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Cloning and Expression in *Escherichia coli* of Genetic Determinants for Production of and Immunity to Microcin E492 from *Klebsiella pneumoniae*

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Microcin E492 is a polypeptide antibiotic that is produced and excreted by *Klebsiella pneumoniae* RYC492. The genetic determinants for microcin synthesis and immunity were cloned in *Escherichia coli* VCS257 into the cosmid vector pHC79, starting from total DNA of *K. pneumoniae* RYC492. The microcin E492 expressed in *E. coli* had the same properties as that of *K. pneumoniae*, i.e., the same molecular weight, the ability to form ionic channels in planar phospholipid bilayers, and essentially identical biological properties. Microcin E492 expression in *E. coli*, like that in *K. pneumoniae*, was mainly in the exponential phase of growth, declining in the stationary phase. The immunity determinant was subcloned into the same vector, and its expression was found to disappear in the stationary phase. This phenomenon is not dependent on *rpoS*, the stationary-phase sigma factor.

Microcins are a family of low-molecular-weight antibiotics produced by members of the family *Enterobacteriaceae*. Microcins, unlike colicins, are not induced by DNA-damaging agents, but like for colicins, bacterial strains producing a specific microcin are immune to the same microcin (3).

Microcin E492 is a polypeptide with an M_r of 6,000 (14) that is produced by *Klebsiella pneumoniae* RYC492 and is active on strains of *Escherichia coli*, *Klebsiella*, *Salmonella*, *Citrobacter*, *Enterobacter*, and *Erwinia* (6). The mechanism of action is through membrane depolarization (9) induced by the formation of pores in the bacterial membrane (14). Studies on channel-forming bacteriocins produced by gram-negative bacteria have been reported only for colicins, all of them of high molecular weight (reviewed in reference 5). The other described bacteriocin which has the cytoplasmic membrane as target is colicin V. This bacteriocin has been reclassified as a microcin because it has a molecular weight of 6,000 and does not have most of the properties which are associated with colicins (10). Despite the fact that microcin E492 has many feature in common with colicin V, they are not closely related, because $ColV^+$ strains that have high-level immunity to colicin V are fully susceptible to microcin E492 (9). Those colicins which act by depolarizing the bacterial energy-transducing membrane in vivo are able to form aqueous channels in both closed liposomes and planar membranes. However, despite the fact that colicin V inhibits the capacity of *E. coli* to carry out active transport of proline and to generate a membrane potential (26), it has not been possible to find an effect of colicin V on asolectin liposomes, and it remains to be established whether this difference reflects a difference in the in vivo mode of action. Thus, so far the only microcin described to be a channel-forming bacteriocin is microcin E492 (14). Nevertheless, low-molecular-weight pore-forming bacteriocins of gram-positive bacteria have been described, among them the lantibiotic nisin and lactococcins A and B, which are nonlantibiotic heat-stable bacteriocins (reviewed in references 1, 13, and 24). The former has unusual amino acid modifications, so far described only for gram-positive bacteriocins, while the latter might be more similar to microcin E492. The study of the structure of microcin E492 as a peptide model that has the ability to recognize a receptor, translocate through the outer membrane, and form an ionic channel is very attractive. However, genetic manipulation is required for overproduction and for structure-function studies. Although the genetics of several microcins have been extensively studied (reviewed in references 3 and 13), there are no reported studies on the genetics of microcin E492, mainly because the producing strain, *K. pneumoniae* RYC492, is a clinical isolate. An approach to this problem is cloning and expression in *E. coli.*

The genetic determinants for microcin production and immunity are thought to be located in the bacterial chromosome, because neither of the two plasmids detected in the producing strain *K. pneumoniae* RYC492 appears to carry determinants for microcin E492 production or immunity (9). Another microcin with genetic determinants located in the bacterial chromosome is microcin H47, and the genetic system was shown to span ca. 10 kb (11). This latter system is more extensive and probably more complex than those of other known microcins, and it includes a silent region which has not been associated with the microcin H47 antibiotic phenomenon (11).

