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Nucleotide Sequence Analysis of the Complement Resistance Gene from Plasmid R100†

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The multiple antibiotic resistance plasmid R100 renders *Escherichia coli* resistant to the bactericidal action of serum complement. We constructed a plasmid (pOW3) consisting of a 1,900-base-pair-long restriction fragment from R100 joined to a 2,900-base-pair-long fragment of pBR322 carrying ampicillin resistance. *E. coli* strains carrying pOW3 or R100 were up to 10,000-fold less sensitive to killing by serum complement than were plasmid-free bacteria or bacteria carrying pBR322. Nucleotide sequencing revealed that 875 of the 1,900 bases from R100 correspond exactly to part of the bacterial insertion sequence IS2. The remaining 1,075 bases contained only one sizeable open reading frame; it covered 729 base pairs (243 amino acids) and was preceded by nucleotide sequences characteristic of bacterial promoters and ribosome binding sites. The first 20 amino acids of the predicted protein showed features characteristic of a signal sequence. The remainder of the predicted protein showed an amino acid composition almost identical with that determined for the *traT* protein from the *E. coli* F factor. Southern blot analysis showed that the resistance gene from R100 does not hybridize to the serum resistance gene from ColV,I-K94 isolated by Binns et al.; we concluded that these genes are distinct.

Complement in mammalian serum constitutes an important host defense mechanism against bacterial infection; individuals deficient in complement frequently suffer recurrent bacterial infections (for review, see reference 2), and bacteria isolated from normal individuals with systemic infections are almost always resistant to killing by complement (e.g., 7, 14, 31). How bacteria become resistant to complement is unclear; however, recent work has demonstrated that, at least in some cases, bacterial plasmids confer resistance (5, 26–29, 42) and that plasmid-borne resistance can directly enhance bacterial pathogenicity (5). Complement resistance, then, augments a wide range of plasmid-borne functions which directly and indirectly increase bacterial virulence (for review, see reference 9).

How do plasmid-encoded functions interfere with complement activity? In the cases studied, both the classical and alternative pathways are attenuated, and resistance does not involve soluble inhibitors or gross depletion of complement (27, 29). An important clue to at least one mechanism of resistance has been provided by Moll et al. (26), who report that with the plasmid R6-5, resistance to killing by complement is mediated by the *traT* protein (traTp), which has previously been identified as an outer-membrane protein responsible for preventing unproductive conjugation between bacteria carrying like plasmids (i.e., surface exclusion; for review, see reference 46). Although it is tempting to draw analogies between the assaults on the bacterial membrane suffered during conjugation and complement lysis, neither process is well understood (46), and no evidence exists to suggest that they are at all alike. Furthermore, the complement resistance gene from the colicin-producing plasmid ColV,I-K94 is apparently unrelated to *traT* (5). A clear understanding of the mechanism(s) of complement resistance as well as of bacterial conjugation will require the isolation and characterization of the components involved in these processes. In this study, we isolated the complement resistance gene from the multiple antibiotic resistance plasmid R100 and determined its nucleotide sequence.

MATERIALS AND METHODS

Enzymes. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Beverly, Mass. Polynucleotide kinase was purchased from New England Nuclear Corp., Boston, Mass.

Plasmids. R100 (in J6-2) was obtained from A. M. Reynard, State University of New York, Buffalo.

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pRR12, a copy number mutant of NR1 (24) previously assumed to be identical with R100, was obtained from S. Silver, Washington University, St. Louis, Mo. In this paper, we refer to the plasmid from Reynard as R100 and to that from Silver as NR1 or pRR12. pBH35 (pBR322:*Bam*HI-A) (5) was obtained from K. Hardy, University of Kent, Canterbury, U.K.

Plasmid DNA was prepared by the method outlined by Tanaka and Weisblum (41). In some cases where a small amount of plasmid was needed for analytical purposes only, the cesium chloride gradient step was omitted.

