JOURNAL OF BACTERIOLOGY, May 1993, p. 2754–2757
0021-9193/93/092754-04$02.00/0

Vol. 175, No. 9

Specific Regions of *Escherichia coli* OmpF Protein Involved in Antigenic and Colicin Receptor Sites and in Stable Trimerization

DIDIER FOUREL,[1] SHOJI MIZUSHIMA,[2] ALAIN BERNADAC,[1] AND JEAN-MARIE PAGÈS[1]*

UPR 9027, GDR 1000, Centre National de la Recherche Scientifique, 31 Chemin Joseph-Aiguier, B.P. 71, 13402 Marseille Cedex 20, France,[1] and Institute of Applied Microbiology, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan[2]

Received 17 November 1992/Accepted 3 March 1993

Four different mutations were obtained by selecting for resistance to colicin N and screening for continued production of the OmpF protein of *Escherichia coli*. Two of them also conferred resistance to colicin A. The substitutions C for R-168 (R168C) and E284K caused the loss of the E21 epitope, while the transition G285D altered the E18, E19, and E20 antigenic sites. The substitution G119D drastically affected the stability of the trimeric conformation.

The major pore proteins of *Escherichia coli* K-12, OmpC and OmpF, which are assembled into trimers in the membrane, interact with peptidoglycan and are tightly bound to the lipopolysaccharide (16, 22). In addition, they form water-filled channels and serve as receptors for various phages and colicins (3, 10, 21, 23). The three-dimensional structure of *E. coli* OmpF shows the existence of a barrel containing 16 antiparallel β-strands which enclose a loop governing the pore diameter (6). The oligomeric organization of native porins has raised several questions concerning the whys and wherefores governing the assembly of the protein into the envelope (1, 26). Two main questions concern the dynamic and conformational aspects and the characterization of the parameters involved during this complex process. The signal sequence is required during the translocation across the inner membrane but not to address the protein to its final location in the outer membrane (13, 29). Moreover, assembly intermediates have been described in the case of OmpF (9, 24). Eiselé and Rosenbusch (7) have shown that all of the information necessary for correct trimerization is contained within the polypeptide. The last residue, which is Phe in every porin sequenced, has been shown to be important for porin assembly (28). We described here the antigenic and colicin receptor characteristics of various mutated OmpF and OmpF-OmpC hybrids. A central domain of OmpF seems to be strategic for stable trimerization of the porin, and the replacement of G-119 in this region reduces trimer stability as well as causes a loss of colicin N receptor activity.

Isolation of *ompF* mutations and effects on colicin sensitivity. To determine the role of specific OmpF residues in colicin receptor activity, we selected mutations in *ompF* that conferred resistance to colicin N. pLG361 DNA encoding *ompF* (13) was incubated overnight at 37°C with 1 M hydroxylamine in 500 mM sodium phosphate, pH 6. The DNA was passed through a spun column (G-50 Sephadex, 4 min, 1,600 rpm) and subsequently precipitated with ethanol. BZB1107 (*ompF*::Tn*5* Km^r) cells were then transformed with mutagenized pLG361 and grown in Luria-Bertani medium at 37°C for 1 h. Colicin N (0.75 mg/ml) was then added, the cells were incubated for 30 min, and the resistant clones were selected on tetracycline-kanamycin plates. The colicin N-resistant clones expressing the OmpF protein were identified by an immunoblotting assay with polyclonal antisera directed against OmpF. Thirty-four colicin N-resistant clones producing an OmpF protein were obtained. DNA sequences were determined by the dideoxy chain termination method (27), and four types of substitutions were identified by sequencing the 34 mutagenized genes. A single base pair transition was identified in each case, and three of them were guanine-to-adenine transitions (Table 1). To analyze the role of OmpF in the first step of colicin N entry, we have previously developed a method using the binding of iodinated colicin N on the surface of intact cells bound to nitrocellulose (8). As shown in Table 1, the four substitutions abolished the colicin N-binding site under these conditions. In addition, two substitutions, D for G-119 (G119D) and R168C, also resulted in resistance to colicin A, whereas plasmids carrying *ompF* E284K or *ompF* G285D restored colicin A sensitivity to the *ompF* cells (Table 1).