Microcin E492 is the only microcin described to date that is produced mainly in the exponential phase of growth (3, 7). A microcin antagonist, identified as enterochelin (19), is produced in the stationary phase and is responsible for changes in microcin activity (8, 19).

In this paper we report the cloning in *E. coli* of the genetic determinants for the synthesis of and immunity to microcin E492 from *K. pneumoniae*. Studies of the expression of the microcin in *E. coli* showed that, like in *K. pneumoniae*, this antibiotic is expressed mainly in the exponential phase of growth. Moreover, studies on expression of the immunity de-

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TABLE 1. Bacterial strains and plasmid

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
K. pneumoniae RYC492	Km^r Mcc$^+$ Imm$^+$	8
E. coli		
BL21(DE3)	F$^-$ *ompT* r_B m_B	23
X8605	F$^-$ *trpR* Δ(*lac*)*U169 strA* Δ(*tonB lacP*)	12
VCS257	DP50 *sup* F[*supE44 supF58 hsd53*(r_B m_B) *dapD8 lacY1 glnV44* Δ(*gal-uvrB*)*47 tyrT58 gyrA29 tonA53* Δ(*thyA57*)]	Stratagene
ZK1000	ZK126 *katF*::*kan*(*rpoS*::*kan*)	Roberto Kolter
Plasmid p11α2	Amp^r, fragment of tubulin cloned in pT7.7	This laboratory

terminant subcloned into the same vector revealed that this determinant is also expressed in the exponential phase of growth, and the expression disappears in the stationary phase. This behavior is not dependent on the expression of the *rpoS* product, the stationary-phase sigma factor.

MATERIALS AND METHODS

Bacterial strains and plasmid. The sources and characteristic of the microorganisms and plasmid employed are listed in Table 1. *E. coli* ZK1000 was kindly provided by R. Kolter (Harvard Medical School).

Microcin E492 assay on plates. To determine the capacity of a recombinant clone to produce microcin E492, 2×10^7 sensitive *E. coli* BL21(DE3)p11α2 organisms were overlaid onto Luria broth (LB)-ampicillin (100 μg/ml) plates with 3 ml of soft agar. Single colonies to be checked were transferred either with a toothpick or by replica plating onto the seeded plates. After overnight incubation, the microcin E492-producing colonies gave a clear growth inhibition zone surrounding the colony.

Microcin immunity test. Aliquots (50 to 300 μl) of cultures to be tested for immunity, either in exponential growth (30 Klett units) or in stationary phase (200 Klett units) and grown in LB-ampicillin, were mixed with 3 ml of top agar and overlaid onto LB plates. Five-microliter portions of serial dilutions of purified microcin were tested. Immune cells did not show any inhibition halo.

Assay in liquid culture of microcin expressed in *E. coli*. Aliquots of cultures were heated at 70°C for 20 min (to kill the cells) and centrifuged in a microcentrifuge for 5 min, 5-μl portions of serial dilutions of the supernatant were overlaid onto the indicator strain *E. coli* BL21(DE3)p11α2 suspended in 3 ml of top agar (19), and the activity was determined by the critical dilution method (16) and expressed in arbitrary units. The increase in microcin activity due to the augmentation in the amount of producer cells was corrected by the optical density expressed in Klett units.

Isolation, purification, and fluorescent labeling of microcin E492. Microcin E492 was extracted from the supernatant of cultures of the producer strain *K. pneumoniae* RYC492, or from the recombinant *E. coli* VCS257 strains grown in M9 minimal medium (17) supplemented with citrate or glucose, and purified as described by de Lorenzo (6). Microcin activity was estimated by the critical dilution method (16), and the activity was expressed in arbitrary antibiotic units per milliliter. When necessary, microcin was concentrated by evaporation in a boiling water bath, without a significant loss of activity, and used immediately for incorporation in planar bilayers. Labeling of microcin with fluorescamine was performed as described by Ortiz et al. (20), while electrophoresis in 20% polyacrylamide–sodium dodecyl sulfate (SDS) was carried out under conditions described by Schägger and von Jagow (22).

