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Molecular Structure and Immunity Specificity of Colicin E6, an Evolutionary Intermediate between E-Group Colicins and Cloacin DF13

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The primary structure of a 3.1-kilobase E6 or E3 segment carrying colicin and related genes was determined. Plasmid ColE6-CT14 showed striking homology to ColE3-CA38 throughout this segment, including homology to the secondary immunity gene, *immE8*, downstream of the E6 or E3 immunity gene. The ColE3-CA38 and ColE6-CT14 sequences, however, contained an exceptional hot spot region encoding both the colicin-active domain (RNase region) and the immunity protein, reflecting their different immunity specificities. On the other hand, some chimeric plasmids were constructed through homologous recombination between colicin E3 and cloacin DF13 operons. The resulting plasmids were deduced to produce chimeric colicins with a colicin E3-type N-terminal part, a cloacin DF13-type C-terminal-active domain, and the DF13 immunity protein. The killing spectra of the chimeric colicins and the immunities of the plasmids were identical to those of colicin E6 and ColE6-CT14, respectively, showing that the colicin E6 immunity specificity is completely equivalent to that of cloacin DF13. Nevertheless, colicin E6 has been found to show a sequence diversity from cloacin DF13 almost to the same extent as that from colicin E3 in their RNase and immunity regions, indicating that only a small number of amino acids defines the immunity specificity for discrimination between colicins E3 and E6 (or cloacin DF13).

E-group colicins are antibacterial proteins which use an outer membrane protein, BtuB, as a common receptor to kill *Escherichia coli* cells. They are further divided into 10 subclasses, colicins A and E1 to E9, according to their immunity specificities (15). The killing actions of some E-group colicins are attributed to their nuclease activities in vitro. Colicins E2 and E8 are DNases, and colicin E3 is a special kind of RNase which effectively acts on the 16S RNA of the 70S ribosome (14, 16, 19). These enzymatic activities are exclusively defined by the C-terminal portion of each colicin molecule, which is referred to as T2A, and the immunity protein specifically binds to the T2A domain of both endogenous and exogenous colicin molecules, conferring immunity on the host cells. Almost the same activity and domain structure as in the case of colicin E3 have been found for cloacin DF13 (4, 6, 15).

Colicins are encoded by Col plasmids and are produced in large amounts in response to the SOS function of the host cells. The operon of the nuclease-type colicins has, as far as has been elucidated, a promoter-operator region containing two units of the SOS box (the LexA-binding site) followed by three kinds of structural genes: the colicin (*col*), immunity (*imm*), and lysis genes (1, 10, 19, 20, 22). Sequence analysis of the ColE2-P9, ColE3-CA38, ColE8-J, and CloDF13 plasmids revealed that there is a unique structural relationship among them, and so we have proposed the following evolutionary pedigree: E2 ↔ E8 → E3 → DF13 (11, 19). More exactly, however, such a model should be drawn in terms of the ancestral bacteriocins. One of the most important bases of the inference given above is that *immE8* is retained downstream of the ColE3-CA38 immunity gene (*immE3*), while the 401-base-pair segment containing *immE8* is deleted from CloDF13 (11). Between ColE3-CA38 and CloDF13, there are two other notable discrepancies in their colicin or cloacin phenotypes. First, CloDF13 does not show immunity to colicin E3, even though it exhibits almost the same in vitro activity toward ribosomes and there is extensive sequence homology between their *imm* genes (9). Second, the structures of their receptor-binding regions are entirely different, corresponding to the different receptors to which they bind. Since *E. coli* K-12 lacks the receptor for cloacin DF13, the immunity phenotype of Col plasmids to cloacin DF13 cannot be examined genetically in K-12 strains, unless the cloacin receptor is artificially introduced into the cells (7), in contrast to the situation of the immunity of CloDF13 to colicins.

