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OmpF Assembly Mutants of *Escherichia coli* K-12: Isolation, Characterization, and Suppressor Analysis

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This paper describes a novel genetic method used to isolate mutations that alter proper assembly of OmpF in the outer membrane. The thermolabile nature of assembly intermediates allowed selection of temperature-sensitive mutations within the *ompF* gene. A variant allele of *ompF* (*ompF*-Dex) was used because it provided a convenient selectable phenotype (Dex^+). Assembly mutants were isolated in two steps. First, amber mutations were obtained that mapped in *ompF*-Dex. This resulted in a Dex^- phenotype. Starting with these Dex^- strains, Dex^+ revertants were isolated. Mutants that displayed a temperature-sensitive Dex^+ phenotype were further characterized. Three such mutants possessed a single substitution within *ompF* that reverted the nonsense codon to a sense codon which replaced W214 with either an E or Q and Y231 with a Q residue in the mature OmpF protein. All three mutant OmpF proteins showed an assembly defect. This defect led to a substantial reduction in the amount of stable OmpF trimers with the concomitant increase of a high-molecular-weight form of OmpF which migrated at the top of the gel. Suppressor mutations were sought that corrected the assembly defect of OmpF. These extragenic suppressor mutations were mapped at 45 min on the *Escherichia coli* chromosome. The suppressor mutations displayed no allele specificity and were recessive to the wild-type allele. In the presence of a suppressor, mutant stable trimers appeared in an almost normal manner. The appearance of stable trimers concurred with a substantial loss of the high-molecular-weight OmpF species. At this stage, it is not clear whether the high-molecular-weight species of OmpF is a normal assembly intermediate or a dead-end assembly product. The results presented in this study raise the intriguing possibility of a chaperone-like activity for the wild-type suppressor gene product.

The cytoplasm of gram-negative bacteria, such as *Escherichia coli*, is surrounded by a complex envelope which consists of an inner and an outer membrane. An aqueous compartment known as the periplasm separates these two membranes. All proteins are synthesized in the cytoplasm. Proteins that are destined for the periplasm or outer membrane exit the cytoplasm with the help of an intragenic *cis*-acting sequence known as the signal sequence (2). The sequence, roughly 20 to 25 amino acids long, sequentially interacts with several cytoplasmic and inner membrane proteins to guide the mature portion of the protein to its extracytoplasmic location (17). While the signal sequence is necessary for protein translocation across the inner membrane, it is not responsible for determining the final cellular location of exported proteins (2). It has been generally assumed that periplasmic proteins remain in the periplasm because of additional informational input. The mechanism(s) by which proteins are incorporated into the outer membrane is poorly understood and is the subject of this study.

Prior to assembling into trimers, mature monomers of certain outer membrane proteins such as OmpF and LamB go through several distinct assembly intermediates (7, 16, 19, 25). These partially folded intermediates have been recognized as either thermolabile dimers (19) or metastable trimers (7, 16, 25). It has been reported that in the case of OmpF, mature monomers are first secreted into the periplasm and are then spontaneously inserted in the outer membrane (11, 21). The site of trimerization remains unclear. In the case of LamB, it was found that monomers remained associated with the membrane through the process of assembly (26). When trimerization of the LamB monomers was conditionally blocked, the accumulated monomers were found in the outer membrane (16). These findings suggested that LamB monomers are targeted to the outer membrane where they then trimerize.

The signal for outer membrane targeting is currently unknown. However, it is clear that the information that directs a protein to the outer membrane lies within the mature sequence of the protein. How then are outer membrane proteins, which have no obvious mature sequence homology, targeted to the same cellular compartment? The notion that there is a common sorting signal represented in the form of a linear sequence within the mature portion of outer membrane proteins has been proposed (18) but is not yet experimentally proven. It is conceivable that the sorting signal is represented in the form of a secondary or tertiary structure, which may be contained within various partially folded intermediates. This hypothesis was tested with a bacterial strain carrying mutant LamB, whose assembly was defective. Consistent with this notion, it was found that the mutation interfered with the assembly of LamB because it affected the proper folding of an assembly intermediate (16). By using epitope-specific OmpF monoclonal antibodies, it was shown that during its assembly from mature monomers to stable trimers, OmpF goes through various structurally distinct intermediates (7). Whether the information that directs this protein to fold resides entirely within the protein sequence itself or whether certain extragenic factors such as chaperone-like proteins are also involved in the process is not known. Since lipopolysaccharide (LPS) is present only in the outer membrane, it was thought that affinity towards LPS could be a major factor in guiding proteins to the outer membrane. This hypothesis is consistent with the finding that mutants producing extremely defective LPS produce reduced amounts of outer membrane proteins (1). It has been shown that in vitro porin trimer formation can be promoted by outer membrane vesicles containing LPS molecules and