Single-channel recordings. Bilayers were formed by using a 4:1 mixture of palmitoyl-oleoyl phosphatidylethanolamine and phosphatidylcholine to give a final lipid concentration of 20 mg/ml in decane (14). The membrane separated two aqueous compartments containing 0.1 M KCl and 0.01 M 2-*N*(morpholino)-ethanesulfonic acid (MES)-KOH, pH 6.7. One of the bilayer compartments (*cis* side) was connected to an adjustable DC potential source that allowed the application of potentials from 0 to ±200 mV. The current flowing through the membrane as a result of the applied potential was measured by a current-to-voltage transducer connected to the other compartment (*trans* side), which therefore was virtual earth. Current was simultaneously monitored on a chart recorder and on an oscilloscope and was recorded on a video tape for subsequent analysis. Microcin was added to the solution in the *cis* compartment. The data presented in Fig. 1 were fitted by a nonlinear regression subroutine of Sigma Plot 5.0.

DNA manipulations. Total DNA from *K. pneumoniae* was extracted as described by Ausubel et al. (2) with the detergent cetyltrimethylammonium bromide. Digestion with restriction endonucleases, dephosphorylation with alkaline phosphatase, ligation with T4 DNA ligase, transformation of competent cells, and agarose gel electrophoresis were performed as described by Maniatis et al. (15). In vitro packaging of the recombinant cosmid pHC79 was performed as recommended by Stratagene, using the Gigapack II Packing Extract. Recombinant cosmid DNA was purified by using the Qiagen system. Endonuclease *Bst*VI (a *Xho*I isoschizomer) was a generous gift of C. Vásquez (Universidad de Santiago de Chile).

RESULTS AND DISCUSSION

Cloning of the genetic determinants of microcin E492 production and immunity. A genomic library of *K. pneumoniae* was prepared in *E. coli* in the following way. Total DNA from *K. pneumoniae* RYC492 was partially digested with *Sau*3A. Fragments of 20 to 30 kb were purified through a 10 to 40% sucrose gradient and ligated to the cosmid pHC79, which was previously digested with *Bam*HI and dephosphorylated with alkaline phosphatase. The ligated mixture was packaged in vitro into λ phage heads and transfected in *E. coli* VCS257. The transfected cells were amplified by growing the cells in LB-ampicillin and plated in the same medium. The resulting colonies were replica plated on plates with the indicator strain, and the microcin producer clones were readily identified by the growth inhibition halo produced around the colonies. From this library, five microcin and immunity producer clones were isolated, indicating that the *K. pneumoniae* genes are expressed in an active form in *E. coli*. Analysis of four of these clones after successive cultures showed that they segregated the microcin character but not the immunity, which was maintained stably. The segregation into non-microcin-producing clones ranged between 5 and 40%, depending on the starting clone. DNA restriction patterns of the nonproducing clones were roughly the same as that of the parental producing clone, so this segregation cannot be explained as major rearrangements on the DNA. A nonproducing segregant clone never reverted to the producing parental form. The segregation character was not dependent on the bacterial host, because when DH5α and XLI-Blue were used as bacterial hosts, the same segregation pattern was found. One clone of the original library (J) was found to maintain the microcin and immunity characters stably and was used for further studies.

Characterization of microcin E492 produced in *E. coli*. *E. coli* VCS257 harboring pJ was found to form a growth inhibition halo on a lawn of the sensitive strain *E. coli* BL21(DE3)p11α2. Mutations of the *E. coli tonB* gene cause the loss of microcin E492 sensitivity, because TonB is required for microcin E492 action (21). Thus, to assess the specificity of this inhibition, *E. coli* X8605 (12), a *tonB* mutant, was used as an indicator, and no inhibition of growth produced by *E. coli* VSC257pJ was observed.

Microcin E492 produced by *E. coli* VCS257pJ was purified as described in Materials and Methods and, like that produced by *K. pneumoniae*, was thermostable; i.e., there was no significant loss of activity after treatment at 100°C for 10 min. Evaporation therefore was used routinely to concentrate the microcin solutions.

