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

By ANTHONY P. PUGSLEY,*† FELIPE MORENO AND VICTOR DE LORENZO‡

Unité de Génétique Moléculaire, Institut Pasteur, 25 rue du Dr Roux, Paris 75724, France
Unidad de Genética Molecular, Servicio de Microbiología, Hospital Ramón y Cajal, Carretera de Colmenar, Km 9, 1000, Madrid 28034, Spain.

(Received 18 April 1986; revised 30 June 1986)

Mutations in three *Escherichia coli* K12 genes, *tonB*, *exbB* and the newly discovered *semA*, reduce sensitivity to the low *M*, 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*, ~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.

† Offprint requests to the Paris address.

‡ Present address: Department of Biochemistry, University of California, Berkeley, Calif. 94720, USA.
glycerol. All cultures were incubated at 37 °C, and broth cultures were well-aerated.

Charlottesville, Va., pBR322 carrying functional and the pACYC184 replication origin) were supplied by E. Groisman (University of Chicago, Ill., USA). pIP27 is onto MacConkey lactose agar containing the same antibiotics together with microcin E492. The presence of resistance and loss of chloramphenicol and ampicillin resistance upon transduction by P1 phage grown on a strain was also used for these tests. In tests for asparagine utilization, the ammonium sulphate in M63 medium was the Lac+ character of potential MudX-generated Sem mutants. The medium used to test for manganese sensitivity was as described by Silver (~1957 rex).

Mutagenesis with MudX was essentially as described by Baker (~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.

The table below lists the strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/other characteristics</th>
<th>Source or reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP488</td>
<td>Δ(pro–lac) rpsL</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>PAP710</td>
<td>semA12::Tn5 gyrA</td>
<td>CGSC</td>
</tr>
<tr>
<td>PAP1611</td>
<td>sem1 gyrA λ⁺</td>
<td></td>
</tr>
<tr>
<td>PAP1612</td>
<td>sem6 gyrA λ⁺</td>
<td></td>
</tr>
<tr>
<td>PAP1613</td>
<td>sem3 gyrA λ⁺</td>
<td></td>
</tr>
<tr>
<td>AB3303</td>
<td>thi-1 pabB3 hisG4 argE3 lacY1 galK2 xyl-5 mtl-1 rpsL700/704 txs-29/358 supE44</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>ZSG113</td>
<td>lacZ833:827 ptsM12 ptsG22 glk-7 rha-4 rpsL223</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>RC63</td>
<td>gal-55 asmA3 relA1 spoT1 thi-1 hsdR4</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>PAP1614</td>
<td>ZSG113 sbmA12::Tn5 via P1 phage grown on strain PAP710</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>C600</td>
<td>thr leu fluA supE44</td>
<td></td>
</tr>
<tr>
<td>GUC41</td>
<td>C600 [Δ(metC–exbB)]</td>
<td></td>
</tr>
<tr>
<td>PAP609</td>
<td>C600 arob::malT::Tn5 malPQ::Tn10</td>
<td></td>
</tr>
<tr>
<td>PAP892</td>
<td>AB3303 Mucts Ap²</td>
<td></td>
</tr>
<tr>
<td>PAP889</td>
<td>araD139 [Δ(araCO1BA–leu)7697 Δ(proAB–argF–lacIPOZYA)XIII rpsL sbmA12::Tn5 M]</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>M1957</td>
<td>trpE lys mal gal ΔlacX74 thiA rpsL txs λ⁺</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>BN905</td>
<td>fur::Tn5 gyrA</td>
<td>Bagg &amp; Neilands (1985)</td>
</tr>
<tr>
<td>BZB1191</td>
<td>gyrA fluA::Tn5</td>
<td>Laboratory strain</td>
</tr>
</tbody>
</table>

*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 Tn5 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% mannone 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. Minimal medium was solidified with 1.6% agar. Minimal medium was solidified with 1.6% agar.

