ABSTRACT

The primary structure and expression of the ColE2-P9 immunity gene (imm) were investigated. The imm gene is located behind the colicin gene (col) in the same orientation with an intergenic space of two base pairs. Although the imm gene was transcribed primarily in response to the SOS function of the host cell as well as the col gene, the immunity phenotype also appeared to be expressed by only a slight level of leaky transcription without an evident promoter. On comparing the ColE2-P9 sequence with those of relevant plasmids, a highly homologous sequence with the immE3 gene was found downstream of the immE3 gene of ColE3-CA38, and thus, an evolitional relationships could be deduced among some E-group Col plasmids.

INTRODUCTION

Colicin E2-P9 is produced as a complex with its immunity protein by E. coli strains harboring plasmid ColE2-P9. Colicin E2 (Mr c.a.60,000) is a kind of DNase whose enzymic activity is located exclusively in its C-terminal portion referred to as T2A (Mr c.a.15,500). The immunity protein (Mr 9,000-10,000) binds to the T2A region nearly stoichiometrically and inhibits the DNase activity (1-3).

Colicin E2 and E3 are homologous colicins which use the same receptor and pathway through the membrane of sensitive cells. They are distinguishable by their final enzymic activities and immunities: E2 is a DNase and E3, an RNase which inactivates ribosomes by cleaving the 16S rRNA at a specific site (4-6). Homology is also evident in their corresponding plasmids ColE2-P9 and ColE3-CA38. Heteroduplex experiments (7,8) and a comparison of restriction maps (9,10) revealed that the major difference between these two plasmids is to be found around the limited region encoding the T2A and immunity protein of each colicin. Colicin E3 is highly homologous to cloacin DF13, which is a bacteriocin specified by plasmid CloDF13 (11,12). E3 and DF13 seem to differ essentially only in their receptor binding regions (13). Nucleotide sequence data showed the homology of the E3 and DF13 genes to continue downstream.
through immunity genes to gene H (hic or the lysis gene), which is responsible for lethality of the host cell and release of colicin (or cloacin) into the medium on its induction (14-19). However, ColE3-CA38 contains an extra segment absent in CloDF13, between the immunity gene and gene H (13,20). Thus, the evolutionary relationships among the E-group Col factors (including ColE2-P9 and ColE3-CA38) and CloDF13 are of much interest.

Production of E-group colicins is induced in response to the SOS signals (21-26). In spite of the apparent operon construction from col through imm up to gene H (18,26), the immunity phenotype toward E2 or E3 seems to depend only partially on the SOS functions (13,21; unpublished data). Thus, we first proposed the possibility of an internal promoter for the immE3 gene within the preceding colicin E3 structural gene (27). The SOS-independent internal promoters were also postulated based on the insertion experiments with transposons into col genes in E2, E3 and E8 (18,26,28). If these internal promoters actually exist, their physiological role must be of much significance for the stable maintenance of colicinogenic strains.

In this paper, we report the cloning and sequence determination of the E2 immunity gene and the flanking regions and examine the possibility of an internal promoter of the immunity gene. Furthermore, we compare the sequence data of ColE2-P9 with those of other plasmids and deduce the evolutionary history among these bacteriocin plasmids.

**MATERIALS AND METHODS**

**Strains and Plasmids**

RR1 (29) and RRlsrlC::Tnl0recA56 (13) were used as recipient strains in the transformation experiments. RR1 was also used as a colicin sensitive indicator. Our original E2 colicinogenic strain was W3110Str [ColE2-P9], provided by H. Yamamoto and T. Beppu. GM3l[pBR327] and GM3l[pBR328] were obtained from F. Bolivar (29). Plasmid pYEJ001, which carries a synthetic strong promoter upstream of cat and tet genes, was constructed in the laboratory of J. Rossi and obtained from P-L Biochemicals. Cells were grown in L-broth at 37° and plasmids were prepared according to (30). Cells were transformed by the method of (31).

