for survival of this mammalian pathogen in macrophages (14). The conservation of these genes in a wide variety of bacteria, including plant and animal pathogens, suggests that functions related to those of BacA/SbmA must confer an important advantage in diverse environments and that an important physiological role might exist for these genes.

Several years ago, we established that the peptide antibiotic microcin J25 is exported by a complex formed by a dedicated ATP binding cassette (ABC) exporter and the outer membrane protein TolC. *E. coli* TolC$^-$ strains harboring multiple copies of microcin-producing genes rapidly lose viability, and we assumed that this was due to the intracellular accumulation of active antibiotic (7). However, we were not able to extract increased amounts of microcin from these cells. More recently, we thought of the possibility that intracellular microcin was inactive and became active during export. If so, the viability loss of TolC$^-$ cells could be due to microcin that reentered the cells and could not be extruded by the efflux system. In the present study, we decided to address this question by constructing an *E. coli* double mutant strain lacking both the TolC and SbmA proteins. The rationale was that SbmA elimination would preclude reentry of microcin into the cells. To construct the double mutant, P1 transduction was performed with strain RYC714 (13), a well-characterized *sbmA*::Tn*5* mutant, as a donor, and MC4100 *tolC*::Tn*10* as the recipient. Despite repeated attempts, we did not succeed in obtaining *sbmA*::Tn*5* *tolC*::Tn*10* transductants on selective agar containing tetracycline (10 μg/ml) and kanamycin (30 μg/ml). We reasoned that the failure could be due to either antibiotic being toxic to the double mutant. Therefore, the experiment was repeated, but this time the transduction mix was plated on LB agar containing either kanamycin or tetracycline. Although a number of clones appeared on the kanamycin plate, no colonies grew on the plate with tetracycline. We concluded that the tetracycline in the selective medium was responsible for the toxicity to the double mutant seen in the first experiment. Several transductants from the kanamycin plates were purified and had the expected SbmA$^-$ and TolC$^-$ phenotypes (resistance to microcins J25 and B17 and sensitivity to 0.05% deoxycholate, respectively). One of these clones, designated REC100, was selected for further study. It was unable to form isolated colonies when streaked on LB plates containing 10 μg/ml of tetracycline but gave single colonies of normal size on either LB or LB-kanamycin plates. Because of the possible instability of the strain, it was necessary to make certain that the Tn*10* was still functional. This was demonstrated by the fact that complementation of the *tolC*::Tn*10* mutation in REC100 by the wild-type *tolC* gene cloned into plasmid pAX629 (12) restored resistance to tetracycline. We concluded that in the *sbmA tolC* background, the transposon was unable to afford resistance to the usual challenge concentrations of the antibiotic (10 to 15 μg/ml). This experiment also demonstrated that the reduced tetracycline resistance was phenotypic. It is striking that the effect of the simultaneous depletion of SbmA and TolC on sensitivity to tetracycline was observed only on plates, since the double mutant grew normally in LB liquid cultures in the presence of the same concentration of the antibiotic. This does not occur

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with the single *tolC* mutant, which grows equally well in liquid or solid medium supplemented with tetracycline (with an upper limit of 40 μg/ml) (6). It was possible that growth in liquid medium was an inoculum-dependent phenomenon. The inoculum effect is a large increase in the MIC occurring with an increase in the inoculum size of a microorganism and occurs primarily with beta-lactam antibiotics (3). To address this possibility, we performed growth experiments with liquid LB medium, using various inocula. Strain REC100 was first grown to stationary phase in LB medium plus 10 μg/ml of tetracycline. This culture was conveniently diluted to achieve inoculum sizes of approximately 10^7 to 10^3 CFU/ml. Inoculum densities were verified by quantitative plating. Cultures were started by adding 100 μl of inoculum to 5 ml of LB medium plus tetracycline (10 μg/ml). After 12 h of incubation at 37°C, all cultures had grown, showing lag phases whose duration depended more or less inversely on the size of the inoculum. From this experiment, we concluded that the inoculum concentration had no influence on the ability of the double mutant to grow in liquid medium.

