JOURNAL OF BACTERIOLOGY, Feb. 1988, p. 528–533
0021-9193/88/020528-06$02.00/0

Vol. 170, No. 2

Isolation and Characterization of OmpC Porin Mutants with Altered Pore Properties

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Received 9 July 1987/Accepted 29 October 1987

The LamB protein is normally required for the uptake of maltodextrins. Starting with a LamB$^-$ OmpF$^-$ strain, we have isolated mutants that will grow on maltodextrins. The mutation conferring the Dex$^+$ phenotype in the majority of these mutants has been mapped to the *ompC* locus. These mutants, unlike LamB$^-$ OmpF$^-$ strains, grew on maltotriose and maltotetraose, but not on maltopentaose, and showed a significantly higher rate of [^{14}C]maltose uptake than the parent strain did. In addition, these mutants showed increased sensitivity to certain β-lactam antibiotics and sodium dodecyl sulfate, but did not exhibit an increase in sensitivity to other antibiotics and detergents. The nucleotide sequence of these mutants has been determined. In all cases, residue 74 (arginine) of the mature OmpC protein was affected. The results suggest that this region of the OmpC protein is involved in the pore domain and that the alterations lead to an increased pore size.

OmpF and OmpC are the two major outer membrane proteins of *Escherichia coli* K-12. These proteins, also known as porins, form relatively nonspecific pores which allow diffusion of nutrients across the outer membrane (23), serve as receptors for various bacteriophages (3), and facilitate the transport of colicins (4). The exclusion limit of pores formed by OmpF and OmpC is estimated to be between 400 and 600 daltons, with pore sizes of 1.2 and 1.1 nm, respectively (26). These proteins are tightly associated with peptidoglycan and lipopolysaccharide (30), and hence a role in membrane integrity has also been proposed (28). The genes encoding OmpF and OmpC have been cloned, and their nucleotide sequences (11, 21) have revealed an extensive homology at both the DNA and protein levels (21).

Over the last decade, the structure and function of the porin proteins have been extensively studied (for reviews, see references 17, 24, and 27). X-ray diffraction and infrared spectroscopic analyses suggested that porins are rich in β-sheet structure and that many of these structures are oriented so that the backbone is roughly perpendicular to the surface of the membrane (7, 8, 12, 30). Electron-microscopic analysis revealed that presence of a trimer unit that contains a triplet of channels on the outside which fuse to form a central channel outlet on the periplasmic face (5). Several other biochemical approaches have also been pursued to define the pore structure (13, 32).

Porin mutants which grow on maltodextrins in the absence of the LamB protein have been described (1). Preliminary studies have indicated that these mutants may have an altered (enlarged) pore size. To date, only one *ompC* mutant of this kind has been isolated and partially characterized (1). In the present study we report the isolation and characterization of additional *ompC*(Dex) mutants and provide the first evidence of the involvement of a specific segment of the OmpC protein in the pore function.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and chemicals. Minimal (M63) and complex (Luria agar or broth) media were prepared as described by Silhavy et al. (34). Maltodextrin was purchased from Pfanstiehl Laboratories, Inc. It was further purified by dialysis in Spectrapor (Spectrum Medical Industries, Inc.) dialysis tubing with a cutoff limit of 1,000 daltons. The dialyzed maltodextrin was titrated such that a LamB$^-$ strain (MCR106) would not grow on this sugar for at least 36 h. Purified maltodextrin sugars (maltotriose to maltohexaose) were purchased from Boehringer Mannheim Biochemicals and used in growth media at a final concentration of 1 mM. Antibiotic disks were purchased from Difco Laboratories or BBL Microbiology Systems. [^{14}C]maltose was purchased from Amersham Corp.

Genetic manipulations. Procedures involving P1 transduction and isolation of a Tn*10* linked to the *ompC* gene were performed by the method of Silhavy et al. (34). The genetic marker *zeiO6*::Tn*10* was isolated from a Tn*10* pool (kindly provided by Nancy Trun) prepared on MC4100. To identify a Tn*10* insertion near the *ompC* gene, the tetracycline resistance (Tc^r) determinant from the Tn*10* pool was moved by P1 transduction into RAM164, which carries an *ompC-lacZ* fusion, and Tc^r transductants were screened for a Lac$^-$ phenotype. One such Lac$^-$ transductant, in which the fusion is presumably replaced by the wild-type *ompC* gene, was purified. The linkage of Tc^r to *ompC* was determined. The Tc^r (*zeiO6*::Tn*10*) marker is approximately 55% linked (by P1 cotransduction) to the *ompC* gene.