The bypass treatment has been shown previously to restore colicin A sensitivity to strains lacking OmpF protein provided that the cells produced another porin (8). All four OmpF variants were found to confer sensitivity to colicins A and N at the same level as cells expressing wild-type OmpF under bypass conditions (Table 1). This indicates that the amino acid substitutions do not affect colicin A or N uptake under receptor bypass conditions. We conclude that two residues, G-119 and R-168, are required for colicin A and N receptor activity while the two others, E-284 and G-285, are required only during colicin N reception and that none of these residues are required for translocation of colicin A or N across the outer membrane. All mutations are located inside the RN regions previously identified as being involved in colicin entry (8).

OmpF regions involved in antigenic determinants. The characterization of the epitopes recognized by four monoclonal antibodies (MAbs) directed against exposed regions of OmpF (17) was carried out with the OmpF-OmpC hybrid porins (11, 19, 30) or the mutated OmpF described above. The *ompF* cells expressing the various modified porins were immobilized on nitrocellulose and incubated with the MAbs. MAbs 18, 19, and 20 bound to cells producing the hybrids 26, 10, 14, 403-10, 403-14, 462-10, 1354-10, and 1354-17 (Fig. 1),

* Corresponding author.

TABLE 1. *ompF* mutations and colicin sensitivities

Plasmid	Transition[a]	Gene product[b]	Colicin N binding[c]	Sensitivity[d] to colicin:			
				N		A	
				Direct	Bypass	Direct	Bypass
pLG361	None	OmpF	+	+	+	+	+
pFD119	G877A	G119D	−	−	+	−	+
pFD168	C1023T	R168C	−	−	+	−	+
pFD284	G1371A	E284K	−	−	+	+	+
pFD285	G1375A	G285D	−	−	+	+	+

[a] According to the sequence from the work of Inokuchi et al. (12).
[b] Numbered from the first residue of the mature sequence.
[c] One microliter of cells (optical density at 600 nm = 1.5) was applied to nitrocellulose. After incubation with buffer (phosphate-buffered saline [PBS] containing 10% bovine serum) for 30 min, the filters were incubated with ^{125}I-colicin N in PBS containing 0.2% Triton X-100 for 3 h. After four washings in the same buffer, the nitrocellulose was dried and the autoradiography was performed.
[d] Cells growing in Luria-Bertani medium (100 μl to an optical density at 600 nm of 0.5) were incubated with a 10^3 dilution of colicins for 20 min at 37°C. After dilution with fresh medium the suspensions were incubated for 2 h, and the percentage of sensitive cells checked by optical density determination was evaluated by comparison with nontreated cells. For bypass experiments, cells were washed three times with sodium phosphate before incubation with colicin A or N in the same buffer.

indicating that they may all detect the respective epitopes. A signal was not observed with other chimeric porins. Hybrids 3703, 10, 14, 403-10, 403-14, 462-10, 1354-10, and 1354-17 were the only ones recognized by MAb 21 (Fig. 1). The region of OmpF located between residues 170 and 313 seems to be necessary for the exposure of E21, while the region located between residues 217 and 340 was required for the exposure of the E18, E19, and E20 epitopes (Fig. 1). Immunogold labelling on ultrathin frozen sections of cells produced similar results (data not shown). Substitution G285D caused the loss of the E18, E19, and E20 sites but not the E21 epitope. The reverse situation was observed with cells with R168C and E284K, which lacked the E21 epitope but retained the E18, E19, and E20 epitopes. The G119D substitution did not affect binding of any of the MAbs. These results indicate that R-168, E-284, and G-285 are required for the binding of colicin N and at least one of the antibodies. Substitutions at residues 284 and 285 produce the opposite effect on the antigenicity, suggesting a specific role of these two residues in their respective epitopes.

Stability of modified trimers. Native OmpF exists as a stable trimer, even in the presence of sodium dodecyl sulfate (SDS), at temperatures below 75°C (20, 25). It has previously been reported that some of the chimeric porins have reduced thermal stability (19). When the junction is around residue 144, e.g., in hybrid 10, the chimeric porin is heat labile. In contrast, stable trimers are formed by hybrids 14 and 403-14, which present the junction at residue 115. Thus, a compatibility between specific regions of OmpF seems to be necessary to form stable trimers, and these porin regions are not totally exchangeable between OmpF and OmpC (11). The four OmpF substitutions were tested under the same conditions. All mutants tested, except G119D, exhibited the same heat stability pattern as wild-type OmpF. In contrast, the G119D OmpF was heat labile and the trimer was dissociated and migrated as a monomer under conditions which caused the dissociation of trimers formed by hybrids containing a junction at residue 144 (data not shown). To determine the level of instability of this trimeric form, analyses were