Determination of the MIC of tetracycline for the double mutant. A colony-forming assay was used to determine the MIC of tetracycline for REC100. LB plates were prepared with increasing concentrations of tetracycline (ranging from 1 to 10 μg/ml, with 1-μg/ml increments). They were inoculated (0.1 ml) with a 10^{-6} dilution of stationary-phase cultures of REC100 grown in LB medium supplemented with 10 μg/ml tetracycline, such that about 2×10^2 to 3×10^2 bacteria were spread per plate, and then incubated overnight at 37°C. The lowest concentration of tetracycline required to completely inhibit colony formation by REC100 was 5 μg/ml.

Effects of single mutations on tetracycline resistance. Earlier, we showed that *E. coli tolC* mutations reduced the high-level resistance to tetracycline afforded by Tn*10* fivefold (6) (Table 1; compare the MICs for MC4100 *thr*::Tn*10* and MC4100 *tolC*::Tn*10*). This led us to propose that high-level resistance conferred by TetA results from its cooperation with the TolC-AcrAB efflux system (6). It was interesting to test whether the *sbmA* mutation alone had a similar effect on tetracycline resistance to that of the *tolC* mutation. Aliquots of a 10^{-6} dilution of strain MC4100 *sbmA*::Tn*5* with Tn*10* inserted in another place on the chromosome (*thr*::Tn*10*) were spread on LB plates supplemented with various amounts of tetracycline (from 10 to 200 μg/ml). While the chromosomal copy of Tn*10* provided the expected high-level tetracycline resistance in the control strain MC4100 *thr*::Tn*10*, the upper limit of this resistance was markedly lower for the *thr*::Tn*10 sbmA*::Tn*5* derivative (Table 1, compare rows 3 and 5). Thus, the single *sbmA* and *tolC* mutations inhibited the expression of high-level tetracycline resistance 40% and 80%, respectively, whereas the combined effect in the double mutant was to reduce resistance 97%. An additive effect of the mutations was evident. Similar results were obtained by constructing the same mutant combination in the reference wild-type *E. coli* K-12 strain MG1655. The new mutant derivative was unable to grow on LB plates supplemented with 10 μg/ml tetracycline, showing that the observed effect is reproducible and is not strain specific.

Complementation of the REC100 phenotype with plasmid-borne *sbmA* and *tolC* genes. It was possible that the observed phenotype of the double mutant was the result of polarity of either of the transposon insertions in the expression of downstream genes. To rule out this possibility, REC100 was transformed with either pAX629 (12) or pMM73.4 (13), carrying only the wild-type *tolC* or *sbmA* gene, respectively. As shown in Table 1 (rows 6 and 7), the plasmids fully complemented the corresponding mutations, and the transformants recovered the ability to form colonies on LB-tetracycline (10 μg/ml) plates. Note that, as expected, either plasmid restored tetracycline resistance only to the level determined by the second, uncomplemented mutation. These results indicated that the phenotype conferred by inactivation of the *sbmA* or *tolC* gene in REC100 was not a result of secondary effects on the expression of neighboring loci. It was concluded that coexistence of the mutations was necessary to give rise to the tetracycline sensitivity phenotype, as complementation of either of the single mutations sufficed to relieve this phenotype.

TABLE 1. MICs of tetracycline

Strain	MIC (μg/ml)[a]
MC4100	2
MC4100 *tolC*::Tn*10*	40
MC4100 *thr*::Tn*10*	200
REC100 (MC4100 *sbmA*::Tn*5 tolC*::Tn*10*)	5
MC4100 *thr*::Tn*10 sbmA*::Tn*5*	120
REC100(pAX629)	120
REC100(pMM73.4)	40
MC4100 *thr*::Tn*10* Δ*acrAB*::Tn*903*	40
MC4100 *thr*::Tn*10* Δ*acrAB*::Tn*903* Δ*sbmA*	5
MC4100 *sbmA*::Tn*5*	2
PB3	0.15
PB3 *sbmA*::Tn*5*	0.15

[a] MICs were determined by an agar dilution method.

Growth properties of REC100. We next investigated the growth properties of the double mutant in comparison with the parent strain MC4100 and the single *sbmA* and *tolC* mutants. Growth was monitored by measuring the absorbance at 600 nm. When grown in LB medium supplemented with the appropriate antibiotic (tetracycline at 10 μg/ml or kanamycin at 30 μg/ml), the *tolC*::Tn*10*, *sbmA*::Tn*5*, and double mutant derivatives had generation times which were essentially identical to that of the parent strain. Thus, both the *sbmA* and *tolC* genes can be inactivated singly or in combination without any apparent effect on bacterial growth.

