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Microcin-E492-insensitive Mutants of *Escherichia coli* K12

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Mutations in three *Escherichia coli* K12 genes, *tonB*, *exbB* and the newly discovered *semA*, reduce sensitivity to the low M_r polypeptide antibiotic microcin E492. The products of the *tonB* and *exbB* genes were previously shown to be involved in the uptake of siderophore-complexed iron and in the action of a number of colicins. Strains mutated at or close to *semA* (collectively referred to as *sem* mutations) remained fully sensitive to these colicins, and grew as well as wild-type strains under conditions of iron starvation. Expression of a number of *sem–lacZ* operon fusions was not affected by iron limitation, and *sem* mutations did not affect the production of iron-regulated outer membrane proteins which are known or thought to be involved in iron uptake. Hfr conjugation and P1 phage transduction experiments indicated that *semA* is located close to *pabB* at 40 min on the *E. coli* K12 chromosome. This places *semA* close to the *mng* locus, wherein mutations result in decreased manganese sensitivity. However, strains carrying the *semA* mutation exhibited increased manganese sensitivity.

INTRODUCTION

Microcin E492 is a polypeptide antibiotic of $M_r \sim 5000$ produced by *Klebsiella pneumoniae* and active against *Escherichia coli* strains, including the laboratory strain K12 (de Lorenzo, 1984). The primary target of microcin E492 seems to be the cytoplasmic membrane, where it causes rapid loss of the transmembrane energy potential (de Lorenzo & Pugsley, 1985). This implies that microcin E492 crosses the *E. coli* outer membrane.

Colicins, which are generally larger than microcins (Baquero & Moreno, 1984; Pugsley, 1984), also have to cross the outer membrane in order to reach their targets. The initial stage in this process is attachment to outer membrane receptors, whose production can be blocked by mutations in the appropriate structural genes, thereby causing the cells to become colicin resistant (Pugsley, 1984). Other mutations, such as *tonB*, *tolC* and those affecting outer membrane porin production, cause the loss of colicin sensitivity by blocking colicin uptake from the receptor-bound state. These mutants are referred to as colicin tolerant (Pugsley, 1984).

Since microcins are apparently structurally different from most colicins (Baquero & Moreno, 1984), it is of interest to determine whether they too must adsorb to cell surface receptors in order to cross the outer membrane. These studies are hindered by the very small quantities of the antibiotic which can be prepared in relatively pure form (Baquero & Moreno, 1984; de Lorenzo, 1984). We have therefore resorted to studies of microcin-insensitive mutants in the hope of identifying cell envelope components involved in microcin uptake. Here we describe the characterization of mutants obtained by selecting for loss of sensitivity to microcin E492.

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Table 1. *E. coli K12 strains*

Strain	Genotype/other characteristics	Source or reference*
PAP488	Δ(*pro–lac*) *rpsL*	Laboratory strain
PAP710	*semA12*::Tn*5 gyrA*	
PAP1611	*sem1 gyrA* λ^+	
PAP1612	*sem6 gyrA* λ^+	
PAP1613	*sem3 gyrA* λ^+	
AB3303	*thi-1 pabB3 hisG4 argE3 lacY1 galK2 xyl-5 mtl-1 rpsL700/704 tsx-29/358 supE44*	CGSC
PAP207	*thr leu* Δ(*proAB-phoE*) *argE3 trpE hisG4 lacZ galK2 xyl-5 mtl-1 rpsL700 gyrA*	Laboratory strain
ZSG113	*lacZ83*:*827 ptsM12 ptsG22 glk-7 rha-4 rpsL223*	CGSC
RC63	*gal-55 asnA3 relA1 spoT1 thi-1 hsdR4*	CGSC
PAP1614	ZSG113 *sbmA12*::Tn*5* via P1 phage grown on strain PAP710	
C600	*thr leu fhuA supE44*	Laboratory strain
GUC41	C600 [Δ(*metC–exbB*)]	Laboratory strain
PAP609	C600 *aroB malT*::Tn*5 malPQ*::Tn*10*	Laboratory strain
PAP892	AB3303 Mucts Ap^R	
PAP889	*araD139* Δ(*araCOIBA–leu*)*7697* Δ(*proAB–argF–lacIPOZYA*)*XIII rpsL sbmA12*::Tn*5* Mu	
M1967	*trpE lys mal gal* Δ*lacX74 thiA rpsL tsx* λ^+	Laboratory strain
BN905	*fur*::Tn*5 gyrA*	Bagg & Neilands (1985)
BZB1191	*gyrA fhuA*::Tn*5*	Laboratory strain

* Strains for which no source or reference is given are described in this paper. CGSC, Coli Genetic Stock Center (curator B. Bachmann), Yale University, New Haven, Conn., USA.