TABLE 1. Bacterial strains and characteristics

Strain	Characteristics	Source or reference
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U139 rpsL150 relA1 flbB5301 ptsF25 deoC1 thi-1 rbsR*	4
MCR106	MC4100 *ΔlamB106*	6
RAM472	MCR106 *ompF205*(Dex⁺)	This study
RAM474	RAM472 *ompF5*; carries an *ompF* amber mutation	This study
RAM478	RAM472 *ompF18*; carries an *ompF* amber mutation	This study
RAM480	RAM474 *ompF35*; Dex⁺ (temperature-sensitive) revertant of RAM474	This study
RAM483	RAM474 *ompF315*; Dex⁺ (temperature-sensitive) revertant of RAM474	This study
RAM485	RAM478 *ompF33*; Dex⁺ (temperature-sensitive) revertant of RAM478	This study
RAM491	RAM472 Φ(*ompC'-lac*⁺)*10-15*	This study
RAM493	RAM480 Φ(*ompC'-lac*⁺)*10-15*	This study
RAM496	RAM483 Φ(*ompC'-lac*⁺)*10-15*	This study
RAM498	RAM485 Φ(*ompC'-lac*⁺)*10-15*	This study
RAM525	RAM496 *sfaA1*; temperature-resistant Dex⁺ derivative of RAM496	This study
RAM533	RAM525 *hisG*::Tn*10*	This study
RAM534	RAM496 *hisG*::Tn*10*	This study
RAM535	RAM533 *recA*::Kan^r	This study
RAM536	RAM534 *recA*::Kan^r	This study
RAM539	RAM535(F'150 *his*⁺)	This study
RAM540	RAM536(F'150 *his*⁺)	This study
RAM541	RAM535(F'500 *his*⁺)	This study
RAM542	RAM536(F'500 *his*⁺)	This study
MH19	MC4100 *pyrD malQ7*	8
PLB3260	MCR106 Φ(*ompF'-lac*⁺)*16-13*	S. Benson
RAM473	PLB3260 *zcb*::Tn*10 pyrD*	This study
RAM507	MCR106 *pyrD zcb*::Tn*10*	This study
RAM555	RAM525 *pyrD zcb*::Tn*10*	This study
RAM552	RAM555 *pyrD*⁺ *ompF205*	This study
RAM553	RAM555 *pyrD*⁺ *ompF35*	This study
RAM554	RAM555 *pyrD*⁺ *ompF33*	This study

not by vesicles prepared from the inner membrane (22). Studies from Nikaido's laboratory showed trimerization of in vitro-synthesized OmpF monomers in the presence of purified LPS (23). However, compared with the trimerization of proteins in intact cells, the extent of trimerization in these in vitro studies was fairly low; thus, these reactions may represent only part of the physiological process. In light of these findings, it is conceivable that other cellular components, in addition to LPS, are required for the efficient targeting and assembly of outer membrane proteins.

In this communication, a novel genetic approach was undertaken to test the hypothesis that folding is required for the proper assembly and targeting of OmpF. An amber reversion approach was employed to isolate missense mutations in *ompF* that rendered a temperature-dependent assembly defect of OmpF. To gain further insight in the assembly process, extragenic suppressors of *ompF* assembly mutants were isolated and characterized. Suppressors are expected to reveal other cellular proteins that may facilitate the assembly and targeting of proteins to the outer membrane.

MATERIALS AND METHODS

Media and chemicals. Minimal medium (M63) and Luria broth were prepared as described previously (24). Maltodextrin was purchased from Pfanstiehl Laboratories, Inc., and was further purified as described previously (13). [^{35}S]methionine was obtained from Du Pont-New England Nuclear. Other chemicals were of analytical grade.

Bacterial strains, mutant isolation, and other genetic techniques. Strains used in this study are listed in Table 1. P1 transductions and Hfr and F' conjugational crosses were performed by the methods of Miller (12) and Silhavy et al. (24).

ompF amber mutations were isolated by selecting for colonies that were resistant to OmpF-specific bacteriophage K20. To rule out mutations in the *ompB* locus, which codes for a positive transcriptional activator of *ompF* and *ompC*, K20-resistant (K20^r) colonies were tested for sensitivity to OmpC-specific bacteriophage Tu1b. Colonies that were sensitive to Tu1b (Tu1b^s) were further characterized. To test for the presence of an amber mutation in *ompF*, K20^r Tu1b^s colonies were cross-streaked against two bacteriophages: the first streak was of a λ phage (Φ80 host range) carrying an amber suppressor tRNA allele (SuIII), and the second streak was that of K20. A passage over λ-SuIII allows lysogenization of this phage in the bacterial cell. If a cell carries an amber mutation, it can now be suppressed by the lysogenized phage, resulting in the production of OmpF and, thus, K20 sensitivity. Thus, K20^r colonies that became K20^s after being streaked over λ-SuIII phage may contain an amber mutation in *ompF*. If an *ompF*(Dex) allele was used to isolate amber mutations, in addition to K20, its maltodextrin utilization (Dex⁺ or Dex⁻) phenotype was also tested. Anywhere between 0 and 8% of the K20^r colonies had an amber mutation present in *ompF*. Once tested positive for the presence of an amber mutation, these *ompF* alleles were moved by P1 transduction into a fresh genetic background. Strains carrying an *ompF* amber mutation had no detectable OmpF present in their envelopes. In some cases, pulse-chase experiments showed the presence of an OmpF amber fragment (data not shown). In strains where an OmpF amber fragment was not detected, lack of detection could be due either to the small size of the amber fragment or to the fact that it was very quickly degraded.

To identify a Tn*10* insertion near *sfaA* (suppressor of OmpF assembly), the tetracycline-resistant (Tc^r) determinant from the Tn*10* pool prepared on a wild-type strain was moved by P1 transduction into RAM525. This strain carries a Su^+ allele of *sfaA* that confers a Dex^+ phenotype in the *ompF315* background. The Tc^r transductants were screened for the Dex^- phenotype. Linkage of the suppressor and Tc^r was determined by using a purified Dex^- Tc^r isolate.

