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NOTES

The *fadL* Gene Product of *Escherichia coli* Is an Outer Membrane Protein Required for Uptake of Long-Chain Fatty Acids and Involved in Sensitivity to Bacteriophage T2

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The *fadL*$^+$ gene of *Escherichia coli* encodes an outer membrane protein (FadL) essential for the uptake of long-chain fatty acids (C_{12} to C_{18}). The present study shows that in addition to being required for uptake of and growth on the long-chain fatty acid oleate ($C_{18:1}$), FadL acts as a receptor of bacteriophage T2. Bacteriophage T2-resistant (T2^r) strains lacked FadL and were unable to take up and grow on long-chain fatty acids. Upon transformation with the *fadL*$^+$ clone pN103, T2^r strains became sensitive to bacteriophage T2 (T2^s), became able to take up long-chain fatty acids at wild-type levels, and contained FadL in the outer membrane.

Long-chain fatty acids (C_{12} to C_{18}) can serve as a sole carbon and energy source to support the growth of *Escherichia coli* (17, 34). Therefore, these hydrophobic compounds must selectively traverse the cell envelope prior to metabolic utilization. The outer membrane of *E. coli* is generally considered to be impermeable to hydrophobic compounds (11, 30). Therefore, the mechanism allowing long-chain fatty acids to traverse this layer is of considerable interest. Recently, Black et al. (4) described the product of the *fadL*$^+$ gene (FadL) as an outer-membrane-bound protein. A functional *fadL*$^+$ gene is required for the uptake of long-chain fatty acids prior to their delivery to the enzymatic machinery involved in energy production (2, 24, 31–33, 37) and phospholipid biosynthesis (16, 24, 35). FadL apparently acts in a highly specific manner to bind long-chain fatty acids to the cell and to allow the passage of these compounds through the outer membrane prior to metabolic transformation.

Strains resistant to bacteriophage T2 are difficult to isolate and occur at low frequencies (13). Hantke identified OmpF (protein Ia) as a receptor for T2 and suggested that lipopolysaccharide was also required (13). Due to the low frequencies of isolating T2-resistant strains, Lenski suggested that bacteriophage T2 resistance occurs by a two-step mutational process (19). The concept of a two-step mutational process giving rise to T2 resistance is in agreement with the hypothesis of Luria (23), who proposed that resistance to T2 may be a combination of two or more mutations resulting from gross chromosomal changes. Morona and Henning (28) identified a new locus, *ttr*, that, like *ompF*, appears to encode receptor activity for bacteriophage T2. These authors showed that the *ttr* locus was closely linked to *fadL* at the 50-min region of the *E. coli* linkage map and encodes an outer membrane protein required for growth on and inducible by the long-chain fatty acid oleate ($C_{18:1}$). The conclusion from these data is that T2 resistance arises as a result of mutations in both *ttr* and *ompF*.

The relationship between *ttr* and *fadL* is not clear, although when judged by genetic criteria they appear to be the same (28). Morona and Henning proposed that one locus is polar to the other and that *ttr*$^+$ encodes an outer membrane component and *fadL*$^+$ encodes an inner membrane component of the fatty acid transport system (28). The observation that *fadL*$^+$ encodes an inner-membrane-bound protein (9) has been shown by Black et al. (4) to be incorrect. Although it is conceivable that *ttr*$^+$ and *fadL*$^+$ both encode outer membrane components of the fatty acid transport system, it is equally conceivable that these loci are the same.

It was important to test the interrelationship between *fadL* and *ttr*, as these loci appear to be closely linked (or identical) and both encode an outer membrane protein involved in long-chain fatty acid uptake. Two bacteriophage T2-resistant (T2^r) strains, E15 and K10 (Table 1), were tested for T2 sensitivity as described by Morona et al. (27) and for ability to grow on oleate as a sole carbon source. These strains were chosen because they are two of the initial strains described as having T2 resistance. Both strains were unable to grow on 5 mM oleate in medium E (26) (Ole$^-$) and were T2^r (Table 2). The Ole$^-$ phenotype was expected for E15, as this strain is *fadL*, but was unexpected for K10. In order to investigate whether *fadL* was involved with T2 resistance, E15 and K10 were transformed with pN103 (*fadL*$^+$) (2) by the $CaCl_2$ procedure of Dagert and Ehrlich (8) and then were retested (Table 2). The plasmids used in these studies were routinely isolated by the cleared lysate-polyethylene glycol precipitation method of Humphreys et al. (15). These data showed that upon transformation with pN103, both E15 and K10 became T2 sensitive (T2^s) and able to grow on oleate as a sole carbon source (Ole$^+$). These results clearly suggested that *fadL* had a role in T2 susceptibility and that this activity was associated with an Ole$^+$ phenotype. As Morona and Henning (28) demonstrated, *ttr* encodes T2 receptor activity as well as being involved in fatty acid uptake. I transformed four of their *ttr* strains (two with Tn*10* insertions and two point mutants; Table 1) with either pN103 or the vector pACYC177 and tested them for their ability to grow on oleate and for sensitivity to T2. These data (Table 2) showed that RMT238, RMT239, RMT253, and RMT254 all became

