JOURNAL OF BACTERIOLOGY, Mar. 1983, p. 1552–1557
0021-9193/83/031552-06$02.00/0

Vol. 153, No. 3

Comparison of Plasmids ColE2-P9 and ColE2-CA42 and Their Immunity Proteins†

ROBERT WATSON,[1] MAYA KONARSKA-KOZLOWSKA,[2] V. N. IYER,[2] MAKOTO YAGUCHI,[3] AND LOUIS P. VISENTIN[3]*

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A OR6[3]; Department of Biology, Carleton University, Ottawa, Ontario, K1S 5B6[2]; and Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, K1A 0C6[1], Canada

Received 7 July 1982/Accepted 2 December 1982

Plasmids ColE2-CA42 (6.1 kilobases) and ColE2-P9 (6.8 kilobases) were found to have homologous (4.3-kilobase) DNA segments which contain their colicin and immunity genes. The relatedness of their immunity proteins was verified by determining their amino acid compositions and N-terminal sequences. These characteristics were compared with those of ColE3-CA38.

In a previous study, we identified a series of ColE plasmids, including five ColE plasmids other than ColE2-P9 (9). One of the plasmids, ColE2-CA42, was noted to differ from ColE2-P9 and the other ColE2 plasmids in that it was smaller in size and did not yield a 0.61-kilobase (kb) DNA fragment after treatment with *Eco*RI. In this communication, we describe comparisons of ColE2-P9 and ColE2-CA42 to determine the relationships of their restriction maps, their regions of DNA homology, and the positions of their colicin and immunity genes. We have also compared their immunity proteins to determine their degree of similarity to each other and to the immunity protein of ColE3-CA38.

Structure of the ColE2 plasmids. Restriction maps of ColE2-P9 and ColE2-CA42 are shown in Fig. 1A and B. ColE2-CA42 was found to be 6.1 kb in size, compared with 6.8 kb for ColE2-P9. Though their restriction maps differ overall, both plasmids have *Pvu*I, *Pvu*II, and *Bgl*II sites (at their 0.0-, 0.23-, and 0.52-kb map positions in Fig. 1A and B) such that the sizes of the intervening plasmid segments are comparable between the two plasmids. The *Bgl*I site at the 5.58-kb map position of ColE2-P9 and that at the 4.86-kb position of ColE2-CA42 are also comparably situated relative to the common *Pvu*I, *Pvu*II, and *Bgl*II sites.

The phenotypic relatedness of ColE2-P9 and ColE2-CA42 suggested that their four commonly situated restriction sites could be due to homology of their DNAs. To test this directly, both plasmids were cleaved at their unique *Pvu*I sites, and the linear species was used for heteroduplex mapping. Electron microscopic visualization of the hybridization products revealed a class of molecules of a heteroduplex type, consisting of a bubble of nonhomologous single-stranded DNA bounded by 1.7- and 2.6-kb double-stranded DNA termini (Fig. 2A). Though a smaller bubble was present in some of these DNA structures, it was not observed consistently. It may be due to a small inverted or partially homologous DNA region.

Plasmids pBB9 and pBB10 were also used for heteroduplex mapping. They were derived by cloning the entire ColE2-P9 (for pBB9, Fig. 1C) or ColE2-CA42 (for pBB10, Fig. 1D) plasmids into the *Pvu*I site of pBR322 (Table 1). In these derivatives, the orientations of the ColE2-derived DNAs relative to that from pBB322 are the same, using the four common ColE2 restriction sites described above for reference. PBB9 and pBB10 have single *Hin*dIII sites within their pBR322 portions which were cleaved before hybridization. The heteroduplex molecules contained a bubble of pBB9/pBB10 nonhomologous DNA with the same dimensions as were noted for the ColE2-P9/ColE2-CA42 heteroduplexes (Fig. 2B). The lengths of the double-stranded DNA arms of the pBB9/pBB10 heteroduplexes allow an unambiguous positioning of the regions of nonhomology on the restriction maps (Fig. 2C, Fig. 1). In ColE2-P9, the nonhomologous DNA is in the 2.6- to 5.1-kb map region compared with the 2.6- to 4.4-kb region for ColE2-CA42.