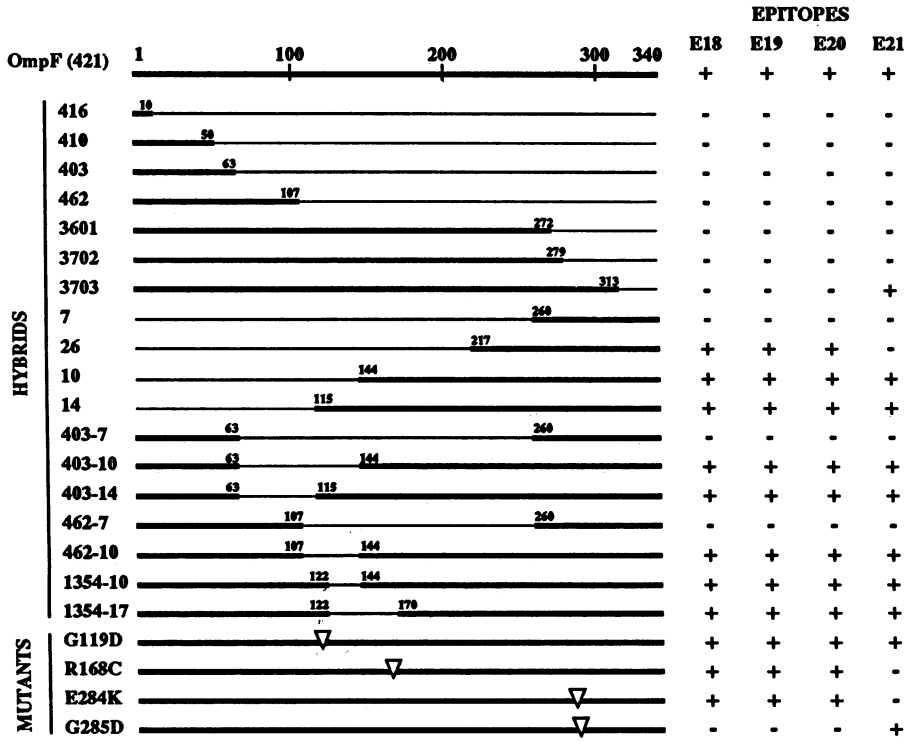

FIG. 1. Antigenic sites on the various modified OmpF proteins. Thick lines correspond to the specific OmpF regions, and thin lines represent the OmpC sequence in the various OmpF-OmpC hybrids. The numbers indicate the first or/and last OmpF residues in the hybrid porins. Triangles show the positions of the substitutions in the mutants. The detection of the various epitopes (E) is indicated.

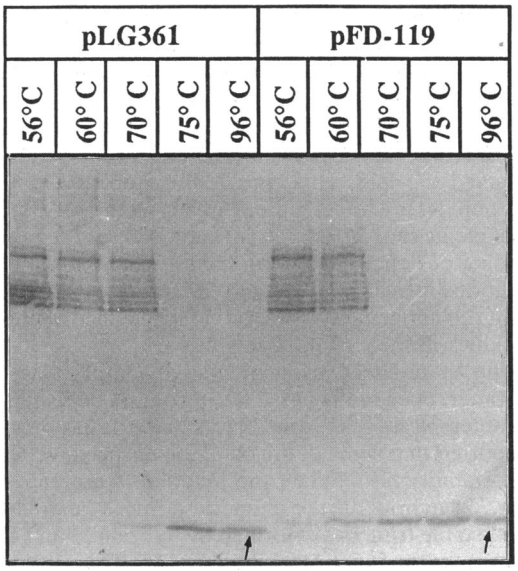

FIG. 2. pFD-119 encoded an OmpF trimer dissociated at low temperatures. BZB 1107 cells were solubilized at various temperatures and loaded on 10% polyacrylamide-SDS gels. After electrotransfer onto nitrocellulose, detections were carried out with polyclonal antibodies directed against OmpF. pLG361, wild-type OmpF; pFD-119, G119D. Arrows show the position of the OmpF monomer.

performed by using various temperatures of solubilization (Fig. 2). Wild-type trimer was dissociated at 75°C, while the monomer corresponding to the mutant protein (G119D) was clearly obtained after treatment at 60°C under the conditions used. In addition, a difference in the migration of monomers from the wild type and G119D was observed (Fig. 2).