Plasmid construction. Strain DB1345 (D. E. Berg) was transformed (20) with *Eco*RI-digested R100 DNA which had been ligated with an excess of *Eco*RI-digested pBR322. After heat shocking, yeast-tryptone broth was added, and the culture was incubated at 37°C for 30 min. Cells were then spun down and suspended in 5 mM Veronal buffer–0.15 M NaCl–0.5 mM $MgCl_2$–0.15 mM $CaCl_2$ (pH 7.5). Guinea pig serum (Pel-Freeze Biologicals, Inc., Rogers, Ark.) was added to a final concentration of 10%, and the suspension was incubated for 1 h at 37°C. Under these conditions, resistant cells are not affected, whereas 99% of sensitive cells are killed (27). Three more cycles of growth in yeast-tryptone broth, followed by serum killing, yielded a cell suspension which was almost totally resistant to complement. The cells were spread onto agar plates containing tetracycline at 20 μg/ml; plasmid DNA from resultant single colonies was digested with *Eco*RI and analyzed on agarose gels.

Blot hybridization experiments were carried out by the method of Southern (37) as modified by Wahl et al. (44) for transfer of large DNA fragments. The probe was purified by agarose gel electrophoresis, followed by chromatography with DEAE cellulose (15), and labeled with ^{32}P by nick translation (30).

DNA sequencing was performed by the partial chemical degradation method of Maxam and Gilbert (22). From base 280 to the end of our sequence, at least two independent sequence runs were made; both strands were sequenced for 90% of this span, and the data include overlaps at all restriction sites.

RESULTS AND DISCUSSION

Complement resistance genes from R100 and R6-5 reside on analogous restriction fragments. Reynard et al. (28, 29) showed that several antibiotic resistance plasmids (R factors), including R100 and R6-5, confer resistance to killing by serum complement. Moll et al. (26) have recently found that the resistance gene from R6-5 resides on a 6-kilobase-pair (kbp)-long *Eco*RI restriction fragment. R6-5 and R100 are closely related R factors (32) with very similar *Eco*RI restriction maps (24, 40, 43). A comparison of the two restriction maps suggests that, by analogy, the complement resistance gene from NR1-R100 should reside on the 6-kbp fragment designated F by Tanaka et al. (40; Fig. 1A). Our results show that this is essentially the case; shotgun cloning of *Eco*RI fragments from R100, followed by selection for complement resistance, gave a collection of resistant bacteria all harboring plasmids carrying an *Eco*RI fragment, F′ (data not shown), which is analogous to fragment F. Our particular isolate of R100 carries an insertion in fragment F giving fragment F′, about 7.3 kbp in length (see Fig. 1A). The Southern blot shown in Fig. 1B demonstrates the homology between fragments F and F′. We show later that the insertion in fragment F′ is insertion sequence IS*2*.

Localizing the resistance gene. To locate the resistance gene in the 7.3-kbp-long *Eco*RI fragment F′, we digested plasmid DNA from one of our clones with restriction enzymes which cut pBR322 only once. These studies revealed that fragment F′ contains single restriction sites for *Hin*dIII and *Bam*HI; these are shown schematically in Fig. 2. Because fragment F′ can insert into pBR322 in two possible orientations with respect to the vector, we analyzed plasmid DNA from four other clones cutting with *Hin*dIII; one of these had F′ inserted in the opposite orientation. Both orientations confer complement resistance (data not shown), suggesting that this fragment contains all of the resistance gene as well as signals required for expression. The resistance gene was then located relative to the restriction enzyme recognition sites as follows. Plasmid DNA carrying F′ in one or the other orientation was cut with *Hin*dIII or *Bam*HI, and the resulting fragments were circularized by ligating at a low concentration. Bacteria transformed with this DNA were then assayed for complement resistance. The results showed that the 3.0-kbp-long DNA segment spanning the *Hin*dIII and *Bam*HI recognition sites (fragment HB in Fig. 2) confers resistance. Fragment HB was then recloned into pBR322 across the *Hin*dIII-*Bam*HI site of the vector (39), giving the 7.0-kbp derivative plasmid pOW2. This plasmid was further digested with *Ava*I or *Pvu*II and recircularized giving plasmids pOW3 and pOW4, which had lost 1,000 and 500 base pairs, respectively, of sequences from R100 as well as essentially all of the *tet* region of pBR322. Both pOW3 and pOW4 confer complement resistance. pOW3, carrying only the 1.9-kbp-long fragment HA (Fig. 2) from R100, was used in all sequencing studies.