Disk sensitivity test. Detergent and antibiotic sensitivity tests were carried out as described previously (1).

[^{14}C]maltose uptake assay. The [^{14}C]maltose uptake assay was performed essentially as described by Benson and DeCloux (1), with the modification that the filters were washed with M63 medium at room temperature. Uptake assays and other growth rate experiments were repeated at least twice.

Preparation of whole-cell envelopes and SDS-polyacrylamide gel electrophoresis. Whole-cell envelopes were prepared from 10-ml overnight cultures by the lysozyme sonication method as described by Morona and Reeves (22). Proteins were analyzed on linear sodium dodecyl sulfate (SDS)-polyacrylamide gels (11% acrylamide) as previously described (16).

DNA methods. Bacterial strains were made competent and transformed with plasmid DNA by the method of Lederberg

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
MC4100	F' *araD139* Δ(*argF-lac*)*U169* *rpsL150 rel-1 flb-5301 ptsF25 deoC1 thi-1*	2
MCR106	MC4100 Δ*lamB106*	6
MH225	MC4100 Φ(*ompC'-lac*$^+$)*10-25*	10
RAM164	MCR106 Φ(*ompC'-lac*$^+$)*10-25*	This study
CS1253	W1485 F$^-$ Δ*ompC178 zei-198*::Tn*10*	33
RAM191	MCR106 Δ*ompC178 zei-198*::Tn*10*	This study
DME553	MCR106 Δ*ompF80*	S. Benson
RAM105	DME553 *zeiO6*::Tn*10*	This study
RAM119	RAM105 *ompC115*	This study
RAM120	RAM105 *ompC119*	This study
RAM121	RAM105 *ompC124*	This study
RAM122	RAM105 *ompC168*	This study
RAM124	RAM105 *ompC200*	This study
RAM125	RAM105 *ompC204*	This study
RAM126	RAM105 *ompC207*	This study
RAM328	RAM105 *ompC122*	This study
RAM330	RAM105 *ompC187*	This study
RAM331	RAM105 *ompC191*	This study
RAM332	RAM105 *ompC194*	This study
RAM333	RAM105 *ompC197*	This study
Plasmids		
pMAN006	Ap^r, vector pSC101 based, cloned genes, *ompC* and *micF*	19
pRAM1001	Ap^r, *Hin*dIII cutdown of pMAN006	This study

and Cohen (15). Plasmid DNA was prepared by the two-step cesium chloride gradient method of Garger et al. (9). Chromosomal DNA was isolated by the method of Nakamura et al. (25). Plasmid pRAM1001 was constructed from pMAN006 (19) by removing the *Hin*dIII piece which contains the *ompC* gene. DNA was treated with restriction enzymes, alkaline phosphatase, and ligase as described by Maniatis et al. (18).

Cloning and sequencing of the mutant *ompC* gene. The *ompC* gene lies within a 2.7-kilobase *Hin*dIII fragment of the chromosome (33). Chromosomal DNA prepared from *ompC* (Dex) mutants was digested with *Hin*dIII and ligated to a low-copy-number plasmid, pRAM1001, which had been digested with *Hin*dIII and treated with alkaline phosphatase. The ligated DNA mixture was transformed into an *ompC* deletion strain (RAM191), and transformants, selected on Luria plates containing ampicillin (50 μg/ml), were screened for the Dex$^+$ phenotype. The presence of the 2.7-kilobase *Hin*dIII insert containing the mutant *ompC* gene was confirmed by restriction analysis of the plasmid DNA prepared from Dex$^+$ colonies and sensitivity to the OmpC-specific phage SS4. The *Hin*dIII fragment (which carries the mutant *ompC* gene) was subcloned into M13mp18 and M13mp19 (20) in the appropriate orientation. The *Hin*dIII piece containing the wild-type *ompC* gene was also subcloned into M13 vectors. The nucleotide sequence (of the 1.1-kilobase coding region of *ompC*) was determined by the dideoxy method of Sanger et al. (31). Primers complementary to the *ompC* gene were synthesized by the DNA synthesis facilities at Princeton University.