***tetA* gene expression is not altered in *sbmA tolC* mutants.** Two genes of the transposon Tn*10*—*tetA*, encoding the inner membrane resistance protein TetA(B), and *tetR*, encoding a repressor—are involved in tetracycline resistance. An explanation for the reduced tetracycline resistance phenotype could be that in the double mutant a regulatory mechanism diminishes expression of the *tetA* gene. To test this possibility, the plasmid pRKH40 (11), harboring a *tetA*::*lacZ* gene fusion whose transcription is under the control of the repressor TetR, was transformed into strains MC4100 and REC100, and β-galactosidase activity was measured as described by Miller (18). Expression of the fusion in the double mutant grown in LB medium (1,520 and 1,450 Miller units in the log and stationary phases, respectively) was similar to that in the parent strain (1,480 and 1,390 Miller units), ruling out an effect of the *sbmA tolC* mutations at the transcriptional or translational expression level of *tetA*. However, since hypersusceptibility to tetracycline is a solid-

phase phenomenon, we determined the activity of the fusion in cells grown on LB agar supplemented with 2 μg/ml tetracycline. We used this low concentration of the antibiotic since the double mutant failed to grow at concentrations above 5 μg/ml; however, this amount of antibiotic is enough to induce expression of the *tetA* gene from the pRKH40 plasmid. A few colonies were scraped off plates inoculated the previous night and resuspended in LB medium, and β-galactosidase activity was measured. The expression level of the fusion in REC100 (pRKH40) (998 Miller units) was comparable to that in the control, MC4100(pRKH40) (886 Miller units) (results are mean values of two independent determinations). We concluded that no regulatory mechanism on *tetA* operates on solid medium as a consequence of the double mutation.

How can we account for the observed effect of the double mutation? Our first working hypothesis was that a physical interaction between SbmA and TolC was somehow required for phenotypic expression of the tetracycline resistance determinant encoded by an introduced Tn*10*. If so, the two single mutations would each be predicted to be mechanistically equivalent (i.e., it would be sufficient to inactivate one of the proteins of the complex to produce the phenotype). This was not observed. Nevertheless, one way to rationalize this observation is that *E. coli* may possess SbmA and TolC functional homologs that can compensate for the loss of each protein in the single mutants and prevent us from observing any phenotype. In connection with this, recent searches of the sequence databases have shown that there is an SbmA-related protein, YddA, which is predicted to be an exporter of unknown function (16) and has been demonstrated to be regulated by the SOS response to DNA damage (4). However, the fact that an *E. coli sbmA* mutant is resistant to microcins B17 and J25 would imply that YddA can perform the same tasks as the SbmA protein except that related to peptide antibiotic penetration. The observation that the simultaneous inactivation of the *sbmA* and *tolC* genes is necessary to make a strain hypersensitive to tetracycline may also suggest that the two genes encode redundant proteins that can functionally replace one another. Thus, in the presence of a wild-type copy of *tolC*, the *sbmA* mutation would have no effect, and vice versa. We consider this functional redundancy unlikely because of the different locations and lack of sequence homology of the TolC and SbmA proteins.

There is another potential explanation for the double mutant phenotype. Eckert and Beck (8) observed that high-level expression of the TetA protein from multiple copies of the *tetA* gene results in partial collapse of the membrane potential, arrest of growth, and killing of the cells. They proposed that the observed effect of overproduced TetA protein on the membrane potential may be a consequence of the absence of sufficient quantities of other cellular components in the membrane and that such components, encoded by the host chromosome, may be involved in Tn*10*-mediated tetracycline resistance. Along these lines, it is tempting to speculate that even though a single-copy Tn*10* can be induced with no apparent harm to a wild-type cell, this induction could be toxic in the double mutant due to the absence of SbmA in the inner membrane. However, the fact that SbmA-depleted, Tn*10*-containing cells grow normally on LB-tetracycline (10 to 120 μg/ml) plates (in contrast to the doubly deficient cells) argues against this proposal.

The *sbmA* gene product and its homologues in other bacterial species are classified as subunits of a putative transporter protein belonging to the ABC superfamily (15). Thus, one appealing possibility is that SbmA is involved in the export of a substance that, in the absence of a functional *sbmA* gene, would accumulate intracellularly and act as an endogenous inhibitor of the TetA pump.