The ability of this recombinant microcin to induce an ion conductance in artificial lipid bilayers was tested as a major feature in the identification of microcin. As seen with the microcin produced by *K. pneumoniae*, the recombinant microcin formed a single ionic channel that has several conductance states, with two preferred ones, of which state 1 is the most visited and state 2 is a transition from state 1 to a lower conductance (14). Figure 1 shows a current-voltage curve from a single channel in state 1, having the typical behavior of a

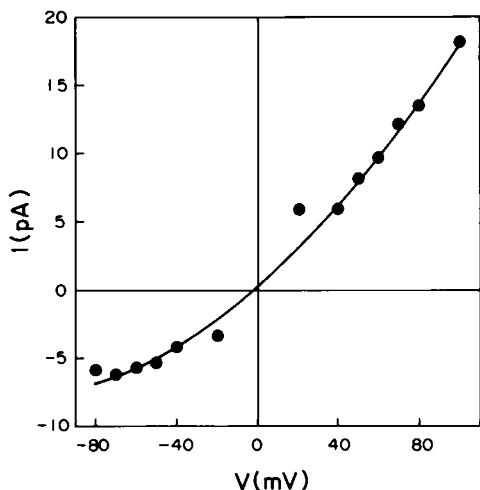

FIG. 1. Single-channel current-voltage curve for microcin E492 from *E. coli* VSC257pJ. Purified recombinant microcin was used for these determinations. The experimental conditions were as described in Materials and Methods. The plot shows a current-voltage curve from state 1, the most frequent state. The conductances for this state were 160 and 95 pS at ±60 mV.

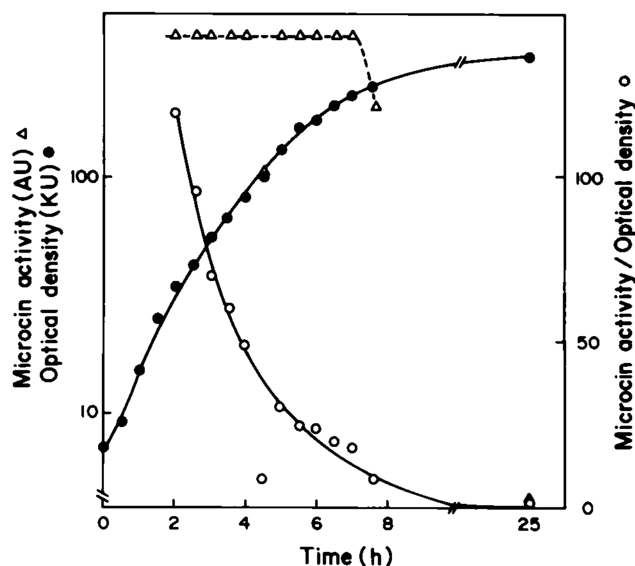

FIG. 2. Expression of microcin E492 during growth of *E. coli* VSC257pJ. The growth curve was initiated with a 1/10 dilution of an overnight culture in LB-ampicillin medium. Growth was monitored by the increase in optical density, expressed in Klett units (KU). Total microcin activity at different times is expressed in arbitrary units (AU) and was determined as described in Materials and Methods. The activity was corrected by the increase in the number of producing cells.

rectifier. The conductance values for this state were 160 and 95 pS at ±60 mV, which were very similar to those found for microcin from *K. pneumoniae* (143 and 95 pS at ±60 mV) (14). As has been discussed previously (14), the rectification of the *I*/*V* curve could represent an asymmetric structure of the channel. Thus, based on this electrophysiological characterization, microcin E492 expressed in *E. coli* has the same behavior as that of *K. pneumoniae* and is suitable for structural studies.

The low molecular weight of this microcin as compared to those of colicins represents an advantage for the analysis of the relationship between its structure and function. The three-dimensional structure of this microcin can be studied in solution through nuclear magnetic resonance, a method that can replace X-ray crystallography when rather small proteins are analyzed. The three-dimensional structures of the pore-forming domains of colicins have been determined after crystallization of proteolytically produced C-terminal fragments (5), and based on these structures and other experimental data, a general mechanism for membrane insertion has been proposed. Pore-forming colicins have molecular masses in the range of 40 to 60 kDa, and the sizes of the channel-forming domains are around 20 to 25 kDa (5), considerably larger than microcin E492. Pore-forming colicins are organized in three functional domains: translocation, receptor-binding, and channel-forming domains (5). Microcin E492 would perform these three functions with the information that is in a peptide of 6 kDa. This characteristic makes microcin E492 an extremely interesting molecule for studying structure and function, especially now that this system can be genetically manipulated.