Plasmid ColE6-CT14 has a region that is structurally similar to the ColE3-CA38 colicin operon and shows immunity to E8 besides the immunity to its own colicin, E6, as in the case of ColE3-CA38 (2, 23). On the other hand, plasmid CloDF13 shows immunity to E6 (8) and a ColE6-CT14 fragment confers immunity to klebicin A1, a phenotypic homolog of cloacin DF13, on *Klebsiella pneumoniae* (5). Thus, when it has been proved that the specificities of colicin E6 and its immunity protein are intrinsically equivalent to those of cloacin DF13 and its immunity protein, respectively, it can be said that colicin E6 is a chimera of colicin E3 and cloacin DF13, suggesting an evolutionary intermediate between E-group colicins and cloacin DF13. Here we report the cloning and complete nucleotide sequence of the colicin E6 operon and discuss the immunity specificities of the E-group colicins and cloacin DF13.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* K-12

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TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a]	Source or reference
E. coli		
RR1	F⁻ λ⁻	17
GM33 (CGSC 5126)	F⁻ λ⁻ *dam*	H. Uchida
Plasmids[b]		
pBR327	*bla tet*	F. Bolivar (17)
pUC18, pUC19	*bla*	24
pSH350	*colE3 immE3 immE8 lys bla*	18
pSH312	*colE3 immE3 bla*	18
pSH131	*immE3 bla*	10
ColE6-CT14	*colE6 immE6 immE8 lys*	R. James (8)
ColE6-Ind8	*colE6 immE6 immE8 lys*	D. P. Brunner (8)
CloDF13	*cloDF13 immDF13 brp*	H. J. J. Nijkamp (22)
CloDF13::Tn*A*	*cloDF13 immDF13 brp mob*::Tn*A*(*bla*)	H. J. J. Nijkamp

[a] *lys*, The lysis gene; *brp*, the bacteriocin release protein gene.
[b] The other plasmids are described in the text.

RR1 was used as both a colicin-sensitive indicator strain and a recipient strain in most of the transformation experiments. Cells were grown at 37°C in modified L broth, containing 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), 5 g of NaCl, and 20 μg of thymine per liter. Plasmid preparation and restriction analysis have been described previously (10, 11).

Transformation with plasmids. Cells were transformed as described previously (11), and transformants were selected with 50 μg of ampicillin per ml, an appropriate concentration of E3 or E6, or both. In some cases, colicin- or cloacin-producing transformants were selected without the help of immunity phenotypes by the triple-layer method, as follows. After postincubation, each transformation mixture was spread with 0.5% soft agar onto an L-agar plate and then overlaid with uninoculated soft agar. The plate was incubated overnight and then overlaid with the third soft agar layer containing sensitive cells. Colicin- or cloacin-producing clones were picked up and purified from the center of the haloes that were formed on the sensitive cell layer.

DNA sequencing. The 2.85-kilobase (kb) *Hin*cII fragment of ColE6-CT14 (*Hin*cIIa-*Hin*cIIb; see Fig. 1) was inserted into the *Hin*cII sites of both pUC18 and pUC19, in such a way that the colicin operon was oriented in the opposite direction to the *lac* promoter. On the other hand, the 1.6-kb *Cla*I-*Pvu*I fragment of ColE6-CT14 (Fig. 1) was inserted into the *Bam*HI site of pUC19 in both orientations, after all the joining sites were filled in with the Klenow fragment of DNA polymerase. The resulting four recombinant pUC derivatives were used as master plasmids to obtain a series of unidirectional deletion plasmids for chain-termination sequencing, as described previously (19). Most of the DNA regions were sequenced from both strands.

Other procedures. Colicins E2, E3, and E8 were purified as described previously (10, 11, 19). E6 was partially purified from W3110(ColE6-CT14) in the 30 to 55% ammonium sulfate precipitation fraction, which was dialyzed against 10 mM Tris hydrochloride (pH 7.6).

Colicin or cloacin production was examined by the stab test. Immunity was checked by the cross-streak test or quantified by the spot test as described previously (10).