**DNA Sequencing**

The sequence of the BamHI fragment of pSH121 was determined by the M13 chain termination method (32). This fragment is a portion of ColE2-P9 and was inserted into the BamHI site of pBR327 (see RESULTS and Figures 1 and 2). For rapid sequencing, we developed the following modification to obtain a subclone
The closed circular DNA of pSH121 was first converted into the open circular form with DNase I in the presence of ethidium bromide, and then into the linear form with nuclease S1. The linear DNA mixture was digested with BamHI, and the fragments with the BamHI cohesive end at one terminus and a non-specific blunt end at the other were subcloned between the BamHI and SmaI sites of M13mp11. Before this BamHI digestion, the DNA mixture was treated with TaqI and alkaline phosphatase to eliminate the possibility of cloning the pBR327 region of pSH121 (there is no TaqI site in the fragment to be sequenced). Thus, the ColE2-P9 fragments of pSH121 deleted various lengths from each BamHI end were subcloned into the sequencing vector. The length and orientation of the subcloned fragments were checked by electrophoresis and appropriate clones were chosen for DNA sequencing by the ordinary method with Amersham Sequencing Kit and \( \left[ S^{35} \right]^{-}5'\)ATP.

Chloramphenicol Acetyltransferase (CAT) Gene Cartridges

To quantify transcription in vivo, we used the structural gene of chloramphenicol acetyltransferase (CAT). According to (33), we constructed pSH101 and pSH102, each containing the BamHI CAT cartridge (originally isolated as a TaqI fragment from pBR328) in the BamHI site of pBR327 (Fig.1). The CAT cartridge is inserted in the same orientation as the tet promoter in pSH101 and in the opposite orientation in pSH102. pSH101 and pSH102 must be identical to pCM4 and pCM4B, respectively (33). The CAT cartridges we used in this study were the TaqI fragment of pBR328 and the BamHI fragment of pSH101 or pSH102. The CAT activity of each cell was assayed according to (34).

Other Procedures

The extent of plasmid immunity to colicin E2 was determined from the apparent titer of E2, the factor of the highest dilution of the purified E2 solution required to make a clear inhibition spot on the top agar initially inoculated with about \( 10^7 \) cells carrying a plasmid concerned. A plasmidless host RRI presented the apparent titer of \( 5 \times 10^6 \). Colicin production was examined as previously described for E3 (13).

Colicin E2 was prepared from W3110Str\( ^{ rf } \)[ColE2-P9] and purified according to (22), except that 0.5M guanidine hydrochloride was used instead of 1M NaCl for the extraction of E2.

RESULTS

Cloning of the E2 Immunity Gene

Plasmid derivations are summarized in Fig.1. ColE2-P9 DNA was cleaved with PvuII, and the larger PvuII fragment was cloned into the PvuII site of
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Figure 1. Construction of plasmids. DNA regions derived from ColE2-P9, ColE3-CA38 and the vector pBR327 or pBR328 are indicated by the thick, boxed and solid lines, respectively. CAT genes are shown by shadowed arrows. The wavy line of pSH301 represents an unexpected short deletion and insertion of unknown origin containing a ClaI site (13).

Ap and Tc indicate bla and tet genes, respectively. Restriction sites are designated as: (P)PvuII, (G)BglII, (C)ClaI, (H)HincII, (F)HinfI, (P)PstI, (E)EcoRI, (B)BamHI, (D)DraI, (X)XhoI and (K)KpnI. For some restriction sites, only those concerned in derivation are shown. The restriction enzymes used in each derivation step are as follows: (a)PvuII, (b)HincII, (c)HinfI and BamHI, (d)BamHI and DraI, (e)BgIII and PstI, (f)TaqI and BamHI, (g)TaqI and ClaI, (h)BamHI, (i)BamHI, (j)PvuII, (k)ClaI.

pBR328. The resulting plasmid pSH201 was then truncated with HincII to give pSH203. Plasmid pSH203, as well as pSH201 and their parental plasmid ColE2-P9, conferred both immunity (Imm+) and SOS-dependent colicin production (Col+) on the host cell.