Locations of the colicin and immunity genes. ColE3-CA38 and ColE2-P9 are 80 to 90% homologous, and their restriction maps are very similar (5, 10). Because the positions of the colicin and immunity genes of ColE3-CA38 are known (R. Watson and L. P. Visentin, submitted for publication), the positions of these genes

† Issued as National Research Council of Canada publication no. 20722 and as Chemistry and Biology Research Institute publication no. 1325.

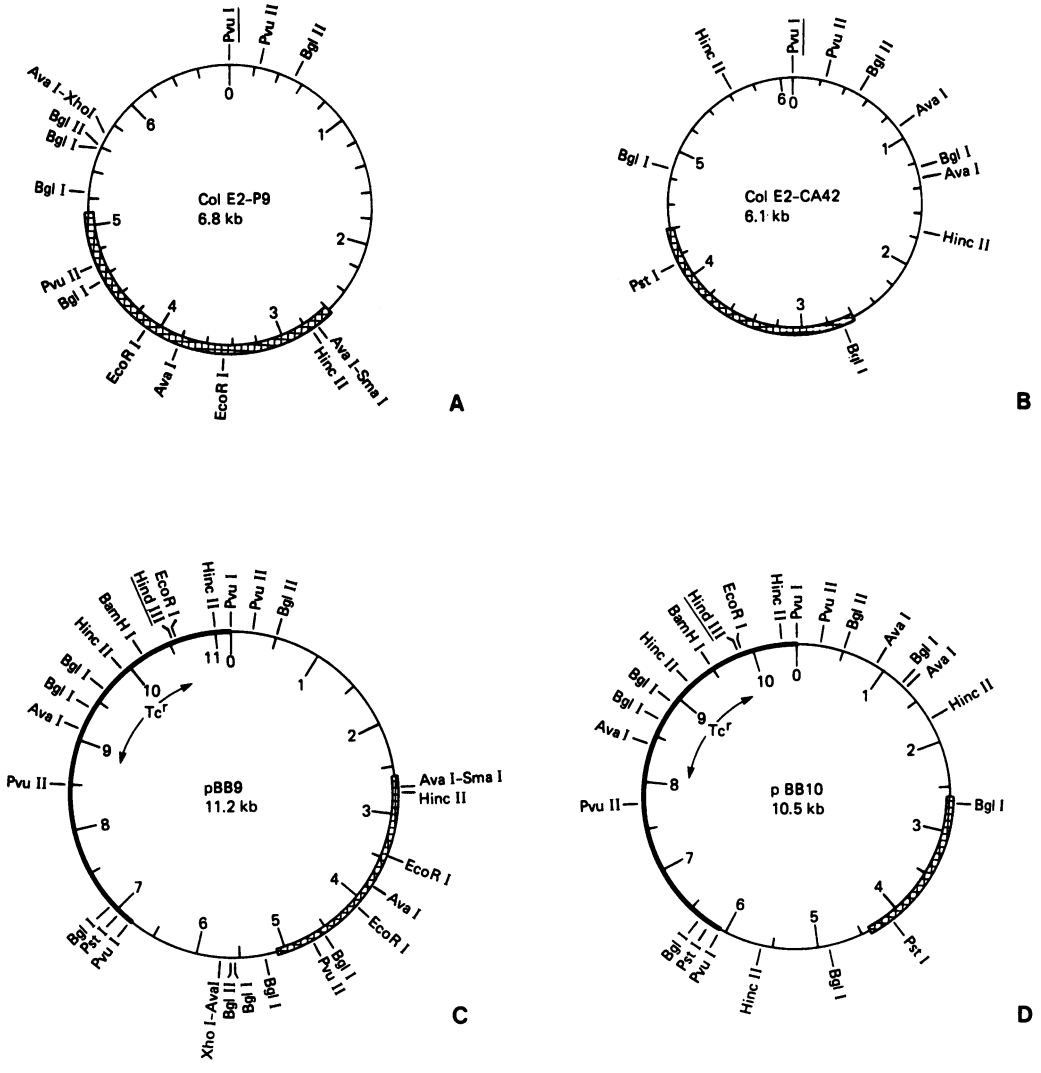


FIG. 1. Restriction maps and regions of nonhomology of ColE2-P9, ColE2-CA42, and ColE2-pBR322 hybrid plasmids. (A) ColE2-P9 (10). (B) ColE2-CA42. (C) pBB9, a hybrid plasmid derived by ligating pBR322 (darker portion) with ColE2-P9 through their unique *Pvu*I sites. The tetracycline resistance gene of pBR322 is indicated. The pBR322 portion is drawn in the opposite directional sense to that which is usually used (8). (D) pBB10, the same as pBB9 except that ColE2-CA42 was used for the construction. For heteroduplex mapping, these plasmids were cleaved at the *Pvu*I or *Hin*dIII sites which are underlined. The regions of nonhomologous DNA are shown as hatched segments of the circles.

for ColE2-P9 and ColE2-CA42 are readily inferred by comparison (Fig. 3). The locations of the E2 colicin and immunity genes have also been tested by cloning portions of the ColE2 plasmids (Table 1; Fig. 3). For ColE2-P9, they have been cloned on a 2.53-kb *Pvu*II-*Hin*cII segment (pBB62, Fig. 3) in agreement with their positions as deduced by comparison with ColE3-CA38. Similarly, the phenotypes of plasmids pBB45 and pBB58 (Fig. 3) localize the ColE2-CA42 colicin and immunity genes to the equivalent region of that plasmid, though these derivatives additionally include about 1.6 kb of DNA adjacent to the presumed 5'-terminal end of the colicin E2-CA42 gene. The E2-immunity genes are at the 3' ends of the colicin genes (pBB46, Fig. 3) such that they might be transcribed together during induction. The E2-immunity genes may contain the *Bgl*II sites in this region of the E2 plasmids, since we have been unable to derive a strain carrying either colicin E2 gene with this site interrupted. The immunity pheno-