RESULTS

Gene localization and identification in ColE6-CT14. The physical map of the 3.1-kb *Hin*cIIa-*Pvu*I segment of ColE6-CT14 is very similar to that of ColE3-CA38, which carries the whole colicin operon (23) (Fig. 1). To localize and identify genes in this ColE6-CT14 segment precisely, we constructed two chimeric plasmids from pSH312 and ColE6-CT14 (Fig. 1). In pSH312 a partial ColE3-CA38 colicin operon was deleted downstream from the *Eco*RI site within the *immE8* gene, and it presented the Ap[r], ColE3^{+}, ImmE3^{+}, ImmE8^{−}, and Lys[−] phenotypes (18). The first chimeric plasmid, pAM361, in which the 1.0-kb *Cla*I-*Eco*RI

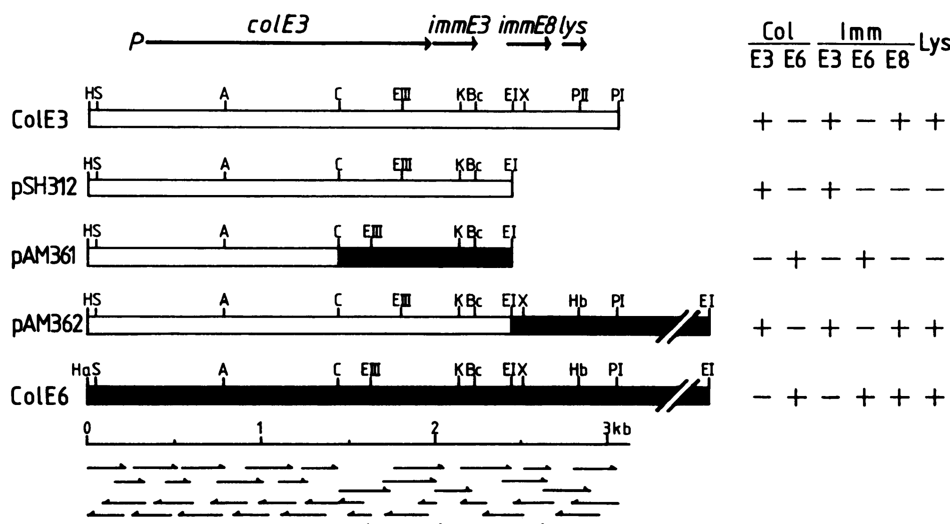


FIG. 1. Restriction maps and phenotypes of ColE3-CA38 (open boxes) and ColE6-CT14 (solid boxes) and their derivatives. The outer regions of the plasmids are not shown, for clarity. The locations and orientations of ColE3-CA38 genes are indicated at the top. *P*, *col*, *imm*, and *lys* are the promoter and the colicin, immunity, and lysis genes, respectively. Arrows at the bottom show the directions and extents of the ColE6-CT14 sequences that were determined. Restriction site abbreviations: H, Ha, and Hb, *Hin*cII, *Hin*cIIa, and *Hin*cIIb, respectively; S, *Sma*I; A, *Aat*II; C, *Cla*I; EIII, *Eco*47III; K, *Kpn*I; Bc, *Bcl*I; EI, *Eco*RI; X, *Xho*I; PII, *Pvu*II; PI, *Pvu*I.

fragment of pSH312 was replaced with that of ColE6-CT14, showed the ColE6$^+$ and ImmE6$^+$, instead of the ColE3$^+$ and ImmE3$^+$, phenotypes. In the second plasmid, pAM362, the ca. 7.5-kb *Eco*RI fragment of ColE6-CT14 was inserted into the *Eco*RI site of pSH312. This insertion regenerated ImmE8$^+$ and Lys$^+$, which was lost in pSH312. These results indicate that the *Cla*I and *Eco*RI sites of ColE6-CT14, and probably the whole *Hin*cIIa-*Pvu*I fragment, functionally coincide with those of ColE3-CA38, including the *immE8* gene. Furthermore, both the colicin and immunity specificities of the two Col plasmids were determined exclusively by the 1.0-kb *Cla*I-*Eco*RI fragments.

Preliminary analysis indicated that there is no difference between the restriction maps of the two types of colicin E6 plasmids, ColE6-CT14 and ColE6-Ind8 (data not shown).

