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

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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 phase-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, *asmA*. 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 *asmA*, whose suppressive effect was not achieved by preventing disulfide bond formation in the mutant OmpC protein.

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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 mutants. 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* 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* Dex+ mutants from SDS* Dex− strains and avoided the isolation of *ompC* null mutations because these alleles would confer a Dex− phenotype. Among SDS* Dex+ revertants, at least two classes of *ompC* mutations were anticipated. In one class, a second alteration in *ompC* could cause SDS* by altering OmpC channel properties. The other class of *ompC* mutations could confer SDS* 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* Dex+ isolates were obtained from two different SDS* 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

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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 asmA4 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-resistance 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::KmR 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::KmR 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::KmR 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 asmA4- 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], G224D [lanes 5 and 6], G223D [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). asmA4 and asmA3 alleles were present in samples analyzed in odd- and even-numbered lanes, respectively. The differential mobilities seen for various OmpC proteins are due to the presence of various alterations in these proteins.](http://jb.asm.org/)

### Table 1. Genetic alterations in SDS'-mutants

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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 G252D 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 asmA4 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 asmA4 was defective. It should be noted that since asmA4 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
background was similar to that observed in an asmA\(^+\) dsbA\(^-\)::Km\(^\prime\) 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\(^\prime\) (EOP = 0.5). Instead, the asmA\(^3\) 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 asmA\(^3\) elevates mutant OmpC levels, the asmA\(^3\)-suppressed mutant OmpC is structurally distinct from either parental OmpC or dsbA::Km\(^\prime\)-suppressed mutant OmpC. In the asmA\(^3\) 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\(^\prime\) 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\(^\prime\)-suppressed OmpC was only moderately affected by these treatments (Fig. 2C).

The structural instability of mutant OmpC in the asmA\(^3\) 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 asmA\(^3\)-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\(^\prime\) background is fundamentally different than that seen in the asmA\(^3\) background.

Assembly of two-cysteine OmpC in a dsbA::Km\(^\prime\) asmA\(^3\) 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\(^\prime\) background, we would expect to see thermostable trimers. On the other hand, if assembly intermediates follow the asmA\(^3\)-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\(^\prime\) background alone (data not shown). These results show that dsbA::Km\(^\prime\) is epistatic to asmA\(^3\). The EOP data of the strain containing dsbA::Km\(^\prime\) and asmA\(^3\) for OmpC-specific bacteriophages were identical to that obtained for the strain containing only dsbA::Km\(^\prime\).

Assembly suppression mechanism. At present, it is not clear how mutations in asmA\(^3\) correct assembly defects, but some speculations can be made. The fact that suppression is achieved in the absence of the asmA\(^3\) gene product implies that the presence of AsmA creates an environment refractory to the assembly of mutant (misfolded) proteins. Moreover, mutations in asmA\(^3\) 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\(^4\) 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.

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REFERENCES