Volume 8 Number 19 1980 Nucleic Acids Research

Molecular structure of the immunity gene and immunity protein of the bacteriocinogenic plasmid Clo DF13

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Received 5 August 1980

ABSTRACT

The nucleotide sequence of the Clo DF13 DNA region comprising the immunity gene has been determined. We also elucidated the aminoacid sequence of the 40 N-terminal and 7 C-terminal aminoacids of the purified immunity protein. From analysis of the data obtained we were able to locate the immunity gene between 11.7 and 14.5% on the Clo DF13 map, and to determine the complete aminoacid sequence of the immunity protein. It was observed that the Clo DF13 immunity gene encodes an 85 aminoacid protein and is transcribed in the same direction as the cloacin gene.

These experimental data support our model, presented elsewhere, which implicates that the cloacin and immunity genes of Clo DF13 are coordinately transcribed from the cloacin promoter. We also present DNA sequence data indicating that an extra ribosome binding site precedes the immunity gene on the polycistronic mRNA. This ribosome binding site might explain the fact that in cloacinogenic cells more immunity protein than cloacin is synthesized.

The comparison of the complete aminoacid sequence of the Clo DF13 immunity protein, with the aminoacid sequence data of the purified, comparable Col E3 immunity protein revealed that both proteins have extensive homologies in primary and secondary structure, although they are exchangeable only to a low extent *in vivo* and *in vitro*. It was also observed that a lysine residue was modified in immunity protein isolated from excreted bacteriocin complexes.

INTRODUCTION

Bacteriocinogenic plasmid Clo DF13 originating from *Enterobacter cloacae* (32) is studied mostly in *Eschericha coli*. This small ($6x10^6$ D,32), non-conjugative plasmid encodes at least 8 proteins (2) and 25 partially overlapping RNA species (7) in minicells. Functions have been assigned to 7 Clo DF13 genes and their gene products (2, 22, 23, 24, 36), but two Clo DF13 proteins and their functions have been studied more extensively. These proteins, cloacin DF13 and Clo DF13 immunity protein have a molecular weight of 58,000 D and 9,000 D respectively (11). The bacteriocin cloacin DF13 causes inhibition of protein synthesis in sensitive bacteria by an

endoribonucleolytic cleavage of a 49 nucleotide fragment from the 3'-terminus of the 16S ribosomal RNA (13). The cloacin protein is excreted as a complex with the immunity protein, in which both polypeptides are present in equimolar amounts (10, 11). This immunity protein was shown (15) to be an inhibitor of the biochemical activity of the cloacin protein.

We reported elsewhere (7), that two overlapping mRNAs, of 2200 and 2400 nucleotides are transcribed from the region coding for the cloacin (15-32% on the physical map) and immunity (11.5-15% on the physical map) proteins. Evidence was presented for a model in which transcription of the cloacin gene and probably also the immunity gene is initiated at a promoter located proximal to the cloacin gene, at 32%. Termination of transcription would occur at two different terminaters distal to the immunity gene. In this model we suggested that both genes are organized in one operon.

In this paper, we present the nucleotide sequence of the immunity gene. We also elucidated part of the aminoacid sequence of the Clo DF13 immunity protein, enabling us to locate the Clo DF13 immunity gene and to determine the direction of transcription of this gene. From the total aminoacid sequence of the Clo DF13 immunity protein and the aminoacid sequence data obtained from the comparable Col E3 immunity protein, we were able to predict the primary structure of the Col E3 immunity protein and the secondary structure of both immunity proteins.

From our results we conclude that the Clo DF13 immunity gene is transcribed in the same direction as the cloacin gene, and that both genes probably are organized in one operon. The differences and similarities in structure between the Clo DF13 and Col E3 immunity proteins are discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

The Eschericha coli minicell producing strain P678-54 (1), was used as a host for the Clo DF13 plasmids. E. Coli W3110, harbouring plasmid Col E3, was obtained from dr. D.R. Helinsky. To isolate the DNA restriction fragment carrying the Clo DF13 immunity gene, we made use of miniplasmid pEV5-cop2. (Stuitje et al; manuscript in preparation).