Nucleotide sequence of the colicin E6 operon. The nucleotide sequence of the ColE6-CT14 *Hin*cIIa-*Pvu*I fragment was determined and compared with the corresponding region of ColE36-CA38 (10) (Fig. 2). Striking sequence homology was evident throughout these two plasmids. There were four major open reading frames in the ColE6-CT14 sequence, implying the *colE6*, *immE3*, *immE8*, and lysis genes, respectively, which is consistent with the results of the gene mapping described above. The promoter-operator regions of ColE3-CA38 and ColE6-CT14 had almost the same structure, and the N-terminal 444 of 551 amino acids deduced from *col* genes were identical. The most outstanding feature was that the about 500-bp region encoding the colicin C-terminal T2A domain and the immunity protein formed a mutational hot spot. Amino acid sequences of this region are presented in Fig. 3 (see also below). Mock et al. (13) have identified another small gene within the *col* gene of ColE3-CA38 in the +1 phase. The initiation codon of this reading frame, however, is replaced by AGG (positions 1673 to 1675) in ColE6-CT14, and a termination codon is found in phase in the downstream region (positions 1748 to 1750), suggesting that the overlapping gene of ColE3-CA38 is of no importance for colicin function or expression.

Amino acid sequences deduced from the *immE8* and lysis genes of ColE6-CT14 are shown in Fig. 4. Although Cooper and James (2) have reported that both ColE3-CA38 and ColE6-CT14 decreased immunity to E8 compared with that in ColE8-J, we could not observe such differences in immunity to E8 among the three plasmids. Thus, we consider that a few nonhomologous amino acids in the three ImmE8 sequences are less essential for the ImmE8 function.

Construction and characterization of chimeric colicins E3::DF13. There has been no direct evidence of whether the immunities of colicin E6 and cloacin DF13 are intrinsically equivalent to each other. These two bacteriocins have different receptor-binding regions at the center of their sequences and similar but different N-terminal sequences which are required for the transfer of the respective bacteriocins across the membranes of sensitive cells (10, 22). To compare their immunity phenotypes on a common protein structural basis, we tried to obtain chimeric colicins composed of the colicin-type membrane transfer and receptor-binding region and the DF13-type T2A region, together with the DF13 immunity protein (Fig. 5).

Plasmid pAM3DF is a pSH312 derivative whose *colE3* and *immE3* genes are followed by the CloDF13 *Bst*NI fragment encoding the nuclease domain of DF13, the immunity protein, and the lysis protein (or bacteriocin release protein [3]). The junction between the ColE3-CA38 and the CloDF13 segments on pAM3DF was cleaved in such a way that *immE3* was also disrupted. Linearized DNA was introduced into strain RR1, with the expectation of intracellular recombination in homologous regions, and colicinogenic transformants were selected for Ap^r and colicin production by the triple-layer method. When RR1 harboring pSH131 (Ap^r ImmE3$^+$) was used as the indicator strain in this experiment, only colicinogenic, i.e., noncloacinogenic, clones with an immunity phenotype other than ImmE3 formed clear inhibition spots.

We isolated seven independent colicinogenic clones and determined the recombination sites of these chimeric plasmids by DNA sequencing. Each of the sequences proved to have changed from that of ColE3-CA38 to that of CloDF13 within one of the three homologous regions (regions marked 1 to 3 in Fig. 3), near the 5′ end of the T2A-coding region. We referred to these three types of plasmids as pAM3D1, pAM3D2, and pAM3D3, with the recombination sites being marked 1, 2, and 3, respectively, in Fig. 3. pAM3D2 and pAM3D3 were deduced to synthesize colicins with the same primary structure.

All the chimera colicins effectively killed *E. coli* cells harboring ImmE3$^+$ plasmids but did not kill cells harboring ColE6-CT14 or CloDF13::Tn*A*. On the other hand, the chimeric plasmids were stably maintained and showed immunity not to E3 but to E6 in the cross-streak test. When examined more quantitatively by the spot test, pAM3D1 and pAM3D3, as well as ColE6-CT14 and CloDF13::Tn*A*, were found to confer perfect immunity on the host cells to the most concentrated E6 preparation, while a 4^{13} times dilution of this E6 preparation could kill sensitive cells harboring pBR327. These results show that the immunity specificity of CloDF13 is exclusively defined by its T2A-*imm* gene region and that the immunity specificity of CloDF13 is completely equivalent to that of ColE6-CT14.