The striking similarity between the restriction map of fragment F′ shown in Fig. 2 and that reported by Moll et al. (26) supports the notion that the complement resistance genes from R6-5 and R100 are closely related. Because transposon mutagenesis studies suggested that the R6-5 resistance gene lies in a region bounded by two *Bst*EII recognition sites, we focused on the apparently analogous region of our DNA fragment and sequenced the 1,200-base-pair-long

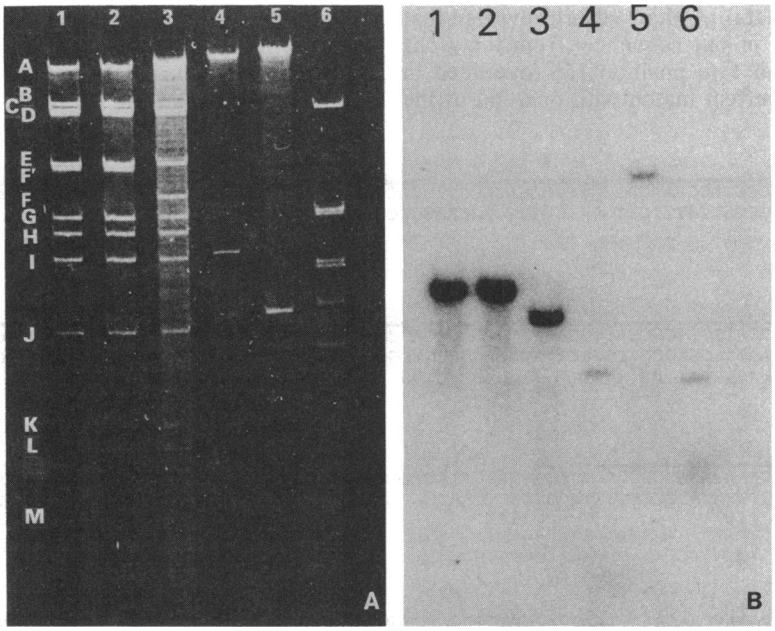

FIG. 1. (A) Ethidium bromide-stained 0.8% agarose gel showing restriction enzyme fragments from R100 digested with *Eco*RI (lanes 1 and 2), pRR12 (NR1) digested with *Eco*RI (lane 3), pBH35 (derived by inserting a 34-kbp-long fragment from ColV,I-K94 containing *iss* into pBR322) digested with *Bam*HI (lane 4), pBH35 digested with *Eco*RI (lane 5), and pBH35 digested with *Eco*RI and *Hin*dIII (lane 6). (B) Autoradiogram of a Southern blot of the gel shown in (A) probed with the 3-kbp-long restriction fragment HB (see text and Fig. 2) labeled with ^{32}P. The probe was slightly contaminated with pBR322, and the faint bands seen in lanes 4 through 6 arise from hybridization to pBR322 sequences in pBH35 (see text and Table 1).

stretch from the leftward *Hpa*I site to the *Ava*I site (shown expanded in Fig. 2). The sequence of this segment is shown in Fig. 3.

Part of the sequence is IS2. Having completed the sequence shown in Fig. 3, we inspected the sequence for open reading frames and also for the presence of insertion elements. Two observations prompted the search for insertion elements. First, the size difference between fragments F and F', 1.3 kbp, fits well with the size of many insertion elements; second, colony hybridization studies had shown that fragment HB hybridizes to DNA from *E. coli* strains which do not carry R100 or related plasmids (J. Tuan,