Media, Chemicals and Enzymes

Brain Heart Infusion medium (Difco) was normally used for growth in liquid; where specified, ampicillin (Amp) was included at a final

concentration of 50 µg/ml. The restriction endonucleases BamHI and ClaI, and T4 polynucleotide kinase and DNA polymerase I were purchased from Boehringer Mannheim (FRG). The restriction enzymes HhaI, HpaII and MboII were obtained from New England Biolabs. Piperidine, hydrazine and dimethylsulfate were obtained from Serva. γ-^{32}P-ATP (>2000 Ci/mmole) was purchased from the Radiochemical Centre, Amersham, England.

Isolation of plasmid DNA and cleavage by restriction endonucleases

DNA from Clo DF13 miniplasmid pEV5-cop2 was isolated from cells, which were grown in BHI-Amp, as described previously (34). This procedure essentially involved CsCl - ethidium bromide gradient centrifugation of cleared lysates prepared with Brij-58 and desoxycholate. Digestion of plasmid DNA with restriction endonucleases was carried out during 2 h at 37°C in 20 µl reaction mixtures containing 0.5-4 µg DNA. Reaction mixtures were prepared as recommended by New England Biolabs.

Isolation of DNA restriction fragments

DNA restriction fragments, longer than 650 base pairs were separated on a 1% agarose slab gel as described (7); DNA restriction fragments shorter than 650 bp were run on a 5% polyacrylamide slabgel and eluted from the gel as described by Stuitje et al (30).

DNA sequence analysis procedures

DNA restriction fragments, labeled at one 5'-end were sequenced using the Maxam and Gilbert degradation procedure (19). Eight percent and twelve percent sequence gels were prepared as described previously (18).

Preparation of Clo DF13 and Col E3 immunity protein

Excess Clo DF13 immunity protein was prepared from E. coli strain p678-54, harbouring Clo DF13-cop3 (35) as described (12). Immunity protein was also isolated from the cloacin DF13-immunity protein complex via chromatography on Sephacryl S200 in 6M guanidinium hydrochloride, followed by dialysis (14). Colicin E3-immunity protein complex was extracted from E. coli W3110 in the same way as described for cloacin DF13-immunity protein complex (12), and purified by ion-exchange chromatography in 0.05 M sodium acetate pH 5.0 on a CM Sephadex column as described for cloacin DF13-immunity protein complex (11). Col E3 immunity protein was prepared from the colicin E3-immunity protein complex by chromatography on Sephacryl S200 in 6M guanidinium hydrochloride.

Aminoacid sequence methodology

Automatic Edman degradation and identification of phenylthiohydantoin-derivatives were performed as described by Gaastra et al (8). Enzymatic cleavage of Clo DF13 immunity protein was carried out by digestion with trypsin, chymotrypsin and thermolysin (9). The proteolytic fragments were isolated and purified by column chromatography on Sephadex G25, by paper electrophoresis at pH 6.5 and 3.5 and by paper chromatography (3). Amino acid analysis of the purified peptides was performed on a Rank Chromaspek amino acid analyser. Dansylation and Edman degradation combined with dansylation were carried out as described by Hartley (14). Identification of dansylated amino acids was achieved by chromatography on polyamide thin layers (14). The sequence of the majority of the purified peptides was however determined by the method of Tarr (13).

Prediction of the secondary structure of the cloacin DF13 and colicin E3 immunity proteins

The secondary structure of the Clo DF13 and Col E3 immunity proteins was predicted from the amino acid sequences by the method of Burgess et al (4), as computerized by Lenstra et al (17).

RESULTS

DNA sequence analysis of a Clo DF13 DNA fragment containing the gene encoding the immunity protein

From the construction and characterization of deletion mutants, it is known that the immunity gene of Clo DF13 is located between 11.5%-15% on the Clo DF13 physical map (36). To analyse the DNA base sequence of this region, we constructed a detailed cleavage map. To facilitate the construction of this map, we made use of miniplasmid pEV5-cop2 (Stuitje et al; manuscript in preparation). From this miniplasmid, we isolated the 9-15% DNA restriction fragment. Restriction cleavage sites were mapped by double digestion, and partial digestion of ^{32}P end-labeled DNA fragments as described by Smith et al (28). Figure 1 shows the detailed cleavage map of the 9-15% part of Clo DF13.