DISCUSSION

Although E3 and DF13, and certainly E6, exhibit almost the same activities toward rRNA in vitro, their immunities, i.e., the binding specificities between colicin (or cloacin) and immunity proteins, are entirely different. Thus, the immunity specificities must be determined by nonhomologous amino acids within the T2A regions and immunity proteins. Only from the phenotypes of the chimeric E3::DF13 plasmids mentioned above could we speculate that the T2A-*imm* region of ColE6-CT14 would have a structure identical to that of CloDF13. Surprisingly, however, ColE6-CT14 was found to have a sequence diversity from CloDF13 that was almost of the same extent as that from ColE3-CA38 (Fig. 3). This finding, however, helped us to exclude some of the nonhomologous amino acids from the list of candidates for immunity determinants.

Of 97 amino acids, 10 were nonhomologous between the E3-T2A and E6-T2A domains. Two of them (at amino acid positions 457 and 531) were nonhomologous between E6 and DF13 as well. The identity of the phenotypes in pAM3D1 and pAM3D2 (or pAM3D3) mentioned above also supports the exclusion of residue 457 from the candidates for immunity determinants. As a first approximation, we can thus restrict the T2A immunity determinants which discriminate ImmE3 and ImmE6 (ImmDF13) to the following eight amino acid pairs; Ala/Asp-467, Asn/Asp-472, Asp/Glu-478, Pro/Glu-481, Ile/Lys-483, Asn/Gly-490, Lys/Ala-496, and Thr/Tyr-499. With the same criterion, there were two other amino acid pairs upstream of the T2A region which were different between the E3 and E6 (DF13) sequences (Asn/Lys-445 and Glu/Asp-448). These can also be excluded from the candi-

