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A ColE1-Encoded Gene Directs Entry Exclusion of the Plasmid

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To detect entry exclusion of the ColE1 plasmid, we established an assay system that was not influenced by incompatibility of extant plasmids in the recipient cells or by the viability of the cells due to the killing action of colicin E1 protein. The assay revealed that *exc1* and *exc2*, assigned as genes directing entry exclusion, had no exclusion activity. Instead, *mbeD*, which had been characterized as a gene for plasmid mobilization, directed the exclusion activity. MbeD was overexpressed and identified as a ^{35}S-labeled protein, which was recovered in both the soluble and membrane fractions, particularly in the inner membrane fraction. An amphipathic helical structure was predicted in the N-terminal region of MbeD as well as in the corresponding homologous proteins of ColA and ColK. These proteins may bind to the inner membrane via the N-terminal amphipathic helix and function in entry exclusion.

The ColE1 plasmid is transmissible from a donor to a recipient cell only in the presence of a conjugative plasmid such as the F plasmid within the donor cell. Entry (surface) exclusion can be defined as the ability of the recipient cells containing a plasmid to reduce the transmission frequency in conjugal matings from other cells containing a closely related or identical plasmid. Inselburg (11) first noted this activity of ColE1, which is unrelated to plasmid incompatibility.

The F plasmid encodes two surface exclusion genes, *traS* and *traT*, which provide exclusion activity by two independent mechanisms (7, 13). The *traS* gene encodes a 18-kDa inner membrane protein. The protein expressed in the recipient cell prevents DNA transfer even when a stable mating aggregate is formed (8). The product of *traT* is a 25-kDa protein found in large amounts in the outer membrane of F-plasmid-containing cells (1). This protein in the recipient cell provides surface exclusion activity probably by reducing stable mating aggregate formation.

The entry exclusion activity of small and non-self-transmissible plasmids such as ColE1 has not been analyzed in detail. Inselburg (11) roughly defined a ColE1 region that encodes exclusion activity. Chan et al. (5) predicted that the genes related to entry exclusion were *exc1* and *exc2*, on the basis of comparison of the lengths of the structural genes with the lengths of *traS* and *traT* of the F plasmid. Upstream of *exc1* and *exc2*, there are genes required for ColE1 mobilization. Boyd et al. (4) have shown that the region which includes *mbeA*, *mbeB*, *mbeC*, and *mbeD* (*mob3*, *mob6*, *mob2*, and *mob7*, respectively, in the terminology of Chan et al. [5]) is essential for ColE1 mobilization with the R64*drd* 11 plasmid.

To detect the entry exclusion ability of ColE1, we established a system in which the entry exclusion assay is not influenced by incompatibility and colicin E1-killing activity. Using this system, we analyzed the gene and its product involved in entry exclusion.

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MATERIALS AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan). [^{35}S]methionine and [α-^{32}P]dATP were obtained from Muromachi Chemical Corp. (Kyoto, Japan) and Amersham International (Amersham, England), respectively.

Bacterial strains and plasmids. The bacteria and plasmids studied are described in Table 1. *Escherichia coli* K-12 strains CA8000 (Hfr) and AB1157R functioned as donor and recipient cells, respectively, in the entry exclusion assay. The donor plasmid was RSF2124Y or pYYCA. Constructs of recipient plasmids, all of which were derivatives of pACYC177 containing the ColE1 fragments, are shown in Fig. 1. DNA was manipulated by conventional means (15). Frameshift mutations were constructed by filling in and ligating the cohesive ends at the restriction sites (27). Plasmids pYY504, pYY505, pYY506, and pYY507 had frameshift mutations at the *Cla*I, *Bss*HII, *Mlu*I, and *Spl*I sites of pYY5, respectively. Plasmids pYY5B15 and pYY5B108, which had deletions of bp 3755 to 3897 and 3549 to 3897, respectively, were generated from pYY5 by exposure to *Bal* 31 exonuclease. The deleted regions were determined by dideoxy sequencing (20). Plasmid pYY501B had an insertion of the *Bam*HI linker (8 bp) at the *Pvu*II site of pYY501, resulting in a frameshift mutation in the *mbeD* gene. To detect the encoded proteins, the various fragments of ColE1 were cloned under the T7 promoter on pVEX11 as shown in Fig. 2.