In this paper, we present the nucleotide sequence of the DNA region between 11.4% (HpaII site) and 15% (Bam HI site). For this purpose pEV5-cop2 DNA restriction fragments were prepared and sequenced as described in

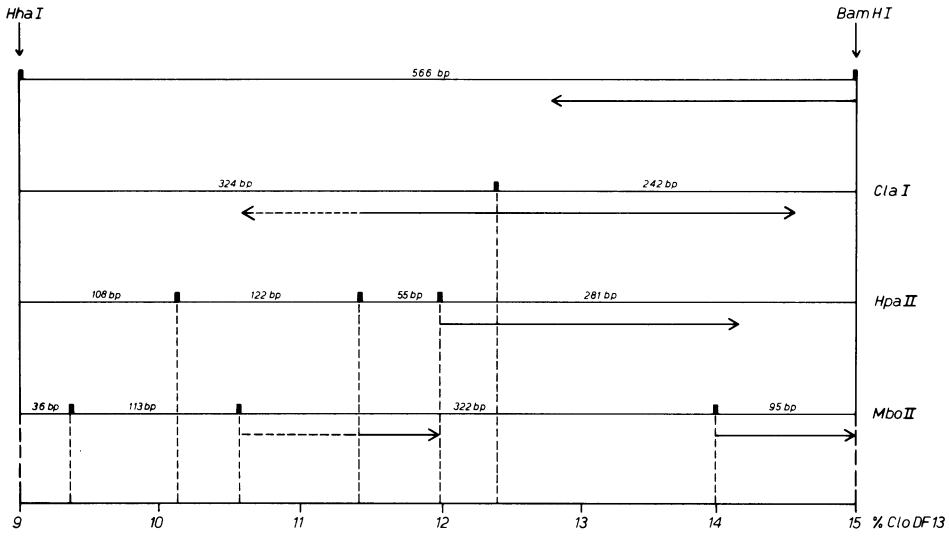

Figure 1. Fine cleavage map of the 9-15% part of plasmid Clo DF13. The direction of the arrows indicates which strands were sequenced; they are aligned in the 5'-3' direction. The nucleotide sequence of the DNA at the right side of 11.4% (HpaII site) is presented in this paper (fig. 3); this is indicated by solid lines.

materials and methods. In figure 1 is indicated in which direction the DNA restriction fragments have been sequenced and the extent of the overlapping sequences obtained. In figure 1 it can be observed that the DNA base sequence between 11.4% and 15% has been determined at least twice. An example of a sequence gel is given in fig. 2, whereas fig. 3 represents the complete DNA base sequence between the HpaII site at 11.4% and the BamHI site at 15%.

Codon analysis of this nucleotide sequence revealed that a protein of 85 aminoacids could be encoded from right to left between triplet ATG at residue 44 and triplet TAA at residue 299. In other reading frames several smaller proteins could be encoded, for instance from left to right (fig.3) between residues 102 and 162, 180 and 240, 288 and 324, and in opposite direction between residues 206 and 176, 141 and 33. The biggest of these proteins would be transcribed, between residues 141 and 33, in opposite direction of the 85 aminoacid protein.

In view of the fact that the Clo DF13 immunity protein consists of at least 70 aminoacids (this paper), only the 85 aminoacid protein might