Characterization of microcin E492 expression in *E. coli*. *K. pneumoniae* produces microcin E492 mainly in the exponential phase of growth (7, 19). The expression pattern of microcin E492 produced by *E. coli* VCS257pJ was monitored as described in Materials and Methods. This strain grew poorly and could not resume growth if the overnight culture was diluted more than 1:15. Figure 2 shows the growth curve of *E. coli* VSC257pJ, the total microcin activity (expressed in arbitrary units) produced during growth, and microcin activity corrected by the number of producing cells, expressed as optical density (Klett units). Microcin activity was detected after 2 h of growth, and it can be observed that this bacteriocin is expressed in the exponential phase of growth, while the activity starts to diminish when the stationary phase is entered and finally disappears. This result can be explained as both a decrease in microcin production while the culture enters the stationary phase and microcin inactivation. This latter effect can be visualized because of the decrease in the total activity level in the stationary phase. This effect cannot be explained as simple microcin inactivation in LB medium, because only a slight inactivation was observed in control experiments in which recombinant microcin was incubated for 24 h in LB medium. The inhibition observed in the stationary-phase culture could be due in part to titration of microcin by the cells, as a consequence of the loss of immunity, as will be presented below. In addition, recombinant microcin purified from stationary-phase cultures had very low activity compared with microcin purified from a culture in exponential phase. It is also possible that, like in *K. pneumoniae* (19), an antagonist of microcin activity is produced in stationary phase. If this is the case, the antagonist would be somehow induced by the recombinant plasmid, because no inhibition of microcin activity was observed when purified recombinant microcin was incubated with supernatant cultures of *E. coli* VSC257pHC79 in stationary phase.

Restriction and deletion mapping of the DNA region encoding microcin production and immunity determinant(s). Electrophoresis of pJ DNA digested with *Bst*VI revealed seven fragments, of 18.8, 15.3, 6.8, 6.0, 4.0, 2.0, and 0.6 kb. By Southern blot analysis and with the cosmid DNA as a probe, the location of the cosmid was found in the 15.3-kb fragment. Ligation of this fragment with the 6.8-kb fragment in both orientations (pJAM229 and pJAM434) resulted in plasmids that conferred the ability for microcin and immunity production, while religation of the 15.3-kb fragment (pJI) resulted in a plasmid that gave the bacterial host the capacity for microcin immunity but not microcin production. Figure 3 shows the