The HinfI recognition sites were mapped on the pSH203 DNA (Fig.2), and the c.a.640 bp HinfI fragment, possibly carrying the E2 immunity gene by analogy with ColE3-CA38 (13), was isolated, treated with nuclease SI and recloned into pBR327 at the BamHI site whose cohesive ends had been filled-in with T4-DNA polymerase prior to ligation. The cloning junctions to the vector thus retained the BamHI sites. The resulting plasmids pSH121 and pSH122 contained this c.a.640 bp BamHI fragment in the opposite orientations to each other. The BamHI fragment was isolated from pSH121, treated with T4-DNA polymerase and recloned into the DraI sites in the bla gene of pBR327, giving rise to pSH123 and pSH124, containing this fragment in the opposite orientations to each other. All the RRI transformants carrying pSH121 to pSH124 were fully immune to colicin E2.

Nucleotide Sequence of the E2 Immunity Gene and the Flanking Regions

The nucleotide sequence of the pSH121 BamHI fragment (equall to the ColE2-
Figure 2. Structure of the ColE2-P9 DNA fragment and sequencing strategy.
a) Restriction map of the HincII-PvuII fragment of pSH203. b) Location and orientation of the SOS-dependent promoter (Pr), col, imm and gene H. c) The BamHI fragment of pSH121 and pSH122 derived from the HinfI fragment of pSH203. d) Arrows indicate the direction and extent of DNA sequences determined. Restriction sites are designated as in Fig.1 except for (A)AatII.

P9 HinfI fragment conferring E2 immunity) was determined and the amino acid sequences were deduced (Fig.3). An open reading frame starting at nucleotide number 320 specified 86 amino acids and the sequence of the first 20 amino acids coincided with the N-terminal sequence of the ColE2-P9 immunity protein determined experimentally (10). A comparison of the amino acid composition deduced from this reading frame with those determined previously (10,35; Table 1) confirmed the immE2 gene to be located from 320 to 577. The molecular weight of the E2 immunity protein was calculated to be 9995.

Just preceding the immunity gene, another long reading frame was found from 3 to 314. This frame encoded the C-terminal portion of colicin E2, since its C-terminal sequence, -His-Arg-Gly-Lys-COOH, was consistent with the C-terminal part of the E2-T2A fragment, -(His,Gly,Arg)-Lys-COOH, determined previously using carboxy peptidases (3). Although the number of amino acids defined by this region in the cloned fragment [104] is smaller than that of the T2A fragment [137], comparison of amino acid compositions showed good correlation between them (36; data not shown). No sequence homology was found in the T2A and immunity regions between ColE2-P9 and ColE3-CA38.

Expression of Immunity

To examine the transcriptional regulation of the col and imm genes, we introduced the CAT gene cartridge into some plasmids. First, a new Col^Imm^ plasmid pSH208 was constructed by the recombination between pSH203 and pSH122 at their BglII and PstI sites. Thus, a unique BamHI site was introduced behind the imm gene in pSH208 (Fig.1). Insertion of the TaqI CAT cartridge into the Clal site within the col gene of pSH208 gave pMT221 and insertion of the BamHI CAT cartridge into the BamHI site of pSH208 gave pMT231 (Fig.1). The CAT activity of the cells harboring these plasmids was measured under several conditions (Table 2-1). While the CAT activity of pMT221 was considerably low.
Figure 3. Nucleotide and deduced amino acid sequences of the ColE2-P9 HinfI fragment conferring E2 immunity. The corresponding regions of ColE3-CA38 (13) and CloDF13 (12) sequences are also presented from the termination codons of their immunity genes, and aligned so as to have much homology in the downstream regions. The numbering of the ColE3-CA38 DNA starts at the 5'-end of the HinfI site. Vertical lines indicate homologous nucleotides. Our sequence data disagree with those reported recently by (20,45) in positions 83 and 182 in ColE2-P9 and 2367-2368 in ColE3-CA38.
Table 1. Amino acid composition of the ColE2-P9 immunity protein.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>This work</th>
<th>Jakes (33)</th>
<th>Watson et al. (12)</th>
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<tr>
<td>Asp</td>
<td>8</td>
<td>10</td>
<td>11</td>
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<tr>
<td>Asn</td>
<td>2</td>
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<td>3</td>
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</tr>
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</tr>
<tr>
<td>Trp</td>
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<td>Total: 86</td>
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under a normal condition (less than 10% that of pSH101 in which transcription must be driven by the tet promoter), the addition of mitomycin C greatly increased the activity as much as about 50 times even by incubation for only four hours. In contrast, the introduction of the chromosome mutation recA56 decreased the CAT activity by about one third in the absence of an inducer. Almost the same phenomenon was observed for pMT231. Although the CAT activity of pMT221 and pMT231 cannot be compared directly because of different physiological backgrounds (Col v.s. Col+), these findings evidently show that both

Table 2. Comparison of CAT specific activities.