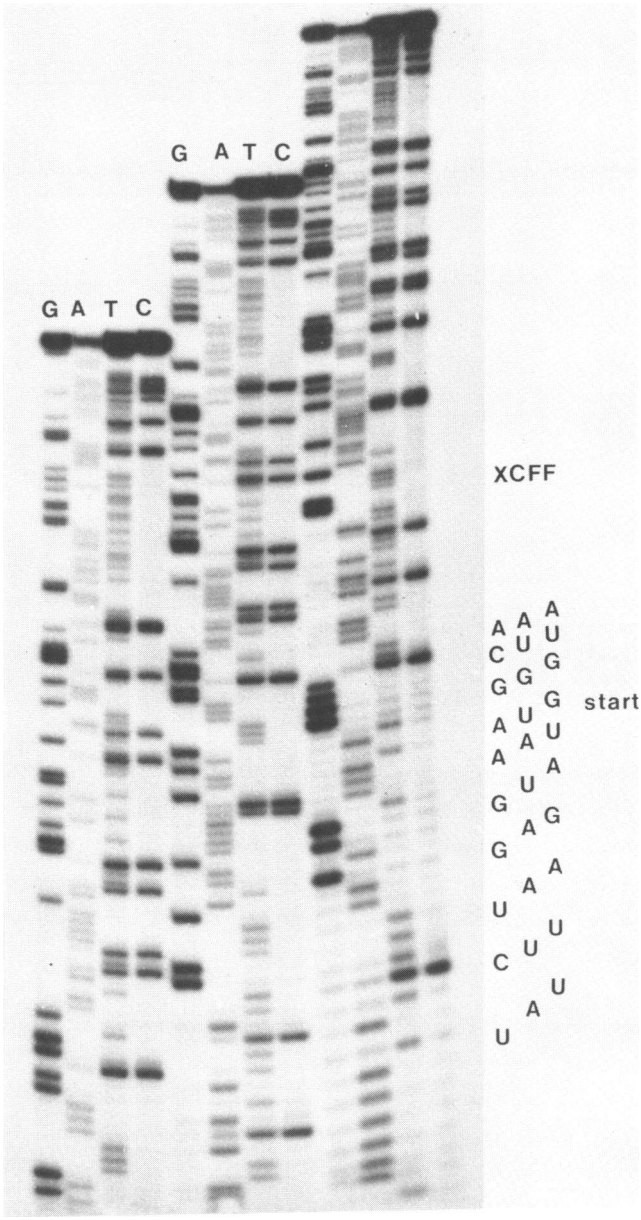

Figure 2.
Eight percent polyacrylamide sequence gel of the DNA fragment from 12.4-15% (ClaI - BamHI), which fragment was labeled at the BamHI site. Indicated are the xylenecyanol FF marker (XCFF) and the AUG startcodon preceding the immunity gene.

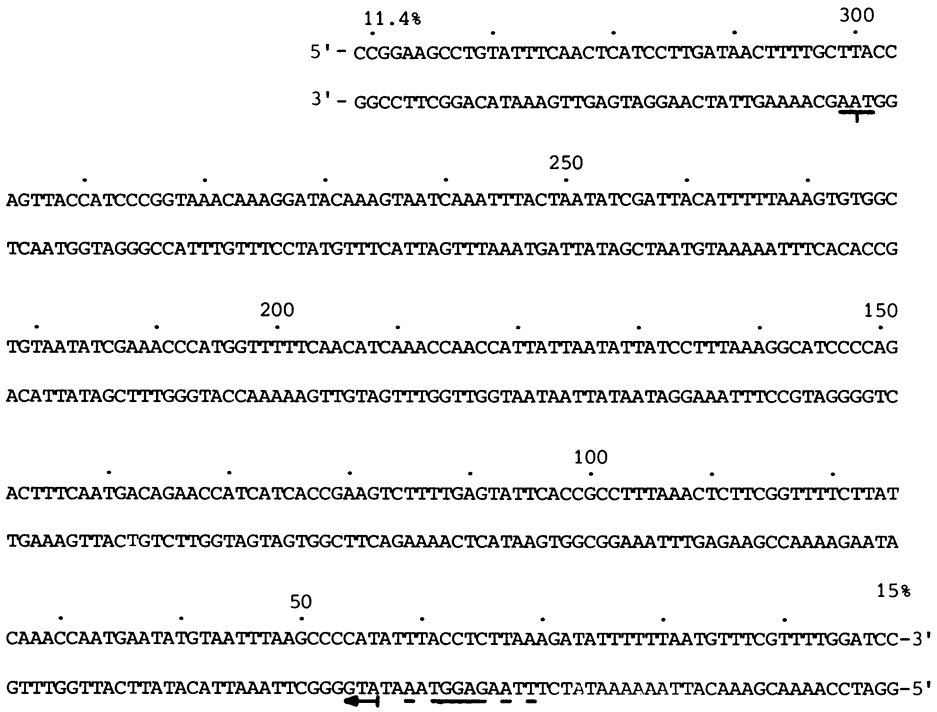

Figure 3. Nucleotide sequence of the Clo DF13 DNA region from 11.4% to 15% Indicated are the nucleotides encoding the AUG startcodon (⊢►) and the UAA stopcodon (⊤) of the immunity gene. The nucleotides which are underlined (residues 41-51) encode RNA bases which are complementary to the 3'-end of 16S ribosonal RNA of E. coli.

represent the immunity protein. Additionally we observed that the messenger RNA transcribed from residue 30 till 41 would have 8 bases, just in front of the AUG startcodon, complementary to the 3'-end of the 16S ribosomal RNA of E. coli. These nucleotides are underlined in the DNA sequence in fig.3. Presumably, this site represents a strong ribosome binding site on a messenger RNA molecule.