DNA sequence analysis. In order to determine the mutational alteration in *ompF*, a 1.1-kb chromosomal DNA encompassing *ompF* was amplified (by polymerase chain reaction) from DNA templates isolated from mutant colonies. The nucleotide sequence of the entire *ompF* gene was determined directly from amplified DNA by using several internal primers complementary to *ompF* sequences.

Purification of OmpF and preparation of OmpF antibodies. OmpF was purified in its native trimeric state from a strain lacking OmpC and LamB by the protocol described for the purification of LamB (16). The purified OmpF preparation was free of any detectable contamination of other outer membrane proteins (data not shown). Antibodies against OmpF trimers were raised in rabbits by using a standard protocol. These antibodies primarily reacted with OmpF trimers. Monomer-specific antibodies recognized OmpF monomers (native or heat denatured). The antigen preparation used to raise OmpF monomer-specific antibodies contained a slight contamination of OmpA; thus, the resulting antibodies also recognize OmpA.

Pulse-chase experiments and immunoprecipitation. Pulse-chase experiments were performed with cells grown on glycerol minimal medium, as described previously (16). When OmpF assembly was examined at a nonpermissive temperature, cells grown overnight at 30°C were diluted and grown at 42°C for at least 2 h prior to labeling. Extraction of OmpF trimers was carried out essentially as described previously (16) with some modifications: proteins were solubilized from EDTA-lysozyme-treated whole cells by adding a buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), and 50 mM NaCl (all final concentrations). Trimer extractions were carried out at 30°C for 30 min. OmpF trimers and monomers were immunoprecipitated from cell extracts with appropriate antibodies. Immunocomplexes were brought down by formalin-killed *Staphylococcus aureus* cells and, after several washes, were analyzed by polyacrylamide gel electrophoresis (PAGE).

Envelope preparations and SDS-PAGE. Envelopes from cultures grown overnight were prepared by the French press lysis procedure as described previously (16). Samples were analyzed on linear SDS-polyacrylamide gels (11%) as previously described (10). In some gels, 4 M solid urea was added to the running gel buffer to achieve better resolution of OmpF from OmpT and OmpA. Protein bands were visualized by staining gels with Coomassie brilliant blue R-250. When [^{35}S]methionine-labeled samples were analyzed, gels were fluorographed at −70°C.

RESULTS

Isolation of *ompF* assembly mutants: the rationale. It is known that prior to assembling in the outer membrane, proteins go through several structurally distinct assembly intermediates (7, 16, 19, 25). The proper folding of these intermediates may be important in ensuring their correct involvement in the assembly process. According to this view, a mutation that interferes with the proper folding of an intermediate would also interfere with its assembly in the outer membrane. Amino acid residues that are essential for the assembly process may not have a role once the protein is properly assembled into its native structure. Thus, simply knowing the crystal structure of a native protein may not reveal which residues are involved during its biogenesis. The following strategy was adopted to unveil amino acid residues within the mature OmpF sequence that play a crucial role in the folding and assembly of the protein in the outer membrane.

The wild-type OmpF protein lacks a positive selectable phenotype, and thus a direct selection of the desired mutants is not straightforward. However, if we start with a slightly altered OmpF that possesses a positive selectable phenotype, the desired mutants can be isolated. One such altered OmpF protein is OmpF(Dex), which allows growth on maltodextrins as the sole carbon source in the absence of LamB, the protein normally required for maltodextrin transport. In this genetic background, a missense mutation in *ompF* that confers a defect in OmpF(Dex) assembly will render a maltodextrin-minus (Dex^-) phenotype. However, since a null mutation will also confer the same phenotype (Dex^-) as an assembly-defective missense mutation in *ompF*, it would be desirable to eliminate this class of mutations. One way of achieving this is by selecting conditional-defective Dex^- mutants of *ompF*. This was accomplished in two steps. First, amber mutations were isolated in *ompF*(Dex) strains. These mutations were obtained from $K20^r$ isolates that simultaneously became Dex^- (see Materials and Methods). In the second step, starting with these Dex^- *ompF*(Dex) amber mutants, Dex^+ revertants were obtained at a permissive temperature (30°C). Among these Dex^+ revertants, isolates were screened for a Dex^- (or Dex-down) phenotype at the nonpermissive temperature (42°C). Employing this selection strategy, several dozen independent mutants were isolated at an estimated frequency of 10^{-9} to 10^{-10}.

Genetic mapping and nucleotide sequence determination of temperature-sensitive *ompF* alleles. Temperature-sensitive *ompF* mutations were genetically mapped in a single step by P1 transduction with a linked genetic marker, *pyrD* (approximately 50% linked to *ompF* by P1 cotransductions). The $pyrD^+$ allele was transduced from mutants into a *pyrD* Φ*ompF'-lacZ*$^+$ recipient strain by selecting for growth on glucose minimal medium. The presence or absence of Φ*ompF'-lacZ*$^+$ among the $pyrD^+$ transductants was monitored by spreading a chromogenic indicator (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) on the plates. $pyrD^+$ transductants that had lost the fusion (white colonies) because of its replacement by an *ompF* allele from the donor were purified and tested for their Dex^+ phenotype at 30 and 42°C. Mutants that displayed a temperature-sensitive Dex^+ phenotype were further characterized.