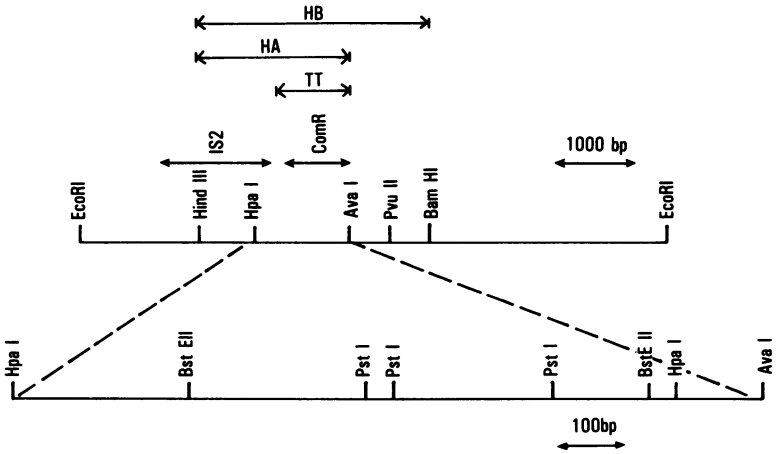

FIG. 2. Restriction enzyme map of the cloned 7.3-kbp-long *Eco*RI fragment (F') from R100 carrying the complement resistance gene. HB, HA, and TT refer to subfragments which have been cloned separately and which confer complement resistance. The positions of the insertion element IS2 and the complement resistance gene were determined by DNA sequence analysis. The complete nucleotide sequence was determined for the *Hpa*I-*Ava*I fragment (shown expanded).

unpublished data). A short search revealed that the beginning of our sequence, from the *Hpa*I site at position 1 to position 156 (overlined in Fig. 3), is a perfect match with one end of the 1.3-kbp-long insertion element IS*2* (12). In addition, the *Hin*dIII site to the left of the *Hpa*I site corresponds to a *Hin*dIII site in IS*2*. Therefore, we infer that fragment F′ carries a complete