<table>
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<th>(I) Plasmid Host</th>
<th>~MMC</th>
<th>+MMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSH208 RR1</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>pSH208 RR1recA56</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>pMT221 RR1</td>
<td>67</td>
<td>3099</td>
</tr>
<tr>
<td>pMT221 RR1recA56</td>
<td>28</td>
<td>ND</td>
</tr>
<tr>
<td>pMT231 RR1</td>
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<td>3555</td>
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<tr>
<td>pMT231 RR1recA56</td>
<td>47</td>
<td>ND</td>
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<table>
<thead>
<tr>
<th>(II) Plasmid Host: RR1</th>
</tr>
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<tbody>
<tr>
<td>none</td>
</tr>
<tr>
<td>pSH101</td>
</tr>
<tr>
<td>pSH102</td>
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<tr>
<td>pSH103</td>
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</tbody>
</table>

unit: n mol/min/mg prot. ND: not determined

Experiment I and II were performed at the same time. 0.03ml of each overnight culture were diluted in the fresh 2ml L-broth and further incubated for 4 hours at 37° in the presence (I) or absence (I,II) of 0.5μg/ml mitomycin C (MMC). The CAT activity of the sonicated cell extract was normalized by the total protein. In experiment II, the values were taken from five (a,b), nine (d) or ten (c) independent cultures.
Figure 4. Structural comparison and evolutionary implication of ColE2-P9, ColE3-CA38 and CloDF13. Shadowed areas show the regions with little sequence homology. The N-terminal regions of colicin E3 and cloacin DF13 share the property rich in glycine in spite of little sequence homology. "immF7-1ike RF" of ColE3-CA38 represents the reading frame homologous to the immunity gene of ColE2-P9 (probably the "pseudo-immE8" gene). Restriction sites are indicated as: (A)AatII, (Bc)BclI, (XH)XhoII, and (S)Sau3AI. Other sites are designated as in Fig.1. Corresponding restriction sites are connected by broken lines. col and imm genes are integrated into a single operon whose transcription is regulated by the SOS function of the host cell. Thus, the transcription of the imm gene seems to depend primarily on the SOS-responding promoter of the col gene. Nevertheless, immunity was completely expressed by our four plasmids pSH121-124, all lacking this promoter and carrying only the last part of the col gene and the imm gene (Figures 1 and 2). Among these plasmids, transcription of the imm genes of pSH121 and pSH123 must be driven by tet and bla promoters, respectively, whereas the imm genes of pSH122 and pSH124 face in directions opposite to these promoters. One explanation for this discrepancy may be the presence of a weak internal promoter within the cloned BamHI fragment. To evaluate the transcriptional activity in vivo within the BamHI fragment of pSH122, the CAT activity of pSH102 and pSH103 was compared. Plasmid pSH103 has the pSH122-derived BamHI fragment preceding the CAT cartridge of pSH102 and the imm gene faces in the same direction as the CAT gene (Fig.1). However, hardly any significant increase in CAT activity could be detected through the BamHI fragment of pSH122 (Table 2-II). Furthermore, we constructed two pSH124-like plasmids, differing from pSH124 only in that the upstream cloning sites were two DraI.
sites numbered 51 and 123 in Fig. 3, respectively. These two plasmids also conferred complete immunity to E2 as in the case of pSH124 (data not shown).

