Journal of Bacteriology, Feb. 1996, p. 1213–1215
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 178, No. 4

Assembly-Defective OmpC Mutants of *Escherichia coli* K-12

XIAOLING XIONG, JACK N. DEETER, and RAJEEV MISRA*
Department of Microbiology, Arizona State University, Tempe, Arizona 85287-2701

Received 30 August 1995/Accepted 11 December 1995

Novel *ompC*(Dex) alleles were utilized to isolate mutants defective in OmpC biogenesis. These *ompC*(Dex) alleles also conferred sensitivity to sodium dodecyl sulfate (SDS), which permitted the isolation of SDS-resistant and OmpC-specific phage-resistant mutants that remained Dex$^+$. Many mutants acquired resistance against these lethal agents by lowering the OmpC level present in the outer membrane. In the majority of these mutants, a defect in the assembly (metastable to stable trimer formation) was responsible for lowering OmpC levels. The assembly defects in various mutant OmpC proteins were caused by single-amino-acid substitutions involving the G-39, G-42, G-223, G-224, Q-240, G-251, and G-282 residues of the mature protein. This assembly defect was correctable by an assembly suppressor allele, *asmA3*. In addition, we investigated one novel OmpC mutant in which an assembly defect was caused by a disulfide bond formation between two nonnative cysteine residues. The assembly defect was fully corrected in a genetic background in which the cell's ability to form disulfide bonds was compromised. The assembly defect of the two-cysteine OmpC protein was also mended by *asmA3*, whose suppressive effect was not achieved by preventing disulfide bond formation in the mutant OmpC protein.

A recent crystallographic study (4) and genetic examinations (2, 3, 8, 9) of porins have provided a clearer picture of the structure and function of these channel-forming proteins of *Escherichia coli* K-12. However, despite these exciting developments, our understanding of how these proteins are targeted to and assembled in the outer membrane remains unclear. In particular, little is known about the cellular components involved in this process. To better understand this complex cellular process, we developed novel genetic selection schemes which led to the isolation of OmpF mutants defective in outer membrane protein assembly (7). These mutants were conditionally defective in assembly and accumulated an intermediate under nonpermissive growth conditions. Suppressor analysis utilizing these mutants revealed several genetic loci that presumably code for proteins involved in the assembly of outer membrane proteins. Detailed analysis of one such suppressor locus, *asmA*, showed that the restoration of mutant OmpF assembly was due to the lack of the *asmA* gene product (10). This finding reflected that *asmA* codes for a protein that either directly or indirectly prevents assembly of mutant OmpF proteins. The deficiency of AsmA in the cell caused no detectable effect on the assembly of wild-type or parental proteins.

In this study, we present our work on assembly-defective OmpC mutants. These mutants were obtained via a genetic scheme that utilized the sodium dodecyl sulfate (SDS) sensitivity (SDS^s) phenotype of previously characterized *ompC*(Dex) alleles (8, 9). As previously seen with OmpF, the assembly defects of mutant OmpC proteins were suppressible by null *asmA* alleles. We extended our study to a unique OmpC mutant that contained two nonnative cysteine residues (6). Disulfide bond formation between the cysteine residues interfered with mutant OmpC assembly. This defect in assembly was reversed when the cell's ability to promote disulfide bond formation was significantly impaired. Moreover, in the presence of a mutant *asmA* allele, the assembly defect of the two-cysteine OmpC protein was also corrected.

* Corresponding author. Phone: (602) 965-3320. Fax: (602) 965-0098. Electronic mail address: aarxm@acvax.inre.asu.edu.

Isolation of assembly-defective OmpC mutants. Since wild-type *ompC* does not provide a positive selectable phenotype, variant *ompC* alleles were used to isolate the desired mutations. Variant *ompC* alleles were obtained from mutants that acquired an ability to grow on maltodextrins (Dex$^+$) in the absence of LamB, which is normally required for maltodextrin transport. These variant *ompC* alleles [*ompC*(Dex)] acquired either base pair substitutions or short in-frame insertions and deletions (8, 9). These alterations led to a functional enlargement of the OmpC channel, such that previously excluded maltodextrins could now diffuse through them. While the channel properties of the mutant OmpC proteins were altered, other features, such as bacteriophage binding, membrane insertion, and trimerization, remained unaffected. One intriguing property of all the *ompC*(Dex) mutants was that they became sensitive to SDS. The SDS and bacteriophage sensitivity phenotypes of *ompC*(Dex) mutants were utilized to isolate a large variety of mutants, including the desired class investigated in this study.