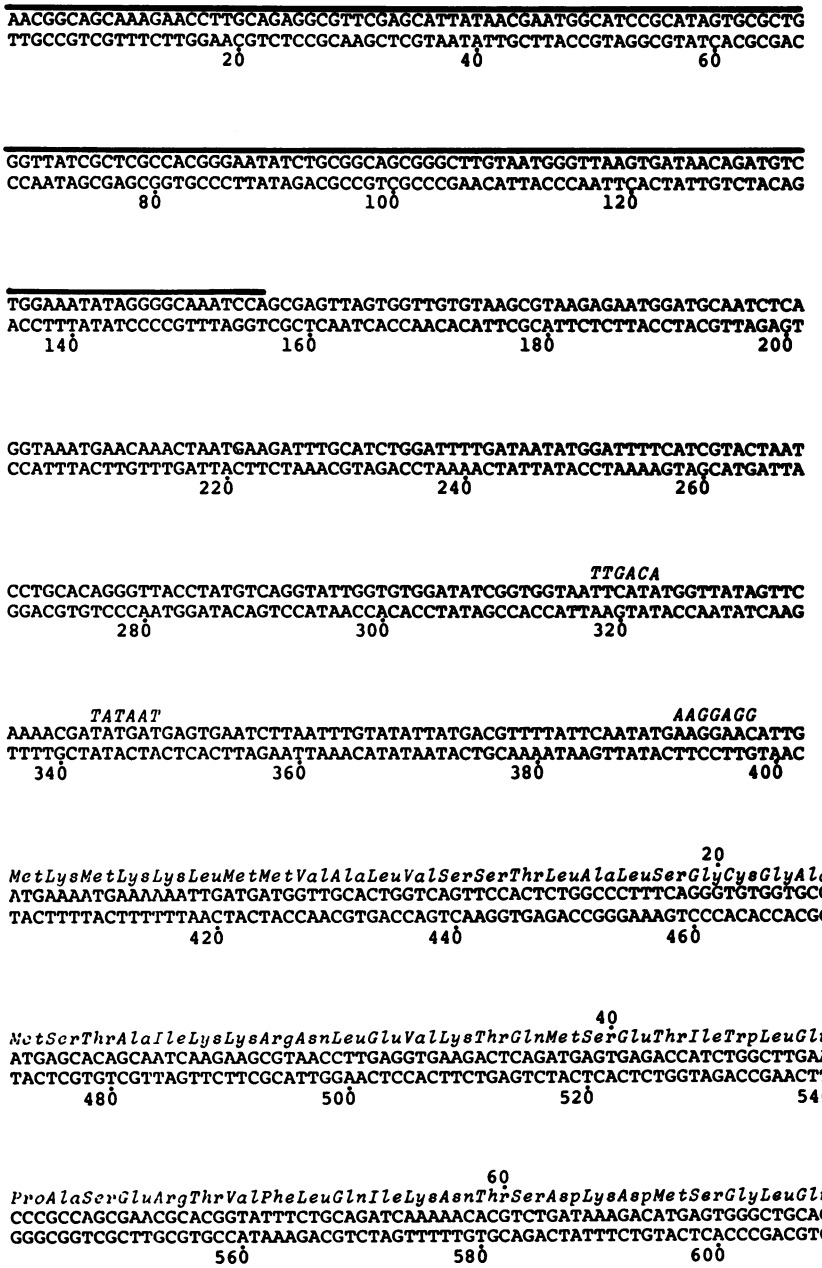

FIG. 3. Nucleotide sequence of the gene for complement resistance (*traT*). The sequence given covers the entire *Hpa*I-*Ava*I restriction enzyme fragment schematically shown in Fig. 2. The overlined sequence corresponds exactly with part of the sequence of the insertion element IS*2* (12). The consensus sequences for the −35 region and the Pribnow box of bacterial promoters (34) are given above the sequence at about bases 320 and 345, respectively. The Shine-Dalgarno sequence is given above the sequence between bases 390 and 400. The proposed amino acid sequence of the *traT* protein is given above the nucleotide sequence starting at base 403; the amino acids are numbered above the protein sequence.

copy of IS2 and that the 1.9-kbp insert in pOW3 carries about 860 bases of IS2. Mickel et al. (23) have shown that R100 does not usually carry IS2; we assume that the R100 plasmid we used is a non-representative variant.

The sequence codes for the *traT* protein. Because IS2 accounts for 860 base pairs of the 1,900-base-pair insert in pOW3, the remaining 1,040 base pairs, beginning at position 157 in our sequence, must harbor the resistance gene. This