METHODS

Bacterial strains, plasmids and bacteriophages. Strains of *E. coli* K12 used in this study are listed in Table 1. The *aroB* mutation was introduced into various strains by P1 transduction using phage grown on strain PAP609 with selection for kanamycin resistance encoded by Tn*5* inserted in the *aroB*-linked *malT* gene. *K. pneumoniae* strain RYC492 was used as the microcin E492 producer. All other colicin and microcin-producing strains were as listed by Pugsley (1985). Bacteriophage MudX was supplied by C. Gross (University of Wisconsin, Madison, Wis., USA). Plasmids pEG109 (Groisman *et al.*, 1984) and pEG5005 (similar to pEG109 but carrying a kanamycin-resistance determinant and the pBR322 replication origin instead of the chloramphenicol resistance determinant and the pACYC184 replication origin) were supplied by E. Groisman (University of Chicago, Ill., USA). pIP27 is pBR322 carrying functional *bla*, *metC* and *exbB* genes (I. Saint-Girons & A. P. Pugsley, unpublished results), and pBJM002 is pACYC177 carrying functional *tet* and *tonB* genes (supplied by R. J. Kadner, University of Virginia, Charlottesville, Va., USA).

Media and culture conditions. L broth and L agar (Miller, 1972) were used for most experiments. Where appropriate, antibiotics were used at the following concentrations: kanamycin and chloramphenicol, 25 μg ml^{-1}; ampicillin, 200 or 25 μg ml^{-1} (for plasmids and MudX respectively); tetracycline, 15 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; and sodium nalidixate, 50 μg ml^{-1}. Minimal medium was M63 (Miller, 1972) containing 0·5% glucose (all % values are w/v) and, where appropriate, amino acids (0·01%) and vitamins (0·007%) (Table 1). Mannose (0·5%) replaced glucose in minimal media in tests for *ptsM* expression. MacConkey 0·5% mannose agar was also used for these tests. In tests for asparagine utilization, the ammonium sulphate in M63 medium was replaced by 1% asparagine. Minimal medium was solidified with 1·6% agar. 100 μM-2,2′-dipyridyl was added to M63 broth cultures to complex iron (Pugsley & Reeves, 1976). MacConkey 0·5% lactose medium was used to test the Lac^+ character of potential MudX-generated Sem mutants. The medium used to test for manganese sensitivity was as described by Silver *et al.* (1972) (0·4% Difco tryptone, 0·25% NaCl) and was supplemented with 0·2% glycerol. All cultures were incubated at 37 °C, and broth cultures were well-aerated.

Genetic procedures. Procedures for P1 phage transduction and for Hfr conjugation were essentially as described by Miller (1972). Tn*5* insertions were selected by mutagenizing various *E. coli* K12 cultures with λTn*5* (*cI857 rex*::Tn*5 029 P80 b21*) and then growing the cultures overnight in L broth containing kanamycin and 10 mM-sodium citrate. The cells were then plated onto L agar containing kanamycin and microcin E492. Mutagenesis with MudX was essentially as described by Baker *et al.* (1983). The mutagenized bacteria derived from strain PAP488 were grown overnight in L broth containing chloramphenicol and ampicillin, and then plated onto MacConkey lactose agar containing the same antibiotics together with microcin E492. The presence of MudX in or close to the *semA* gene in Lac^+ clones was confirmed by the simultaneous acquisition of kanamycin resistance and loss of chloramphenicol and ampicillin resistance upon transduction by P1 phage grown on a strain

carrying the *semA12*::Tn*5* mutation. All transductants remained insensitive to microcin E492. The *sem*::MudX mutations could also be transduced into other bacteria with selection for MudX-encoded chloramphenicol resistance. Mini-Muduction with pEG109 was essentially as described by Groisman *et al.* (1984). Mixed Mu lysates grown on a microcin-E492-sensitive strain of *E. coli* K12 were used to infect strain PAP892, which was then plated out on minimal medium containing chloramphenicol and thiamin but devoid of *p*-aminobenzoic acid. Colonies appearing after 2–3 d incubation were purified on the same medium. Procedures for the isolation of plasmids by the Holmes and Quigley method, and for transformation, were as described by Maniatis *et al.* (1982).