SDS^r revertants were isolated by placing paper discs soaked with SDS (67 mg/ml) and nitrosoguanidine (0.83 mg/ml) on plates containing minimal medium supplemented with maltodextrin (0.2%). This selection strategy sought SDS^r Dex$^+$ mutants from SDS^s Dex$^+$ strains and avoided the isolation of *ompC* null mutations because these alleles would confer a Dex$^-$ phenotype. Among SDS^r Dex$^+$ revertants, at least two classes of *ompC* mutations were anticipated. In one class, a second alteration in *ompC* could cause SDS^r by altering OmpC channel properties. The other class of *ompC* mutations could confer SDS^r by lowering OmpC levels in the outer membrane. The reduced OmpC levels must still allow transport of maltodextrins at a rate high enough to sustain growth but not sufficient for the diffusion of lethal amounts of SDS. Although many types of intragenic mutations can reduce OmpC levels, including those affecting synthesis, there should be some which lower OmpC levels by interfering with OmpC assembly. It is this latter class of mutations that are studied here in some detail.

A total of 62 independent SDS^r Dex$^+$ isolates were obtained from two different SDS^s Dex$^+$ parents bearing either a short in-frame insertion (50 isolates) or a deletion (12 isolates) in *ompC*. Many isolates displayed unstable phenotypes and were

TABLE 1. Genetic alterations in SDS^r mutants

ompC allele	Mutational alteration[a]	
	Nucleotide	Amino acid
ompC124 (parent)[b]		
ompC500	G753A	G251D
ompC501	G126A	G42D
ompC502	G846A	G282D
ompC504	G669A	G223D
ompC505	G117A	G39D
ompC509	G672A	G224D
ompC511	ND[c]	ND
ompC168 (parent)[d]		
ompC510	A720C	Q240P

[a] Nucleotides and amino acid residues corresponding to those of mature OmpC.
[b] Dex^+ parent carrying an in-frame insertion (8).
[c] ND, not determined.
[d] Dex^+ parent carrying an in-frame deletion (9).

discarded. Genetic mapping with *ompC*-linked markers was performed with the remaining 43 isolates. In 25 of the 33 isolates from an insert-bearing parent and 5 of the 10 isolates from a deletion-bearing parent, mutations were mapped at *ompC*. Examination of envelopes from these isolates revealed at least three classes of mutants on the basis of OmpC protein levels: (i) mutants containing the same level of OmpC as those of parents, (ii) mutants containing lower levels of OmpC than those of the parents, (iii) or mutants producing completely undetectable levels of OmpC. Six isolates from an insert-bearing parent and one isolate from a deletion-bearing parent that produced reduced levels of OmpC were analyzed in this study. These mutants contained single-base-pair alterations, thus causing G39D (substitution of glycine for aspartic acid at position 39), G42D, G223D, G224D, Q240P, G251D, and G282D substitutions (Table 1).

Examination of mutant OmpC proteins in an assembly suppressor background. We examined the ability of an assembly suppressor allele, *asmA3*, to restore mutant OmpC levels in the outer membrane. *asmA3* was isolated as an extragenic suppressor mutation of the assembly-defective OmpF315 protein (7, 10). We reasoned that since OmpF is very similar to OmpC in every aspect of its biogenesis, it is conceivable that an OmpF assembly suppressor would also suppress OmpC assembly defects. Indeed, if the *ompC* mutations affected assembly at a level different than that affected by the *ompF* assembly mutation, *asmA3* may not be able to correct their defects.

ompC alleles were moved by P1 transduction into strains containing either a wild-type or mutant *asmA* allele. Envelopes prepared from these strains were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Only a sample of this analysis is shown in Fig. 1. The results showed that in some cases (lanes 4, 6, and 8), the presence of *asmA3* elevated OmpC levels. There were also mutants in which the presence of *asmA3* did not make any significant difference in OmpC levels (Fig. 1, lanes 9 and 10). For mutants showing an increase in mutant OmpC levels in the presence of *asmA3*, there exists a strong possibility that the defect in biogenesis is at the level of assembly. For mutants in which OmpC levels were unaffected by *asmA3*, either synthesis was affected or assembly at a step not involving *asmA* was defective. It should be noted that since *asmA3* is a null allele, lack of suppression cannot be due to allele specificity.