Once genetically mapped to *ompF*, DNA sequence analysis of the mutant allele was carried out to reveal the genetic alteration. This was performed by first generating template DNA from the mutant colony by the polymerase chain reaction method and then sequencing the entire *ompF* gene by using several internal primers. Five primers complementary to the *ompF* sequence were used to sequence the approximately 1,100-bp-long *ompF* gene. In all cases, a single nucleotide substitution was found at the site of the previous amber codon. The nonsense codon was changed to a sense codon that was different from that present in the wild-type *ompF* allele. A summary of the mutants and their genetic characteristics is given in Table 2.

Characterization of *ompF* assembly mutants. Three mu-

TABLE 2. Characteristics of *ompF*(Dex) mutants

ompF allele	Substitution(s)[a]	Growth on maltodextrins at:		Phage sensitivity to K20 at[b]:	
		30°C	42°C	30°C	42°C
ompF205	R82>S	+	+	S	S
ompF5	R82>S, W214>Am (TGG>TAG)	−	−	R	R
ompF18	R82>S, Y231>Am (TAC>TAG)	−	−	R	R
ompF315	R82>S, Am214>E (TAG>GAG)	+	−	S	R
ompF35	R82>S, Am214>Q (TAG>CAG)	+	−/+	S	PR
ompF33	R82>S, Am231>Q (TAG>CAG)	+	−/+	S	PR

[a] Abbreviations: R, arginine; S, serine; W, tryptophane; Y, tyrosine; E, glutamic acid; Q, glutamine; Am, amber codon. Numbers after the single-letter amino acid code correspond to the residue of the mature OmpF protein. Nucleotide changes are shown in parentheses.

[b] Phage sensitivity tests were carried out on glucose minimal medium. Abbreviations: S, sensitivity; R, resistance; PR, partial resistance.

tants that displayed a strong temperature-sensitive phenotype were studied further. The mutant and parent strains were tested by a cross-streak method for sensitivity against an OmpF-specific bacteriophage, K20. All strains were sensitive to K20 when tested at 30°C on complex (Luria agar), glycerol minimal, and glucose minimal media. At 42°C, pseudorevertants remained sensitive to K20 when tested on glycerol and Luria agar but showed partial resistance against the phage on glucose minimal medium plates; the mutant carrying the *ompF315* allele was the most resistant. Growth on glucose has been reported to reduce OmpF levels, which could explain the partial-resistance nature of the pseudorevertants that already have reduced levels of OmpF (see below). As expected, the parent strain was sensitive to the phage under all conditions tested. Since the pseudorevertants showed sensitivity to K20, the altered residues in the protein could not be directly involved in phage binding.

The amount of OmpF present in the envelope of various strains grown at permissive and nonpermissive temperatures was examined (Fig. 1). The level of OmpF present in the parent strain did not alter at either growth temperature. This constitutive level of OmpF is observed in strains lacking OmpC. However, in the mutant strains, OmpF levels dropped sharply when cells were grown at 42°C compared with the level present at 30°C. The strain carrying the *ompF315* allele had the severest effect on OmpF levels,

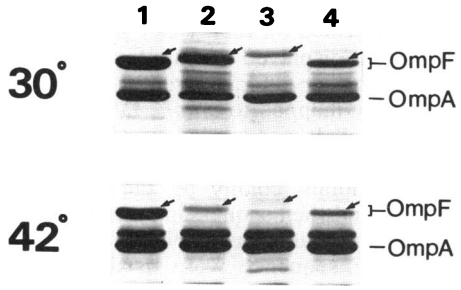

FIG. 1. SDS-PAGE (polyacrylamide-urea gel) of envelopes prepared from strains carrying various *ompF* alleles. Lanes 1 to 4 contain samples from strains carrying *ompF205*, *ompF35*, *ompF315*, and *ompF33* alleles, respectively. Envelopes were prepared from strains grown at either 30 or 42°C. The positions of OmpF are indicated by arrows. Protein bands were visualized by Coomassie blue staining. Growth temperatures are shown.

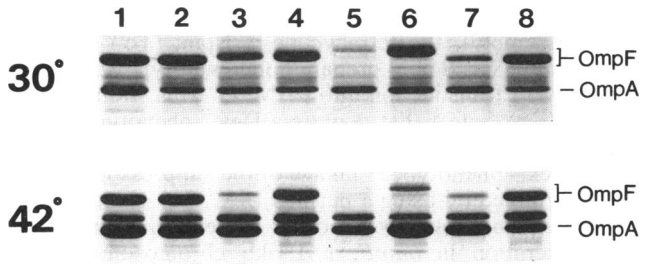

FIG. 2. SDS-PAGE (polyacrylamide-urea gel) of envelopes prepared from various *ompF* mutants in a wild-type (Su^-) or mutant (Su^+) background. Lanes 1, 3, 5, and 7 contain samples from strains carrying *ompF205*, *ompF35*, *ompF315*, and *ompF33* alleles, respectively, in a Su^- background. Lanes 2, 4, 6, and 8 contain samples from strains carrying *ompF205*, *ompF35*, *ompF315*, and *ompF33* alleles, respectively, in a Su^+ background. Protein bands were visualized by Coomassie blue staining. Growth temperatures are shown.

which were significantly down even at 30°C. These results were consistent with the phenotypic data obtained above. Note the difference in mobilities of OmpF in various strains. Altered mobility of porin proteins due to a single amino acid substitution is common and has been previously observed (3, 14).

