Four Plasmid Genes Are Required for Colicin V Synthesis, Export, and Immunity

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The colicin V production and immunity genes were isolated from plasmid pColV-K30. A HindIII-to-SalI fragment of 9.4 kilobases was cloned into the compatible vectors pBR322 and pACYC184. Mutants defective in colicin production were generated by Tn5 insertions and by constructing deletions in vitro. Physical analysis of these mutations identified a 4.4-kilobase region of this DNA which contains all the plasmid genes (cwa) needed for the production of colicin V. The colicin V immunity determinant (cvi) is in a 700-base-pair fragment located within one end of this region. Complementation tests identified three genes, called cwaA, cwaB, and cwaC, required for colicin production. Analysis of the proteins labeled in minicells harboring various Tn5 insertions allowed us to identify protein products for the cwaA and cwaC genes. Mutations in cwaA and cwaB eliminated colicin activity in culture supernatants, but not within the cells. Mutations in cwaC, however, eliminated all detectable activity. From these results we conclude that the cwaC gene codes for the structural gene for colicin V, while cwaA and cwaB are apparently needed for the normal export of the colicin.

Colicin V is a small, proteinaceous toxin whose activity along with an immunity determinant is encoded on large, low-copy-number plasmids (14). ColV plasmids have been found naturally occurring in many strains of Escherichia coli and other members of the family Enterobacteriaceae. These bacteria also define the activity range for colicin V. Its target for growth inhibition is thought to be the cytoplasmic membrane, whereas it prevents the formation of membrane potential (32). ColV plasmids are often associated with E. coli invasiveness and pathogenicity (29, 30). These plasmids also often carry genes which may enhance the ability of cells to proliferate within the host. Examples of these are the arobaclitc mutants (31) and a gene for increased serum resistance (5). In addition, an enhanced adherence to intestinal epithelial cells has been noted in strains harboring ColV plasmids (11). Colicin V production does not appear to be a virulence determinant, but it has been hypothesized that it may help to selectively maintain these genes (26, 31).

The colicin V toxin is distinguished from other colicins by the small size of the active protein (13) and by its constitutive, rather than SOS-inducible, synthesis (15). There is also no evidence that, like many colicins, colicin V accumulates in the cell before its release or has a lysis gene product responsible for its release (25).

Frick et al. (13) cloned a 900-base-pair (bp) region of the pColV-B188 plasmid which included the colicin V immunity gene (cvi) and an apparent colicin V structural gene (cwa). However, cells harboring this cloned fragment did not produce growth inhibition zones on a lawn of sensitive cells, and culture supernatants did not contain assayable amounts of colicin. The killing activity coded by this 900-bp fragment could only be assayed after lysing the cells and appeared to be fourfold less potent than that coded by a much larger fragment (13). In this paper we show that a 4.4-kilobase (kb) region of DNA containing cvi and at least three other genes is necessary for the normal production and export of killing activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and culture conditions. Media were prepared as described by Miller (24). Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 150; chloramphenicol, 20; and kanamycin, 50.

DNA manipulations and colicin V assays. Plasmid DNA preparation, digestions, ligations, transformations, and electrophoresis were performed as described previously (6, 12, 21). Colicin V activity and immunity were determined as described previously (13). Activity was quantitated by the critical dilution method (22).

Tn5 mutagenesis. Transposition of Tn5 into pHK11 and pHK22 was accomplished by introducing these plasmids into strain DB1358 (28). To ensure independent insertions into many different sites, 20 colonies of DB1358 harboring each plasmid were grown independently, and plasmid DNA was prepared. This DNA was then used to transform MC4100, selecting for resistance to kanamycin and ampicillin for the pHK11 derivatives and to kanamycin and chloramphenicol for the pHK22 derivatives. About 10% of these colonies no longer produced colicin V. To map the Tn5 insertion sites, BglII, HindIII and BamHI digests were done, thereby using the precisely known restriction sites within the IS50 of Tn5 (16) and the unique sites in pHK11 or pHK22.