In cases where mutant OmpC levels were improved by *asmA3*, a defect in assembly was anticipated at a step involving the conversion of metastable trimers to stable trimers. This is because a similar defect is seen in OmpF315 (7). To examine this, trimerization of mutant and parental OmpC was examined in radioactively labeled cells and OmpC trimer-specific antibodies were used to immunoprecipitate trimers. In the parental strain, stable OmpC trimers accumulated kinetically, while in mutant strains containing the wild-type *asmA* allele, little or barely detectable levels of stable trimers were observed (data not shown). The levels of metastable trimers in mutant strains were found to be similar to that present in the parental strain. Thus, it appears that the step from metastable trimer to stable trimer was defective. When the assembly of parental and mutant OmpC proteins was examined in a genetic background containing *asmA3*, mutant OmpC proteins were now able to form stable trimers. Thus, the elevated levels of mutant OmpC proteins seen in envelopes coincided with an increase in stable trimer formation.

Assembly of the two-cysteine OmpC protein. We have previously reported the isolation of an OmpC-specific bacteriophage-resistant mutant bearing two nonnative cysteine residues in OmpC (6). The first substitution of R74C was obtained via a genetic selection involving the isolation of *ompC* mutants with altered channel properties (Dex^+ phenotype; 9). The second cysteine alteration (G154C) was obtained when this *ompC*(Dex) mutant was used to isolate OmpC-specific bacteriophage-resistant mutants (6). Further investigation showed that the presence of these two nonnative cysteine residues resulted in bacteriophage resistance by lowering OmpC levels in the outer membrane instead of bringing about a structural change specifically affecting the receptor region. The two cysteine residues engaged in the formation of disulfide bonds, thus causing a defect in OmpC biogenesis at a posttranslational level: pulse-chase experiments showed a complete lack of stable trimers (data not shown).

In a *dsbA*::Km^r background, where the cell's ability to form extracytoplasmic disulfide bonds is drastically impaired, the mutant OmpC level was restored and stable trimers accumulated with the kinetics indistinguishable from that seen for the parental protein (data not shown). The presence of free and cross-linked cysteine residues was examined with iodoacetamide as described previously (1). As expected, in a *dsbA*::Km^r background, the mutant OmpC protein existed primarily in its reduced state. Thus, the two-cysteine OmpC protein assembled properly because of the lack of disulfide bonds in a *dsbA*::Km^r background and not as an indirect consequence of the lack of DsbA in the cell.

Suppression of two-cysteine OmpC assembly in an *asmA3* background. The ability of *asmA3* to suppress the assembly defect seen in the two-cysteine OmpC protein was examined.

The presence of *asmA3* elevated the two-cysteine OmpC level, making it equal to that of the parental protein level present in either an *asmA*$^+$ or *asmA3* background. The elevated level of two-cysteine OmpC in an *asmA3 dsbA*$^+$ genetic

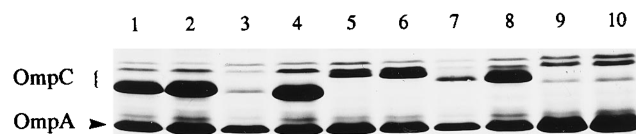

FIG. 1. Effect of *asmA3* on OmpC levels. Envelopes prepared from parent (lanes 1 and 2) and various *ompC* mutant strains (Q240P [lanes 3 and 4], G42D [lanes 5 and 6], G224D [lanes 7 and 8], and synthesis down mutant [lanes 9 and 10]) were analyzed by a SDS-polyacrylamide (11%)-urea (4 M) gel as described previously (7). *asmA*$^+$ and *asmA3* alleles were present in samples analyzed in odd- and even-numbered lanes, respectively. The different mobilities seen for various OmpC proteins are due to the presence of various alterations in these proteins.