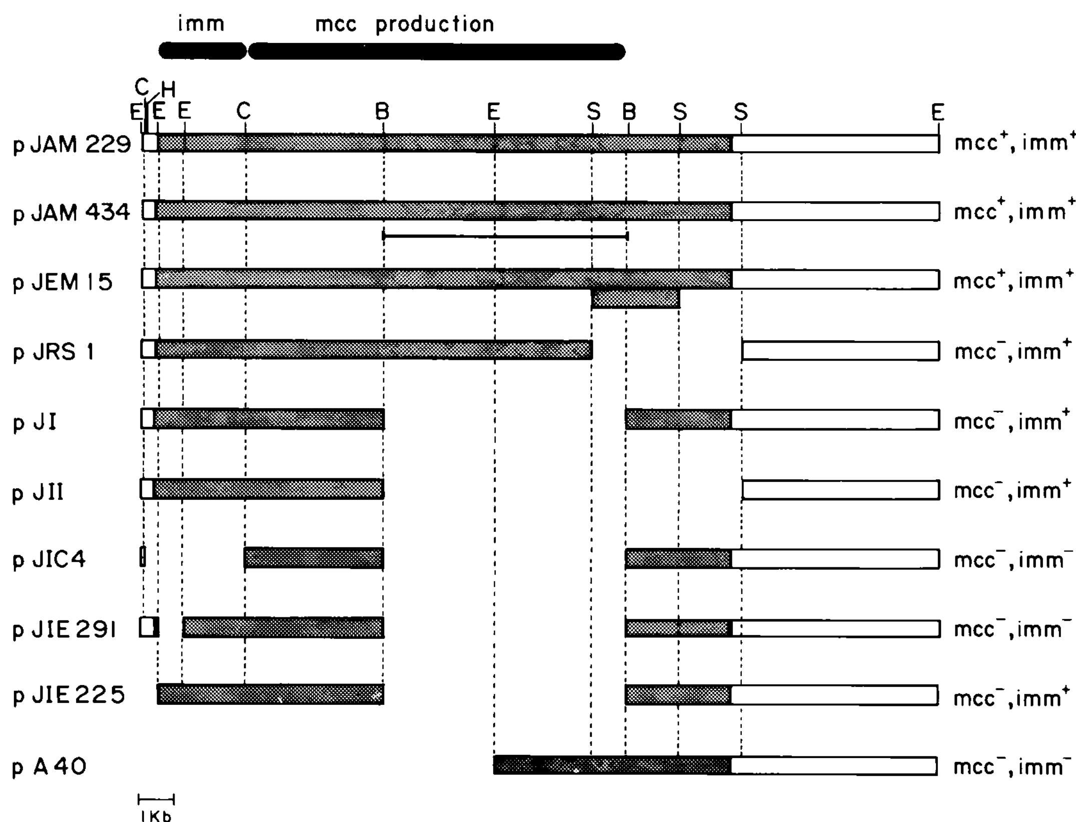

FIG. 3. Physical and deletion maps of the genetic determinants for the production of and immunity to microcin E492. The maps were constructed by single and double digestion with restriction endonucleases. The white boxes represent the DNA of the cosmid pHC79, and the stippled boxes represent the DNA from *K. pneumoniae* RYC492. The 6.8-kb *Bst*VI fragment was cloned in both orientations, and the inversion is underlined. Abbreviations: mcc, microcin; imm, immunity; E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; B, *Bst*VI; S, *Sal*I.

restriction patterns of the derivatives constructed starting from pJAM229 and pJI. After generation of a restriction map for pJAM229 (Fig. 3), deletions and insertions in the plasmid were made by cutting with endonucleases and religation. Thus, pJEM15 and pJRS1 were constructed by random religation after digesting pJAM229 with *Sal*I. pJEM15 has a duplication of the 2.5-kb *Sal*I fragment, and cells transformed with this plasmid grew better and produced 4 or 5 times more active microcin than the parental strains. On the other hand, pJRS1 has a deletion of the two small *Sal*I fragments, and the cells transformed with this plasmid did not produce microcin but were immune to its action, indicating that it is most likely that the 1-kb *Sal*I/*Bst*VI fragment (internal to the 6.8-kb *Bst*VI segment) is necessary for microcin activity. The region encompassing the second *Bst*VI site (from left to right) to the *Sal*I site into the cosmid was discarded as being involved in microcin production, because in the parental plasmid pJ there are inserts of DNA of more than 30 kb next to this *Bst*VI site.

pJI, the 15.3-kb *Bst*VI fragment ligation product, was the starting plasmid for all of the following deletion constructs. pJI was digested with *Bst*VI and *Sal*I (compatible cohesive ends), and ligation of the fragment containing the vector gave rise to pJII, which also conferred immunity to the bacterial host. pJI was also digested with *Cla*I (18- and 3-kb fragments), and religation of the larger fragment gave pJIC4, a plasmid that had lost the immunity determinant(s). Cloning of the 3-kb *Cla*I fragment in Bluescript resulted in a plasmid that conferred immunity to microcin E492 (25). The immunity was lost in pJIE291, the product of digestion with *Eco*RI (16-, 0.8-, and 0.4-kb fragments) and religation of the 16- and 0.4-kb fragments. However, if the religation included the 0.8-kb fragment (instead of the 0.4-kb fragment), the resulting plasmid, pJIE225, encoded microcin E492 immunity. This result indicates that immunity is totally or partially encoded in the 0.8-kb *Eco*RI fragment. The clones lacking the immunity (i.e., harboring pJIE291 and pJIC4) were tested with both recombinant microcin E492 and microcin E492 purified from *K. pneumoniae*. The lack of immunity was also observed in both exponential- and stationary-phase cultures.