Hybrid Colicins between E2 and E3

ColE2-P9 and ColE3-CA38 are homologous plasmids as described above. As far as the regions around colicin genes are concerned, these two plasmids could be distinguished physically by restriction sites between ClaI-BglII of ColE2-P9 and ClaI-Sau3AI of ColE3-CA38 (Fig. 4). To verify this domain structure in the light of their functions, we tried to construct hybrid colicins between E2 and E3; pSH201 (a ColE2-P9 derivative) and pSH301 (a ColE3-CA38 derivative; 13) were cleaved with ClaI, and ClaI fragments were exchanged between them and ligated. Consequently, E2 and E3 were recombined in their coding regions at the ClaI sites (Fig. 1). The resulting plasmid pSH631 contained the upstream PvuII-ClaI segment from ColE2-P9 and the downstream ClaI-PvuII segment from ColE3-CA38, and pSH621, vice versa. These plasmids were maintained stable and showed the phenotypes Col and Imm, both completely specified by the new downstream ClaI-PvuII segments.

DISCUSSION

A small fraction of colicinogenic cells spontaneously produce colicin (37, 38), and the culture medium of E2 or E3 colicinogenic strains usually contains a sufficient amount of colicin molecules to kill sensitive cells even in the absence of the SOS inducer. Thus, colicinogenic cells must always protect themselves from the killing action of colicin in the medium. Nevertheless, the E2 and E3 immunity genes are integrated in an SOS operon, and in fact, synthesis of immunity proteins is induced by the addition of mitomycin C (23, 26, 39). Our data provide an answer to the question as to how inducible immunity proteins protect the host cell in uninduced state. The BamHI fragment carrying imm and the last part of col of ColE2-P9 conferred complete immunity to E2 even when there was virtually no transcription from the vector plasmid. It is conceivable that an internal promoter precedes the imm gene within the cloned fragment. A comparison of the CAT activity of pSH102 and pSH103, however, showed no significant increase in transcription by this BamHI fragment (Table 2-II), and thus, participation of an internal promoter was not probable. Table 2-II also shows that a slight but significant level of transcription can be still detected at the BamHI site of pBR327 in the opposite direction to the tet gene (about one per cent of that in the normal direction; 33). This leaky transcription from the vector region should be responsible for the complete immunity of pSH122, and probably for that of pSH124 (33).
The above mentioned results may not completely exclude the possibility of a very weak internal promoter of the imm gene in the native ColE2-P9 plasmid. In any event, however, it may be concluded that the immunity phenotype to E2 can be sufficiently expressed by only a slight level of leaky transcription of the imm gene without any definite internal promoter.

As to the discrepancy between the constitutively expressed phenotype of E3 immunity and the inducible feature of colicin E3 and lysis genes, Jakes and Zinder postulated an SOS-independent promoter and terminator set for the imm gene within the SOS-dependent col-imm-lysis operon (18). But we consider this idea to be unnatural. The different thresholds in transcription required for phenotypes between immunity and colicin production (and lysis) is probably responsible for this discrepancy.

The recombination experiment between pSH201 and pSH301 clearly showed the specificities of colicin activity and immunity of both E2 and E3 to be defined exclusively by the Clal-PvuII segments in these two plasmids. From characterization of the proteolytic fragments of E2 and E3, their receptor binding activities appear to be carried on the central parts of these molecules and translocation activities across the membrane on the N-terminal parts (3). The Clal site is assumed to be in the receptor binding region in the E3 gene (13), and thus, above finding also implies that colicin E2 and E3 molecules are exchangeable with each other in the domains responsible for both binding to the receptor and translocation across the membrane.

Of eight classes of the E-colicins (A,E1,---,E7) and one cloacin (DF13), colicins A and E1 seem to differ entirely from the others in both structural and functional aspects (25,40). The other bacteriocins show good homology with each other and appear to be roughly divided into two types (E2-type and E3-type) based on physiological properties, though structural and biochemical information is very limited. Thus, we compared available data on the E2, E3 and DF13 sequences (12,13). The promoters and structural genes of E3 and DF13, as well as those of E1 and E3, were compared previously in detail (13). Although the boundary between homologous and nonhomologous regions may be difficult to determine precisely only from the sequences, and our interpretation of the homology between ColE3-CA38 and CloDF13 is somewhat different from that recently proposed by Watson et al. (20), we consider the following to be most probable. As seen in Fig.3, the homologous region continues downstream for 71 bp behind the termination codons of the imm genes. Following the 401 bp extra DNA segment of ColE3-CA38, absent in CloDF13, a highly homologous sequence starts again at 13 bp upstream of the initiation codon of gene H.
Thus, ColE3-CA38 may have derived from CloDF13 by the 401 bp insertion or CloDF13 from ColE3-CA38 by deletion.