RESULTS

Isolation and mapping of the *ompC*(Dex) mutants. To isolate and map the *ompC*(Dex) mutants, we started with a LamB$^-$ OmpF$^-$ strain (RAM105) which carries *zeiO6*::Tn*10* adjacent to the *ompC* gene (55% linked by P1; see Materials and Methods). Approximately 5×10^{10} cells were plated on dextrin-minimal plates. Dex$^+$ colonies were obtained, after 3 to 6 days of incubation, at a frequency of 10^{-10}. A total of 70 spontaneous Dex$^+$ colonies were purified and 20 of them were characterized. The *ompC* region from these mutants was transduced into DME553 and RAM164 by using the linked selectable marker, i.e., *zeiO6*::Tn*10*. The Tc^r transductants, selected on Luria-tetracycline plates, were screened for the Dex phenotype. If the mutation which confers the Dex$^+$ phenotype lies within or near the *ompC* gene, about 50 to 60% of the Tc^r transductants would be expected to become Dex$^+$. Moreover, with RAM164, which carries an *ompC-lacZ* fusion, introduction of the *ompC* gene from the mutant would result in the loss of the fusion (LacZ$^-$). Consequently, if the Dex$^+$ lesion lies within the *ompC* gene, all of the LacZ$^-$ transductants would have a Dex$^+$ phenotype.

P1 transduction results (Table 2) of 12 mutants met both criteria; i.e., the phenotypes Tc^r and Dex$^+$ were linked by approximately 53%, and the loss of the *ompC-lacZ* fusion was always associated with the acquisition of the Dex$^+$

TABLE 2. P1 mapping of the Dex$^+$ mutants

Donor	Recipient							
	RAM164 (Tc^s Dex$^-$ Lac$^+$)					DME553 (Tc^s Dex$^-$)		
	Selected marker (Tc^r)[a]	Unselected marker			% Linkage of Tc^r to Dex$^+$ Lac$^-$	Selected marker (Tc^r)[a]	Unselected marker (Dex$^+$)	% Linkage of Tc^r to Dex$^+$
		Dex$^+$ Lac$^-$	Dex$^-$ Lac$^+$	Dex$^+$ Lac$^+$				
RAM119	315	176	139	0	56	210	109	52
RAM120	179	87	92	0	49	155	77	50
RAM121	210	107	103	0	51	169	89	52
RAM122	151	77	74	0	51	142	71	50
RAM124	309	173	136	0	56	242	123	51
RAM125	268	166	102	0	62	234	132	56
RAM126	250	132	118	0	53	230	115	50
RAM328	238	128	110	0	54	247	128	52
RAM330	271	150	121	0	55	232	123	53
RAM331	246	154	92	0	63	221	120	54
RAM332	182	89	93	0	49	224	116	52
RAM333	269	140	129	0	52	238	126	53

[a] Number of colonies tested.

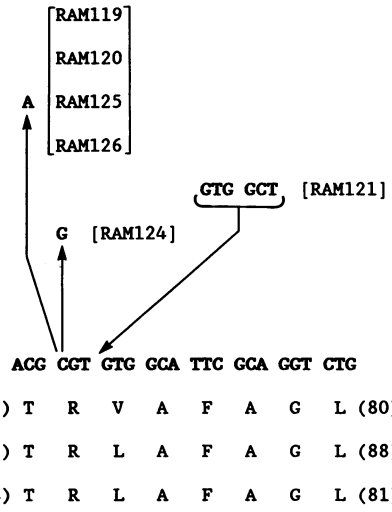

FIG. 1. Nucleotide sequence of part of the *ompC* gene shown by boldface letters. The corresponding amino acid sequence (in single-letter code) of the mature OmpC protein is shown below the nucleotide sequence. Mutational changes are indicated by arrows, and the mutants are shown in brackets. Amino acid sequences (in single-letter code) of the OmpF and PhoE proteins, homologous to OmpC, are shown in the bottom two lines. Numbers in parentheses represent the number of amino acid of the mature protein.

phenotype. These results suggest that in 12 mutants (listed in Table 2), the Dex^+ mutation lies within or very close to the *ompC* gene. The remaining eight mutants did not meet the above criteria (data not shown), and hence the Dex^+ mutation in these mutants maps elsewhere in the chromosome. Six of the *ompC*(Dex) mutants (RAM119, RAM120, RAM 121, RAM124, RAM125, and RAM126) were further characterized.