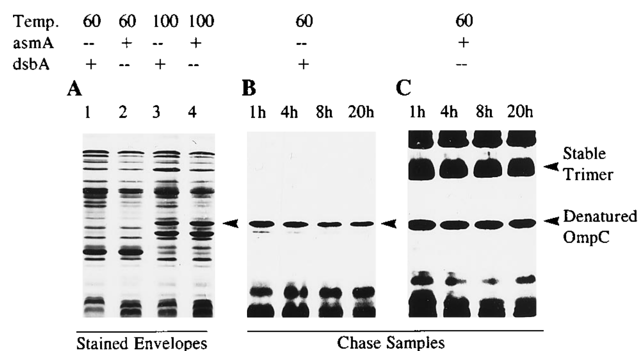

FIG. 2. (A) Thermostability of the two-cysteine OmpC protein in envelopes obtained from *asmA3 dsbA*$^+$ (lanes 1 and 3) and *asmA*$^+$ *dsbA*::Km^r (lanes 2 and 4) strains. These strains did not produce OmpF because of the presence of a null Φ(*ompF′-lacZ*$^+$) allele. Envelope samples were heated at 60 or 100°C prior to their analysis on a SDS-polyacrylamide (11%) gel. (B and C) Pulse-chase experiments followed by immunoprecipitation of the two-cysteine OmpC protein with OmpC trimer-specific antibodies. Cells were grown to an optical density at 600 nm of 0.3 in minimal medium (M63-based) containing glycerol (0.4%), labeled with [^{35}S]methionine-cysteine (40 μCi/ml) for 20 s, and chased with excess nonradioactive methionine-cysteine. Samples, withdrawn at the chase time intervals shown above the lanes, were immediately chilled by placing tubes in a dry ice-ethanol bath. OmpC trimers were extracted as described previously (7) and immunoprecipitated with OmpC trimer-specific antibodies. Immunoprecipitates were heated at 60°C prior to SDS-PAGE analysis. After the completion of electrophoresis, gels were dried and fluorographed. Trimers were obtained from either *asmA3 dsbA*$^+$ (B) or *asmA*$^+$ *dsbA*::Km^r (C) background. The positions of denatured OmpC monomers and stable trimers are shown.

background was similar to that observed in an *asmA*$^+$ *dsbA*::Km^r background. Surprisingly, however, in the former genetic background, the efficiency of plaquing (EOP) of OmpC-specific bacteriophages (Hy2 or SS4) was not restored to either the parental level (EOP = 1) or that seen in strains carrying *dsbA*::Km^r (EOP = 0.5). Instead, the *asmA3 dsbA*$^+$ strain containing two-cysteine OmpC showed a 10-fold-lower EOP than strains containing the parental one-cysteine OmpC protein in the above genetic backgrounds. It is likely that even though *asmA3* elevates mutant OmpC levels, the *asmA3*-suppressed mutant OmpC is structurally distinct from either parental OmpC or *dsbA*::Km^r-suppressed mutant OmpC. In the *asmA3* background, assembly of mutant OmpC appeared defective, as no thermostable trimers were detected even after 20 h of chase (Fig. 2B). In contrast, the mutant OmpC protein present in envelopes, prepared from a culture grown overnight, was as thermostable as that found in the *dsbA*::Km^r background. The thermolabile species seen during pulse-chase and trimer assays could be due to treatments involving trimer extraction followed by immunoprecipitation. The thermostability of the *dsbA*::Km^r-suppressed OmpC was only moderately affected by these treatments (Fig. 2C).

The structural instability of mutant OmpC in the *asmA3 dsbA*$^+$ background could be due to the existence of disulfide bonds in the protein. This possibility was confirmed by utilizing iodoacetamide (data not shown). The presence of disulfide bonds in the *asmA3*-suppressed mutant OmpC protein must alter its structural properties such that OmpC-specific bacteriophages only partially recognize this protein as their proper receptor, resulting in a lower EOP. These results show that the mechanism of suppression of two-cysteine OmpC in the *dsbA*::Km^r background is fundamentally different than that seen in the *asmA3* background.