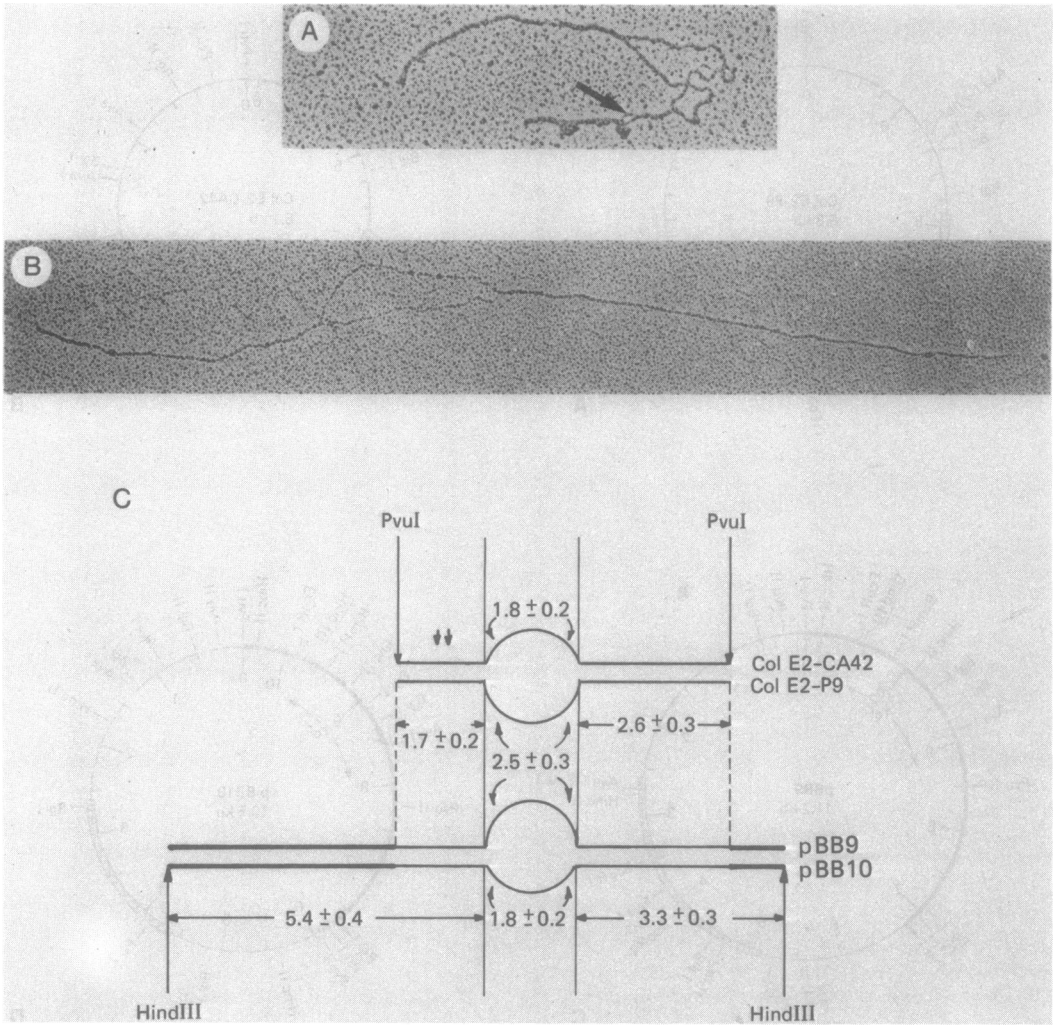


FIG. 2. Heteroduplexes of ColE2 plasmids and ColE2-pBR322 hybrid plasmids. (A) Heteroduplex between the DNAs of ColE2-P9 and ColE2-CA42, showing the large substitution loop and the suspected small region of nonhomologous or inverted DNA (arrow). The latter loop was seen only in some heteroduplexes. (B) Heteroduplex between the DNAs of pBB9 and pBB10; the small region of nonhomologous or inverted DNA was not observed. (C) Interpretation of measurements of heteroduplex molecules of the above two types (14 and 30 molecules, respectively). The numerals shown are the molecular lengths in kikobases (kb), calculated from the relationship 1.0 μm of DNA = 3.3 kb and using pBR322 (4.36 kb) as the standard. The two small arrows on the shorter heteroduplex indicate the position of the small loop that was observed occasionally (1.0 kb from the *PvuI* site on the short arm; loop size, 0.2 kb). Right to left in this diagram corresponds to the restriction maps of the plasmids shown in Fig. 1 taken clockwise from their *PvuI* or *Hin*dIII sites. The heteroduplex mapping techniques used have been described elsewhere (3, 12).

type is not displayed by strains carrying the cloned 1.74-kb *Bgl*II fragment of ColE2-P9 (pBB17, pBB18, Fig. 3).

Comparison of the ColE2-P9 and ColE2-CA42 immunity proteins. It is of interest to determine to what degree the immunity proteins of ColE2-CA42 and ColE2-P9 have deviated in structure while retaining the same functional specificity and to compare them with the ColE3-CA38 immunity protein. Colicins E2-P9 and E2-CA42 were purified from mitomycin C-induced cultures of WA802(pBB9) and WA802(pBB10), respectively, as described by Herschman and Helinski (4) through the DEAE-Sephadex chromatography step. Fractions active against WA802 were pooled, dialyzed against distilled water, and freeze-dried. The protein was dissolved in 44% formic acid and applied to a