Primary and secondary structure of the Clo DF13 and Col E3 immunity proteins

For the aminoacid analysis of Clo DF13 immunity protein, excess immunity protein, not complexed with cloacin DF13, was extracted directly from E. coli cells harbouring Clo DF13-cop3. The total aminoacid composition of this immunity protein is given in table 1.

Table 1. Amino acid analysis of cloacin DF13 immunity protein.

Amino acid	Number of residues
asp	10.8
thr	+
ser	4.3
glu	6.1
pro	2.6
gly	7.1
ala	-
cys	+
val	5.1
met	1.1
ile	4.9
leu	5.7
tyr	2.9
phe	6.3
lys	8.2
his	3.7
arg	1.1
trp	2.0

The conclusion can be drawn that Clo DF13 immunity protein consists of at least 70 aminoacids. With the automatic Edman degradation the aminoacid sequence of excess immunity protein could be followed up to residue 40. In contrast to Sidikaro and Nomura (27), who prepared a tryptic digest of excess Col E3 immunity protein, we were not able to recover all tryptic peptides from the Clo DF13 immunity protein. The same holds for the digestion of Clo DF13 immunity protein with chymotrypsin and thermolysin. In fig. 4 the covalent structure of the N-terminal part of Clo DF13 immunity protein obtained by automatic Edman degradation is given. In this figure we also present the structural data obtained from the manual Edman degradation of purified peptides, which data also resulted in the elucidation of the covalent structure of the 7 C-terminal aminoacids. We also analysed the aminoacid composition and sequence of immunity protein isolated from cloacin DF13-immunity protein complex. From these experiments, it could be concluded that the aminoacid sequence (untill residue 40) of this immunity protein is the same as the aminoacid sequence of the excess immunity protein, extracted directly from the cloacinogenic cells, as given in fig. 4; except that lysine at position 12 is modified, probably methylated.

The knowledge about the covalent structure of the 40 N-terminal and 7 C-terminal aminoacids of the Clo DF13 immunity protein enabled us to identify the immunity gene in the DNA base sequence. The 85 aminoacid coding region between residue 44 and 299 (fig. 3), turned out to encode a protein with