Entry exclusion assay. Donor cells and recipient cells grown overnight in LB medium (1.0% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl) were diluted 100-fold in the medium and incubated at 37°C to an A_{600} of 0.5. The same volume of the donor and recipient cells was then mixed and incubated at 37°C for 2 h with very gentle agitation. The mixture was diluted and spread on three LB agar plates containing 50 μg of kanamycin or ampicillin per ml or both antibiotics. The total numbers of recipients (kanamycin resistance), transconjugants (ampicillin resistance), and recipients that accepted RSF2124Y (kanamycin and ampicillin resistance) were counted. The ratio of transconjugants is the number of ampicillin- and kanamycin-resistant cells divided by the number of kanamycin-resistant cells. The entry exclusion index is expressed as the ratio of transconjugants in the presence of the entry exclusion negative plasmid, pYY2, divided by the ratio of transconjugants in the presence of a test plasmid. When the index was $\leq$1, entry exclusion was negative; entry exclusion was positive when the index was $>$1.

In vivo protein synthesis. In vivo protein synthesis was analyzed by a modification of the method of Anderson and Rodwell (2). BL21(λDE3) cells harboring a pVEX11 recombinant plasmid and pLysS were grown at 37°C in LB supplemented with ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml) to an A_{600} of 0.5. Cells (1-ml culture) were harvested by centrifugation, washed twice, and suspended in 1 ml of M9 medium (15) containing each of 19 amino acids (without methionine) at 0.01%. Cells were shaken at 37°C for 30 min and incubated for 30 min in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside to induce the synthesis of T7 RNA polymerase. Rifampin was then added to a final concentration of 200 μg/ml, and incubation was continued at 37°C for an additional 30 min. Subsequently, [^{35}S]methionine (370 kBq) was added, and after incubation at 37°C for 5 min, the cells were harvested, washed once with cold M9 medium, and suspended in 100 μl of 10 mM Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS), 0.5% 2-mercaptoethanol, 10% sucrose, and 1 mM EDTA. After boiling at 100°C for 5 min, an aliquot of the solution was resolved by 6 M urea–SDS–polyacrylamide gel electrophoresis (12) followed by autoradiography to detect labeled proteins.

Subcellular fractionation. The outer and inner membranes were fractionated by the method of Hayashi and Wu (9). Cells labeled with [^{35}S]methionine in a

TABLE 1. Strains and plasmids used

Name	Genotype and description	Source or Reference
E. coli strains		
CA8000	Hfr Hays Sm^s $B1^-$	J. Beckwith
AB1157	F^- *thr-1 ara-14 leuB16* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2* λ^- *rac hisG4*(Oc) *rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	3
AB1157R	Spontaneous colicin E1-resistant mutant from AB1157	This study
BL21	*hsdS gal*	24
Plasmids and phage		
RSF2124	ColE1, Amp^r	23
pYYCA	ColA, Amp^r, ColE1 with insertion of Tn*3* fragment at the *Sma*I site in the *caa* gene	This study
RSF2124Y	RSF2124 derivative with a frameshift mutation at the *Eco*RI site in the *cea* gene	This study
pACYC177	Amp^r, Km^r	19
pYY2	Km^r, pACYC177 with deletion of a small *Bam*HI-*Pst*I fragment	This study
pYY5	Km^r, pYY2 with insertion of the ColE1 *Nae*I-*Pst*I fragment (3.1 kb)	This study
pYY4	Km^r, pYY2 with insertion of the ColE1 *Mlu*I-*Pst*I fragment (1.7 kb)	This study
pYY501	Km^r, pYY5 with deletion of the ColE1 *Mlu*I-*Pst*I fragment (1.7 kb)	This study
pVEX11	Amp^r, pBR322 with T7 promoter of ϕ10 gene and its terminator	24
pLysS	pACYC184 with T7 lysozyme and ϕ3.8 promoter	24
λDE3	λ*c*I857 *ind1 S*am7 *nim5 lac* UV5-T7 gene 1	24
pUCGCD1	*gcd* gene	26
pVEX11 *gcd*	pVEX11 with *gcd* gene	This study