TABLE 1. Plasmids used[a]

Plasmid	Description
ColE2-P9	ColE2 plasmid native to *Shigella sonnei* p9 (10).
ColE2-CA42	ColE2 plasmid native to *E. coli* CA42 (9).
ColE3-CA38	ColE3 plasmid native to *E. coli* CA38 (10).
pBR322	Cloning vehicle (1). Phen: Ap^r Te^r. Size: 4.36 kb.
pACYC177	Cloning vehicle (2). Phen: Ap^r Km^r. Size: 4.0 kb.
pBB9	ColE2-P9-pBR322 hybrid plasmid derived by ligation of *Pvu*I-cleaved ColE2-P9 into the *Pvu*I site of pBR322 such that the ColE2-P9 *Pvu*II site is proximal to the pBR322 *Pst*I site (Fig. 1C). Phen: Tc^r Col^+ Imm^+. Size: 11.2 kb.
pBB10	ColE2-CA42-pBR322 hybrid plasmid analogous in derivation and structure to pBB10 (Fig. 1D). Phen: Tc^r Col^+ Imm^+. Size: 10.5 kb.
pBB51	The *Pvu*II to *Eco*RI segment of ColE2-P9 spanning its map coordinates 0.23 to 3.47 kb↔clockwise (Fig. 1A) ligated into pBR322 such that it replaces its *Eco*RI to *Pvu*II segment which contains the tetracycline resistance gene. Phen: Ap^r Col^+ Imm^+. Size: 5.5 kb.
pBB62	The *Pvu*II to *Hin*cII segment of colE2-P9 spanning its map coordinates 0.23 to 2.77 kb clockwise (Fig. 1A) replacing the segment of pBR322 from its *Pvu*II site, through the tetracycline resistance gene and *Eco*RI site, to the *Hin*cII site within the ampicillin resistance gene. Derived from pBB51 by removal of its 1.16-kb *Hin*cII segment. Phen: Col^+ Imm^+. Size: 4.3 kb.
pBB17, 18	The 1.74-kb *Bgl*II segment of ColE2-P9 cloned into the *Bam*HI site of pBR322. In pBB17, the ColE2-P9 *Bgl*II segment is orientated such that its *Pvu*I site is in the position farthest from the pBR322 *Eco*RI site. In pBB18, that *Pvu*I site is closer to the *Eco*RI site. Phen: Ap^r Col^- Imm^-. Size: 6.1 kb.
pBB58	The *Pvu*I to *Pst*I segment of ColE2-CA42 spanning its 0.0- to 4.14-kb map coordinates clockwise (Fig. 1B) cloned in the Ap^r gene of pBR322 such that it replaces the small *Pvu*I-*Pst*I segment therein. Derived from pBB10 by removing its 2.1-kb *Pst*I segment. Phen: Tc^r Col^+ Imm^+. Size: 8.4 kb.
pBB45	ColE2-CA42-pACYC177 hybrid plasmid derived by blunt-end ligation of *Pvu*II-cleaved ColE2-CA42 into the *Sma*I site of pCYC177. Phen: Ap^r Col^+ Imm^+. Size: 10.1 kb.
pBB46	The 2.22-kb ColE2-CA42 *Hin*cII fragment ligated into the *Hin*cII site of pACYC177. Lacks one of the two expected *Hin*cII sites, but no deletion of DNA detectable. Phen: Km^r Col^- Imm^+. Size: 6.2 kb.

[a] Plasmids were constructed by ligation of mixtures of the ColE2 plasmid and cloning vehicle after cleavage with the appropriate restriction endonucleases and were transformed into WA802 by using standard techniques (1, 2, 9, 10). Transformants carrying the desired plasmids were selected by using the antibiotic resistance imparted by the cloning vehicle, followed by testing for the absence of the antibiotic resistance expected to be insertionally inactivated or removed, and then by testing for colicinogenic phenotypes. The transformant carrying pBB62 was selected by using immunity to colicin E2 (9), followed by testing for the absence of ampicillin resistance. Plasmids were then purified and characterized by analysis of their restriction endonuclease cleavage fragments. Phen, Phenotype.