Assembly of two-cysteine OmpC in a *dsbA*::Km^r *asmA3* double mutant background was examined. If newly synthesized assembly intermediates of two-cysteine OmpC assembled without forming disulfide bonds, as is the case in the *dsbA*::Km^r background, we would expect to see thermostable trimers. On the other hand, if assembly intermediates follow the *asmA3*-mediated suppression pathway, the thermolabile species will accumulate. The results showed that in the double mutant background, the mutant OmpC protein assembled into stable trimers with kinetics similar to that seen in the *dsbA*::Km^r background alone (data not shown). These results show that *dsbA*::Km^r is epistatic to *asmA3*. The EOP data of the strain containing *dsbA*::Km^r and *asmA3* for OmpC-specific bacteriophages were identical to that obtained for the strain containing only *dsbA*::Km^r.

Assembly suppression mechanism. At present, it is not clear how mutations in *asmA* correct assembly defects, but some speculations can be made. The fact that suppression is achieved in the absence of the *asmA* gene product implies that the presence of AsmA creates an environment refractory to the assembly of mutant (misfolded) proteins. Moreover, mutations in *asmA* do not confer any detectable effect on the assembly of wild-type proteins, suggesting that correctly folded proteins assemble independently of AsmA activity (10). We have recently observed that bacterial strains lacking AsmA display significantly increased sensitivity toward hydrophobic antibiotics (5). These antibiotics are known to penetrate the outer membrane via a lipidic pathway (11). Increased antibiotic sensitivity seen in *asmA* mutants reflects an altered state of the outer membrane in these mutants. This altered state of the outer membrane may now allow mutant proteins to assemble without drastically affecting the assembly of wild-type or parental proteins. Which specific component of the outer membrane is affected in strains lacking AsmA is currently unknown, but our future efforts should provide some insight.

It is not clear why the specified changes in the primary sequence of OmpC would influence protein assembly. To understand the role of these residues during assembly, it may be more important to know their location on partially folded assembly intermediates (such as metastable trimers), but presently nothing is known about their structure.

We thank our laboratory colleagues and Leanne Misra for critically reading the manuscript.

This work was supported by grants from the National Institutes of Health (GM48167) and Arizona Disease Control Research Commission.

REFERENCES

1. **Bardwell, J. C. A., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation in vivo. Cell **67:**581–589.
2. **Bauer, K., M. Struyve, D. Bosch, R. Benz, and J. Tommassen.** 1989. One single lysine residue is responsible for the special interaction between polyphosphate and the outer membrane porin PhoE of *Escherichia coli*. J. Biol. Chem. **264:**16393–16398.
3. **Benson, S. A., J. L. Occi, and B. A. Sampson.** 1988. Mutations that alter the pore function of the OmpF porin of *Escherichia coli* K-12. J. Mol. Biol. **203:**962–970.
4. **Cowen, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghose, 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.
5. **Deng, M., and R. Misra.** Unpublished data.
6. **Misra, R.** 1993. A novel *ompC* mutation of *Escherichia coli* K-12 that reduces OmpC and OmpF levels in the outer membrane. Mol. Microbiol. **10:**1029–1035.
7. **Misra, R.** 1993. OmpF assembly mutants of *Escherichia coli* K-12: isolation, characterization, and suppressor analysis. J. Bacteriol. **175:**5049–5056.
8. **Misra, R., and S. A. Benson.** 1988. Isolation and characterization of OmpC porin mutants with altered pore properties. J. Bacteriol. **170:**528–533.
9. **Misra, R., and S. A. Benson.** 1988. Genetic identification of the pore domain of the OmpC porin of *Escherichia coli* K-12. J. Bacteriol. **170:**3611–3617.
10. **Misra, R., and Y. Miao.** 1995. Molecular analysis of *asmA*, a locus identified as the suppressor of OmpF assembly mutants of *Escherichia coli* K-12. Mol. Microbiol. **16:**779–788.
11. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.