Nature of the *ompC*(Dex) mutation. The genetic mapping of the *ompC*(Dex) mutants (see above) suggested that lesions resulting in the Dex^+ phenotype were within the *ompC* gene. To confirm this and determine the exact nature of the alteration, we cloned and sequenced (see Materials and Methods) the *ompC* gene from *ompC*(Dex) mutants. All six *ompC*(Dex) mutants have an alteration at a specific region of the *ompC* gene (Fig. 1). In four mutants (RAM119, RAM120, RAM125, and RAM126), a C-to-A transversion of the first base of codon 74 resulted in the amino acid at position 74 of the mature protein being changed from arginine (R-74) to serine (S). In RAM124, a C-to-G transversion of the first base of codon 74 resulted in R-74 being changed to a glycine (G)residue. In RAM121, a 6-base-pair DNA sequence (GTG GCT) is located immediately after codon 74, which resulted in valine (V) and alanine (A) residues being inserted between R-74 and V-75.

Characterization of the *ompC*(Dex) mutants. We tested the *ompC*(Dex) mutants for their sensitivity to antibiotics and detergents. The results (Table 3) show that the *ompC*(Dex) mutants exhibit a significant increase in their sensitivity to ampicillin, benzylpenicillin, chloramphenicol, and SDS, but remained indifferent in their sensitivity to novobiocin, erythromycin, rifampin, Triton X-100, and deoxycholate. There was no dramatic difference in the sensitivity pattern among the mutants, except for RAM121, which exhibited a slightly greater sensitivity than the others.

Whole-cell envelopes prepared from the mutant and parent strains were analyzed by SDS-polyacrylamide gel electrophoresis. No apparent difference in the membrane protein profile of the mutant and parent strains was observed (data not shown). The OmpC protein from mutants did not show any qualitative or quantitative difference from the protein from the parent strain. The mutants were tested for their sensitivity to the OmpC-specific bacteriophages SS4 and Hy2 by cross-streaking against them. All mutants remained sensitive to these phages.

Growth of the *ompC*(Dex) mutants on different sugars. The maltodextrin substrate used to isolate Dex^+ mutants contained a mixture of maltooligomers ranging from maltotriose to maltoheptaose. To investigate the size-dependent diffusion of nonelectrolyte sugars across the outer membrane, we used purified maltodextrin substrates of defined lengths. These sugars were used at a very low concentrations, so that their diffusion across the outer membrane became rate limiting. The mutant and parent strains were grown on minimal medium containing glucose, maltose, maltotriose, maltotetraose, maltopentaose, or maltohexaose. These sugars have molecular weights of 180, 340, 504, 666, 829, and 991 respectively. The growth rates were plotted against the molecular weight of the corresponding sugar (Fig. 2). The results show that MC4100 ($LamB^+$ $OmpF^+$ $OmpC^+$) grew well on all sugars owing to the presence of LamB, a maltoporin, which allows diffusion of maltooligomers of up to eight units (35). The presence of the OmpF porin in MCR106 ($LamB^-$ $OmpF^+$ $OmpC^+$) and RAM191 ($LamB^-$ $OmpF^+$ $OmpC^-$) allowed growth on maltotriose, although at a much lower rate than was observed in the presence of the