General procedures. β-Galactosidase was assayed according to Miller (1972). Cross streak tests for microcin, colicin and phage sensitivity were essentially as described by Davies & Reeves (1975*b*). Microcin E492 was prepared and assayed as described previously (de Lorenzo, 1984; de Lorenzo & Pugsley, 1985). Outer membranes were prepared from 5 ml cultures grown to OD_{600} = 1·0 in L broth or minimal medium. The cells were harvested, washed once and resuspended in 1 ml 25 mM-Tris/HCl (pH 7·4) containing 1 mM-Mg^{2+}, and disrupted by sonication. Cell debris was removed by low-speed centrifugation. Triton X-100 (final concentration 2%) was added to the broken cell suspension and the mixture was incubated on ice for 20 min. The outer membrane (Triton-insoluble wall) was then pelleted by centrifugation for 30 min at 13000 *g* in a microcentrifuge. The pellet was drained and resuspended in 100 μl sample buffer for SDS polyacrylamide gel electrophoresis (SDS-PAGE) in gels containing 9% acrylamide with or without 8 M-urea. The Tris/glycine buffer system (Pugsley & Schnaitman, 1979) was used. The gels were stained with Coomassie brilliant blue.

RESULTS

Isolation of microcin-E492-insensitive mutants

E. coli K12 cultures and pooled Tn*5*-mutagenized cultures derived therefrom were plated onto L agar containing varying amounts of microcin E492 and incubated at 37 °C. Isolated colonies appearing after 1–2 d were purified and tested for sensitivity to microcin E492 and to other microcins, colicins and bacteriophages (Pugsley, 1985). Three classes of mutants were obtained, corresponding to the previously described TonB$^-$ and ExbB$^-$ phenotypes (Pugsley & Reeves, 1976; Pugsley, 1985), and to a novel phenotype which we refer to as Sem$^-$ (for Sensitivity to E microcin). Unlike the TonB$^-$ and ExbB$^-$ mutants, which exhibited extensive cross-resistance to several colicins and, in the case of TonB$^-$, to bacteriophage φ80 (Pugsley & Reeves, 1976; Pugsley, 1985), Sem$^-$ mutants exhibited reduced sensitivity solely to microcin E492. The TonB$^-$ and ExbB$^-$ mutants were insensitive to the highest concentration of microcin available (about 1000 antibiotic units: de Lorenzo, 1984), whereas the Sem$^-$ mutants retained a residual level of microcin E492 sensitivity (sensitive to 100–250 antibiotic units; wild-type bacteria were sensitive to 1 antibiotic unit under these assay conditions).

Map location of the sem gene(s)

In order to confirm that the mutations in strains exhibiting the TonB$^-$ phenotype were indeed located in the *tonB* gene, P1 phage grown on these strains was used to transduce strain M1967 (*trpE*) to Trp$^+$. The *trpE* locus is close to *tonB*. In the three cases studied, 30–40% of the Trp$^+$ transductants were insensitive to microcin E492. Furthermore, sensitivity to the microcin was recovered upon introduction of pBJM002, a high-copy-number plasmid carrying the *tonB*$^+$ allele. These results confirmed that the mutations were in *tonB*. Similarly, ExbB$^-$ mutants recovered their sensitivity to microcin E492 upon introduction of pIP27, a high-copy-number plasmid carrying the *exbB*$^+$ allele. This result, together with the fact that strain GUC41 [Δ(*metC–exbB*)] was also microcin-E492-insensitive, whereas the parent strain (C600) was not, confirmed that the ExbB$^-$ mutants carried mutations in *exbB*.

In order to map the mutations conferring the Sem$^-$ phenotype, P1 phage grown on an Sem$^-$ mutant (strain PAP710) derived from a Tn*5*-mutagenized stock of *E. coli* K12 was used to introduce the Tn*5*-generated mutation into a number of *E. coli* Hfr strains. Loss of sensitivity to microcin E492 was always 100% cotransduced with resistance to kanamycin. This mutation, henceforth referred to as *semA12*::Tn*5*, is the prototype of the *sem* mutations studied here. These strains were then conjugated with strain PAP207 with selection for transfer of Tn*5*-encoded kanamycin resistance and for retention of streptomycin or nalidixate resistance. Transconjugants were checked for loss of auxotrophic or antibiotic resistance markers of strain PAP207. The site of insertion of Tn*5* was found to be between *trpE* (28 min) and *gyrA* (48 min).

