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The Peptide Antibiotic Microcin 25 Is Imported through the TonB Pathway and the SbmA Protein

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Selection of spontaneous mutants for insensitivity to the peptide antibiotic microcin 25 led to the isolation of five categories of mutants. Phenotypic and mapping studies showed the mutations to be located in the *fhuA*, *exb*, *tonB*, and *sbmA* genes. The latter encodes a cytoplasmic membrane protein which is also required for the penetration of microcin B17.

Microcin 25 (Mcc25) is a plasmid-encoded peptide antibiotic produced by *Escherichia coli* AY25 when cultures approach the stationary phase (24). The level of free iron in the culture medium is at least one of the factors controlling its synthesis (26). The peptide appears to interfere with cell division, since susceptible cells filament when exposed to it (24). Recently, we have demonstrated that a Tn*5*-generated mutation at *fhuA*, a gene encoding a multifunctional outer membrane protein, caused a complete loss of sensitivity to Mcc25 (25). Thus, FhuA is most likely the receptor for the antibiotic. Subsequently, Moeck et al. (18) identified a region of FhuA which is important for recognition of Mcc25.

In the present paper, we describe the characterization of spontaneous Mcc25-resistant mutants and demonstrate that the *tonB*, *exb*, and *sbmA* genes play a role in microcin uptake.

Strains of *E. coli* and plasmids used in this work are listed in Table 1. All strains are *E. coli* K-12 derivatives, except for AY25 and RYC492, the natural producers of microcins 25 and E492.

For the isolation of Mcc25^r mutants, 50 μl of an overnight culture of *E. coli* AB259 was plated onto Luria-Bertani agar containing Mcc25 as selective agent. Fifty microcin-insensitive colonies were picked up, purified, and confirmed as resistant to Mcc25 in a cross-streaking assay. When grown on M9 agar plates, some of the mutants hypersecreted enterochelin. In *E. coli*, several mutants which overproduce this siderophore have been described previously, among them mutants at the *tonB*, *exbB*, *exbC*, and *fepA* genes, which are insensitive to various group B colicins (3, 4, 8–10, 23); in addition, TonB$^-$ mutants are resistant to bacteriophages ϕ80 and T1 (11). Therefore, all the Mcc25^r mutants were checked for their resistance to colicins B, V, and M and to phage ϕ80, which allowed the differentiation of five phenotypic classes of mutants (Table 2).

Mutants of group 1 were typical *fhuA* mutants (2, 4, 29). They were expected in view of our previous finding that an insertional mutation in *fhuA* causes a complete loss of sensitivity to Mcc25 (25). These mutants were not further analyzed.

Mutants of group 2 showed the properties of classical *tonB* mutants (4, 11). Growth of the type strain SBG251 under conditions of iron stress (Tris-buffered medium of Simon and Tessman, with iron omitted) (27) was considerably retarded, compared with that of AB259 (Fig. 1). This is characteristic of *tonB* mutants, which are completely defective in iron transport (6). When SBG251 was transduced to Tc^r with P1 grown on BW7622 (*trpB*::Tn*10*), we found that the microcin resistance marker was 77% linked to *trp*, suggesting that the mutation in strain SBG251 was indeed in *tonB*. Moreover, *tonB* mutants of strain AB259, obtained in our laboratory by selecting for resistance to phage ϕ80, were found to be resistant to Mcc25.

The group 3 mutants showed properties pointing to an *exb* mutation. They were resistant to colicin M and partially sensitive to colicin B (the titer of a colicin B preparation was 64-fold lower on the type mutant strain SBG206 than on the wild-type AB259) (1, 13). The plating efficiency of phage ϕ80 on the mutant was reproducibly reduced by 20% compared with that on the parent strain. This has been described before for *exb* mutants (1). Strain SBG206 grew normally in iron-deficient medium and hypersecreted enterochelin (but to a lesser extent than the *tonB* mutants of group 2) (8, 10, 22). Mapping of the mutation was achieved by transducing strain AT2446 (MetC$^-$) with a P1 phage stock prepared on SBG206. Most MetC$^+$ transductants (98%) also inherited the Mcc25^r character, strongly suggesting that the mutation was indeed in *exb*. The *exb* locus, which has been mapped at min 65 of the *E. coli* linkage map (10, 23), consists of two genes, termed *exbB* and *exbD*, which are probably transcribed as an operon (5). In fact, because of polarity effects or the presence of short deletions, we cannot distinguish between loss of ExbB and loss of ExbD, and the microcin resistance phenotype may be due to loss of ExbB, ExbD, or both proteins. We have isolated some Mcc25-resistant mutants showing the *exb* phenotype which had a requirement for methionine. These mutants may contain a deletion encompassing *exb* and the nearby *metC* locus. Many *exb* mutants which are methionine auxotrophs have been described previously (4, 7, 10). Finally, strains HE2 and KP1040, two well-characterized *exbB*::Tn*10* mutants (1, 16), were also insensitive to Mcc25. Absolute linkage between the Tc^r and Mcc25^r phenotypes was shown by P1 transduction.