**Genetic procedures.** Procedures for P1 phage transduction and for Hfr conjugation were essentially as described by Miller (1972). Tn5 insertions were selected by mutagenizing various *E. coli* K12 cultures with ΔTn5 (cI857 rec : Tn5 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.
Microcin E492 resistance

Microcin E492 was always 100% cotransduced with resistance to kanamycin. This mutation, mutant (strain PAP710) derived from a Tn5-mutagenized stock of [A(metC-exbB)] encoded kanamycin resistance and for retention of streptomycin or nalidixate resistance. Confirmed that the ExbB- mutants carried mutations in recovered their sensitivity to microcin E492 upon introduction of pIP27, a high-copy-number plasmid carrying the corresponding to the previously described TonB- and ExbB- phenotypes (Pugsley, 1985). Three classes of mutants were obtained, corresponding to the Trp+ allele. These results confirmed that the mutations were in 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 [A(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 Tn5-mutagenized stock of E. coli K12 was used to introduce the Tn5-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 : Tn5, is the prototype of the sem mutations studied here. These strains were then conjugated with strain PAP207 with selection for transfer of Tn5-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 Tn5 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

<table>
<thead>
<tr>
<th>Strain on which the P1 phage was grown</th>
<th>Recipient</th>
<th>Selection</th>
<th>Cotransduced marker</th>
<th>Percentage cotransduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP710 (<em>semA12::Tn5</em>)</td>
<td>AB3303</td>
<td>Pab⁺</td>
<td>Km⁺</td>
<td>98 (98/100)</td>
</tr>
<tr>
<td>PAP1611 (<em>semI</em>)</td>
<td>AB3303</td>
<td>Pab⁺</td>
<td>Km⁺</td>
<td>100 (215/215)</td>
</tr>
<tr>
<td>PAP1612 (<em>semD</em>)</td>
<td>AB3303</td>
<td>Pab⁺</td>
<td>Sem⁻</td>
<td>84 (54/64)</td>
</tr>
<tr>
<td>PAP1613 (<em>sem3</em>)</td>
<td>AB3303</td>
<td>Pab⁺</td>
<td>Sem⁻</td>
<td>73 (62/85)</td>
</tr>
<tr>
<td>PAP710 (<em>semA12::Tn5</em>)</td>
<td>ZSG113</td>
<td>K₄M⁺</td>
<td>Pab⁺</td>
<td>86 (88/97)</td>
</tr>
<tr>
<td>PAP1614 (<em>semA12::Tn5 ptsM</em>)</td>
<td>AB3303</td>
<td>Pab⁺</td>
<td>Pst⁻</td>
<td>38 (38/100)</td>
</tr>
<tr>
<td>AB3303 (<em>pabB</em>)</td>
<td>PAP1614</td>
<td>PtsM⁺</td>
<td>K₄M⁺</td>
<td>62 (62/100)</td>
</tr>
<tr>
<td></td>
<td>AB3303</td>
<td>Pab⁺</td>
<td>PtsM⁺</td>
<td>96 (96/100)</td>
</tr>
</tbody>
</table>

For more precise mapping, P1 phage grown on strains carrying *semA12::Tn5* 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 Tn5 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::Tn5–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::Tn5* (Table 2). Two other, independently isolated *sem::Tn5* 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, 1975b; 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
Microcin E492 resistance

2.0 t

0 1 2 3 4 5 6 7 8 9 10

Time (h)

Fig. 2. Effect of manganese on growth of strain PAP710 (semA12::Tn5) (filled symbols and dashed lines) and BZB191 (fluA::Tn5) (this strain, which is isogenic with PAP710, was used in preference to the parent of PAP710 in order to compensate for any effects of Tn5 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-MnSO₄ (△, ▲) or 1.5 mM-MnSO₄ + 1.5 mM-MgSO₄ (○, ●). Growth was measured as increases in OD₆₀₀.

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, 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–lac2 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::Tn5 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 Tn5-generated sem mutations were more sensitive than parent strains, or other isogenic strains, to 1.5–15 mM-MnSO₄ in liquid culture tests (Fig. 2). The effects of Mn²⁺ on mutant and wild-type strains were always overcome by including 1.5–15 mM-Mg²⁺ in the growth medium (Fig. 2). The Sem− mutants were also more sensitive to MnSO₄ 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 Mucts Ap8); 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 microcin 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-Mutants 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 (Lavina *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, 1975a) in its requirement for tonB and exbB gene products, whereas microcin B17 resembles the group A colicins (Davies & Reeves, 1975b), and requires the OmpF porin for efficient killing (Lavina *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 (Lavina *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. Lavina & F. Moreno, unpublished data; see also Lavina *et al.*, 1986), but sbmA+ can be stably maintained in high-copy-number plasmids (Lavina *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.

We are grateful to Maxime Schwartz for his interest in this work. Financial support was provided by the Centre National de la Recherche Scientifique (UA 04 1149 and ATP CP96), by the Ministère de la Recherche et de la Technologie (82 V 1279), by the Fondation pour la Recherche Médicale, and by a joint French-Spanish government scientific exchange program. V.deL. was the recipient of an EMBO short-term fellowship.

REFERENCES