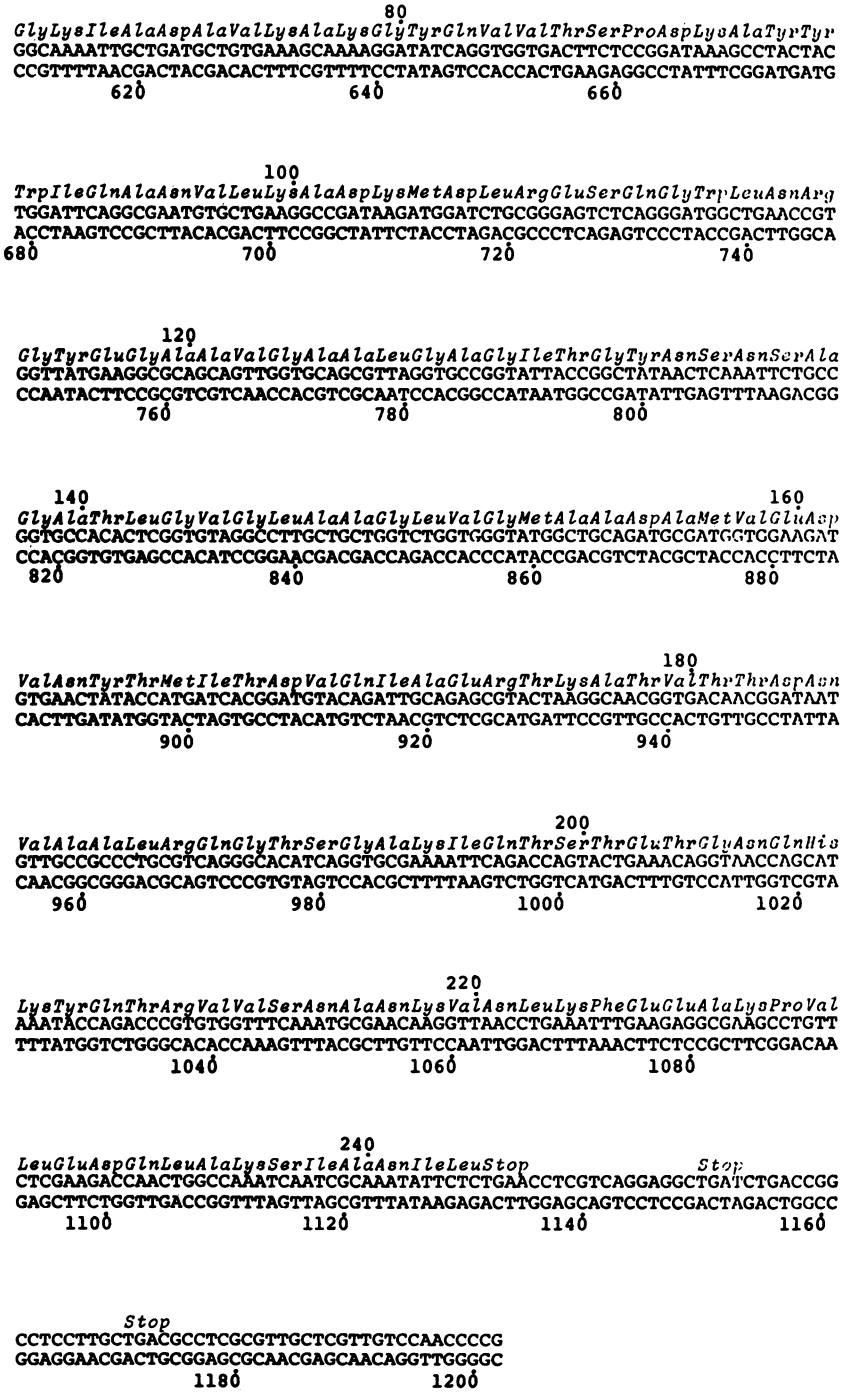

FIG. 3. *Continued*

sequence contains only one reading frame of appreciable length on either strand. Beginning at the first AUG codon, it codes for a peptide 243 amino acids long; the sequence of the proposed peptide is shown above our DNA sequence in Fig. 3. The second-largest reading frame containing a start codon (beginning at position 191 in Fig. 3) encodes a peptide 56 amino acids long; we later show that this region can be excised without affecting complement resistance. Therefore, the large reading frame must encode the resistance gene.

Sequences upstream from the open reading frame support the idea that it is expressed as a protein. The sequence beginning about 80 base pairs upstream from the proposed resistance gene resembles a bacterial promoter (34): our sequence TTCATA, in the top strand starting at base 318, matches reasonably well with the consensus sequence TTGACA (shown above our sequence in Fig. 3) for the −35 homology region, and our sequence TATGAT, beginning at base 343, is an excellent match with the consensus sequence TATAAT for the Pribnow box. Nineteen base pairs separate these two sequences; this is more than the norm but within the range of known promoters. The sequence AAGGAAC lies 43 base pairs downstream from the putative Pribnow box and immediately precedes the coding region; this sequence matches closely with the Shine-Dalgarno sequence (33), AAGGAGG (shown above our sequence), characteristic of ribosome binding sites.

The putative gene is almost certainly the R100 *traT* gene. The serum resistance gene is coincident with *traT* in the closely related plasmid R6-5 (26), and our sequence shows no other open reading frames; we can therefore exclude the possibility of two distinct but overlapping genes which frequently occur in extrachromosomal elements. The putative resistance gene codes for a protein the size of *traT* protein, 25,000 daltons (1, 25), and the amino acid composition of the predicted protein is almost identical with that determined by Minkley (submitted for publication) for the *traT* protein from the F sex factor. In addition, cells carrying pOW3 express an outer-membrane protein antigenically similar to *traT* protein (6). The first stop codon used here, UGA, is relatively inefficient (45) and is closely followed by two other inphase UGA codons. Therefore, distinct *traT* and resistance proteins might arise, differing only at their carboxy termini. However, there is no evidence for heterogeneity in traTp (21).