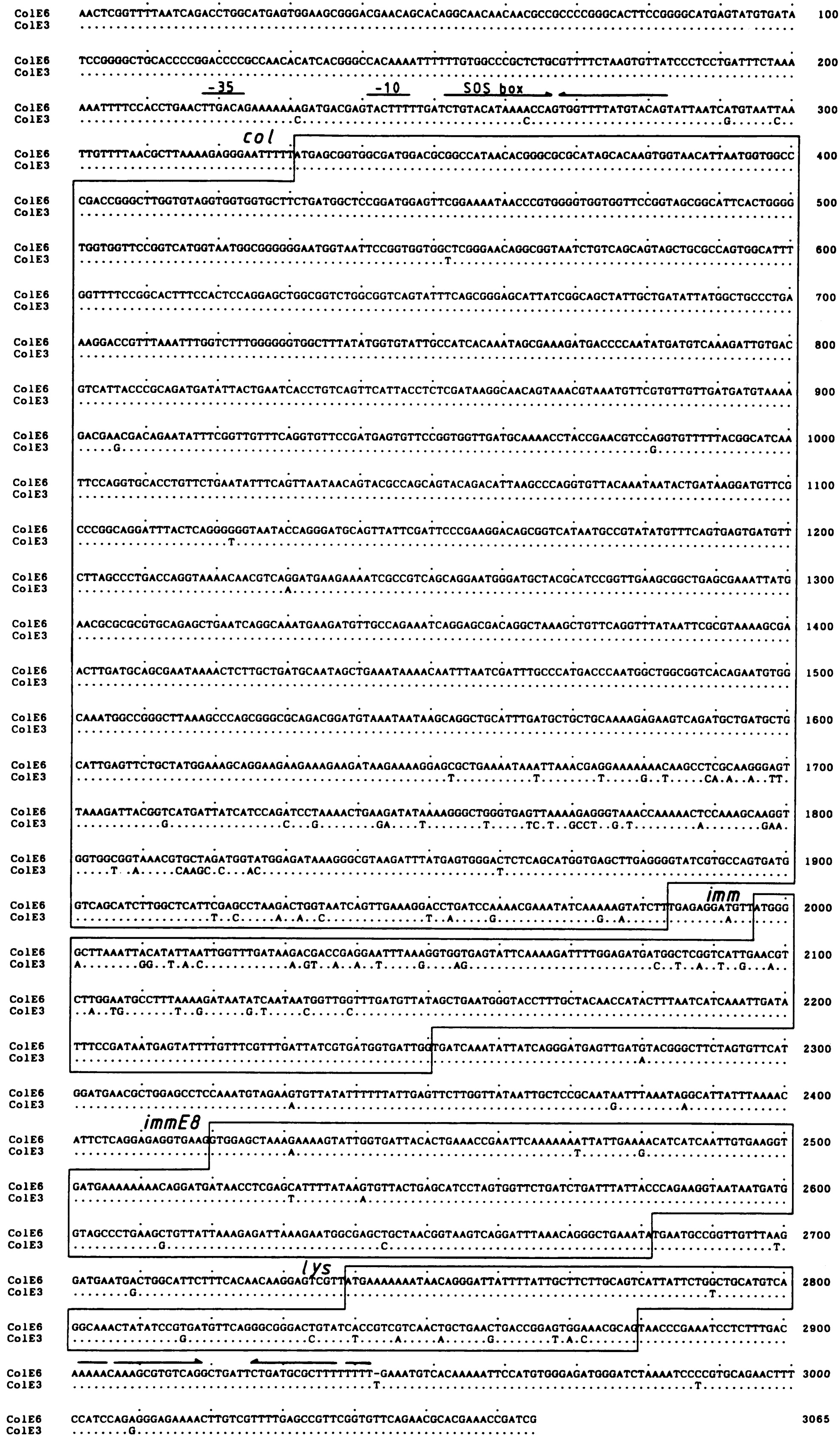


FIG. 2. Nucleotide sequence of the *Hin*cIIa-*Pvu*I fragment of ColE6-CT14 aligned with the ColE3-CA38 sequence; only nonidentical nucleotides are shown. Our previous ColE3-CA38 sequence (10) had an error, i.e., an extra T residue at position 133, and this has been rectified here. Putative −35 and −10 regions of the promoter and two units of the SOS box are shown. The *colE6* (*colE3*), *immE6* (*immE3*), *immE8*, and lysis genes are boxed. The inverted repeat at the 3′ end is a putative [*rho*]-independent transcriptional termination signal.