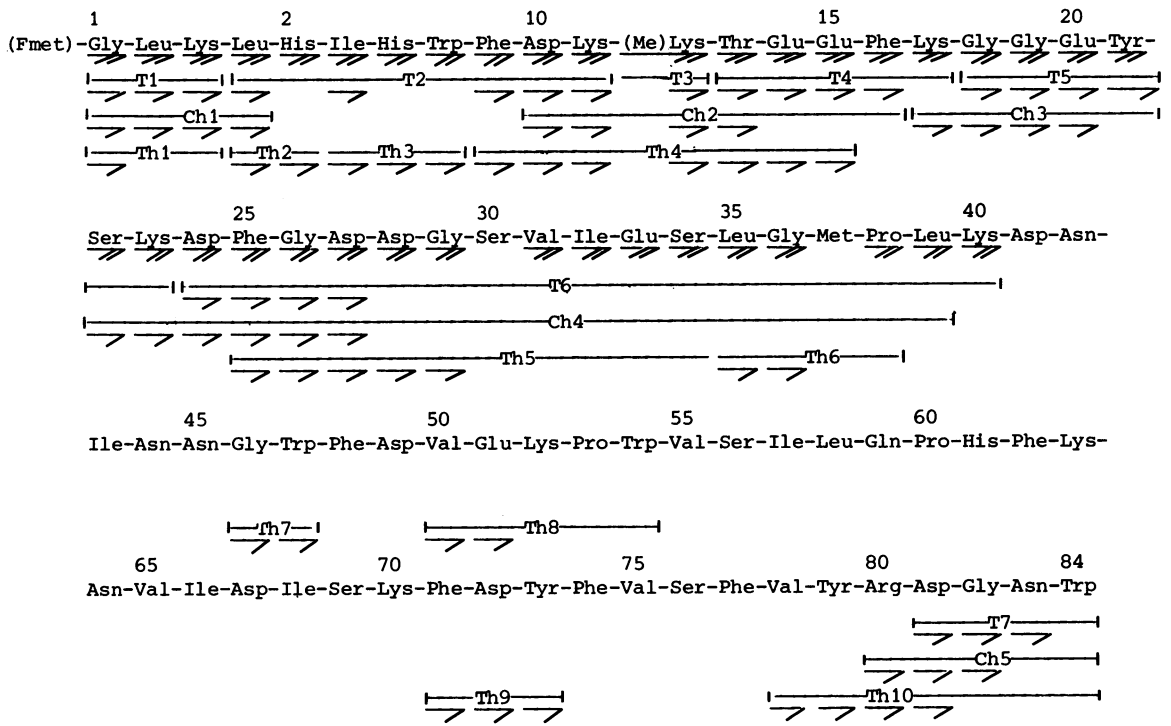

Figure 4. Complete aminoacid sequence of the Clo DF13 immunity protein including Fmet, as determined by DNA sequence analysis. Those aminoacid residues, which were also identified as phenylthiohydantoin derivatives by automatic Edman degradation are indicated (⟶//), as well as those identified by manual Edman degradation (⟶). Tryptic peptides are prefixed T, chymotryptic peptides Ch, and thermolysin peptides Th. (Me)Lys at position 12 represents a lysine residue which is probably methylated in the Clo DF13 immunity protein isolated from the cloacin DF13-immunity protein complex.

identical N-terminal and C-terminal ends as the immunity protein (fig. 4), indicating that this region indeed represents the Clo DF13 immunity gene. From the DNA sequence of the immunity gene, the complete aminoacid sequence of the corresponding protein could be derived (fig. 4).

In fig. 5, the covalent structure of the Col E3 immunity protein is compared with that of the Clo DF13 immunity protein. The primary structure of the Col E3 immunity protein, isolated from the colicin E3 - immunity protein complex, was determined by automatic Edman degradation up to residue 40. The remainder of the sequence was derived from the aminoacid analysis of tryptic peptides from Col E3 immunity protein obtained by Sidikaro and Nomura (27). Their tryptic peptides T7 (residues 1-3), T5 (residues 4-11), T6 (residues 13-23), T2 (residues 24-40), T3 (residues 41-52), T4 (residues 53-80) and T1 (residues 81-84) can easily be accomodated. The lysine residues at positions 12 and 17 however are unlikely if one considers the aminoacid analysis obtained by Sidikaro and Nomura (27). At position 12, like in the case of Clo DF13 immunity protein, isolated from the cloacin DF13-immunity protein complex, we found a modified lysine residue, probably methylated. At position 17 lysine was determined unequivocally by three different identification methods. Furthermore, in peptide T4, Sidikaro and Nomura determined more Asx residues, than we could accommodate for in the sequence of Clo DF13 immunity protein; the presence of two trytophane residues in T1 is also not clear.

The secondary structure of Clo DF13 immunity protein and Col E3 immunity protein was predicted from the aminoacid sequence by the method of Burgess et al (4) as computerized by Lenstra et al (17). As can be seen in fig. 5, this method predicted an α- helix structure for the region between aminoacid residues 8 and 17 in both proteins, a β-sheet structure in the regions between aminoacids 30 till 37, and 70 till 77. Both β-sheet regions are followed and preceded by β-turns. In the Col E3 immunity protein, there is an additional region of β-sheet structure between residues 54 and 58.

DISCUSSION

In a previous paper, we proposed a model for transcription of the Clo DF13 region involved in replication control, cloacinogenity and immunity. This model, represented in fig. 6, implicates that the genes coding for cloacin DF13 and immunity protein are organized in one operon, with one promoter (P1 at 32%) and two terminators (T1 and T2 a ± 12 and ± 9%, respectively). The model was based upon the following experimental data (7). (I) Two RNAs are transcribed from the regions between 32% and 9%, respectively 32 and 11.5%.