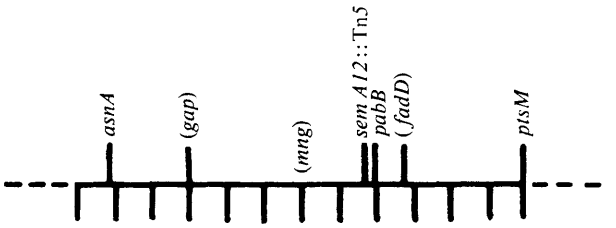

Fig. 1. Partial linkage map of the 40 min region of the *E. coli* K12 chromosome redrawn from Bachmann (1983) and showing linkages between genes around *semA*. Loci in parentheses were not mapped in the present study; their positions are redrawn directly from Bachmann (1983). The precise position of the *mng* locus is not known.

Table 2. *Cotransduction data for sbmA12::Tn5 and adjacent genes and mutations*

Strain on which the P1 phage was grown	Recipient	Selection	Cotransduced marker	Percentage cotransduction
PAP710 (*semA12*::Tn*5*)	AB3303	Pab$^+$	Km^R	98 (98/100)
		Km^R	Pab$^+$	100 (215/215)
PAP1611 (*sem1*)	AB3303	Pab$^+$	Sem$^-$	84 (54/64)
PAP1612 (*sem6*)	AB3303	Pab$^+$	Sem$^-$	73 (62/85)
PAP1613 (*sem3*)	AB3303	Pab$^+$	Sem$^-$	86 (88/97)
PAP710 (*semA12*::Tn*5*)	ZSG113	Km^R	PtsM$^+$	38 (38/100)
		Pst$^+$	Sem$^-$	33 (33/100)
PAP710 (*semA12*::Tn*5*)	RC63	Km^R	Asn$^+$	62 (62/100)
PAP1614 (*semA12*::Tn*5 ptsM*)	AB3303	Pab$^+$	Sem$^-$	96 (96/100)
			PtsM$^+$	48 (48/100)
AB3303 (*pabB*)	PAP1614	PtsM$^+$	Sem$^+$	39 (45/115)
			Pab$^-$	51 (51/100)

For more precise mapping, P1 phage grown on strains carrying *semA12*::Tn*5* were used to transduce the kanamycin resistance marker into strains carrying mutations between 28 min and 48 min on the *E. coli* K12 chromosome. In this way, we demonstrated that the Tn*5* insertion was linked to *pabB*, *ptsM* and *asnA* (Table 2), but not to *aroD*, *man-4*, *lpp-6*, *pdxH15* or *pheS* (data not shown). Further, two- and three-point crosses were used to complete the linkage map (Table 2) and to establish the following clockwise gene order on the *E. coli* K12 chromosome: *asnA–semA12*::Tn*5–pabB–ptsM* (Fig. 1).

To determine whether the mutations in other Sem$^-$ derivatives mapped at the same site, P1 phage grown on selected spontaneous mutants were used to transduce strain AB3303 to Pab$^+$. In every case, the mutation was closely linked to *pabB*, although co-transduction frequencies between these *sem* mutations and *pabB* were not as high as with *semA12*::Tn*5* (Table 2). Two other, independently isolated *sem*::Tn*5* mutations were also closely linked to *pabB* (data not shown).

Characterization of SemA$^-$ mutants

Because we suspected that the product(s) of the *sem* gene(s) might function as the microcin E492 receptor, we first looked for changes in outer membrane protein composition in the SemA$^-$ mutants. Outer membrane protein profiles of wild-type and mutant strains were identical in two different SDS-PAGE systems (Methods). The observation that the products of the *exbB* and *tonB* genes are required for microcin E492 sensitivity, and the fact that enterochelin affords low level protection of sensitive cells against the microcin (unpublished observation) suggested that the *sem* gene product(s), like the receptors for some group B colicins (Davies & Reeves, 1975*b*; Pugsley & Reeves, 1976, 1977), might be overproduced during growth under iron-limiting conditions. We therefore used SDS-PAGE to examine outer membrane proteins from parent and mutant bacteria after iron starvation resulting from the presence of an *aroB* mutation, which prevented production of the siderophore enterochelin, and growth in minimal medium in the