FIG. 3. Locations of the colicin and immunity genes of ColE3-CA38, ColE2-P9, and ColE2-CA42. The top line is a linear restriction map of the 3.78-kb *Pvu*I-*Eco*RI segment of ColE3-CA38 which contains its colicin and immunity genes. A 0.3-kb portion of the map is omitted between the oblique lines to adjust its length to the comparable ColE2-P9 and ColE2-CA42 map segments shown below it. The ColE3-CA38 and ColE2-P9 maps are aligned through their common *Sma*I, *Hin*cII, and *Eco*RI sites; ColE2-P9 and ColE2-CA42 are aligned through their common *Pvu*I, *Pvu*II, and *Bgl*II sites. The approximate position of the region of nonhomology between ColE3-CA38 and ColE2-P9 is indicated, as is part of the region of nonhomology between ColE2-P9 and ColE2-CA42. The ColE3-CA38 colicin and immunity genes were position as described elsewhere (Watson and Visentin, submitted for publication). The equivalent genes of the ColE2 plasmids are shown in the comparable positions. The position of the 5′ end of the colicin E2-CA42 gene is speculative, since the molecular weight of this colicin is not known. The ColE2 immunity genes could be positioned farther to the left, as indicated by the arrows, since they are not within a region of DNA which is homologous to ColE3-Ca38 and thus their positions cannot be compared directly with the immunity gene of ColE3-CA38. The portions of ColE2-P9 and ColE2-CA42 DNA carried by some of the derived plasmids listed in Table 1 are shown. The short vertical lines mark ends of the cloned DNA segments; the arrowheads indicate that the cloned DNA extends outside the plasmid region shown. The phenotypes conferred by the plasmids carrying these segments are indicated at the left.

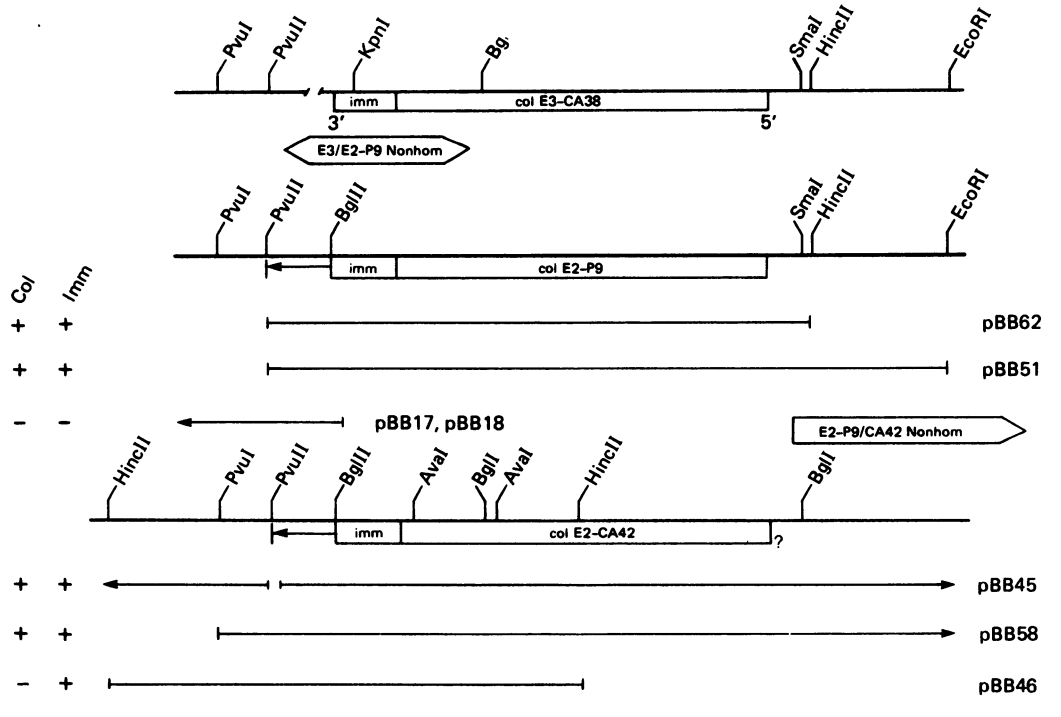
TABLE 2. Amino acid compositions of the colicin E2-P9 and E2-CA42 immunity proteins[a]