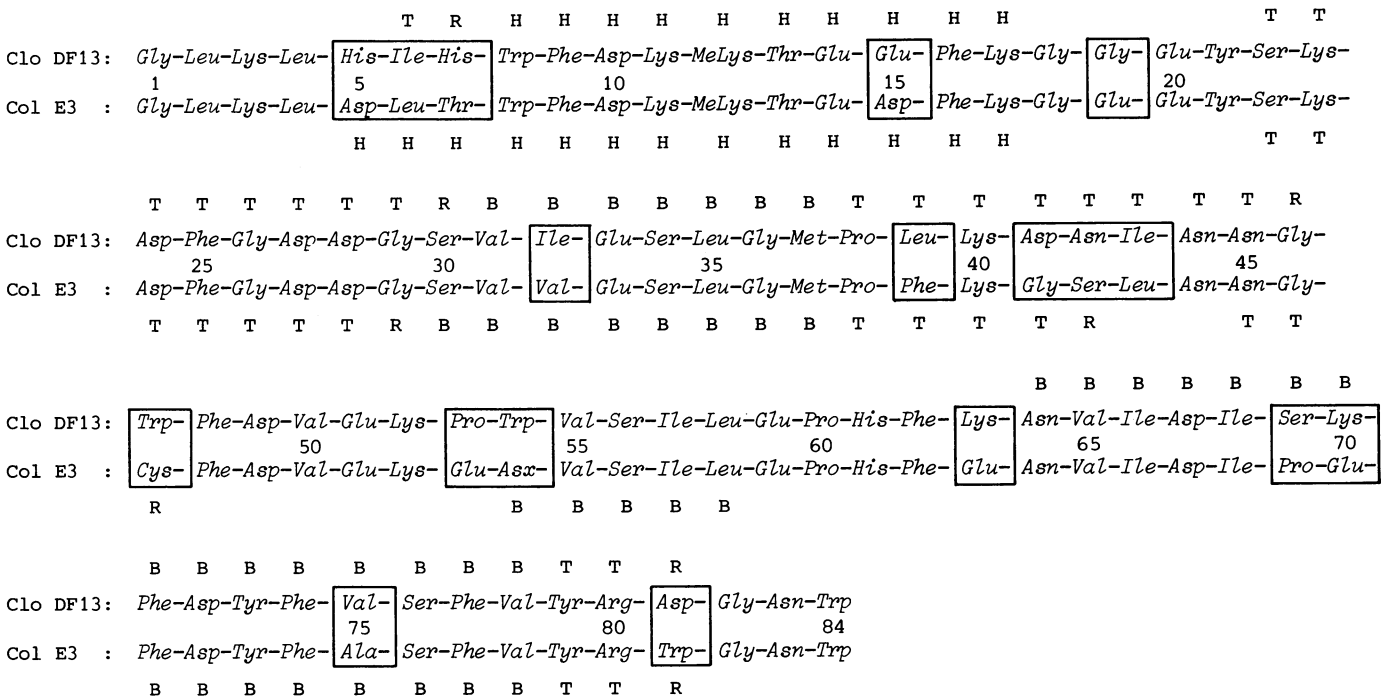

Figure 5. Comparison of the covalent structure of Clo DF13- and Col E3- immunity proteins. The sequence of Col E3 immunity protein from residue 41 to 84 is derived from the aminoacid analyses of tryptic peptides obtained by Sidikaro and Nomura (27). The aminoacid residues were positioned in such a way that maximal homology in the DNA base sequence was obtained. A prediction of the secundary structure of the Clo DF13 and Col E3- immunity protein is also given, as indicated by H(α-helix), B(β-sheet) and T--R(β-turn).

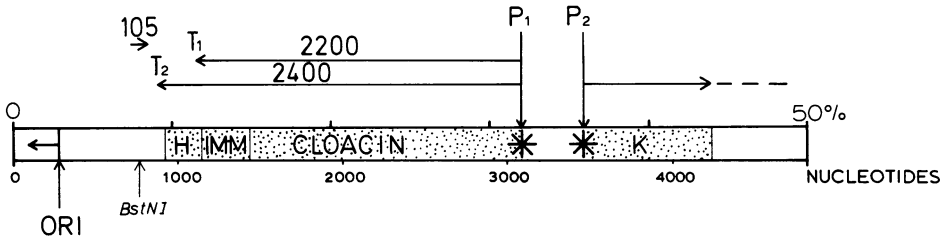

Figure 6.
Model for transcription of the 0-50% DNA region of plasmid Clo DF13; as presented elsewhere (7). Promoters (P1 and P2) and terminaters (T1 and T2) are indicated, as well as the origin of replication (ORI).