The *traT* gene lies in a large operon, the *traY→Z* operon, carried by F-like conjugative plasmids; this operon encodes most of the functions necessary for conjugation. The *traT* product (traTp) mediates surface exclusion. Although traTp is quite similar among the F-like plasmids, small functional differences do exist which doubtless reflect structural variations in traTp from these plasmids. In the following discussion, unless specifically stated, we refer to a generalized traTp.

traT protein is an *Escherichia coli* outer-membrane protein which is exposed at the bacterial surface and is associated with peptidoglycan (10, 21). Our amino acid sequence of the putative traTp from R100 (Fig. 3) exhibits features expected of such a protein. Its first 20 amino acids resemble a signal peptide (for review, see reference 18): it has basic residues at the amino end, a hydrophobic middle, and an amino acid with a small side chain at its carboxy end. We have arbitrarily assigned the end of the signal to residue 20 because this generates a signal of average size and because Gly-Cys is the signal cleavage site in lipoprotein, another *E. coli* outer-membrane protein (16). The putative protein also contains a central 36-amino acid-long hydrophobic region, residues 119 through 155, which likely spans the membrane. Part of this region, residues 131 through 137, is somewhat hydrophilic and may be involved in aggregation of traTp in the membrane (1, 10).

Achtman et al. (1) have previously reported that traTp from F sex factor (F traTp) does not undergo post-translational processing. However, this result has recently been questioned by Ferrazza and Levy (11) and by Minkley (submitted for publication). Minkley has isolated a precursor form of F traTp, pro F traTp; he estimates that pro F traTp is 2,000 to 3,000 daltons larger than the mature protein and has determined the amino acid compositions of both proteins. A comparison of the amino acid compositions predicted for R100 traTp and experimentally determined for F traTp is quite striking. Our data supported the contention that traTp has a precursor form: the compositions matched well only when we excluded the first 20 amino acids in our sequence, and these 20 residues showed an unusually high content of methionines, leucines, and lysines, in agreement with the composition of Minkley's putative signal peptide. The fit between the compositions of the signal peptides from R100 and F factor is not nearly as good as it is for the rest of the protein. This may be owing to errors inherent in calculating the signal composition by taking the difference between the compositions of the precursor and mature forms, as Minkley did, or it may be owing to real differences between the signals of R100 *traT* and F *traT*. If the latter is the case, there has been considerably more divergence of the signal sequence than of the mature protein; precedents for this exist for eucaryotes (18) as well as procaryotes (13). Efforts to sequence

directly the amino terminus of traTp from R100 (R100 traTp) have been unsuccessful (Finn et al., unpublished data).

***traT* from R100 carries a functional promoter.** The *traY→Z* operon, which includes the *traT* gene, is subject to complex positive and negative controls (for review, see reference 46). Expression of *traT* itself, however, appears to be somewhat anomalous: synthesis of traTp is linked to operon expression, but levels of traTp appear to be disproportionately high (17, 25). Similarly, expression of R100 *traT* has been reported to be both coordinate with (25) and independent of (10) *traY→Z* operon expression. In addition, cloned DNA fragments carrying F *traT* but not other promoter proximal sequences express high levels of F traTp; in these cases, mRNA synthesis has been assumed to initiate at promoters in the cloning vehicle (4, 17). We do not believe that expression of R100 *traT* in pOW3 initiates at a promoter in pBR322, because none of the promoters in this plasmid are in the proper position (38), and plasmids carrying fragment F' in either orientation confer complement resistance.

Although the *traT* coding sequence follows sequences which resemble bacterial promoters, this does not prove the presence of a functional promoter. Because of the possibility that the *tet* promoter from pBR322 might contribute to *traT* expression (although insertion at the *Hin*dIII site disrupts the *tet* promoter) and because part of IS2 also precedes *traT* in an orientation known to promote gene expression downstream (12), we constructed another plasmid, pSL1, to test the function of the putative *traT* promoter. pSL1 was constructed by cutting pOW3 to completion with *Eco*RI and then only partially with *Bst*EII. Staggered ends in the resulting fragments were filled in with *E. coli* DNA polymerase I. The fragments were then circularized with T4 DNA ligase and transfected into *E. coli*. Transformants resistant to ampicillin harbored plasmids 4,800, 3,800, and 3,100 base pairs in length, corresponding to the three possible products of the partial *Bst*EII digestion. The 3,800-base-pair-long plasmid was designated pSL1; it carries the 920-base-pair-long fragment (TT) containing all of *traT*, including the putative promoter region, but none of IS2 (see Fig. 2 and 3) or the *tet* promoter region. pSL1 confers the same level of complement resistance to *E. coli* strain J6-2 as does pOW3 (data not shown). We concluded that R100 *traT* carries its own promoter. Because the complement resistance test does not accurately reflect cellular levels of traTp and because pSL1 is a high-copy-number plasmid, the promoter may be somewhat inefficient. Our data are consistent with the idea that *traT* expression involves an inducible component superimposed on a base level of constitutive synthesis independent of *traY→Z* (10). Some constitutive synthesis might explain the observation that complement resistance is only slightly affected by turn-on of *traY→Z* expression (29).