TABLE 1. Bacterial strains

Strain	Characteristics[a]	Source (reference)
K-12	Prototrophic	
RS3010	*fadR*	Simons et al. (38)
LS6164	*fadR* Δ*fadL*	Ginsburgh et al. (9)
E15	Hfr PO2A *tonA22* Δ*phoA8 ompF627 fadL701 relA1 pit-10 spoT1* $T2^r$	A. Garen via CGSC[b]
K10	Hfr PO2A *tonA22 ompF627 relA1 spoT1* $T2^r$	A. Garen via CGSC
RMT209	F^- *fhuA pryD*$^+$ *ompF680*	Morona and Henning (28)
RMT238	F^- *fhuA ompF680 ttr*::Tn*10*	Morona and Henning (28)
RMT239	F^- *fhuA ompF627 ttr*::Tn*10*	Morona and Henning (28)
RMT253	F^- *thi argE proA thr leu mtl xyl galK lacY rpsL supE non ompF680 ttr*	Morona and Henning (28)
RMT254	F^- *thi argE proA thr leu mtl xyl galK lacY rpsL supE non ompF680 ttr*	Morona and Henning (28)

[a] Nomenclature is according to Bachmann (1).
[b] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Ole$^+$ and $T2^s$ upon transformation with pN103. Total cellular protein and outer membrane protein (isolated as described by Crowlesmith et al. [7] and as modified by Hall et al. [10]) from *fadL*$^+$ and *fadL* transformants were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels (3, 18) and subjected to immunoblotting as described by Burnette (6). FadL was absent in all four *ttr* strains and in the $T2^r$ stains E15 and K10 either alone or harboring the plasmid vector pACYC177 (Table 2). FadL, a protein with an M_r of 43,000, was detected only when these strains were transformed with the *fadL*$^+$ plasmid pN103 (Table 2). These data

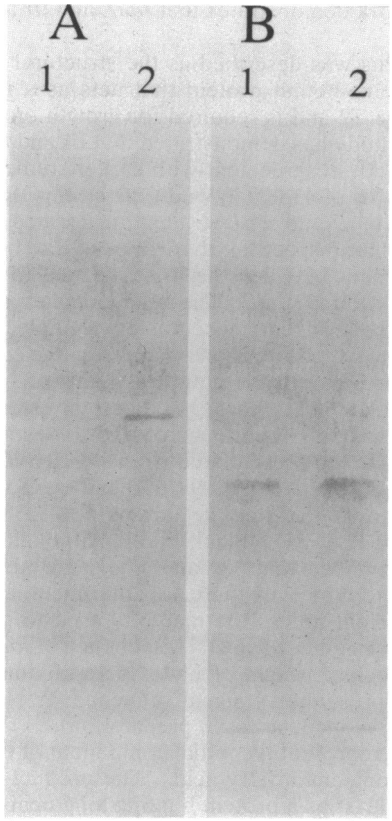

FIG. 1. Immunoblots of outer membrane proteins insoluble in 2% SDS–0.7 M 2-mercaptoethanol and probed with anti-FadL (A) or anti-OmpF (B). Lanes: 1, strain LS6164 (Δ*fadL*); 2, strain RS3010 (*fadL*$^+$). FadL migrated with an M_r of 43,000 and OmpF migrated with an M_r of 37,000 on 12% SDS–polyacrylamide gels.

showed a clear correlation between the presence of FadL, T2 sensitivity, and the ability to grow on oleate as a sole carbon source (Table 2). These data strongly suggest that *ttr*$^+$ and *fadL*$^+$ are the same gene. Both *ttr* and *fadL* map at 50 min, encode an outer membrane protein required for growth on oleate, have a role in bacteriophage T2 susceptibility, and are complemented by the *fadL*$^+$ plasmid pN103.