Complementation testing. Each cwa::Tn5 mutant from pHK11 was used to cotransform ZK4 in combination with each cwa::Tn5 mutant from pHK22. Transformants were selected as being simultaneously resistant to kanamycin, ampicillin, and chloramphenicol. From each transformation, six colonies were tested for colicin V production. The ability to form a zone of growth inhibition was interpreted as positive complementation. No cases of partial complementation were observed. In every case, all six colonies tested gave the same results. In addition, we confirmed the presence of two separate plasmids on several of the double transformants.

Preparation of minicells, protein labeling, and protein analysis. Strain P678-54T was transformed with pHK22 and
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MC4100</td>
<td>F’ araD139 3lacU169 rpsL relA thiA</td>
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<tr>
<td>ZK4</td>
<td>MC4100 recA56</td>
<td>This work</td>
</tr>
<tr>
<td>DB1358</td>
<td>F’ ilv::Tn5</td>
<td>28</td>
</tr>
<tr>
<td>P678-54T</td>
<td>thr leu thiA supE lacY fhuA</td>
<td>1</td>
</tr>
<tr>
<td>JM83</td>
<td>gal mal syl ara mtl thy min ara Δ(lac-proAB) rpsL 330 lacZM15</td>
<td>33</td>
</tr>
<tr>
<td>71-18</td>
<td>Δ(lac-proAB) thi supE F’ lacF lacM15</td>
<td>33</td>
</tr>
<tr>
<td>pColV-K30</td>
<td>ColV’, 90 kb</td>
<td>31</td>
</tr>
<tr>
<td>pBR222</td>
<td>Ap’ Te’, 4.3 kb</td>
<td>7</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm’ Te’</td>
<td>9</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap’ LacZa’</td>
<td>33</td>
</tr>
<tr>
<td>pMK16</td>
<td>Km’ Te’</td>
<td>17</td>
</tr>
<tr>
<td>M13tg131</td>
<td>LacZa’</td>
<td>18</td>
</tr>
</tbody>
</table>

Appropriate pHK22::Tn5 insertion mutants. Minicells were isolated and labeled for 10 min as described previously (23). Labeled proteins were electrophoresed on either 21 or 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (19) at 130 V. Prestained size standards were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

RESULTS

Cloning of colicin V genes. HindIII fragments from a complete digest of pColV-K30 DNA were ligated with HindIII-cut pBR222. This ligation mixture was used to transform strain MC4100. Ampicillin-resistant transformants were selected and screened for colicin V production on the basis of zones of growth inhibition surrounding the colonies. Apparent colicin-producing clones were further purified and tested for colicin V activity and immunity. Plasmid DNA was then subjected to restriction analysis. For each of the clones tested, the pBR222 plasmid derivative contained a 20-kb HindIII fragment insertion. The two possible orientations of the fragment inserted were designated pHK3 and pHK4.

After a restriction map of pHK4 was generated (Fig. 1), deletions were made in the plasmid by cutting with endonucleases and religating. One deletion, a SalI digestion followed by religation, still maintained both colicin V production and immunity. This plasmid was called pHK11 (Fig. 1). The growth inhibition zones generated by pHK11 and other high-copy-number clones were larger than those observed with the wild-type pColV-K30.

Subcloning of pHK11. Three fragments of pHK11 were subcloned separately into pMK16, and the resultant constructs were assayed for colicinogenic and immunity properties (Fig. 2). The plasmids containing 2.2- or 1.5-kb BamHI-EcoRI fragments did not confer colicin production or immunity properties on the cells. Cells which contained the SalI-BamHI (5.2-kb) fragment did not produce colicin V but were immune to colicin V (Cva- Cvi+). Similarly, the KpnI-BglII (4.2-kb) fragment cloned into pUC19 resulted in cells (strain JM83) with a Cva+ Cvi− phenotype. However, cells (strain 71-18) with M13tg131 containing the HindIII-BglII (7.2-kb) fragment were Cva+ Cvi−.