1-ml culture were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C, washed, and disrupted by sonic oscillation. The crude extract was centrifuged at 200,000 × *g* for 2 h at 4°C. The membranes were suspended by a bath sonicator in 100 μl of 10 mM sodium phosphate (pH 7.0). The membrane suspension (75 μl) was mixed with 125 μl of distilled water and 200 μl of 1% Sarkosyl and then incubated for 1 h at room temperature. The mixture was centrifuged at 200,000 × *g* for 2 h at 4°C. The supernatant was collected, precipitated with trichloroacetic acid (final concentration of 10%), washed once with acetone, and dissolved in 100 μl of 10 mM Tris-HCl (pH 8.0) containing 1% SDS, 0.5% 2-mercaptoethanol, 10% sucrose, and 1 mM EDTA. The proteins that were soluble and insoluble in Sarkosyl after these procedures were considered inner and outer membrane proteins, respectively. Aliquots of the solution were resolved by 6 M urea–SDS–polyacrylamide gel electrophoresis.

Computer analysis. The secondary structures of MbeD, MbaD, and MbkD proteins were analyzed as described by Schiffer and Edmundson (21), using GENETYX-MAC (analytical software containing genetic information; Software Development Co. Ltd. Japan).

RESULTS

Construction of the ColE1 entry exclusion system. An Hfr strain (CA8000) and a colicin E1-resistant strain (AB1157R) were used as the donor and recipient, respectively, for the entry exclusion assay. The donor plasmid was RSF2124Y, a ColE1 derivative that has a frameshift mutation in the *cea* gene. Consequently, the plasmid is defective in producing active colicin E1. As a result of a Tn*3* insertion, this donor

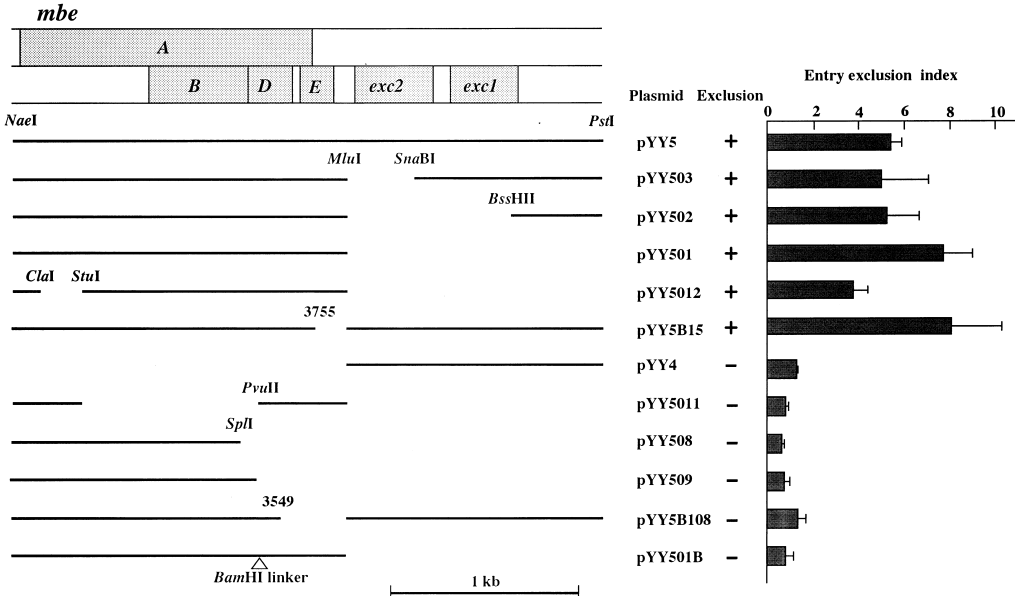

FIG. 1. Structures of deletion mutants in the region responsible for the entry exclusion activity of ColE1. Various deletion mutants were constructed from pYY5, in which the *Nae*I-*Pst*I fragment of ColE1 was inserted at the *Bam*HI-*Pst*I site of pACYC177, and then assayed for entry exclusion. Open reading frames, including *mbeA*, *mbeB*, *mbeD*, *mbeE*, *exc2*, and *exc1* around the *mbe* region, are shown at the top. Heavy lines represent the ColE1 fragments in pYY5 and its derivatives. Entry exclusion activity is shown on the right. Entry exclusion indices are averages of those of 3 to 10 assays. + and − indicate more and less than one entry exclusion index unit, respectively.