The Mcc25^r mutation in strain SBG247 (group 4) was 41% linked to *trp*, as shown by P1 transduction analysis, with this strain as donor and IY13 (Trp$^-$) as recipient. This suggested that SBG247, which hypersecreted enterochelin in amounts comparable to those of the *tonB* mutant SBG251, carried a mutated *tonB* gene. Interestingly, the phenotype of this class of mutants differed from that of typical *tonB* mutants in that they were partially sensitive to colicin V, showed a residual sensitivity to phage ϕ80 and colicin B (Table 2), and grew in a

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference[a]
Strains		
AY25	Natural isolate (not K-12), Mcc25$^+$	24
AB259	HfrH *supQ80* λ$^-$ *relA1 spoT1 thi-1*	CGSC
HE2	*tolQ exbB*::Tn*10*	V. Braun
TO4	Carries pTO4 (*cma cmi* in pBR322), ColM$^+$	V. Braun
ES965	Carries pES7 (*cba cbi* in pACYC184), ColB$^+$	V. Braun
MC4100 (pHK11)	pHK11 is pBR322 with cloned *cvaA* to -*C cvi* genes, ColV$^+$	R. Kolter
RYC1000 (pMM39)	pMM39 is pBR322 with cloned *mcbA* to -*G* genes, MccB17$^+$	F. Moreno
RYC492	*Klebsiella pneumoniae*, MccE492$^+$	M. Laviña
AT2446	*metC69*	CGSC
IY13	*trp*	30
BW7622	*trpB114*::Tn*10*	CGSC
N3033	*lacZ98*::Tn*10*	CGSC
RYC726	Δ(*phoA-sbmA*)*14*	F. Moreno
RYC714	*sbmA11*::Tn*5*	F. Moreno
GM247	*proC*	F. Moreno
SBG206	AB259 *exb*	This study
SBG251	AB259 *tonB*	This study
SBG247	AB259 *tonB*	This study
SBG203	AB259 *sbmA*	This study
Plasmids		
pRZ540	Kan^r ColE1 derivative carrying *tonB*	K. Postle
pKP298	pACYC184 carrying *exbBD*	K. Postle
pMM73.4	pBR322 carrying *sbmA*	F. Moreno

[a] CGSC, *E. coli* Genetic Stock Center.

similar fashion as the wild-type strain AB259 under conditions of iron deprivation (Fig. 1). These could be an exceptional type of *tonB* mutants, carrying a point mutation which affects the uptake of Mcc25 but influences the other *tonB*-related processes to a lesser extent. *tonB* mutants that remain fully sensitive to phages ϕ80 and T1, and to colicin V, but which have lost other *tonB*-dependent functions have been described previously (12).

Preliminary mating experiments indicated that the locus mutated in group 5 mutants (which comprised 50% of the mutants isolated in this work) was near *lac*. Therefore, a P1 phage stock

TABLE 2. Phenotypic groups of Mcc25-resistant mutants

Group	Type strain	Sensitivity or resistance[a] to:						
		Phage ϕ80	Colicin			Microcin		
			B	M	V	25	B17	E492
1		R	S	R	S	R	S	S
2	SBG251	R	R	R	R	R	S	R
3	SBG206	S	P	R	S	R	S	R
4	SBG247	(R)	(R)	R	P	R	S	R
5	SBG203	S	S	S	S	R	R	S
Parent	AB259	S	S	S	S	S	S	S

[a] Sensitivity to phage ϕ80 was determined by spotting a 5-μl sample of a phage stock (10^{10} PFU/ml) onto Luria-Bertani plates seeded with the test strains. Sensitivity to colicin B was determined by dropping series of different dilutions of a crude extract (prepared from strain ES965) onto Luria-Bertani plates seeded with the test strains. Sensitivity to colicins M and V and to microcins was tested by cross-streaking against strains TO4 (ColM$^+$), MC4100 (pHK11) (ColV$^+$), AY25 (Mcc25$^+$), RYC1000 (pMM39) (MccB17$^+$), and RYC492 (MccE492$^+$), which had been pregrown in M9 plates. S, sensitive; R, resistant; P, partially resistant; (R), nearly resistant (turbid zones of growth inhibition with undiluted phage or colicin).