TABLE 3. Disk sensitivity test of the *ompC*(Dex) mutants to various compounds

Strain	Sensitivity[a] to following compound[b]:								
	Nov (5 μg)	Ery (10 μg)	Rif (5 μg)	Cml (5 μg)	Ap (5 μg)	Pen (10 U)	SDS (1 mg)	Triton (1 mg)	DOC (1 mg)
MC4100	0	0	7	10.5	6.5	0	0	0	0
MCR106	0	0	6.5	10.5	8	0	0	0	0
RAM191	0	0	7	10.5	11.5	6	0	0	0
RAM105	0	0	7.5	8	8.5	0	0	0	0
RAM119	0	0	7	15	14	9	13	0	0
RAM120	0	0	7	15	14	9.5	13	0	0
RAM121	0	0	8	17	17	12	19	0	0
RAM124	0	0	7	15.5	15	9.5	15	0	0
RAM125	0	0	7	14	13	9	13	0	0
RAM126	0	0	6.5	15	13	9.5	13	0	0

[a] Sensitivity was determined as described in Materials and Methods. The values are the zone of inhibition in millimeters.

[b] Abbreviations: Nov, novobiocin; Ery, erythromycin; Rif, rifampin; Cml, chloramphenicol; Ap, ampicillin; Pen, penicillin G (benzylpenicillin); Triton, Triton X-100; DOC, deoxycholate.

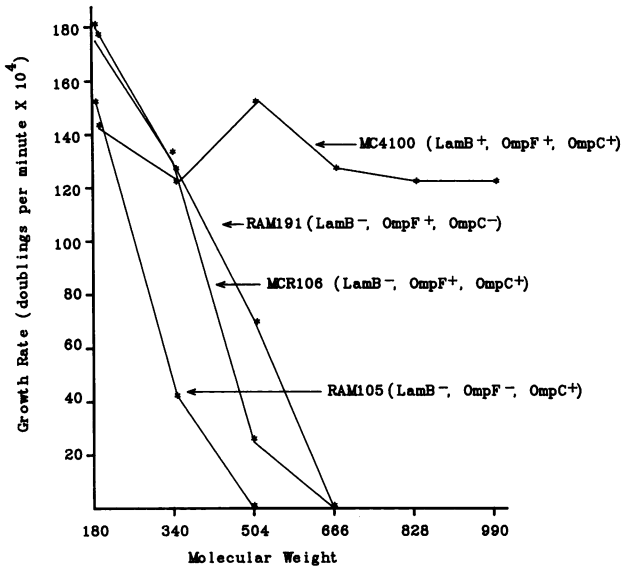

FIG. 2. Growth rates of strains with different porin composition on various sugars. Cultures were grown in M63 minimal medium containing maltose. Cells were washed twice in M63 salts, resuspended in M63, and diluted to an optical density of 0.1 at 600 nm in M63 minimal medium containing glucose (molecular weight, 180), maltose (molecular weight, 340), maltotriose (molecular weight, 504), maltotetraose (molecular weight, 666), maltopentaose (molecular weight, 828), or maltohexaose (molecular weight, 990). Cultures were grown with shaking at 37°C, and the optical density at 600 nm was noted at various times. The growth rate was calculated as the inverse of the doubling time in minutes.

LamB maltoporin. Interestingly, RAM191, which carries an *ompC* deletion, grew twice as well on maltotriose as MCR106 did. We believe that this is due to an increase in the amount of OmpF in RAM191 (data not shown). At this stage it is not clear why MC4100 grows more slowly than other strains on glucose. RAM105 (LamB$^-$ OmpF$^-$ OmpC$^+$) had the lowest growth rate on maltose and did not grow on maltotriose or higher-molecular-weight sugars. Under these growth conditions, the *ompC*(Dex) mutants showed a significantly higher growth rate than did the parent strain, RAM105, on maltose and maltotriose and were able to grow on maltotetraose, albeit at a lower growth rate than that of MC4100 (Fig. 3). These mutants were unable to grow on maltopentaose and maltohexaose.

[^{14}C]maltose uptake assays of the *ompC*(Dex) mutants. The ability of the *ompC*(Dex) mutants to grow on maltotriose and maltotetraose suggests that the mutant OmpC protein has an altered pore structure which allows the diffusion of larger substrates. We reasoned that if the size of the diffusion pore (OmpC) is enlarged, the rate of [^{14}C]maltose uptake should increase. When [^{14}C]maltose uptake is assayed at a micromolar concentration, the rate-limiting step is its diffusion across the outer membrane (24). The results (Fig. 4) show that the [^{14}C]maltose uptake rate in the *ompC*(Dex) mutants was 5- to 10-fold higher than that of the parent strain. Interestingly, the mutants showed a noticeable difference in their [^{14}C]maltose uptake rates. RAM119, RAM120, RAM 125, and RAM126 showed a rate of uptake that was approximately 5-fold higher than that of the parent strain, whereas RAM121 and RAM124 showed an approximately 10-fold increase in the uptake rate.