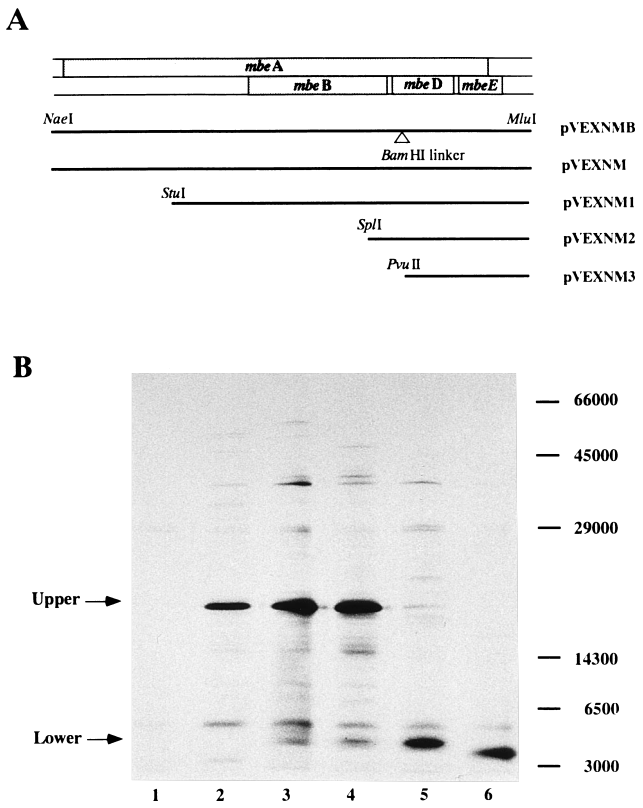

FIG. 2. Subcloning of the *mbe* genes into the T7 expression vector (A) and identification of their products (B). (A) Plasmid pVEXNM was constructed by cloning the *Nae*I-*Mlu*I fragment of ColE1 under the control of T7 promoter in pVEX11. Plasmids pVEXNM1, pVEXNM2, and pVEXNM3 were deletion mutants of pVEXNM. Plasmid pVEXNMB was constructed by inserting a *Bam*HI linker at the *Pvu*II site of pVEXNM. (B) Cells harboring the recombinant plasmids were incubated in the presence of [^{35}S]methionine after T7 RNA polymerase induction and resolved by 6 M urea–SDS–19.3% acrylamide gel electrophoresis. Lanes 1 through 6 show the labeled proteins from cells harboring pVEX11 (T7 vector), pVEXNMB, pVEXNM, pVEXNM1, pVEXNM2, and pVEXNM3, respectively. Molecular weights are shown on the right.

plasmid confers ampicillin resistance on the donor cells. Neither modification affected plasmid mobilization. pYY plasmids having various ColE1 fragments were constructed as extant plasmids in the recipient. These were derivatives of pA CYC177, which is compatible with RSF2124Y and has the kanamycin resistance gene. The ampicillin resistance gene of pACYC177 was disrupted by inserting the ColE1 fragments at its *Bam*HI-*Pst*I locus. This combination of hosts and plasmids freed the assay from the influence of incompatibility and colicin E1-killing activity and allowed convenient monitoring of the entry exclusion activity after counting of drug-resistant colonies.