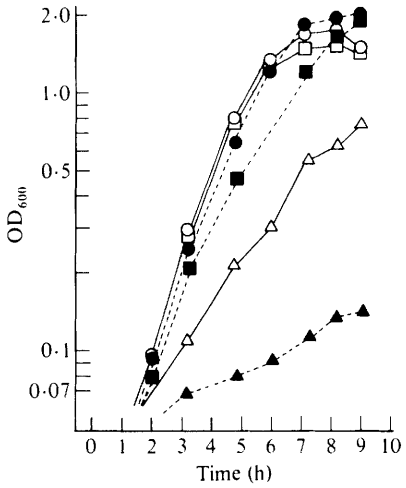

Fig. 2. Effect of manganese on growth of strain PAP710 (*semA12*::Tn*5*) (filled symbols and dashed lines) and BZB1191 (*fhuA*::Tn*5*) (this strain, which is isogenic with PAP710, was used in preference to the parent of PAP710 in order to compensate for any effects of Tn*5* on bacterial growth) (open symbols and solid lines). Cells were grown overnight in manganese sensitivity test medium and then diluted 1:100 into fresh medium (□, ■) or fresh medium containing 1·5 mM-$MnSO_4$ (△, ▲) or 1·5 mM-$MnSO_4$ + 1·5 mM-$MgSO_4$ (○, ●). Growth was measured as increases in OD_{600}.

presence of the iron chelator 2,2′-dipyridyl (Pugsley & Reeves, 1976). The parent *E. coli* K12 strain produced five iron-starvation-induced outer membrane proteins (M_r 70000–84000), as did all Sem$^-$ mutants tested (data not shown). We also observed that the Sem$^-$ mutants grew as well as the parent strains under iron-limiting conditions (data not shown), which indicated that they do not have a major defect in iron uptake.

In a further attempt to determine whether the *sem* gene(s) was(were) regulated by the level of iron in the growth medium, we constructed a series of *sem–lacZ* operon fusions (Methods). In all of the eight independently isolated fusion strains tested, fusion-encoded β-galactosidase reached 150–200 units ml^{-1}, irrespective of the presence or absence of dipyridyl in the minimal glucose growth medium in the presence of an *aroB* mutation in the chromosome. Likewise, the level of β-galactosidase was not affected by the introduction of the *fur*::Tn*5* mutation, which is known to derepress iron-regulated genes in *E. coli* (Bagg & Neilands, 1985). We therefore concluded that *sem* is not regulated by the level of free iron in the medium, and does not code for one of the iron-regulated outer membrane proteins. We noted, however, that strains carrying presumed *sem–lacZ* operon fusions formed darker red colonies (i.e., more strongly Lac$^+$) when plated on MacConkey lactose agar containing dipyridyl.

The *semA* gene is located close to the *mng* locus, wherein previously isolated mutations were found to confer increased manganese resistance (Silver *et al.*, 1972; Bachmann, 1983). We therefore tested Sem$^-$ mutants to see if they exhibited altered sensitivity to manganese. Mutants carrying spontaneous or Tn*5*-generated *sem* mutations were more sensitive than parent strains, or other isogenic strains, to 1·5–15 mM-$MnSO_4$ in liquid culture tests (Fig. 2). The effects of Mn^{2+} on mutant and wild-type strains were always overcome by including 1·5–15 mM-Mg^{2+} in the growth medium (Fig. 2). The Sem$^-$ mutants were also more sensitive to $MnSO_4$ in plate tests (data not shown).