DISCUSSION

In this paper, we report the isolation and characterization of *ompC* mutants which grow on maltodextrin in the absence of LamB. The preliminary characterization of these mutants suggests that they have an altered OmpC pore structure.

Both in vivo and in vitro studies (24) have shown that the pores formed by OmpC are smaller than those formed by OmpF. The growth experiments performed in this study, involving the use of maltodextrin sugars of various sizes,

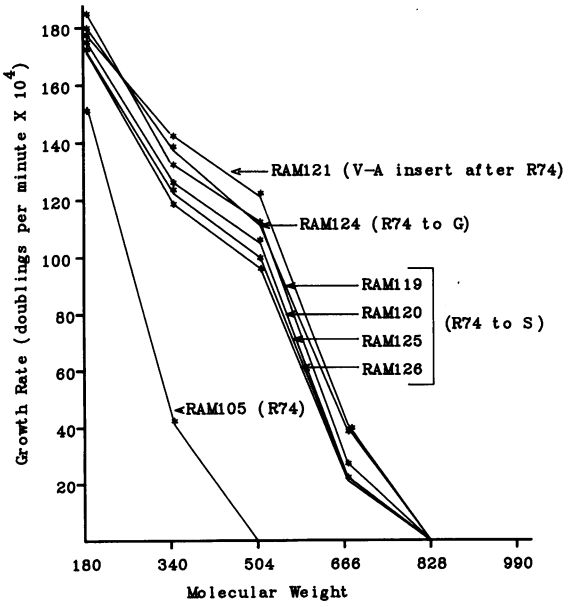

FIG. 3. Growth rate curves of the parent (Dex$^-$) and mutant (Dex$^+$) strains grown on various sugars. Strains were grown as described in the legend to Fig. 3. Mutational changes are shown in parentheses.

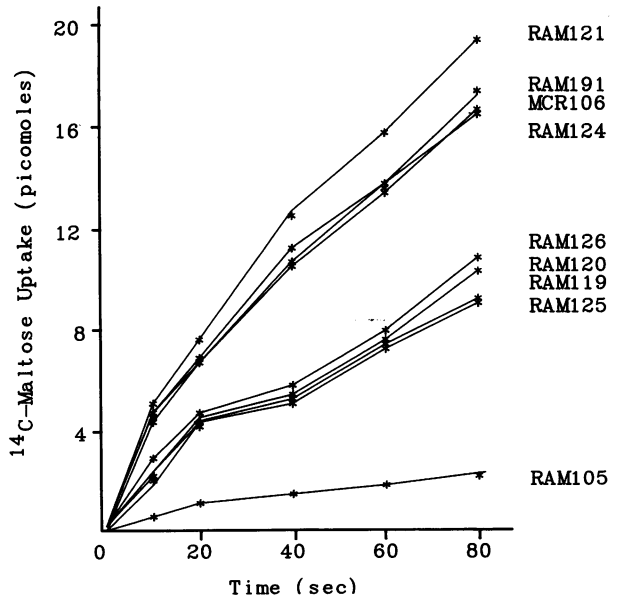

FIG. 4. [^{14}C]maltose uptake assays of parent and *ompC*(Dex) mutant strains. Assays were performed as described in Materials and Methods with 3.3 μM [^{14}C]maltose.

support this notion. The inability of a LamB$^-$ OmpF$^-$ OmpC$^+$ strain (RAM105) to grow on maltotriose or higher-molecular-weight maltooligomers at a concentration of 1 mM suggests that OmpC pores exclude sugars with a molecular weight of 504 or higher. In contrast, a LamB$^-$ OmpF$^+$ OmpC$^+$ strain (MCR106) or a LamB$^-$ OmpF$^+$ OmpC$^-$ strain (RAM191) grew on maltotriose but not on maltotetraose, suggesting that the exclusion limit of the OmpF pore is between 504 and 666 molecular weight. These results also suggest that in the absence of LamB, the ability of bacteria to grow on various malto sugars is influenced by the type of porin present.