A functional *fadL* gene is required for the uptake of long-chain fatty acids. Therefore, I was interested in evaluating long-chain fatty acid uptake in the $T2^r$ and *ttr* strains in an effort to further evaluate the relationship between *fadL* and *ttr*. Long-chain fatty acid uptake experiments were performed as previously described (24) with the original T2-resistant strains (alone or harboring the *fadL*$^+$ clone or vector) and with several of the *ttr* strains (alone or harboring the *fadL*$^+$ clone or vector). The data from these experiments (Table 2) showed that the $T2^r$ strains were unable to take up long-chain fatty acids (>5 pmol/min per mg of protein). Furthermore, these data showed that the original $T2^r$ strains and the *ttr* strains, upon transformation with *fadL*$^+$ (which made all the strains $T2^s$), were able to take up long-chain fatty acids at levels comparable to wild-type levels (~800 pmol/min per mg of protein). From these data it is apparent that mutations in the *ompF* locus do not affect the uptake of long-chain fatty acids, because the levels of uptake in E15(pN103), K10(pN103), RMT238(pN103), RMT253 (pN103), LS6164(pN103), and RS3010 were essentially the same (Table 2). The data imply that, although *ompF* and *fadL* both encode receptor activity for T2, both gene products are not required for the uptake of long-chain fatty acids.

Many of the proteins of the outer membrane span the membrane (30). Notable among these are the Omp proteins, LamB, and PhoE. Thus, it was of interest to determine whether FadL was also a transmembrane protein. Rosenbusch demonstrated that OmpF is associated with the underlying peptidoglycan layer following solubilization of most of the membrane proteins in 2% SDS at room temperature (36). By using the procedure of Rosenbusch (36), total cell envelope from the *fadL* strain LS6164 and its isogenic parent RS3010 was solubilized in 2% SDS–0.7 M 2-mercaptoethanol at room temperature. After this treatment, the insoluble (peptidoglycan-associated) material was pelleted at 45,000 × *g* and analyzed by immunoblotting with both anti-FadL and anti-OmpF (Fig. 1). As expected, OmpF was peptidoglycan associated in both LS6164 and RS3010. FadL appeared to be associated with the peptidoglycan layer in strain RS3010 and, as expected, was absent from LS6164 (Fig. 1). Although the nature of the peptidoglycan association was not deter-

TABLE 2. Complementation patterns of T2^r strains following transformation with pN103 (*fadL*$^+$)

E. coli strain(plasmid)	Relevant bacterial genotype	Plasmid genotype[a]	Growth on oleate[b]	Mean $C_{18:1}$ transport ± SD[c]	FadL[d]	T2 resistance after transformation[e]
K-12		None	+	891 ± 127	+	s
LS6164	*fadL*	None	−	>1 ± 12	−	s
LS6164(pN103)	*fadL*	Ap^r *fadL*$^+$	+	927 ± 65	+	s
LS6164(pACYC177)	*fadL*	Ap^r Kn^r	−	>1 ± 7	−	s
E15	*ompF fadL* T2^r	None	−	12 ± 6	−	r
E15(pN103)	*ompF fadL* T2^r	Ap^r *fadL*$^+$	+	701 ± 74	+	s
E15(pACYC177)	*ompF fadL* T2^r	Ap^r Kn^r	−	>1 ± 4	−	r
K10	*ompF* T2^r	None	−	8 ± 5	−	r
K10(pN103)	*ompF* T2^r	Ap^r *fadL*$^+$	+	917 ± 82	+	s
K10(pACYC177)	*ompF* T2^r	Ap^r Kn^r	−	>1 ± 3	−	r
RMT209	*ompF*	None	+	786 ± 29	+	s
RMT253	*ompF ttr* T2^r	None	−	5 ± 4	−	r
RMT238(pN103)	*ompF ttr* T2^r	Ap^r *fadL*$^+$	+	846 ± 148	+	s
RMT238(pACYC177)	*ompF ttr* T2^r	Ap^r Kn^r	−	43 ± 3	−	r
RMT238	*ompF ttr* T2^r	None	−	>1 ± 5	−	r
RMT253(pN103)	*ompF ttr* T2^r	Ap^r *fadL*$^+$	+	733 ± 144	+	s
RMT253(pACYC177)	*ompF ttr* T2^r	Ap^r Kn^r	−	69 ± 53	−	r

[a] Ap^r, Ampicillin resistant; Kn^r, kanamycin resistant.
[b] −, No growth on 5 mM oleate; +, growth on 5 mM oleate.
[c] Expressed in picomoles per minute per milligram of protein (average of at least three separate experiments); protein was determined by the method of Lowry et al. (20).
[d] −, FadL absent; +, FadL present as determined by immunoblotting.
[e] r, Resistant to bacteriophage T2; s, sensitive to bacteriophage T2.

mined, it can be concluded that FadL was likely to be associated with the peptidoglycan layer.