Suppressors of the temperature-sensitive *ompF* alleles. Among the three *ompF* mutants studied, *ompF315* conferred the most dramatic phenotypic defect and resulted in no detectable OmpF protein when envelopes from cultures grown at a nonpermissive temperature were examined (see above). Experiments described below showed that *ompF315* exerted a strong assembly defect. Suppressors of *ompF315* were sought to reveal genes whose products might be involved in the assembly process. Dex^+ revertants were obtained at an estimated frequency of 10^{-7} to 10^{-8}. Revertants that simultaneously became sensitive to phage K20 were further examined. Envelopes prepared from strains carrying the suppressor or wild-type allele were analyzed by SDS-PAGE (Fig. 2). The results showed that OmpF levels in strains carrying the suppressor allele were indeed higher than that in the strain carrying the wild-type gene (Fig. 2). However, the amount of OmpF present was lower than that present in the parent strain.

P1 transduction mapping analysis revealed that in four independent Dex^+ revertants, the suppressor alleles did not map at or near *ompF*. Hfr conjugational crosses showed that the suppressor allele in all four revertants mapped between 40 and 46 min on the chromosome. The suppressor mutation was mapped with P1 cotransductions by using linked Tn*10* markers. In all four cases, the mutation was mapped close to 45 min: P1 cotransductional linkages were approximately 10, 50, and 70% to *his*G::Tn*10*, *non*::Tn*10*, and *cps*::Tn*10*, respectively. Genetic mapping and phenotypic characterization suggested that all four independently isolated suppressor alleles were very similar to each other and could map to one gene. The results from only one such suppressor allele are shown in the following experiments.

The suppressor mutation was selected by its ability to suppress a temperature-sensitive Dex^+ phenotype rendered by *ompF315*. To test the allele specificity of the suppressor, other *ompF* alleles were moved into this genetic background. At 42°C, the presence of the suppressor allele resulted in a Dex^+ phenotype in all of the mutant (temperature-sensitive) *ompF* strains. The suppressor had no obvi-

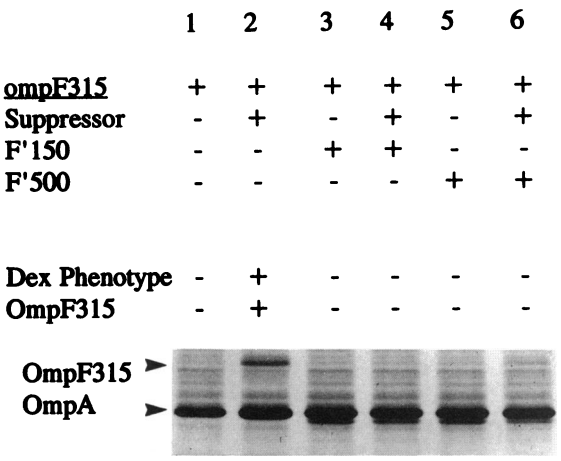

FIG. 3. Diploid analysis of the suppressor mutation. This test was performed at 42°C. Envelopes of various haploid and partially diploid strains were analyzed as described in the legend to Fig. 1. The wild-type or mutant suppressor alleles, F'150, F'500, and the resulting Dex phenotypes are indicated. Protein bands were visualized by Coomassie blue staining.

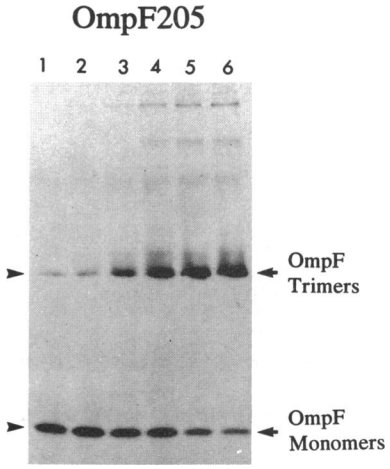

FIG. 4. OmpF trimers assayed from a strain carrying the *ompF205* allele. Freshly grown cells (grown at 42°C for roughly 2 h; for details, see Materials and Methods) were labeled with [^{35}S]methionine for 20 s and chased with excess of nonradioactive methionine. Samples were withdrawn at various chase times (0.25, 1, 5, 10, 30, and 60 min in lanes 1 through 6, respectively), and proteins were immunoprecipitated with OmpF trimer-specific antibodies. Prior to SDS-PAGE analysis, immunoprecipitates were heated to 60°C. The gel was dried and fluorographed. Positions of OmpF trimers and monomers are shown.

ous phenotypic effects on a strain carrying the parent *ompF* allele. Envelopes prepared from various *ompF* strains carrying the suppressor or wild-type allele grown at 30 and 42°C were analyzed by SDS-PAGE (Fig. 2). The results showed that the presence of the suppressor significantly increased OmpF levels in all mutant strains but not in the parent strain. It should be noted that while the suppressor restores the OmpF level in strains carrying weaker *ompF tsf* alleles back to the parental level, it only partially restores the amount of OmpF in the strain carrying a strong *ompF tsf* allele, *ompF315*. These results showed no clear allele specificity by the suppressor mutation.

Diploid analysis was carried out to further characterize the suppressor mutation. Two different F′ elements carrying the wild-type 45-min region of the chromosome were introduced into strains carrying the suppressor allele. The Dex$^+$ phenotype of diploid strains was tested both at permissive and nonpermissive temperatures. At 42°C, diploid strains showed a Dex$^-$ phenotype, indicating that the suppressor mutation is recessive to the wild-type allele (Fig. 3). This was also confirmed biochemically; the OmpF315 level in diploid strains was similar to that present in a strain lacking the suppressor allele (Fig. 3).