Amino acid	E2-P9			E2-CA42			E3-CA38
	24 h	72 h	Proposed no. of residues	24 h	72 h	Proposed no. of residues	No. of residues
Asp	10.8	11.1	11	9.8	9.6	10	18
Thr	3.2	3.3	3	3.8	3.4	4	2
Ser	4.6	4.0	5	4.0	3.4	5	6
Glu	14.5	14.8	15	13.6	13.6	14	8
Pro	3.2	3.3	3	3.6	3.1	3	3
Gly	6.1	6.3	6	7.0	6.9	7	7
Ala	6.9	7.1	7	5.8	6.0	6	1
Cys[b]	1.0		1	1.0		1	1
Val	4.1	4.5	5	3.5	3.9	5	6
Met	0.7	0.5	1	0.9	0.7	1	1
Ile	4.0	4.4	5	4.4	4.8	5	3
Leu	5.0	5.0	5	5.0	5.0	5	6
Tyr	2.1	1.8	2	2.0	1.9	2	4
Phe	3.5	3.3	3	4.1	4.1	4	8
His	1.7	1.8	2	1.8	2.0	2	1
Lys	7.1	7.4	7	7.3	7.4	7	5
Arg	5.7	5.9	6	5.2	5.0	5	1
Trp[c]	0.0		0	0.0		0	3

[a] The amino acid compositions of the ColE2 immunity proteins determined after 24 and 72 h of HCl hydrolysis were calculated relative to leucine (5 mol per mol of 8,800-molecular weight protein). The number of amino acid residues proposed for each protein is given, as well as the known number of each residue for the immunity protein of ColE3-CA38 (11). Amino acid compositions were determined by using a Durram D-500 amino acid analyzer as described previously (7).

[b] Determined as cysteic acid after performic acid oxidation.

[c] After hydrolysis with methanesulfonic acid.



	4	8	12
E2-P9	Met-Glu-Leu-Lys-	His-Ser -Ile -Ser-	Asp-Tyr-Thr-Glu-
E2-CA42	Met-Glu-Leu-Lys-	His -Ser -Ile -Ser-	Asx-Tyr-Thr-Glx-
E3-CA38	(Met)-Gly-Leu-Lys-	Leu-Asp-Leu-Thr-	Trp-Phe-Asp-Lys-
	16	20	24
E2-P9	Ala-Glu-Phe-Leu-	Glu-Phe-Val-Lys-	
E2-CA42	Ala-Glx-Phe-Leu-	Glx-Phe-Val-Lys-	Lys-Ile-Ala-Arg
E3-CA38	Ser-Thr-Glu-Asp-	Phe-Lys-Gly-Glu-	Glu-Tyr-Ser-Lys-

FIG. 4. N-terminal amino acid sequence of immunity proteins of ColE2-P9 and ColE2-CA42. The amino acid sequences of the first 20 residues of the ColE2-P9 immunity protein and the first 24 residues of the ColE2-CA42 immunity protein are shown, beginning with their N-terminal methionines. The first 23 residues of the ColE3-CA38 immunity protein are also shown for comparison. They are aligned such that the N-terminal glycine corresponds to the second residues (*Glu*) of the E2-type immunity proteins. The *Met* residue in parentheses is that coded for by an ATG initiation codon in the corresponding position of ColE3-CA38 DNA, though it is not present in the protein (unpublished data). N-terminal sequence determination by automatic degradation with a Beckman model 890C Protein Sequenator and identification of the thiazolinone or phenylthiohydantoin derivatives have been described previously (7).