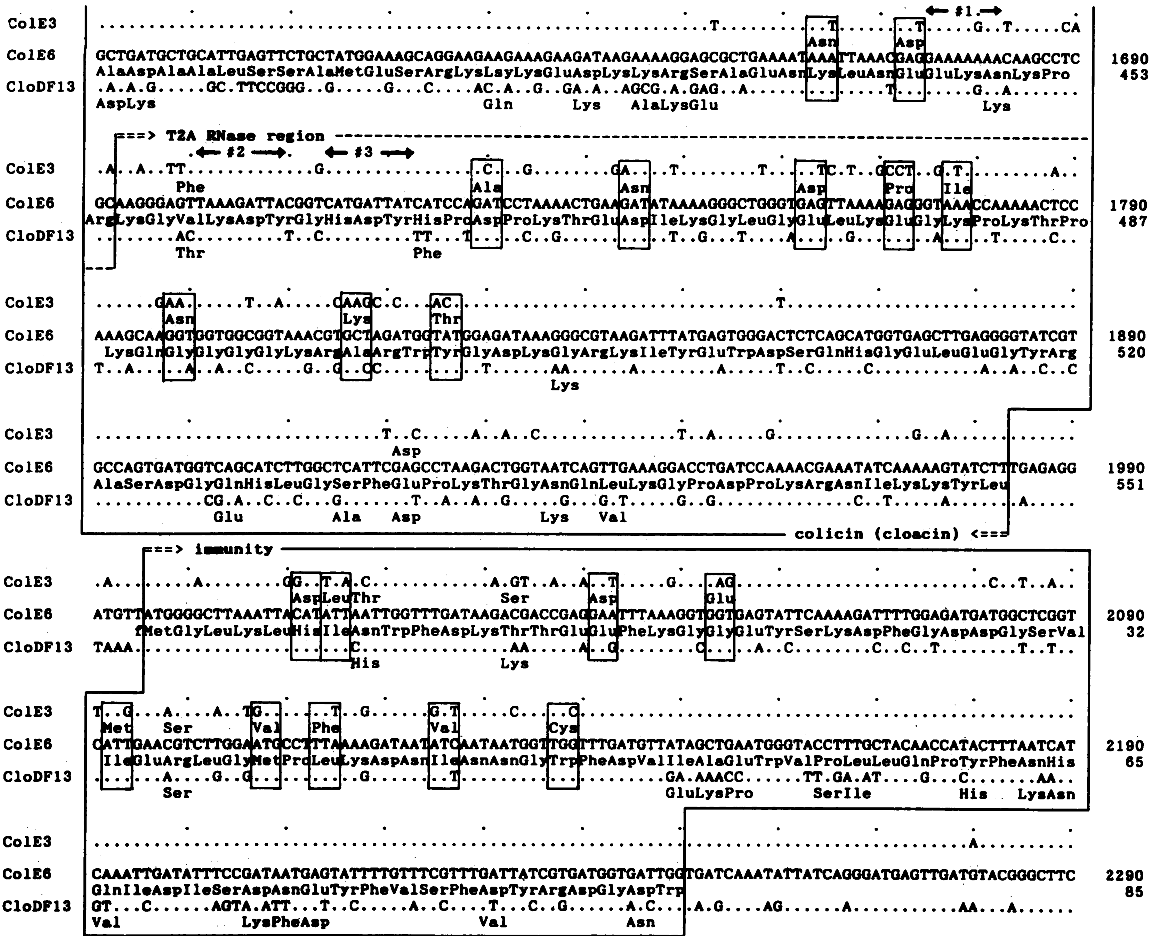


FIG. 3. Sequence comparison of the T2A-*imm* regions of ColE3-CA38, ColE6-CT14, and CloDF13. For the ColE3-CA38 and CloDF13 sequences, only nucleotides and amino acids that were not identical to those in the case of ColE6-CT14 are presented (9, 22). Homologous recombination from the *colE3* to *cloDF13* sequences marked 1, 2, and 3 gave rise to the chimeric plasmids pAM3D1, pAM3D2, and pAM3D3, respectively. Possible determinants of the E3 and E6 (or DF13) immunity specificities are boxed. Numbering starts at the *Hinc*IIa site for the ColE6-CT14 DNA and at the N-terminal methionine for colicin E6 and the E6 immunity protein. The purified E3 and DF13 immunity proteins were reported to lack the N-terminal methionine (12, 21). Amino acid sequences at the N-terminal side were identical between colicins E3 and E6.

dates for immunity determinants since all the E3::DF13 chimeric colicins mentioned above had an E3-type sequence in this region, even though they had E6-type specificities.

In the same way, the immunity determinants of Imm proteins which discriminated E3 and E6 (DF13) could be restricted to the following 9 of 84 amino acid pairs: Asp/His-6, Leu/Ile-7, Asp/Glu-16, Glu/Gly-20, Met/Ile-33, Val/Met-38, Phe/Leu-40, Val/Ile-44, and Cys/Trp-48.

ImmE8

```
ColE6    MELKKSIGDYTETEFKKIIENIINCEGDEKKQDDNLEHFISVTEHPSGSD  50
ColE3                      F D              Y N
ColE8        N S           D

ColE6    LIYYPEGNNDGSPEAVIKEIKEWRAANGKSGFKQG                 85
ColE3                G
ColE8
```

Lysis

```
ColE6     MKKITGIILLLLAVIILAACQANYIRDVQGGTVSPSSTAELTGVETQ   47
ColE2/E3                       S                  V  LA
ColE8                                             V  LA
```

FIG. 4. Amino acid sequences deduced from the *immE8* and lysis genes of ColE6-CT14 aligned with those of the closely related Col plasmids. Only residues that were not identical to those of ColE6-CT14 are shown.