Conclusions and discussion. By comparison with the three-dimensional structure (6), three loops, L6, L7, and L8 (Fig. 3), seem to be necessary for the exposure of E18, E19, and E20. The substitution G285D in L7 eliminates these epitopes. E21, detected only on the trimer (9), involves loops L4, L5, L6, and L7. The substitutions at R-168 and E-284 positively identify L4 and L7 as a part of the epitope. Loop L2 of one monomer interacts with loops L2, L*3, and L4 of the adjacent subunit (6). The substitution at R-168, located inside L4, could affect the trimeric conformation altering E21 without affecting the other epitopes. Sequence comparisons show that a consensus sequence, from 116 to 120 (PEFGG), is conserved in the *E. coli*, *Enterobacter cloacae*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* porins (14). This region has been described as important in pore function (2, 6, 15, 18). The substitution G119D affects a residue that is located in the internal constriction zone delimiting the pore. This transition, altering the colicin binding and the thermal stability without affecting the antigenic profile, probably disturbs the conformation. Taking into account the antigenic map; the kinetics of the exposure of the E18, E19, and E21 epitopes (9); and the OmpF structure indicating a salt bridge between the N and C termini (6), the folding of the COOH-terminal part of OmpF would appear to be an early event during the assembly. This step can explain the requirement for the integrity of the COOH domain as previously reported for OmpF (4) and PhoE (5). In addition, the interaction between loops L4, L5, L6, and L7 of each monomer and between the loops belonging to the three subunits occurs rapidly since the epitope E21 is detected early during the OmpF assembly.

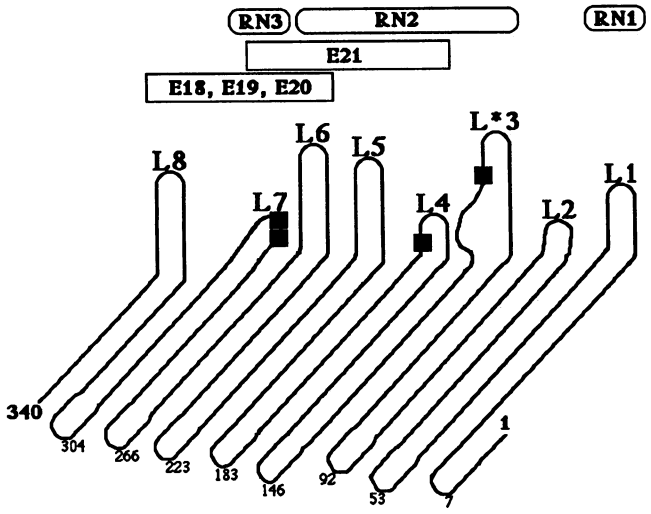

FIG. 3. Regions involved in antigenic sites and in the entry of colicin N. The OmpF topological model (6) presents the various cell surface-exposed regions (L1, L2, L4, L5, L6, L7, and L8) and the internal loop (L*3). RN1, RN2, and RN3 correspond to regions involved in the binding of colicin N that were previously determined (8). Black squares indicate the substitutions isolated in this work.

We thank Immunotech for carrying out the preparation of the MAbs. We gratefully acknowledge J.-M. Bolla, D. Cavard, A. P. Pugsley, and T. Schirmer for helpful discussions and M. Green for careful reading.

This work was supported by the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale (CRE 893011).

REFERENCES

1. **Baker, K., N. Mackman, and I. B. Holland.** 1987. Genetics and biochemistry of the assembly of proteins into the outer membrane of *E. coli*. Prog. Biophys. Mol. Biol. **49**:89–115.
2. **Benson, S. A., J. L. L. Occi, and B. A. Sampson.** 1988. Mutations that alter the pore function of the OmpF porin of *E. coli* K-12. J. Mol. Biol. **203**:961–970.
3. **Benz, R., and K. Bauer.** 1988. Permeation of hydrophilic molecules through the outer membrane of gram-negative bacteria: review on bacterial porins. Eur. J. Biochem. **176**:1–19.
4. **Bolla, J.-M., A. Bernadac, C. Lazdunski, and J.-M. Pagès.** 1990. A genetic engineering approach to study the mode of assembly of the OmpF porin in the envelope of *E. coli*. Biochimie **72**:385–395.
5. **Bosch, D., M. Scholten, C. Verhagen, and J. Tommassen.** 1989. The role of the carboxy-terminal membrane spanning fragment in the biogenesis of *E. coli* outer membrane protein PhoE. Mol. Gen. Genet. **216**:144–148.
6. **Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch.** 1992. Crystal structures explain functional properties of two *E. coli* porins. Nature (London) **358**:727–733.
7. **Eiselé, J.-L., and J. P. Rosenbusch.** 1990. *In vitro* folding and oligomerization of a membrane protein. Transition of bacterial porin from random coil to native conformation. J. Biol. Chem. **265**:10217–10220.
8. **Fourel, D., S. Hikita, J.-M. Bolla, S. Mizushima, and J.-M. Pagès.** 1990. Characterization of OmpF domains involved in *E. coli* K-12 sensitivity to colicins A and N. J. Bacteriol. **172**:3675–3680.
9. **Fourel, D., S. Mizushima, and J.-M. Pagès.** 1992. Dynamics of the exposure of epitopes on OmpF, an outer membrane protein of *E. coli*. Eur. J. Biochem. **206**:109–114.