Tn5 mutagenesis and complementation analysis. To better localize the colicin production region and to perform complementation experiments, we constructed pHK22, a derivative of pACYC184 which contains the same SalI-HindIII (9.4-kb) fragment as pHK11 and is compatible with pHK11. Inhibition zones from strains harboring pHK22 were approximately the same size as those from strains containing pHK11. Both pHK11 and pHK22 were mutagenized with Tn5 as described in Materials and Methods. Only two phenotypes with respect to colicin V production were obtained: colonies which still produced normal-sized growth inhibition zones and colonies which completely failed to produce growth inhibition zones.

The insertion sites for 11 cva::Tn5 mutants in pHK11 and for 13 such mutants in pHK22 were determined by restriction-site analysis (Fig. 2). All Cva− insertions mapped within the BglII-KpnI (4.2-kb) fragment. The results of complementation experiments with all possible combinations of mutants indicated that there were three distinct complementation groups. Insertion mutations pHK11-5, -7, -10, and -8 and

FIG. 1. Restriction map of pHK4 and its derivatives. Plasmids derived from pHK4 were made by single restriction enzyme digests followed by religation. Vector pBR322 is represented by a heavy line.
pHK22-8, -2, and -13 did not complement each other but were complemented by all other Tn5 mutations. Likewise, pHK11-11, -4, -3, -6, and -2 and pHK22-1, -5, -9, -10, -4, -14, -7, and -11 formed another complementation group, and a third group was formed by pHK11-9 and -1 and pHK22-6.

We have designated these contiguous genes cvaA, cvaB, and cvaC (Fig. 2). The left-hand boundary of cvaA must lie between pHK22-3, a Cva+ Tn5 insertion, and the KpnI site, while the right-hand boundary lies between pHK11-8 and pHK11-11. The boundaries for cvaB are between pHK11-8 and pHK11-11 on the left and between pHK11-2 and pHK11-9 on the right. The cvaC gene boundaries must lie between pHK11-2 and pHK11-9 on the left and between pHK22-6 and the BglII site on the right (Fig. 2). The approximate gene sizes are estimated as 850 to 1,450 bp for cvaA, 1,900 to 2,550 bp for cvaB, and 150 to 1,050 bp for cvaC.

Localization of colicin V activity. Colicin V activity in culture supernatants and lysed cells was quantitated for pHK11 and pHK11 cva::Tn5 mutants to determine whether any of the cva genes are involved in the release of colicin V (Table 2). While mutations in cvaA (pHK11-8) and cvaB (pHK11-4) resulted in an approximately twofold reduction in colicin V activity in lysed cells, they prevented any detectable activity in the supernatant. A mutation in cvaC (pHK11-1) eliminated activity in both the supernatant and the cells. The colicin V structural gene therefore appears to be cvaC, while cvaA and cvaB are responsible for its release.

Mapping of the immunity region. All cva::Tn5 insertion mutants were tested for immunity to colicin V and were Cvi+. A BamHI-BglII deletion in pHK11 (Fig. 2) gave a Cvi- phenotype. We then used the HindIII site from within the ISS50 of pHK11-1 to clone a 1.9-kb HindIII-BglII fragment containing 1,195 bp from Tn5 and 700 bp of ColV DNA into another vector, pUC19. When JM83 cells were transformed with this pUC19 derivative, the cells were Cvi+. Therefore, the cvi gene(s) lies within the 700-bp region between the BglII site and the rightmost Tn5 insertion in pHK11, pHK11-1 (Fig. 2).