Determination of the genes responsible for ColE1 entry exclusion. The results of the entry exclusion assay in the presence of the ColE1 fragments included in the pYY plasmids are shown in Fig. 1. Plasmid pYY5, which contains the *mbeA*, *mbeB*, *mbeD*, *mbeE*, *exc1*, and *exc2* genes, showed entry exclusion activity. pYY503, pYY502, and pYY501, lacking the *exc1* and *exc2* regions which were thought to be responsible for ColE1 entry exclusion (5), retained the activity. Entry exclusion indices obtained from these plasmid were 5.5 to 7.8, comparable to the index of 13.9 reported for a cloned *traS* and *traT* plasmid (7). On the other hand, pYY4, lacking the *mbeA*, *mbeB*, *mbeD*, and *mbeE* genes, had no entry exclusion activity. Thus, to identify the genes responsible for entry exclusion, we constructed frameshift and further deletion mutants from pYY5 and pYY501. Plasmids pYY504, pYY505, and pYY507, which had frameshift mutations at the *Cla*I site in the *mbeA* gene, at the *Bss*HII site in the *exc1* gene, and at the *Spl*I site in the *mbeB* gene, respectively, did not lose exclusion activity (data not shown). Plasmid pYY5012 had the activity despite deletion of part of the *mbeA* gene, but pYY5011, pYY508, and pYY509, with deletions of the *mbeB* and *mbeD*, *mbeB*, *mbeD*, and *mbeE*, and *mbeD* and *mbeE* genes, respectively, did not. To determine which of *mbeD* and *mbeE* contributes to entry exclusion, an 8-bp *Bam*HI linker was inserted at the *Pvu*II site located in the *mbeD* gene of pYY501. The resultant plasmid, pYY501B, lost the activity. Furthermore, pYY5B108, lacking the *mbeD* gene, lost the activity, but pYY5B15, lacking the *mbeE* gene, retained the activity. These results indicated that ColE1 entry exclusion was caused by the *mbeD* gene.

Given the sequence homology (70%) between the *mbeD* gene of ColE1 and the *mbaD* gene of ColA, which corresponds to the *mbeD* gene, we thought that the *mbeD* gene would function against ColA transfer. RSF2124Y was replaced with pYYCA, a ColA derivative having the ampicillin resistance gene inserted at the *Sma*I site in the *caa* gene. Transfer of pYYCA from the Hfr strain was excluded by pYY5 and pYY501 in the recipient cell with entry exclusion indices of 14.8 and 25.7, respectively, but not by pYY501B (entry exclusion index, 0.36).

Identification of proteins related to ColE1 entry exclusion. To identify the products of the *mbe* genes, we subcloned selected ColE1 fragments into an expression vector, pVEX11, downstream of the T7 promoter. Plasmid pVEXNM, constructed by subcloning the *Nae*I-*Mlu*I fragment including the *mbeA*, *mbeB*, and *mbeD* genes into the vector, is shown in Fig. 2A together with other deletion mutants of pVEXNM. Two major bands, as indicated by the upper and lower bands, appeared to be derived from the inserted ColE1 genes. The upper band (16 kDa) from cells harboring pVEXNMB (Fig. 2B, lane 2), pVEXNM (lane 3), or pVEXNM1 (lane 4) seemed to be the *mbeB* gene product, because most of it disappeared in cells harboring pVEXNM2, which lacks most of the *mbeB* and *mbeA* genes (lane 5). In lane 5, a weak 16-kDa band was found at the same position of the *mbeB* gene product, but this band also appeared in some cells harboring the vector, indicating the presence of a vector-derived protein in this position. The lower band (5.7 kDa) seen in lanes 3 to 5 was designated the *mbeD* gene product, though its molecular weight calculated from the DNA sequence is 9,299 (5). To confirm the identity of the *mbeD* gene product, an 8-bp *Bam*HI linker was inserted at the *Pvu*II site in the *mbeD* gene of pVEXNM, since this insertion diminished ColE1 entry exclusion, as shown in Fig. 1. The result showed that the 5.7-kDa band disappeared after this insertion (lane 2). When the N-terminal region of the *mbeD* gene was deleted in pVEXNM3, a 5-kDa band appeared (lane 6), which was considered to be a truncated *mbeD* gene product. A short alternative N terminus was added after ligation between parts of the ϕ10 gene on pVEX11 and the *mbeD* gene in this deletion construct. The amount of the *mbeD* gene product in lane 5 was much larger than those in lanes 3 and 4, probably because the T7 promoter was located just upstream of the *mbeD* gene in pVEXNM2. The *mbeE* gene next to the *mbeD* gene was also included in pVEXNM constructs, but its gene product was undetectable. The other weak bands seen in each lane could not be definitely identified.