All microcin and colicin genetic systems so far described are organized in operons, so the genetic determinants for the microcin E492 production and immunity are expected to be contiguous. The genetic organization of microcins encoded by plasmids, like C7, B17, and colicin V, is rather compact, having up to seven genes in a 6-kb DNA segment (13). However, the situation for chromosomally encoded microcins might be different. The other described microcin with genetic determinants located chromosomally is microcin H47. In that case, the genetic determinants span a DNA region of ca. 10 kb, with a 3-kb silent region inside the system (11). This system showed a complex transcriptional organization, and six genes for the production of the antibiotic and one for immunity production were identified (11). The DNA region encoding microcin E492 genetic determinants seems to be at least as large as that for microcin H47. The true orientation of the 6.8-kb *Bst*VI fragment remains to be determined; since plasmids with this fragment in both orientations confer a microcin-producing phenotype, the genes involved in microcin production in this DNA

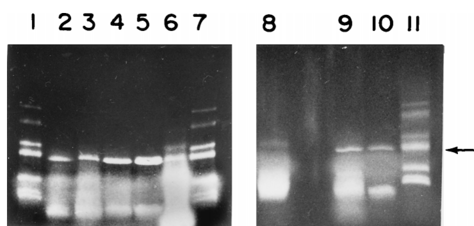

FIG. 4. Electrophoresis of microcin E492 purified from *E. coli* VSC257 harboring different constructs. SDS-polyacrylamide gel electrophoresis of microcin E492 covalently labeled with fluorescamine is shown. Microcin was purified from *E. coli* VSC257 harboring plasmids pJAM229 (lane 3), pJEM15 (lanes 4 and 9), pJAM434 (lane 5), pJ (lane 6), and pJRS1 (lane 8) and from *K. pneumoniae* RYC492 (lanes 2 and 10). Molecular weight markers (lanes 1, 7, and 11) were myglobin fragments with M_rs of 16,950, 14,440, 8,160, 6,210, 3,460, and 2,510. The arrow indicates the protein band corresponding to microcin.

segment probably have independent transcription units. Supposing that microcin E492 is organized in contiguous operons, the correct orientation could correspond to pJAM434, because the *Sal*I/*Bst*VI fragment is very distant from the immunity determinant(s). In contrast, if this genetic system has silent regions, pJAM229 could be the true orientation. To determine the number of genetic determinants and if there is a silent region, mutagenesis experiments will be performed. Currently, we are sequencing the 3-kb *Cla*I fragment in which the immunity is encoded.

Microcin produced by *E. coli* VCS257 transformed with pJ, pJAM229, pJAM434, and pJEM15 was purified as described by de Lorenzo (6), and fluorescent labeling was performed as described in Materials and Methods. Figure 4 shows electrophoresis of microcin covalently labeled with fluorescamine in an SDS-polyacrylamide system. It can be observed that the migrations of microcin E492 produced by *K. pneumoniae* (lanes 2 and 10) and by *E. coli* (lanes 3, 4, 5, 6, and 9) are very similar, corresponding to the expected molecular weight of around 6,000 (14). The slight difference in migration observed for JAM229 seems to correspond to an anomaly in lane 3, because usually this difference was not observed. As shown previously (14), this 6-kDa band corresponds to the biologically active microcin, whereas all of the components present in the smear of low-molecular-weight fragments are inactive (14). The same purification procedure was performed with Mcc^- strains, i.e., strains transformed with pJI, pJII, and pJRS1, and electrophoresis with these samples was carried out. While no microcin band was detected in cells transformed with pJI and pJII (not shown), a band with a higher molecular weight than microcin was produced by VSC257pJRS1 (Fig. 4, lane 8). This band could correspond to an inactive microcin precursor, but identification of this protein has to await sequencing data or immunological identification. If this band corresponds to a microcin precursor, the *Sal*I/*Bst*VI fragment missing in pJRS1 could be important for microcin processing. It is noteworthy that when this segment is duplicated, as happens in pJEM15, the cells harboring this plasmid grow better and produce more active microcin. Thus, the gene(s) encoded in this segment would be restrained in its expression, and higher gene dosage would not only render more active microcin but would also prevent the accumulation of toxic intermediates.