The ability of the *ompC* mutants to grow on maltotriose and maltotetraose can then be explained by proposing that these mutants have functionally enlarged OmpC pores. This possibility is supported by the observation that the mutants have a higher rate of [^{14}C]maltose uptake than the parent strain does. In addition, the *ompC* mutants exhibited a significant increase in sensitivity to only certain antibiotics, namely, ampicillin, benzylpenicillin, and chloramphenicol, which are thought to penetrate the cell through porin proteins (14, 27, 29). Furthermore, these mutants are unaffected in their sensitivity to hydrophobic antibiotics, such as novobiocin, erythromycin, and rifampin, which are thought to enter the cell in ways other than through porins (27). We therefore favor the possibility that the Dex$^+$ phenotype, differential increase in the permeability to antibiotics, and the increased rate of [^{14}C]maltose uptake of the *ompC* mutants are due to enlarged OmpC pores. Alternatively, it may be possible that the mutant OmpC protein, like LamB, has acquired a specificity for maltose compounds. We believe this contention to be unlikely, since the *ompC* mutants undergo size-dependent growth on various sugars which extends only up to maltotetrose and furthermore does not account for the increased sensitivity to specific antibiotics and SDS. In addition, LamB$^+$ strains grown on maltose, fully induced for LamB, do not exhibit an increase in sensitivity to SDS or other antibiotics (data not shown).

We have previously reported (1) an *ompC*(Dex) mutation (*ompC3256*). This mutation, unlike the mutations characterized here, confers a dramatic increase in the sensitivity to a variety of antibiotics and detergents and alters the mobility of OmpC in SDS-polyacrylamide gels (1). In addition, it increases the rate of [^{14}C]maltose uptake 10- to 20-fold over that in the *ompC*(Dex) mutants described in this study. In light of these findings it appears possible to confer a Dex$^+$ phenotype via the OmpC porin in at least two ways. We suggest that the phenotype conferred by the *ompC3256* Dex$^+$ allele may reflect changes in the OmpC protein structure that alter not only pore property but also other functions of the protein. In contrast, the *ompC* Dex$^+$ alleles studied here appear to affect only the pore function.

The frequency of obtaining *ompC*(Dex) mutants in a LamB$^-$ OmpF$^-$ OmpC$^+$ background is approximately 10^{-10}. This low frequency suggests that the *ompC* mutations resulting in the Dex$^+$ phenotype are highly specific. All six mutants have an alteration at a specific amino acid residue, R-74, of the mature OmpC protein. In five mutants, a 1-base-pair substitution resulted in R-74 being replaced by a serine or glycine residue. In the sixth mutant, an insertion of a 6-base-pair DNA sequence resulted in the addition of valine and alanine residues immediately after R-74.

If the mutant OmpC protein is indeed altered in its pore function (as we suggested above), then the region of the protein encompassing R-74 may be involved in the pore structure. Further biochemical analysis of these mutants is necessary to gain an insight into the pore structure. The role of the R-74 residue in pore function remains to be determined. On the basis of results with six mutants, it is premature to speculate on the pore structure. However, it seems unlikely that a single residue is responsible for pore size determination. Current research is directed toward obtaining additional mutants to test this possibility. Clearly, one approach we are considering is the use of site-specific mutagenesis to evaluate side-chain characteristics such as charge, chain length, and hydrophobicity at residue R-74.

It is interesting that in all three porin proteins (OmpC, OmpF, and PhoE), this region of the protein is highly conserved (Fig. 1) (21), which may suggest its involvement in the pore structure of porin proteins. This notion was supported by the finding that in some of the *ompF*(Dex) mutants, the mutation altered the R-82 (homologous to R-74 of OmpC) residue of the OmpF protein (S. Benson and J. Occi, unpublished data).

ACKNOWLEDGMENTS

We are grateful to Tom Ferenci for valuable suggestions and to other laboratory colleagues for reading the manuscript. Thanks to Susan DiRenzo for typing the manuscript.

This work was supported by Public Health Service grant GM-34810 from the National Institutes of Health.

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