R100 and ColV,I-K94 encode distinct resistance genes. Plasmids producing colicin V show a strong association with bacterial pathogenicity (35, 36). Binns et al. (5), using cloned fragments from one of these plasmids, ColV,I-K94, have shown that enhanced pathogenicity can be traced to increased serum resistance, and they have located a complement resistance gene, designated *iss* (for increased survival in serum). The *iss* locus maps well outside of the transfer genes of ColV,I-K94, and it is probably distinct from *traT*. To confirm this, we examined *iss* and *traT* for homologous sequences by the Southern blotting method. As a probe, we used the 3-kbp-long restriction fragment spanning the *Hin*dIII and *Bam*HI recognition sites shown in Fig. 2. We cloned this fragment in pBR322, digested the recombinant plasmid with *Hin*dIII and *Bam*HI enzymes, and purified the fragment by agarose gel electrophoresis. The preparation was slightly contaminated with linearized pBR322, and this served as a convenient internal control in our experiment. With this probe, we examined sequences in pBH35, a plasmid containing *iss* (a gift from M. Binns and K. Hardy; referred to as pBR322:*Bam*HI-A in reference 5). pBH35 consists of a 33.7-kbp-long *Bam*HI restriction endonuclease fragment from ColV,I-K94 cloned into pBR322. Figure 1A shows the restriction fragments generated by digestion of pBH35 with *Bam*HI, *Eco*RI, and both *Hin*dIII and *Eco*RI. Table 1 identifies the fragments carrying *iss* and pBR322 in these digests (M. Binns, Ph.D. thesis, University of Kent, Canterbury, England, 1980). Figure 1B shows a Southern blot of the gel, using the probe described above. The probe hybridizes strongly to sequences in R100 and NR1, as discussed earlier. Weak hybridization is seen to pBH35, but all hybridizing bands contain pBR322, owing to contaminating vector in the probe. We conclude that *traT* and *iss* do not hybridize and are thus distinct genes. Since the products of these genes are also immunologically distinct (6), these results suggest that at least

TABLE 1. Sizes of restriction fragments from pHB35 carrying *iss* or pBR322 or both

Enzyme	Size (kbp)	
	iss	pBR322
*Bam*HI	33.7	4.4
*Eco*RI	34.4	34.4
*Hin*dIII + *Eco*RI	10.5	4.0

two separate mechanisms of plasmid-encoded complement resistance exist.

What is the mechanism of complement resistance? Unfortunately, the sequence itself does not provide clear clues. Except for hydrophobic regions at the beginning and in the middle of the molecule, the amino acid sequence does not show any distinctive features. In addition, a computer search through a data base of about 1,400 protein sequences failed to uncover any strong homologies with other proteins (8). The fact that traTp mediates complement resistance as well as surface exclusion suggests that complement lysis and conjugation involve similar mechanisms and possibly even similar proteins. Indeed, the membrane adhesion zones described by Bayer (3) have been suggested as focal points for both processes (46, 47). This is an attractive idea, given that complement must otherwise breach the outer membrane and cross the periplasmic space to reach the inner membrane. However, apparent inconsistencies do exist. For example, stationary-phase bacteria, which have fewer adhesion zones than log-phase cells (3), show more resistance to complement lysis (47), but less surface exclusion (19) and the same content of traTp (1). Nevertheless, an understanding of the mechanism of traTp-mediated resistance to complement may clarify the function of the Bayer adhesion zones as well as the mechanisms of conjugation, surface exclusion, and complement killing of bacteria.

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