An alternative explanation for the phenotype displayed by the double mutant, and the one we favor, is that SbmA and TolC work independently of each other. As we have noted, inactivation of *sbmA* alone partially inhibited high-level tetracycline resistance, from resistance at 200 μg/ml to resistance at 120 μg/ml, an effect similar to, though less severe than, that resulting from *tolC* mutation. This might be explained by assuming that SbmA could influence the activity of the TetA pump such that, in the absence of SbmA, the pump is less efficient. It is unknown how this is accomplished mechanistically. One possibility is that both proteins interact and that a lack of SbmA may result in insertion of the TetA protein into the membrane in an abnormal, less active conformation. Alternatively, the absence of SbmA may cause a disturbance of the structure or function of the bacterial membrane, affecting TetA function in an indirect manner. Whatever the mechanism involved, in doubly deficient cells the effects of each mutation may be compounded sufficiently to diminish tetracycline resistance by 97% (5 μg/ml versus 200 μg/ml for wild-type cells), precluding growth of a colony at the usual challenge concentrations of the drug. This hypothesis implies that the tetracycline sensitivity of the *tolC sbmA* strains is at least partly due to inactivation of the TolC-AcrAB efflux system. However, TolC mutants are pleiotropic (1, 9, 19, 20, 21), and we cannot exclude the possibility that another mechanism might contribute to the hypersensitivity phenotype. One way to address this issue was to generate an *sbmA acrAB* double mutant strain and to see whether it displayed the same phenotype as the *sbmA tolC* strain. However, this construction was hindered by the fact that the only available *sbmA* and *acrAB* alleles were both marked with a kanamycin resistance gene. To circumvent this problem, we obtained strain JW0368, in which the *sbmA* gene has been replaced by a kanamycin resistance cassette via a λ Red recombinase-mediated gene replacement, from the KEIO collection (2, 5). The Δ*sbmA* mutation was transduced into strain MC4100, and the kanamycin cassette was subsequently removed using the FLP recombinase produced by a conditionally replicating plasmid (5), thus creating an unmarked *sbmA* deletion. The Δ*acrAB*::Tn*903* (Kan^r) (from strain AG100A [22]) and *thr*::Tn*10* mutations were then transduced successively. The MIC of tetracycline for the *sbmA acrAB* strain was exactly the same as that for REC100 (Table 1, rows 4 and 9), indicating that the *tolC* mutation indeed acts solely through inactivation of the AcrAB efflux system.

The above results implicate a requirement for SbmA for full expression of tetracycline resistance by Tn*10*-encoded TetA. This raises the question of whether, in the absence of TetA, SbmA contributes to intrinsic resistance to the drug. As shown in Table 1, inactivation of *sbmA* alone did not increase susceptibility above that of the parent strain (compare MC4100 and MC4100 *sbmA*::Tn*5*). To examine the effect of the *sbmA* mu-

tation in the absence of a functional AcrAB-TolC pump, the *sbmA*::Tn*5* allele was transduced into strain PB3 (Δ*tolC5*). The resulting transductant demonstrated no alteration of the intrinsic susceptibility to tetracycline compared to PB3 (Table 1). Note that in these strains, the defective AcrAB-TolC pump accounts for the decreased MIC of tetracycline, as this efflux system is a key determinant of intrinsic resistance to this antibiotic (24). Furthermore, we have tested the resistance of strain MC4100 *sbmA*, in both the presence and absence of *tolC*, to another antibiotic, chloramphenicol, for which, as occurs with tetracycline, the AcrAB-TolC drug efflux pump plays an important role in determining the intrinsic level of resistance in *E. coli* (22, 24). The MICs for MC4100 *sbmA*::Tn*5* and REC100 were the same as those for MC4100 (3 μg/ml) and MC4100 *tolC* (0.3 μg/ml), respectively. These data support the concept that the *sbmA* effect is more likely specific for TetA-mediated resistance to tetracycline than a general phenomenon.

In summary, these findings present clear evidence that in a *tolC* background, lack of SbmA produces a strong decrease in transposon Tn*10*-encoded tetracycline resistance. To our knowledge, this is the first report associating the loss of *E. coli sbmA* with a phenotype other than resistance to peptide antibiotics. Understanding the precise mechanism involved may be important to both clinical applications and basic studies of tetracycline resistance.

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