Identification of cva gene products. The protein products of pHK11, pHK22, and the Tn5 insertion derivatives were analyzed in minicells. Two or three mutants in each complementation group were analyzed in this way with identical results. The results shown in Fig. 3 and 4 are from pHK22, pHK22-6, pHK22-9, and pHK22-8. The 12.5% SDS-polyacrylamide gel shows two proteins, with masses of 27 and 43 kilodaltons (kDa), which are affected by a disruption of the cvaA gene (Fig. 3). In Fig. 4, the 21% gel shows a protein of approximately 6 kDa as a gene product for cvaC. Lane 5 of Fig. 4, which contains plasmid proteins from cvaC

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Colicin V activity (U)* in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lysed cells</td>
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<tr>
<td>pHK11-1</td>
<td>cvaA</td>
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<td>pHK11-4</td>
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<td>pHK11-1</td>
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<tr>
<td>pHK11ΔBamHI-BglII</td>
<td>cvaABC</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Units determined by the critical dilution method (22) as described in Materials and Methods.
**DISCUSSION**

We have identified a 4.2-kb region of plasmid pColV-K30 which is responsible for the production of colicin V and immunity to it. Tn5 mutagenesis and complementation analysis defined three complementation groups involved in normal colicin V production. The three genes defined by the complementation groups, cvaA, cvaB, and cvac, are encoded contiguously (Fig. 2) and could be transcribed as part of an operon. The lack of strong polarity by the Tn5 insertions suggests that each gene has its own transcriptional promoter. However, instances in which transposon insertions do not show polarity have been described (27). These instances could be due to outward promoters from the ends of transposons (4, 10) or to transcription antitermination (20). The immunity gene, cvi, is within a 700-bp region adjacent to, or possibly overlapping, the cvac gene (Fig. 2).

Mutations in cvaA abolished the production in minicells of two proteins of approximately 27 and 43 kDa. It is possible that these proteins are the products of overlapping genes. No obvious product change was apparent in the cvaB mutants. Mutations in cvac abolished a protein of approximately 6,000 MW. A deletion in the cvi region resulted in the disappearance of a 7,000-MW protein which can be seen in preparations from a plasmid with the cloned cvi gene.

In an earlier study of the genes necessary for colicin V activity and immunity, Frick et al. (13) obtained a plasmid essentially identical to pHK11 from pColV-B188. In that study, the structural gene for colicin V and the immunity gene were mapped to the EcoRI-BgII fragment (Fig. 2). The fragment carrying these genes was then reduced to a 900-bp HaeIII-RsaI fragment. These 900 bp would cover the region of cvac and cvi defined in the present study. While the 900-bp fragment coded for colicin V activity, this activity could only be detected in extracts of lysed cells. In our study, lysed-cell extracts from cvaA and cvaB mutants showed activity, while those from cvac mutants did not. Supernatants from all three mutant cultures also showed no detectable activity. Since strains harboring plasmids with the wild-type HindIII-SalI fragment yielded active colicin V both within the cells and in the supernatant, these results suggest that cvac is the structural gene for colicin V and that the products of the other two genes, cvaA and cvaB, are necessary for the proper export or release of the colicin. Comparison of the cloned region of pColV-B188 (13) with that of pColV-K30 shows extensive homology. There are some minor differences in the number of and distance between several restriction sites, which could reflect polymorphisms between the two parent ColV plasmids.

Most colicin plasmids contain a cluster of three genes involved in the production and release of the colicin: the colicin structural gene, the colicin immunity gene, and a lysis gene (25). The results described here indicate that the genes involved in colicin V production do not follow this pattern. Our work on the genetic arrangement of colicin V production and immunity genes, along with the evidence that colicin V is not induced by DNA damage (15), supports the suggestion that colicin V can be considered a microcin (32). Microcins constitute a family of low-molecular-weight antibiotic substances produced by many *Enterobacteriaceae* (2, 3). Unlike the colicins, none of the microcins is inducible by DNA-damaging agents.

Further research into the production and function of colicin V and microcins in general should prove to be useful in the understanding of peptide and small-protein transport in *E. coli*. It is likely that colicin V must cross the double membrane and cell wall of the producer cell and must then...
span at least part of the envelope barrier of the susceptible cell. The export and uptake of this protein could be different from that of the larger colicins, which are thought to first accumulate in the producing cell and then to rely on a lysis gene product for export (25).

ACKNOWLEDGMENTS

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LITERATURE CITED