Characterization of the expression in *E. coli* of immunity to microcin E492. The expression of the immunity in *E. coli* was studied with *E. coli* VCS257pJI, a derivative that expresses immunity to microcin E492 but has impaired microcin production. This strain was found to be more suitable for the study of the immunity than a producing one, because changes in immunity expression may be overlooked because of the strong selection on the immunity condition that the presence of microcin imposes. The expression of the immunity, like the expression of microcin E492, also depends on the growth phase. The immunity was assayed as described in Materials and Methods, basically by using the *E. coli* recombinant strain as an indicator strain and testing different dilutions of purified microcin. Microcins purified from both *E. coli* and *K. pneumoniae* were used, and the results were the same irrespective of the microcin source employed. Table 2 summarizes these results. Immunity is expressed only in the exponential phase of growth and is completely lost in the stationary phase. This effect is not due to a metabolite that accumulates in the medium, because when cells in the exponential phase of growth were resuspended in spent medium from the stationary phase, and vice versa, the immunity still was expressed only in cells in the exponential phase of growth. The loss of the immunity was not due to an effect of high cellular density, because under conditions of nutrient deprivation (LB diluted 1:5), the loss of immunity was observed at a low cellular density as cells entered the stationary phase. The regulation of the expression in the stationary phase did not involve the sigma factor RpoS, because no changes were observed when this factor was induced in the exponential phase of growth by sodium benzoate (18) or when a host defective in *rpoS* (4) was used (Table 2). The lack of expression of immunity during the stationary phase may account for the difficulties in growing *E. coli* VCS257pJ, especially in resuming growth after dilution of an overnight culture.

TABLE 2. Immunity of *E. coli* pJI[a]

E. coli strain	Condition(s)	Immunity
VSC257pJI	Exponential phase	+
	Stationary phase	−
	Exponential phase with supernatant of spent medium in stationary phase	+
	Stationary phase with supernatant of spent medium in exponential phase	−
	Exponential phase with nutrient deprivation	+
	Stationary phase with nutrient deprivation (low number of cells)	−
	Exponential phase with benzoic acid	+
	Stationary phase with benzoic acid	−
ZK1000 pJI	Exponential phase	+
	Stationary phase	−

[a] Cells were grown in LB-ampicillin medium. Immunity was assayed using the *E. coli* strain shown as an indicator. *E. coli* ZK1000 is defective in *rpoS.*

Regarding the expression of immunity to microcin E492 in *K. pneumoniae* RYC492, cultures in stationary phase are fully immune to high concentrations of microcin E492. However, the partial protection conferred by the microcin antagonist that is produced in the stationary phase (19), plus the abundant capsule that is made by this strain, could mask an eventual loss of immunity in the stationary phase in *K. pneumoniae.*

The expression of immunity and the expression of microcin E492 in *E. coli* seem to be coordinated, because both are not expressed in the stationary phase. Both genes could be coupled in the same transcriptional unit, as happens with microcins B17 and C7 and colicin V (13), or in the case that they are not under control of the same promoter, they might share regulatory features that coordinate the expression of these genetic determinants.

Like most antibiotics, microcins (with the exception of mic-

rocin E492) are synthesized when cultures enter the stationary phase. Regulation of both microcins B17 and C7 has been studied, and even though both sets of genes are induced by the cessation of growth, the mechanisms to accomplish this are different. While the stationary-phase expression of microcin B17 is independent of *rpoS*, the opposite occurs with the operon of microcin C17, in which the expression in stationary phase depends on a functional *rpoS* product (13).

Regulation of expression of immunity and microcin E492 production is different from that in the other described systems, and again, a microcin may serve as a model system for studying a general question, in this case regarding gene down regulation in stationary phase, providing new insights into the regulatory mechanisms that command this process.

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