10. **Hancock, R. E. W.** 1987. Role of porins in outer membrane permeability. J. Bacteriol. **169**:929–933.
11. **Hikita, C., Y. Satake, H. Yamada, T. Mizuno, and S. Mizushima.** 1989. Structural and functional characterization of the OmpF and OmpC porins of the *E. coli* outer membrane. Res. Microbiol. **140**:177–190.
12. **Inokuchi, K., N. Mutoh, S. Matsuyama, and S. Mizushima.** 1982. Primary structure of the *ompF* gene that codes for a major outer membrane protein of *E. coli* K-12. Nucleic Acids Res. **10**:6957–6968.
13. **Jackson, M. E., J. M. Pratt, N. G. Stoker, and I. B. Holland.** 1985. An inner membrane protein N-terminal signal sequence is able to promote efficient localization of an outer membrane protein in *E. coli*. EMBO J. **4**:2377–2383.
14. **Jeanteur, D., J. H. Lakey, and F. Pattus.** 1991. The bacterial porin superfamily: sequence alignment and structure prediction. Mol. Microbiol. **5**:2153–2164.
15. **Lakey, J. H., E. J. A. Lea, and F. Pattus.** 1991. *ompC* mutants which allow growth on maltodextrins show increased channel size and greater voltage sensitivity. FEBS Lett. **278**:31–34.
16. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *E. coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737**:51–115.
17. **Lupi, N., A. Bourgois, A. Bernadac, S. Laboucarié, and J.-M. Pagès.** 1989. Immunological analysis of porin polymorphism in *E. coli* B and K-12. Mol. Immunol. **26**:1027–1036.
18. **Misra, R., and S. A. Benson.** 1988. Genetic identification of the pore domain of the OmpC porin of *E. coli*. J. Bacteriol. **170**:3611–3617.
19. **Mizuno, T., H. Kasai, and S. Mizushima.** 1987. Construction of a series of *ompC-ompF* chimeric genes by in vivo homologous recombination in *E. coli* and characterization of their translational products. Mol. Gen. Genet. **207**:217–223.
20. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *E. coli* K-12. J. Biochem. **80**:1411–1422.
21. **Nikaido, H.** 1992. Porins and specific channels of bacterial outer membranes. Mol. Microbiol. **6**:435–442.
22. **Nikaido, H., and M. Vaara.** 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
23. **Pugsley, A. P.** 1984. The ins and outs of colicins. Part I: production, and translocation across membrane. Microbiol. Sci. **1**:168–176.
24. **Reid, J., H. Fung, K. Gehring, P. Klebba, and H. Nikaido.** 1988. Targeting of porin to the outer membrane of *E. coli*. J. Biol. Chem. **263**:7753–7759.
25. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *E. coli*. J. Biol. Chem. **249**:8019–8029.
26. **Saier, M. H., P. K. Werner, and M. Müller.** 1989. Insertion of proteins into bacterial membranes: mechanism, characteristics, and comparisons with the eucaryotic process. Microbiol. Rev. **53**:333–366.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Struyvé, M., M. Moons, and J. Tommassen.** 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. **218**:141–148.
29. **Tommassen, J., H. van Tol, and B. Lugtenberg.** 1983. The ultimate localization of an outer membrane protein of *E. coli* K-12 is not determined by the signal sequence. EMBO J. **2**:1275–1279.
30. **Yamada, H., N. Oshima, T. Mizuno, H. Matsui, Y. Kai, H. Noguchi, and S. Mizushima.** 1987. Use of a series of OmpF-OmpC chimeric proteins for locating antigenic determinants recognized by monoclonal antibodies against the OmpC and OmpF proteins of the *E. coli* outer membrane. J. Biochem. **102**:455–464.