Assembly of OmpF. The *ompF* mutations described above conditionally reduced the level of OmpF. The examination of total OmpF from pulse-chase experiments showed that the low levels of OmpF present in mutant strains were not due to a defect in the synthesis of mature monomers. Thus, transcription, translation, and translocation of OmpF across the inner membrane were not affected. However, some degradation of OmpF315 was observed at later chase times, indicating a possible defect in the assembly process or that the assembled trimer is somewhat unstable. No degradation of OmpF315 was noted in the suppressor background. As expected, the parental OmpF205 did not degrade. Trimer assays were carried out to examine OmpF assembly. Fresh bacterial cultures grown either at permissive or nonpermissive temperatures were labeled with [^{35}S]methionine and chased with an excess of nonradioactive methionine in the presence of chloramphenicol. OmpF was extracted from samples withdrawn at various chase times and was immunoprecipitated with either OmpF trimer-specific antibody or a mixture of trimer- and monomer-specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE (Fig. 4 to 8).

When samples were heated to 60°C prior to SDS-PAGE analysis, OmpF trimers obtained from the parent strain (OmpF205) migrated at a distinct position in the gel (Fig. 4). The band that migrated at the monomeric position contained thermolabile OmpF assembly intermediates, which were gradually chased into stable trimers. The true monomeric form of OmpF205 was seen only when monomer-specific antibodies were used during immunoprecipitation and samples were not heated prior to SDS-PAGE analysis (data not shown). When the mutant strains were grown at a nonpermissive temperature, thermolabile OmpF intermediates were synthesized normally, but either none (e.g., OmpF315; Fig. 5) or only a fraction (e.g., OmpF35 and OmpF33, Fig. 6 and 7, respectively) of these assembly intermediates were chased into stable trimers. Instead, they were chased into a form that accumulated at the top of the gel (Fig. 5 to 7, panel A). These high-molecular-weight forms together with OmpF trimers migrated in the gel at a monomeric OmpF position when the above samples were heated to 100°C prior to SDS-PAGE analysis (Fig. 5 to 7, panel B). The high-molecular-weight forms were not detected when monomer-specific antibodies were used, indicating that these forms of OmpF have a conformation similar to that of trimers. The high-molecular-weight OmpF species did not accumulate when OmpF205 was examined (Fig. 4) and were absent in strains lacking OmpF (data not shown). These results showed that at a nonpermissive temperature, the assembly of mutant OmpF was defective at a step after the synthesis of early thermolabile intermediates but preceding the formation of heat-stable trimers. The inclusion of OmpF monomer-specific antibodies during immunoprecipitation did not result in the appearance of any new OmpF-related bands (data not shown).

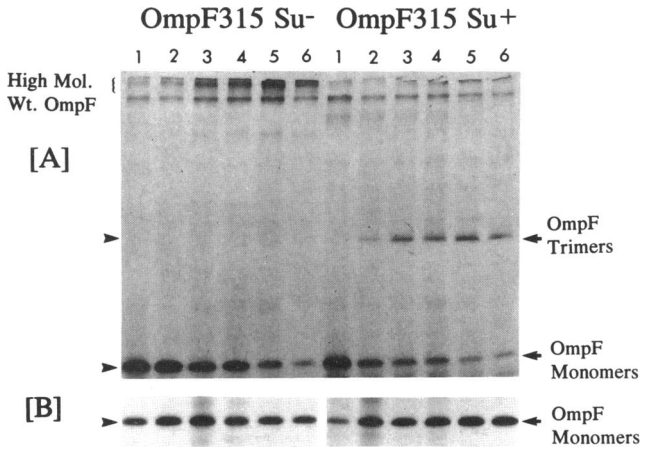

FIG. 5. OmpF trimer assays from strains carrying the *ompF315* allele in Su^- or Su^+ background. Growth and labeling conditions were similar to those described in the legend to Fig. 4. Immunoprecipitates were either heated to 60°C (A) or 100°C (B) prior to SDS-PAGE analysis. Gels were fluorographed. Positions of high-molecular-weight OmpF, trimers, and monomers are indicated.

At a permissive temperature, assembly of OmpF315 improved dramatically compared with that observed at a nonpermissive temperature (compare Fig. 5 and 8). The assembly of OmpF35 and OmpF33 was not affected when examined in cells grown at a permissive temperature (Fig. 8). In all three mutants, trimers that assembled at a permissive temperature were functionally and structurally intact at a nonpermissive temperature. This was tested with [^{14}C]maltose uptake assays (3, 13) and by examining the thermal stabilities of trimers (data not shown).