Subcellular localization of MbeD. To determine where in the cellular compartments this protein functions for entry exclusion, the subcellular localization of MbeD was determined

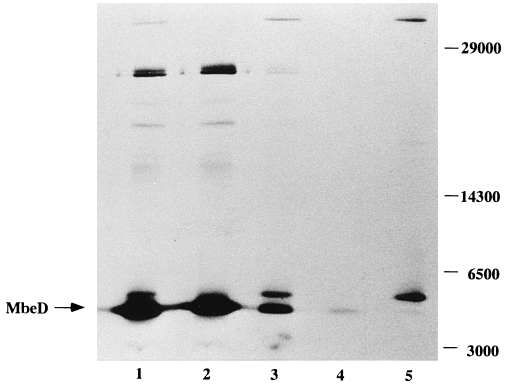

FIG. 3. Subcellular localization of MbeD. Cells harboring pVEXNM2 were labeled with [^{35}S]methionine and then subjected to subcellular fractionation as described in Materials and Methods. The fractionated materials were analyzed by 6 M urea–SDS–19.3% acrylamide gel electrophoresis and autoradiography. Lanes 1 through 5 show total proteins and soluble, membrane, inner membrane, and outer membrane fractions, respectively. The intensities of the bands are comparable because equivalent amounts of total protein were applied in all lanes. Molecular weights are shown on the right.

according to the Sarkosyl solubility of membrane proteins (9), by which inner membrane proteins are solubilized but outer membrane proteins are not. The products from pVEXNM2 were solubilized and resolved by urea-SDS-polyacrylamide gel electrophoresis (Fig. 3). Lane 1 shows the total products from pVEXNM2. Lanes 2 and 3 show the soluble and membrane fractions, respectively. When the membrane fraction was further fractionated, MbeD was recovered predominantly in the inner membrane fraction (lane 4). The low recovery (about 10%) of MbeD protein may be due to proteolytic degradation during membrane fractionation. Since MbeD was detected in both the soluble and membrane fractions, it seems to be located and to function in both the cytoplasm and the inner membrane. The 27-kDa band in lanes 1 and 2 is β-lactamase expressed from the *bla* gene on the vector, since it was always present in cells harboring the vector.

By means of the same procedure, the inner and outer membrane fractions were obtained from BL21(λDE3) cells harboring pVEX11*gcd*, which encodes inner membrane glucose dehydrogenase (26). We determined the distribution of the overexpressed dehydrogenase by Western blotting (immunoblotting) using an antibody against the enzyme. Over 90% of the dehydrogenase was recovered in the inner membrane fraction.

Secondary structure analysis of MbeD, MbaD, and MbkD. Boyd et al. (4) have compared the amino acid sequences of open reading frames located in the ColE1 *mbe* region with those of the closely related *mba* and *mbk* regions of ColA and ColK and shown that MbeA, MbeB, MbeC, and MbeD from ColE1 are homologous to the corresponding proteins from ColA and ColK. Since the amino acid identity of MbeD with the corresponding proteins is quite high (ColE1-CoA, 70%, ColE1-ColK, 66%; and ColA-ColK, 61%), these proteins may be important for plasmid functions. The subcellular localization indicated that MbeD possesses hydrophobic or membrane-binding domains. A hydropathy analysis, however, indicated that MbeD has no hydrophobic domains (data not shown). Thus, we tried to plot helical wheels by the method of Schiffer and Edmundson (21). As shown in Fig. 4, N-terminal regions from amino acid residues 7 to 21 of MbeD, MbaD, and MbkD show amphipathic helical structures. In these regions, the amino acid identities between MbeD and MbaD, MbeD and MbkD, and MbaD and MbkD are 80, 100, and 80%, respectively.

