

## CLONING OF STRUCTURAL GENES FOR COLICIN V AND THEIR ROLE IN PATHOGENICITY OF INVASIVE *Escherichia coli*

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### ABSTRACT

The colicin V structural genes of plasmid pMV<sub>14</sub> were cloned into the vector pYP 328. These genes were isolated on a 2.4 kb deoxyribonucleic acid fragment which did not transport or express the virulence genes of strain UEL 14. Insertion of the transposon Tn5 into plasmid pMV14 led to the construction of a mutant plasmid which did not produce colicin V but conferred virulence to one-day-old chicks. From these results we conclude that the colicin V activity is not essential to ColV plasmid-mediated virulence enhancement in *Escherichia coli* of avian origin.

### INTRODUCTION

Colicin V is a proteinaceous toxin encoded by a large low-copy-number plasmid (Col V plasmid) (Hardy, 1975) that occurs in many strains of *Escherichia coli* and other members of the family *Enterobacteriaceae*. This protein has a MW of 4,000 Da (Frick *et al.*, 1981) and it inhibits bacterial growth, interfering with membrane potential formation (Yang and Konisky, 1984).

Smith (1974) observed that in experimental animals *E. coli* strains were more prone to provoke sepsis after acquisition of the Col V plasmid, and that pathogenicity decreased after elimination of the Col V plasmid. Smith and Huggins (1976), then reported a significant correlation in *E. coli* between the possession of a Col V plasmid and the ability to cause septicemia, particularly in domestic animals such as cattle and

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chickens. These observations were confirmed by Ozanne *et al.* (1977). Milch *et al.* (1984), observed that in *E. coli* strains isolated from meningitis and sepsis cases from a hospital outbreak in Hungary, the virulence determining gene could be attached to either the Col V or R plasmids. These authors reported that colicin V activity is not essential to Col V plasmid-mediated virulence enhancement in *E. coli*, in agreement with the findings of Quackenbush and Falkow (1979).

We have found that *E. coli* 045 strains causing colisepticemia in poultry (Vidotto *et al.*, 1990) were producing colicin V-I. In the present investigation we cloned structural genes for colicin V to determine whether colicin V activity was necessary for the enhancement of the virulence of these strains.

## MATERIAL AND METHODS

### *Bacterial*

#### *Strains and plasmids*

The *E. coli* strains and plasmids used are listed in Table I. *E. coli* UEL 14, serotype 045, which was recovered from chickens with colisepticemia in Londrina-PR., carries a non-self transferable Col V-I plasmid that is known to contain genes encoding for virulence enhancement, pMV14, that was used in cloning experiments. The *E. coli* UEL 15, non-pathogenic to one day-old chicks, plasmidless, was used as a recipient in transformation experiments (Vidotto, 1988). *E. coli* HB 101 (PCBT 01::Tn 5) carries the plasmid pCBT 01 which is a derivative of R 621a, with the resistance gene Tc and containing the transposition element Tn 5, that confers resistance to Km.

#### *Colicins assays*

To determine the capacity of whole cells to produce colicin, single colonies were transferred to tryptone plates with tooth-picks. After incubation at 37°C for 12 to 18 h, the cells were killed with chloroform vapors, and the plates were overlaid with 3 ml of soft tryptone agar (0.7% agar), containing 1 ml of an overnight culture of strain MA335 diluted 10<sup>-1</sup>. Colicin-producing colonies gave a clear killing zone surrounding the cell colony (Azevedo and Costa, 1973).

Immunity to colicin V was defined as insensitivity of a strain to colicin V (20R915), but sensitivity to colicin Ia and/or Ib (22R81, 22R82, 22R83).

Table I - Bacterial strains and plasmids.

Strain or plasmid	Relevant properties or genotype	Sources
UEL 14	Natural isolate carrying pMV14, pathogenic	This laboratory
UEL 15	Natural isolate plasmidless, non-pathogenic	This laboratory
HB101	F <sup>hdsS20 recA13 ara 14 proA<sub>2</sub> lacY1 leu gal K2 rpsL20 xyl5 mlt1 thi supeE44</sup>	Boyer and Rouland- Dussoix, 1969
Ma 335	pro met trp his lacI lacZ Nal <sup>r</sup>	M.L.M. Silva <i>et al.</i> , 1978
K <sub>12</sub> -711	F(λ) lac28 his 51 trp 30 proC 23 phe Nal <sup>R</sup>	S. Falkow, 1974
pMV14	Col V <sup>+</sup> Tc <sup>r</sup> (120-kb)	This Laboratory
pCBT01::Tn5	Tc <sup>r</sup> Km <sup>r</sup>	Diógenes S. Santos (1985)*
pYP 328	Ap <sup>r</sup> Tc <sup>r</sup> Cm <sup>r</sup> (6.0-kb)	Santuza M.R. Teixeira and Spartaco Filho (1985)

\* Provided by Diógenes S. Santos

### Pathogenicity Tests

The pathogenicity of *E. coli* strains was evaluated in susceptible day-old broiler chickens. Five birds per isolate were inoculated subcutaneously with 0.5 ml of nutrient broth culture containing  $1.0 \times 10^8$  colony-forming units (CFU) of *E. coli*. Birds were maintained for 7 days postinoculation and monitored daily for mortality (Harry, 1964).