The effect of the suppressor mutation on the assembly of mutant OmpF proteins was examined. Pulse-chase experiments showed a dramatic increase in the amount of mutant OmpF stable trimers in the presence of the suppressor allele

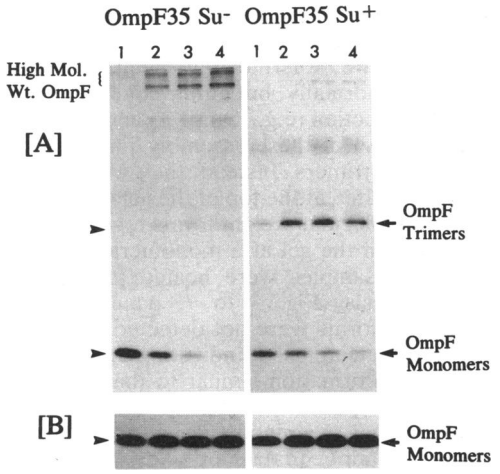

FIG. 6. OmpF trimer assays from strains carrying the *ompF35* allele in either the Su^- or Su^+ background. Cells were grown and labeled as described in the legend to Fig. 4. Chase samples were withdrawn after 1, 10, 30, and 60 min (lanes 1 through 4, respectively) and were subsequently treated as described in the legend to Fig. 5. Positions of high-molecular-weight OmpF, trimers, and monomers are indicated.

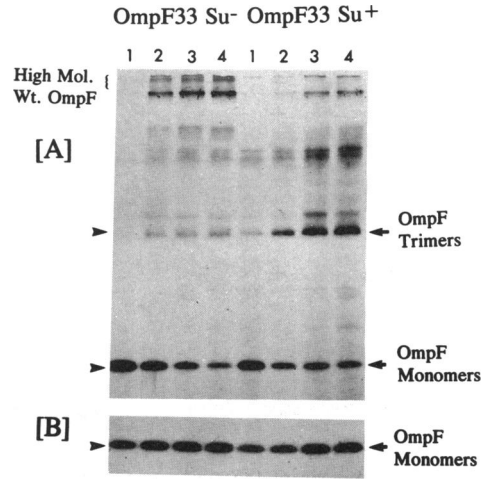

FIG. 7. OmpF trimer assays from strains carrying the *ompF33* allele in either the Su^- or Su^+ background. Cells were grown and labeled as described in the legend to Fig. 4. Chase samples were withdrawn after 1, 10, 30, and 60 min (lanes 1 through 4, respectively) and were treated as described in the legend to Fig. 5. Positions of high-molecular-weight OmpF, trimers, and monomers are indicated.

(Fig. 5 to 7, panel A). An increase in stable trimers with a concomitant decrease in the amount of higher-molecular-weight OmpF assembly intermediates indicated that the accumulation of the latter is seen only when the synthesis of the former is blocked. While the presence of the suppressor mutation had a positive effect on the formation of mutant OmpF stable trimers, it exerted no effect on the assembly of wild-type OmpF (data not shown).

DISCUSSION

In this study, a novel method to examine the assembly and targeting of an *E. coli* outer membrane protein is presented. A selection scheme was devised to isolate mutations in *ompF* that conferred a conditional defect in OmpF assembly. The basic premise behind the selection was the assumption that proper folding of the mature sequence (signal sequence

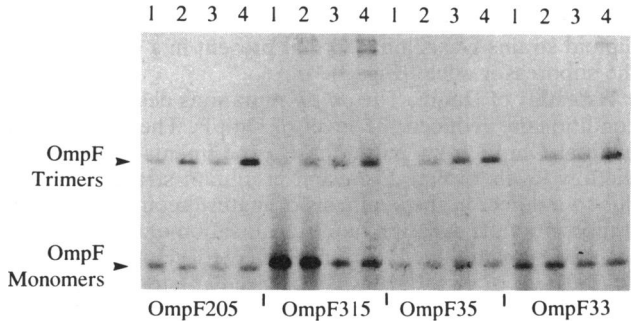

FIG. 8. Assembly assays of the parental and mutant OmpF proteins performed at a permissive temperature (30°C). Freshly grown cells were labeled as described in the legend to Fig. 4. Chase samples were withdrawn after 1, 10, 30, and 60 min (lanes 1 through 4, respectively). Proteins were extracted as described in Materials and Methods, and OmpF was immunoprecipitated with OmpF trimer antibodies. Samples were heated to 60°C prior to SDS-PAGE analysis.

removed) of an outer membrane protein determines its correct assembly and targeting. Since it is not presently known which residue or region of the protein might be involved in the folding process, a random amber reversion approach was utilized. First, various *ompF* amber mutations were isolated that prevented the proteins from functioning. In the second step, conditional heat-sensitive revertants were obtained from strains carrying the *ompF* amber mutations. The conditional nature of these revertants ensured the screening out of true or pseudorevertants that possess normal OmpF function and thus presumably normal assembly. Since the mutation in desired conditional revertants altered a residue in the mature portion of the protein, it was not anticipated that the cellular processes such as transcription, translation, or translocation (signal sequence processing) would be affected. A rapid determination of the mutational alteration within the gene was possible because the locations of the amber mutations were known, although the entire *ompF* gene was sequenced to examine other possible alterations.

Of the six different amber mutations utilized in this study, two resulted in missense mutations that conferred a heat-sensitive phenotype. These mutations were termed temperature sensitive for folding (*tsf*) because the resulting substitution most likely caused misfolding of the protein in a temperature-dependent manner. It is anticipated that not all amber sites will yield *tsf* mutations. Furthermore, when the amber codon is present at a critical folding site, not all substitutions are expected to yield *tsf* mutations. This limited the frequency of obtaining the desired mutations (from amber mutations present at a critical folding site) to approximately 10^{-9} to 10^{-10}. In all three of the *tsf* mutations studied here, an aromatic residue (W or Y) was replaced by a nonaromatic residue. The most dramatic effect was observed when a tryptophane was changed to a charged glutamic acid residue. While it is not clear exactly how these substitutions affect protein structure, they did allow us to determine residues and sites within the polypeptide chain that may play a critical role in the folding and assembly processes. A three-dimensional crystal structure of OmpF has recently been determined (5). In this structure, W214 and Y231 are located on the external surface of the monomer and are not involved in subunit interactions.