(II) The cloacin gene is transcribed from right to left. (III) A relatively strong RNA polymerase binding site has been localized around 32%. (IV) In vitro, it could be demonstrated that RNA synthesis is initiated around 31%. To get more evidence for this model, we have determined the DNA base sequence of the region containing the immunity gene. From these data and the partial aminoacid sequence of the immunity protein, presented in this paper, it can be concluded that transcription of the immunity gene proceeds in the same direction as transcription of the cloacin gene, i.e. from right to left in fig. 6. Additionally, proximal to the immunity gene, between residues 31-41 (fig. 3) 8 bases are observed, which are complementary to the 3'- end of the 16S ribosomal RNA of E. coli. This probably means that RNA containing these bases possesses a very efficient ribosome binding site (29).
This ribosome binding site would provide the Clo DF13 plasmid with an extra possibility to regulate the synthesis of immunity protein. Indeed, previously it has been reported that significantly more immunity protein than cloacin is synthesized in cloacinogenic cells (16).

Another possibility to regulate the synthesis of Clo DF13 immunity protein might be the presence of an additional promoter proximal to the immunity gene. It should be noted that the only possible Pribnow box (25,26), within the DNA sequence from residue 1-45, is the sequence TATCTTT (residue 26-32). This promoter however would lack a -35 recognition region (26). Moreover, if RNA synthesis starts at ± residue 36, the resulting mRNA would have a rather weak ribosome binding site.

When we compare the mode of transcription of the cloacin and immunity genes, with that of the comparable genes of the Col E1 plasmid, a remarkable difference can be observed. The immunity gene of Col E1 seems to be trans-

cribed in opposite direction of the colicin gene (20,21), which is not the case for the described Clo DF13 genes. Induction, with mitomycin c, of cells harbouring plasmids with comparable bacteriocinogenic activity such as CLO DF13 (6), Col E2 or Col E3 (33), causes an increased synthesis of at least the bacteriocins and immunity proteins. Induction however, of cells harbouring Col E1 does only result in an increased synthesis of colicin E1. The induction phenomena can be explained very well from the fact that the Clo DF13 genes coding for cloacin and immunity are organized in one operon, whereas this seems not to be the case for the genes encoding colicin E1 and the corresponding immunity protein. These differences between Col E1 and Clo DF13 are particularly interesting because of the considerable similarity in arrangment of the genes (5) involved in bacteriocinogenity, immunity, replication control and mobilization. Also, the origin of replication of both plasmids not only is located on similar sites, but there is also a great homology in DNA base sequence between these plasmids in the origin of replication and the replication control area (between 0% and 10% on the Clo DF13 map, (30; Stuitje et al; manuscript in preparation).

As indicated by boxes in fig. 5, 18 differences have been found between the aminoacid sequence of Clo DF13 immunity protein and Col E3 immunity protein. Most of these substitutions can be accounted for by point mutations. Six of the substitutions are conservative, namely ile -> leu, glu -> asp, ile -> val, leu ->phe, ile -> leu and val ->ala at positions 6,15,32,39,43, and 75 respectively. In eight other substitutions, namely his -> asp (position 5), his ->thr (pos. 7), gly ->glu (pos. 19), asp ->gly (pos. 41), lys ->glu (position 63 and 70), asp ->trp (pos. 81) and possibly trp -> asx (pos. 54) changes in the charge of the aminoacid residue are involved. Since there is no major effect of the 18 aminoacid substitutions on the predicted secondary structure, it is likely that the 8 substitutions, involving a change in charge, affect the binding of immunity protein with its homologues bacteriocin. A remarkable difference was observed between immunity protein isolated from the excreted bacteriocin complexes and excess immunity protein isolated directly from the bacteriocinogenic cells. Only in immunity protein isolated from excreted bacteriocin complex, the lysine residue at position 12 was modified. This observation indicated that modification of immunity protein probably occurred during its translocation through the cell membrane.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J.M. Walker and Dr. J.R.B. Hastings

(Chester Beatty Institute, London) for performing the amino acid analysis and their help with the automatic Edman degradations; they are also very grateful to Drs. A.R. Stuitje for his very helpful suggestions during the DNA-sequence experiments. W.G. wishes to thank the Federation of European Biochemical Societies for a short-term fellowship. The help of Dr. J.A. Lenstra with the prediction procedures and the excellent technical assistance of Mrs. Pia Klaasen-Boor are appreciated very much.

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