Interestingly, in both the T2A and Imm regions, the immunity specificity determinants mentioned above were concentrated into their N-terminal halves. On the contrary, most of the colicin (E3 and E6)-specific and cloacin-specific amino acids within the T2A regions were concentrated into the C-terminal regions. Also, for immunity proteins, all the colicin- and cloacin-specific residues were restricted to the C-terminal halves. One possible explanation is that the C-terminal regions of the T2A domains and immunity proteins interact with each other, as in the case of the N-terminal regions which determine the E3 and E6 (DF13) specificities. However, this may not be the case, since colicin E6 is perfectly blocked by, i.e., has a good affinity to, the DF13 immunity protein, as is the chimeric colicin E3::DF13 to the E6 immunity protein. We obtained two additional hybrid plasmids; one had the colicin E3::DF13 gene (derived from pAM3D3) and the *immE6* gene, and the other had the *colE6* and *immDF13* genes. Both plasmids were also stably maintained and produced colicin, as did their parental plasmids, ColE6-CT14 and pAM303 (data not shown). Thus, it is more

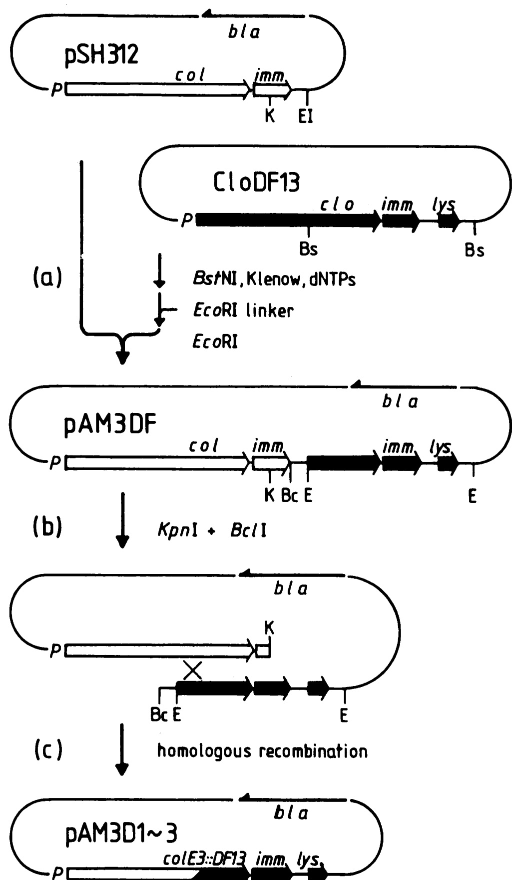
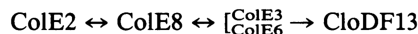


FIG. 5. Construction of chimeric colicin E3::DF13 plasmids. ColE3-CA38- and CloDF13-derived genes are denoted by open and solid arrows, respectively. (a) The *Bst*NI fragment of CloDF13 was treated with the Klenow enzyme, ligated with the *Eco*RI linker, and then inserted into the *Eco*RI site of pSH312. (b) The resulting plasmid, pAM3DF, was digested with *Kpn*I and *Bcl*I. (c) The linearized plasmid was subjected to in vivo homologous recombination, and successful chimeric plasmids were selected for Ap^r and the ability to kill an *E. coli* strain carrying an *immE3* plasmid. The bacteriocin release protein gene (*brp*) of CloDF13 is designated *lys* here.

likely that the colicin- and cloacin-specific amino acids in both the C-terminal regions of T2A and the immunity proteins are involved in the folding of each protein. Recently, we succeeded in determining the contributions of some amino acids of ImmE3 and ImmE6 to the respective immunity specificities (manuscript in preparation).

We have shown here that the colicin E6 operon is structurally homologous to the colicin E3 operon and that E6 immunity is functionally equivalent to that of cloacin DF13. This dual feature of E6 seems to extend our previous evolutionary model of plasmids (11, 19) as follows:



The *immE8* gene, which is closely homologous to *immE2*, is retained in ColE3-CA38 and ColE6-CT14 and has been deleted from CloDF13. The immunity specificity of ColE6-CT14 has already changed (evolved) to the cloacin DF13 type.

Recent findings for three group A klebicins by James et al. (5), however, suggested that the evolutionary pedigree is, in fact, more complicated. According to their immunity spectrum analysis, the klebicin A2 that they isolated is possibly a klebicin counterpart of colicin E3, just like klebicin A1 (a phenotypic homolog of cloacin DF13) is a klebicin counterpart of colicin E6. We believe that a more exact drawing of the evolutionary history of the plasmid will be possible on further structural and functional analyses of A-group klebicins and E-group colicins.

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