Sephacryl 200 column (1.5 by 150 cm) preequilibrated with 44% formic acid and then was eluted with this same solution. The immunity proteins were identified among the fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). They were of molecular weight 8,800 to 9,000, compared with 8,200 for immunity protein from WA802 (ColE3-CA38).

The amino acid compositions and N-terminal amino acid sequences determined for the two E2-immunity proteins are shown in Table 2 and Fig. 4, along with corresponding data for the E3-immunity protein. The E2-immunity proteins have very similar amino acid compositions and identical N-terminal sequences, with the possible exception of the glutamic acid/glutamine or aspartic acid/asparagine residues not differentiated for the ColE2-CA42 protein. The similarity of these proteins may reflect a stringent amino acid sequence requirement to provide complementarity to the colicin E2 molecule, or it may be due to a more recent evolutionary separation of the two E2 plasmids.

In contrast, the E2-immunity proteins are very different in amino acid composition and sequence from the immunity protein of ColE3-CA38, as expected from their different immunity types and their positions within portions of the plasmids which are not homologous. The E3 protein lacks an N-terminal methionine residue, even though the E3-immunity gene has an ATG codon immediately preceding the DNA sequence coding for the amino acids shown in the table (unpublished data). The only similarity of the two immunity protein types is that the glu-leu-lys sequence at residues 2 to 4 of the E2-immunity proteins compares to the sequence of the first three amino acids, gly-leu-lys, of the E3-immunity protein. The glu/gly difference could be due to a single base difference in the DNAs, for example GAA/GGA.

We thank G. Masson, F. Rollins, W. Rowsome, and C. Roy for their skilled technical assistance.

Research at Carleton University was supported by grants from the Natural Sciences and Engineering Council and the Medical Research Council of Canada.

LITERATURE CITED

1. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene (Amst) 2:95–113.
2. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141–1156.
3. **Fiandt, M., A. Hradecna, H. A. Lozeron, and W. Szybalski.** 1971. Electron microscopic mapping of deletions, insertions, inversions and homologies in the DNAs of coliphages lambda and Phi80, p. 329–354. *In* A. E. Hershey (ed.), The bacteriophage lambda. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Herschman, H. R., and D. R. Helinski.** 1967. Comparative study of the events associated with colicin induction. J. Bacteriol. 94:691–699.
5. **Inselburg, J., and V. Johns.** 1975. Mapping of colicin E2 and colicin E3 plasmid deoxyribonucleic acid *Eco*R1-sensitive sites. J. Bacteriol. 121:381–389.
6. **Mochitate, K., K. Suzuki, and K. Imahori.** 1981. Amino acid sequence of immunity protein (B subunit) of colicin E3. J. Biochem. (Tokyo) 89:1609–1618.
7. **Nazar, R. N., M. Yaguchi, G. E. Willick, C. F. Rollin, and C. Roy.** 1979. The 5-S RNA binding protein from yeast (*Saccharomyces cerevisiae*) ribosomes. Evolution of the eukaryotic 5-S RNA binding protein. Eur. J. Biochem. 102:573–582.
8. **Sutcliffe, J. G.** 1978. pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acids Res. 5:2721–2728.
9. **Watson, R., W. Rowsome, J. Tsao, and L. P. Visentin.** 1981. Identification and characterization of Col plasmids from classical colicin E-producing strains. J. Bacteriol. 147:569–577.
10. **Watson, R., and L. P. Visentin.** 1980. Restriction endonuclease mapping of ColE2-P9 and ColE3-CA38. Gene (Amst) 10:307–318.
11. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412.
12. **Westmoreland, B., W. Szybalski, and H. Ris.** 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163:1343–1348.