A comparison of the E2 and E3 sequences showed the latter c.a.350 bp region of the ColE2-P9 HindII fragment to be highly homologous to ColE3-CA38 (Fig. 3). To our surprise, the immE2 gene was completely included within this homologous region, that is, ColE3-CA38 retained an immE2-like reading frame following its own immE3 gene. This frame starts by GTG (Val) as compared with ATG of the true immE2 gene, and possibly encodes 85 amino acids, one residue fewer than that of the true E2 immunity protein. This frame is also preceded by GAGCTG, complementary to the 3'-terminal sequence of the 16S rRNA (41). To examine the immunity activity of this region, we constructed a recombinant plasmid carrying the BclI-PvuII fragment of ColE3-CA38 within the BamHI-NruI sites of the tet gene of a synthetic promoter vector pYEJ001 which must promote the transcription of the cloned fragment. However, this recombinant plasmid, as well as its parental plasmid ColE3-CA38, showed hardly any significant immunity toward E2 (data not shown). This reading frame thus does not function as an immE2 gene. Furthermore, deletion of this region from ColE3-CA38 derivatives had no influence on either E3 production or E3 immunity (13; unpublished data).

Recently, James' group reported that ColE3-CA38 confers immunity to both E3 and newly isolated colicin E8-J, and also showed that the plasmid region between the immunity and lysis genes of ColE3-CA38 is responsible for the immunity toward E8 (42,43). Thus, the immE2-like sequence of ColE3-CA38 is more likely the pseudo-immE8 gene than the pseudo-immE2 gene. Our discovery of this pseudogene strongly suggests that ColE3-CA38 was derived from ColE8-J, and in turn, ColE8-J from ColE2-P9 or ColE2-P9 from ColE8-J. ColE2-P9 and ColE8-J must be closely related and one may evolved from the other through the accumulation of point mutations within the plasmid domain of the imm gene and the corresponding col region. Then, plasmid ColE8-J and a "proto-E3 gene" may possibly have recombined asymmetrically in such a way that the region encoding the C-terminal part of E8 was displaced by the E3-T2A and its inhibitor (immunity) regions, giving rise to the colE3 and immE3 genes followed by the pseudo-immE8 gene of ColE3-CA38 (Fig. 4). Since E8 can be considered an E2-type colicin entirely different from the E3-type with respect to physiology and immunity, just at the moment of the segregation of ColE3-CA38 from its parent ColE8-J, the remaining pseudo-immunity gene may have been of some advantage for ColE3-CA38 to survive in the vast population of the E8-colicinogenic cells.
The evolutional relationships among E2, E8 and E3 necessarily lead to the speculation that ColE3-CA38 did not evolve from CloDF13 by insertion, but CloDF13 from ColE3-CA38 by deletion of the 401 bp region containing the pseudo immE8 gene. Males and Stocker reported that K12[CloDF13] was immune to colicin E6-CT14 (44). This suggests another relationship that ColE6-CT14 is positioned between ColE3-CA38 and CloDF13 in evolution. ColE6-CT14 confers partial immunity toward E8, as in the case of ColE3-CA38 (42). Thus, ColE6-CT14 may possibly still retain the pseudo-immE8 gene, while the immE6 and the corresponding col regions have already been altered to the CloDF13 type. Finally, deletion of the pseudo-immE8 region, together with alteration of the receptor binding region, may have given rise to CloDF13.

The regulation and specificity of immunity must be of great significance to the survival of bacteriocinogenic cells in a limited intestinal environment. It is of special interest that these E-group Col factors have developed various countermeasures by modifying limited regions on each plasmid DNA. A detailed structural and functional investigation of all these Col plasmids should clarify the evolutionary history.

REFERENCES