It should be noted that the isolation of *tsf* mutations in *ompF* was possible because the native trimeric OmpF is extremely thermostable (requires heating above 65°C in 2% SDS solution for denaturation into monomers), whereas its assembly intermediates are thermolabile (19). Thus by lowering the melting temperature of the thermolabile intermediates below the growth temperature, one can isolate temperature-sensitive assembly mutants. Native OmpF trimers are structurally very stable, and minor changes (single amino acid substitutions) usually do not lower their characteristic thermostable nature. More dramatic structural alterations (in-frame deletions, insertions, or multiple substitutions) in the polypeptide chain may lower the dissociation temperature of native trimers below the growth temperature. The three *ompF* mutations described here affected the assembly process in a temperature-dependent manner. When the mutant OmpF protein was permitted to trimerize at a permissive temperature, it remained functionally and structurally intact at a nonpermissive temperature, suggesting that it is not the assembled trimer but the process of trimerization which is conditionally defective. Similar types of *tsf* mutations that affect the assembly process of LamB and tail spike protein of bacteriophage P22 have been described previously (9, 16).

Trimer assays revealed that the mutant proteins are defective in the formation of stable trimers. Since synthesis of these proteins was not affected, it can be concluded that the mutations exerted their effects at a step after synthesis but before stable trimer formation. Thus, these mutations affect the proper assembly of OmpF. Starting with these *ompF tsf* mutations, second-site suppressor mutations that restored the Dex^+ phenotype at a nonpermissive temperature were isolated. In the suppressor background, the trimerization of OmpF was restored, albeit not to the extent seen in a strain carrying the parental OmpF protein (OmpF205). The Dex^+ phenotype of mutants was dependent on the synthesis of OmpF, therefore, the extragenic suppressor is not a bypass mutation that renders a Dex^+ phenotype by creating a new pathway for maltodextrin entry; our previous studies have shown that in a $LamB^-$ background, Dex^+ revertants that bear mutations in one of four different genetic loci can be isolated (3, 13, 15, 20).

When mutant OmpF failed to form stable trimers, a high-molecular-weight species of OmpF was kinetically accumulated. Upon heating above 60°C prior to SDS-PAGE analysis, these high-molecular-weight forms of OmpF dissociated into monomers. This species of OmpF did not accumulate in the parental strain carrying OmpF205. In the suppressor background, almost all of the high-molecular-weight species of mutant OmpF disappeared with the concomitant appearance of stable trimers. At this stage, it is not known whether the high-molecular-weight species of OmpF is a normal assembly intermediate or a dead-end misfolded assembly product. It is apparent from these experiments that the synthesis of the high-molecular-weight form blocks the synthesis of heat-stable trimers. Immunological assays with specific antibodies suggest that the high-molecular-weight forms are structurally related to OmpF trimers. In the case of LamB, a temperature-dependent block in assembly resulted in the transient accumulation of a monomeric intermediate (16). We showed that this effect was partly reversible; i.e., a fraction of the accumulated monomers could be converted into trimers upon the temperature shift (16). These kinds of reversion experiments have not been successful so far with the OmpF mutants. The wild-type suppressor gene has been cloned by simple genetic complementation. Further characterization of the suppressor gene product will reveal its role in the assembly and targeting of outer membrane proteins.

The suppressor mutation displayed no allele specificity, although it should be noted that all three *ompF* mutations studied here were clustered in a small area of the protein sequence and thus could impose a similar structural alteration. In the suppressor background, the absence of the high-molecular-weight form of OmpF with the concomitant appearance of stable trimers suggested that the suppressor gene product is involved in the assembly process. The suppressor protein could interact with the mutant OmpF protein and drive it to the proper assembly pathway. This interaction could prevent the formation of high-molecular-weight OmpF species. It is also conceivable, although less likely, that the suppressor interacts with OmpF in a postassembly manner. In such a case, the suppressor perhaps resolves the misassembled OmpF aggregates into native trimers. These hypothetical roles of the suppressor gene product will be similar to that of a molecular chaperone.

The extragenic suppressor mutations were obtained at a relatively low frequency (10^{-7} to 10^{-8}), which is consistent

with the notion of an altered suppressor gene product. But the fact that the suppressor mutation is recessive to the wild-type allele argues that the suppressor may be a null or loss-of-function mutation. Thus, in the absence of the suppressor gene product, the mutant and parental OmpF proteins assemble normally, but the presence of the wild-type suppressor protein interferes with the assembly of the mutant OmpF protein. In other words, the wild-type suppressor protein acts as an inhibitor of mutant OmpF assembly. This will make the wild-type allele dominant over the mutant suppressor allele in regard to the assembly of mutant OmpF. This would imply that the normal role of the wild-type suppressor protein is to prevent assembly of certain structurally altered OmpFs, a role generally performed by a class of proteins known as chaperones. At this stage, these alternative theories have not been resolved. Recently, we isolated additional extragenic suppressors of the OmpF assembly mutants. One such suppressor mutation maps close to 2 min on the *E. coli* chromosome (9a). Currently we are analyzing the role of the suppressor gene product and further characterizing the high-molecular-weight OmpF species.

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