DISCUSSION

Inselburg (11) demonstrated the phenomenon of incompatibility in the ColE1 plasmid, which confers resistance to conjugal transfer of DNA between mating pairs carrying homologous ColE1 plasmids, and located the region necessary for the phenomenon in the *Hae*IIB fragment of the plasmid. Since the name generates confusion with the incompatibility that results from inhibition by RNA I in synthesizing primer RNA at the first stage of replication, Chan et al. (5) adopted the alternative designation entry exclusion, which was originally described by Naumova et al. (16). Two open reading frames, *exc1* and *exc2*, which are comparable in size to the surface exclusion genes *traS* and *traT* of the F plasmid, were found in the *Hae*IIB fragment. However, there was no apparent significant homology in amino acid sequences between these gene products in the ColE1 and F plasmids. It was not certain which gene is responsible for entry exclusion, since the *Hae*IIB fragment contains four genes, *mbeD*, *mbeE*, *exc1*, and *exc2*. In this study, we established an assay system and subjected various constructs containing ColE1 fragments to the assay. The results indicated that the *mbeD* gene participates in ColE1 entry exclusion.

To understand the mechanism of entry exclusion, we determined the subcellular localization of overexpressed MbeD protein. MbeD protein was recovered in the soluble and inner membrane fractions. The data suggested that MbeD is able to

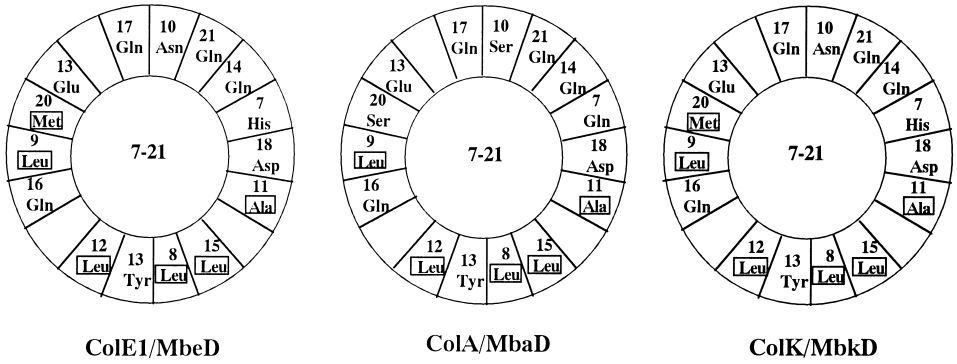

FIG. 4. Edmundson helical wheel analysis of the N-terminal amino acid sequences of MbeD in ColE1, MbaD in ColA, and MbkD in ColK. Hydrophobic residues are boxed.

bind to the inner membrane. The hydropathy analysis of Kyte and Doolittle (14) revealed no membrane-spanning segment in the protein, but it possessed a possible amphipathic helix at its N terminus (Fig. 4). Prostaglandin H_2 synthase 1 is a monotropic membrane protein, in that it integrates into only one leaflet of the lipid bilayer (18). This protein lacks a membrane-spanning segment but has four amphipathic helices which can interact with the membrane. Thus, it is possible that MbeD binds to the inner membrane via its amphipathic helix. MbaD of ColA and MbkD of ColK, corresponding to MbeD, have similar amphipathic helices at their N termini (Fig. 4), and their primary structures are quite similar to that of MbeD. Therefore, these proteins probably play similar roles in the entry exclusion of the plasmids in the inner membrane. In fact, the ColA plasmid was shown to be a target of the ColE1 entry exclusion mechanism in this study.

Willetts (25) argued that the ColE1 plasmid dose not require the products of F the *traI*, *traM*, and *traZ* genes for its transfer, because these F *tra* products are replaced by products of the *mbe* genes of ColE1. The TraI and MobA proteins of various plasmids are included in the same family and function in the initiation of plasmid transfer (10, 17). On the other hand, TraM had characteristics similar to those of MbeD; both had low isoelectric points (pIs of MbeD and F TraM are 4.5 and 5.1, respectively), faster migration in SDS-polyacrylamide gels, an N-terminal amphipathic helix, and the ability to associate with the inner membrane (6, 22). Moreover, F TraM binds to one site at *oriT* of the ColE1 plasmid (6). Therefore, it is assumed that MbeD also binds to this site of ColE1 presumably via the N-terminal amphipathic helix.

Thus, MbeD would play a role in initiating plasmid transfer in the donor cells. In the recipient cells, however, it is still unclear how MbeD interferes with ColE1 DNA transfer. From the fact that F TraS in the inner membrane functions in the surface exclusion, the ability of MbeD protein to adhere to the inner membrane of the recipient cells may provide a clue to its role in the entry exclusion mechanism.

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