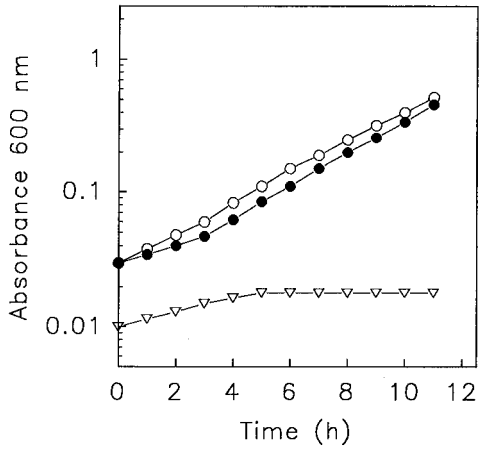

FIG. 1. Growth in Tris-buffered medium of strains AB259 (wild type [○]), SBG247 (*tonB* [●]), and SBG251 (*tonB* [▽]). Cultures were inoculated with bacteria that had been pregrown in the same medium, and growth was measured as an increase in A_{600}.

was prepared on strain N3033 (*lacZ*::Tn*10*) and used to transduce strain SBG203 to Tc^r. Ninety percent of the transductants examined remained Mcc25 resistant. In a subsequent experiment, by using P1 prepared on SBG203 to transduce GM247 (ProC$^-$) to ProC$^+$, the microcin resistance marker was found to be 96% linked to *proC*, which is located at 9 min on the *E. coli* genetic map. Close to *proC*, at 8.7 min, there is a locus designated *sbmA*, whose product functions as the specific inner membrane transport protein for MccB17 (14, 17). Therefore, we tested all group 5 mutants, including SBG203, for resistance to MccB17 and found that they were fully resistant to this microcin. Conversely, strains RYC726 and RYC714 (two authentic *sbmA* mutants) (17) showed complete resistance to Mcc25. These results strongly suggested that the *sbmA* gene product was also implicated in the action of Mcc25. Moreover, a significant proportion (12%) of the *sbmA* mutants isolated in the present study required proline for growth in minimal medium and did not produce alkaline phosphatase, indicating that they probably carried deletion mutations covering the *sbmA-phoA-proC* region. Two other *sbmA* mutants were PhoA$^-$ but could grow in minimal medium. Most likely, this resulted from a short deletion covering only the *sbmA* and *phoA* loci. Finally, spontaneous *sbmA* mutants of strain AB259, generated in our laboratory by using MccB17 as a selective agent, were resistant to Mcc25.

When plasmids with cloned *tonB*, *exb*, and *sbmA* genes were available, we did complementation tests to corroborate the preceding results. Mutations in SBG247 and SBG251 were complementable by plasmid pRZ540 (*tonB*) (20). On the other hand, full sensitivity to Mcc25 was restored in strains SBG203 and SBG206 when transformed with plasmids pMM73.4 (*sbmA*) (17) and pKP298 (*exbBD*), respectively. The latter also conferred microcin sensitivity on KP1040 and the putative *exb-metC* deletion mutants isolated in the present study. These results convincingly demonstrate that the mutations are indeed in the stated genes.

Mcc25 resembles the group B colicins in its requirement for the *tonB* and *exb* gene products to enter *E. coli* (4), and in addition, it shares with colicin M a receptor, FhuA (2). Another microcin, E492, also requires functional *tonB* and *exb* genes to enter *E. coli* (Table 2) (21).

Finally, we propose a model for uptake of Mcc25 which

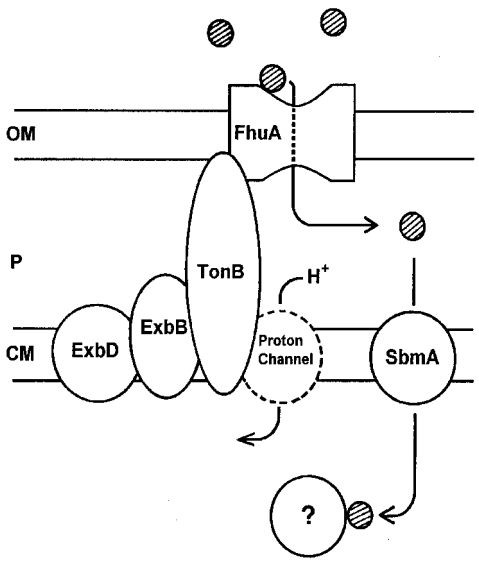

FIG. 2. A model for Mcc25 uptake. Mcc25 (denoted by the hatched circles) first binds to the FhuA receptor protein, which forms a closed channel in the outer membrane (15). TonB is postulated to form a complex with ExbB, ExbD, and, possibly, with a proton channel (drawn with broken lines, since its existence and physical interaction with TonB are hypothetical) (19, 28). Energy from the proton motive force across the cytoplasmic membrane causes a conformational change, which is transmitted via TonB to FhuA. This, in turn, would induce the opening of the FhuA channel (15) and the release of bound Mcc25 into the periplasmic space, whereupon it would be translocated across the cytoplasmic membrane by a process requiring the SbmA protein. The protein labelled with a question mark represents the putative cytoplasmic target of the antibiotic. OM, outer membrane; P, periplasm; CM, cytoplasmic membrane.

integrates the preceding results and our previous observation that FhuA serves as a receptor for the antibiotic (Fig. 2).

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