### Plasmid isolation

For preparation of plasmid DNA the rapid method of Birnboim and Doly, 1979, was used. Preparation of large plasmids was carried out according to the method of Kado and Liu, 1981.

### *Restriction, ligation and transformation*

Digestion of DNA with restriction endonucleases was usually carried out under the conditions suggested by the commercial enzyme source (Bethesda Research Laboratories, Rockville, Md). Digestions were arrested by heating for 10 min at 65°C. Ligation of DNA fragments was performed in 6 mM Tris-hydrochloride (pH 7.9-6 mM MgCl<sub>2</sub>-6 mM mercaptoethanol-0.2 mM ATP), using T4-induced DNA ligase (New England Biolabs). Transformation of competent cells and selection of transformed bacteria were done as previously described (Maniatis *et al.*, 1982).

### *Electrophoresis of DNA*

The sizes and digestion patterns of DNA fragments were analyzed by electrophoresis in 0.8 to 1.0% agarose gel with Tris-borate buffer (Meyers *et al.*, 1976).

### *Mapping of restriction sites*

Restriction sites were mapped by examination of the sizes of singly or doubly digested whole plasmids (Maniatis *et al.*, 1982).

### *Tn5 mutagenesis*

Transposition of Tn5 into pMV14 was achieved by mating *E. coli* HB101 (PCBT01::Tn5) with UEL 14 (pMV14) in TSB (Tryptic Soy broth). Transconjugants were selected by inoculation on minimum agar plates containing kanamycin (20 µg/ml) and incubating for 24 h at 37°C.

## RESULTS

### *Cloning of colicin V genes*

Sau 3A fragments from a partial digest of pMV14 DNA were ligated with Bam HI-cut YP328. This ligation mixture was used to transform strain HB101. Ampicillin-resistant and tetracycline sensitive transformants were selected and screened for Colicin V production on the basis of zones of growth inhibition surrounding the indicator colonies. The colicin-producing clones were tested for colicin V immunity.

One of the colicin V-producing, but colicin sensitive, transformants was found to harbor an 8.3 kb plasmid. This plasmid, designated YP14, encoded colicin

V production. It was not cleaved by Bam HI, but as the YP328 vector linearized upon Bam HI digestion was 5.9 kb in length, the fragment inserted was 2.4 kb.

The restriction map of YP14 was obtained through digestion with Eco RI, Bam HI, Sal I, Hind III and Cla I (Figure 1).

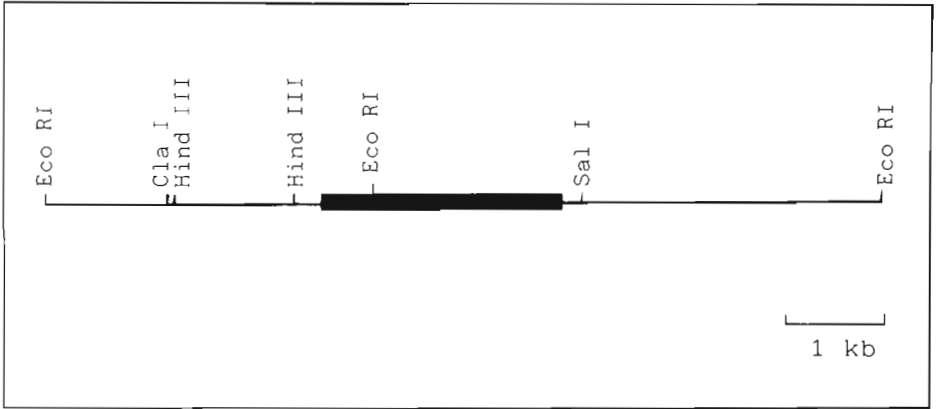


Figure 1 - Restriction map of pYP14. The solid bar represents 2.4 kb cloned fragment from plasmid pMV14, and the thin line represents the DNA of the vector plasmid.

The YP14 plasmid was also utilized to transform the UEL 15 strain, that is prototrophic, Col V and non-pathogenic to one-day-old chicks. The UEL 15/YP14 transformant was grown in nutrient broth and inoculated ( $10^8$  cells) in five, one-day-old chicks. The recombinant was not virulent. This result suggests that the inserted fragment did not contain or express the virulence gene of the UEL 14 strain.