Cloning of semA

We made use of the close proximity of the *semA* and *pabB* alleles to clone the wild-type *semA* allele by selecting for Pab$^+$ mini-Muductants of a derivative of strain AB3303 [PAP892 (*pabB* Mu *cts* Ap^R); Methods]. Representatives of six of the eight series of Pab$^+$ clones carried plasmids which were able to restore microcin E492 sensitivity to normal, wild-type levels when

introduced into strain PAP889. This confirmed the genetic linkage between *semA* and *pabB* and demonstrated that the wild-type *semA* allele was dominant over the mutated allele. The fact that the presence of the recombinant plasmids did not increase microcin sensitivity to levels higher than in wild-type bacteria suggests that the *semA* gene product was not overproduced, or else that it is not normally limiting for microcin E492 sensitivity. However, each cycle of plasmid extraction and retransformation produced a number of clones which did not recover micocin sensitivity. Furthermore, microcin-resistant clones segregated from the microcin-sensitive transformants of strain PAP889 despite continued selection for plasmid-encoded chloramphenicol resistance. The analysis of plasmids derived from these clones indicated that they had sustained deletions which had presumably removed the *semA* gene. Some of the deletions also removed the *pabB* gene. We also noted that *semA*$^+$ plasmids were never obtained among Pab$^+$ mini-Muductants of PAP892 when pEG109 was replaced by pEG5005, which has a higher copy number.

DISCUSSION

We have adopted a genetic approach for the study of factors which affect the microcin sensitivity of *E. coli* K12. We have identified two possible candidates for genes encoding hypothetical microcin receptors, the *semA* gene described here and the *sbmA* gene affecting sensitivity to microcin B17 (Laviña *et al.* 1986). Although there are some obvious parallels between the results reported here and those reported previously with microcin B17, there are also some striking differences which may help us to understand how microcins reach their targets. Microcin E492 resembles the group B colicins (Davies & Reeves, 1975*a*) in its requirement for *tonB* and *exbB* gene products, whereas microcin B17 resembles the group A colicins (Davies & Reeves, 1975*b*), and requires the OmpF porin for efficient killing (Laviña *et al.*, 1986). This almost certainly reflects the use of distinct uptake pathways by the two microcins (Pugsley, 1984), which have different targets [microcin B17 inhibits chromosome replication whereas microcin E492 affects the cytoplasmic membrane energy potential (Herrero & Moreno, 1986; de Lorenzo & Pugsley, 1985)].

There is no direct evidence that either the *sbmA* or the *semA* gene products actually codes for a microcin receptor protein, or indeed that they are involved in translocating the microcin across the outer membrane, although there is indirect evidence for the latter in the case of *sbmA* (Laviña *et al.*, 1986). *sbmA* and *sem* mutations cause specific loss of sensitivity to a single lethal agent, which is also sometimes the case with mutations which affect colicin receptors (Pugsley, 1984). However, unlike *sbmA* and most of the mutations preventing synthesis of colicin receptors, *sem* mutations do not cause the complete loss of microcin sensitivity. This may indicate that there are two pathways for microcin uptake. Only TonB$^-$ mutations were obtained by selecting for high-level microcin E492 resistance in strains carrying an *sem* mutation.

Studies with operon fusions indicated that both *sbmA* and *semA* genes are poorly expressed (this study and M. Laviña & F. Moreno, unpublished data; see also Laviña *et al.*, 1986), but *sbmA*$^+$ can be stably maintained in high-copy-number plasmids (Laviña *et al.*, 1986) whereas *semA*$^+$ (or a closely linked gene) cannot. SbmA$^-$ mutants do not have any recognizable phenotype other than their loss of sensitivity to microcin B17, whereas Sem$^-$ mutants are more sensitive to manganese than parent strains. This latter phenomenon may be useful in further studies into the mechanism of microcin E492 uptake and the physiological role of the *sem* gene product(s). For example, it may be that a *sem* gene codes for a manganese efflux pump (Silver *et al.*, 1972). This pump may have been more efficient or present in increased amounts in the Mng$^-$ mutants, whereas it may be completely absent from Sem$^-$ mutants. Alternatively, *sem* mutations may exert a polar effect on the expression of another gene whose product is involved in manganese transport. The results presented here do not completely exclude a possible interaction between iron transport and microcin E492 action. The evidence that the *tonB* and *exbB* gene products are required for microcin E492 action, and the fact that enterochelin affords low-level protection against this microcin, are both indicative of such an interaction. Enterochelin may protect against microcin E492 in a manner similar to that by which it protects against colicins G, H, Ia, Ib and V, rather than by preventing the interaction of microcin E492

with a cognate receptor, as is the case for colicins B and D (Pugsley & Reeves, 1976). The absence of any indication of iron regulation of *semA* regulation except on MacConkey lactose agar may indicate a more complex interaction between microcin E492 uptake and ion transport than is the case for other TonB-dependent agents.

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