### *Tn5* mutagenesis

Four hundred clones resistant to kanamycin were screened for ability to produce colicin V. Approximately 6% of the clones presumed to carry Tn5 insertions did not produce detectable levels of Col V activity.

Four of the putative colicin V - negative, Col V::Tn5 containing strains derived from *E. coli* UEL 14 were screened for the presence of plasmid deoxyribonucleic acid as described by Meyers *et al.* (1976). All four clones still carried plasmids of similar molecular mass (approximately 120 kb) to the Col V plasmid, indicating that inactivation of colicin V was caused by mutations mediated by Tn5 insertion events.

The virulence of the colicin V-negative mutants and related strains was tested in one-day-old chicks by injections of 0.5 ml of culture ( $10^8$  cell) administered subcutaneously. The strains carrying the Col V plasmid but lacking colicin V activity produced mortality rates in chicks that did not differ from mortality rates produced by the parental strains *E. coli* UEL 14 (Table II).

Table II - Pathogenicity tests in one-day-chicks.

Samples	Nº Killed	Nº Inoculated
UEL 14 (Col V <sup>+</sup> )	5	5
UEL 14::Tn5 (Col V <sup>-</sup> )	5	5
K <sub>12</sub> 711	0	5

## DISCUSSION

The discovery by Smith (1974) of a plasmid coding for colicin V production and associated with lethality led Ozanne *et al.* (1977) to determine conditions under which colicin V could be readily obtained *in vitro* to be used in studies of pathogenicity. These authors suggested that colicin V is active against mouse peritoneal phagocytic cells, acting as a virulence factor in some *E. coli* strains. However, Quackenbush and Falkow (1979) reported that colicin V activity was not essential to Col V plasmid mediated virulence enhancement, when the colicin V synthesis was inactivated by transposon insertion. This experiment was repeated by using an *E. coli* Col V strain 045, isolated from chickens in Londrina, PR. Two colicin V negative strains due to Tn-5 mediated insertional inactivation were still virulent for one-day-old chicks. This gives further evidence for the hypothesis.

Colicin production seems not to be the virulence determinant, however virulence is associated with Col V plasmids. It is probable that the genetic determinants of virulence enhancement are closely linked to colicin V-related genes and the colicin V production acts in the maintenance of these genes.

Gilson *et al.*, 1987, identified a 4.2-kb region of plasmid pCol V-k30 which is responsible for the production of and immunity to colicin V. Three contiguous genes *cvaA*, *cvaB* and *cvaC*, were found. These genes could be transcribed as part of an operon. Gene *cvi* is in a 700-bp region adjacent to or overlapping with the *cvaC* gene. These authors also verified that the structural gene for colicin V is the *cvaC* gene, and that the other two genes, *cvaA* and *cvaB*, are necessary for the proper export or release of the colicin.

The structural gene for colicin V and the immunity gene were mapped to the Eco RI-Bgl II fragment and then this fragment was reduced to a 900-bp Hae-Rsa I fragment (Frick *et al.*, 1981). These 900 bp would cover the region of *cvaC* and *cvi* defined in the study of Gilson *et al.* (1987). Comparison of the cloned region of pCol V-B188 (Frick) with that of pCol V-K30 showed extensive homology, with some minor differences in the number of and distance between several restriction sites (Gilson *et al.*, 1987).

In the present study, a 2.4 kb region of plasmid pMV14 responsible for production of colicin V but not immunity was identified. This fragment seems to include only the *cvaB* and *cvaC* genes and does not have the restriction sites reported by Frick *et al.* (1981). We have demonstrated that the genes for colicin V production did not affect the pathogenicity of *E. coli* for chicks, by inoculating UEL 15 strains carrying the YP14 plasmid which contained the cloned fragment. These strains did not cause the death of inoculated chicks.

The cloned fragment should be useful in the construction of a probe for cloning of the region around Col V to pick up the virulence genes in the plasmid. The probe containing these virulence genes could be utilized for identification of other strains of *E. coli* pathogenic for poultry and other animals.

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### RESUMO

Os genes estruturais para colicina V do plasmídeo pMV14 foram clonados no vetor pYP328. Estes genes foram isolados em um fragmento de ácido deoxirribonucleico de 2.4 kb o qual não transportou ou não expressou os genes de virulência da amostra UEL 14. A inserção do transposon Tn5 no plasmídeo pMV14 levou a construção de um plasmídeo mutante, o qual não produziu colicina V, mas conferiu virulência para pintos de um dia.

Destes resultados, nós concluímos que a atividade da colicina V não é essencial para a virulência mediada pelo plasmídeo Col V em *E. coli* de origem aviária.

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