The data presented above provided compelling evidence that *ttr* and *fadL* were the same locus. Morona and Henning (28) demonstrated that Ttr was sensitive to the action of proteinase K. If *fadL* and *ttr* are the same locus, FadL should also be sensitive to proteinase K. Whole cells [RS3010 (*fadL*$^+$), E15(pN103) (*ompF fadL* p*fadL*$^+$), and E15(pACYC177) (*ompF fadL* plasmid vector)] were treated with proteinase K as described by Morona et al. (27), and total cellular protein was analyzed by immunoblotting with anti-FadL. These data demonstrated that FadL was sensitive to the action of proteinase K (data not shown), which suggested that a portion of FadL is exposed at the surface of the cell. These data clearly corroborate the results of Morona and Henning. In order to evaluate this relationship further, I determined whether T2 could be inactivated by FadL as it can be by Ttr (28). Both chloroform-treated cells and total cell envelope prepared from strain E15 harboring pN103 (*ompF fadL* p*fadL*$^+$) were able to inactivate T2 (data not shown). As the only difference in the outer membrane preparations was the presence or absence of FadL, it can be concluded from these data that T2 uses FadL as a receptor.

The present study has defined FadL as an outer membrane protein that, in addition to being required for the uptake of long-chain fatty acids, acts as a receptor for bacteriophage T2. These conclusions are based on several lines of evidence: (i) all bacteriophage T2-resistant strains tested were unable to take up and therefore to grow on the long-chain fatty acid oleate ($C_{18:1}$) as a sole carbon and energy source; (ii) T2^r strains became sensitive to bacteriophage T2 and able to take up and grow on oleate as a sole carbon and energy source following transformation with the *fadL*$^+$ clone pN103; (iii) strains that were Ole$^+$ and T2^s contained FadL, as a 43,000-M_r polypeptide, in the outer membrane and had T2 receptor activity; (iv) FadL was sensitive to the action of proteinase K in intact cells, which implied that a portion of this protein is exposed at the cell surface; and (v) FadL appeared likely to be peptidoglycan associated following solubilization of total membrane with 2% SDS. Furthermore, this work demonstrates that *fadL* and *ttr* are the same locus.

The *ttr* locus was described as the structural gene for an outer-membrane-bound protein that acts as a receptor for bacteriophage T2 and is required for growth on oleate (28). The present study has demonstrated that *ttr* and *fadL* are the same locus and, in agreement with earlier studies (2, 4) that FadL has an M_r of 43,000 in wild-type strains (RMT209 and RS3010) and in *ttr* and *fadL* strains transformed with pN103 (*fadL*$^+$). On first inspection, there appeared to be a substantial disagreement between the M_rs assigned to the protein products of *ttr* and *fadL*. The discrepancies may be explained by the inherent inaccuracies of molecular weight determination by SDS-polyacrylamide gel electrophoresis and by the heat-modifiable nature of FadL (2). At 100°C in the presence of SDS, the M_r of FadL is estimated to be 43,000, whereas at 37°C, the M_r of FadL is estimated to be 33,000 (2, 4). In the present study, I have identified FadL as a 43,000-M_r protein in RMT209 (*ttr*$^+$) and in RS3010 (*fadL*$^+$) and have shown that the *ttr* strains RMT238, RMT239, RMT253, and RMT254 all lack FadL. Although the present study demonstrates that Ttr and FadL are identical, it is difficult to determine why there are differences in the relative molecular weight unless the methods used to generate the standard curves differ. The DNA sequence of *fadL* indicates that the molecular weight of FadL is approximately 47,000 (including a presumptive signal sequence; P. N. Black and C. C. DiRusso, manuscript in preparation).

FadL may represent the initial component of the transport system for long-chain fatty acids. The product of the *fadL* gene acts at least as a protein binding long-chain fatty acids when fatty acid utilization is blocked by mutation (*fadD*) (31). FadL may have a role(s) beyond the binding of long-chain fatty acids. I propose as a working model that this protein is responsible for both the initial binding of long-chain fatty acids to and the passage of long-chain fatty acids

through the outer membrane. One can only speculate how this protein functions as a component of the long-chain fatty acid transport system. FadL must act as a specific, high-affinity protein binding long-chain fatty acids. This protein must also specifically allow the passage of long-chain fatty acids through the outer membrane while preventing the passage of many other hydrophobic compounds (e.g., hydrophobic antibiotics) (30).

There are a number of different proteins of the outer membrane specifically involved in the uptake of metabolically useful compounds. These proteins generally represent one component of a multicomponent transport system. Notable among these are LamB (maltodextran selective channel) (5, 21), TonA (involved in ferrichrome uptake) (22, 25), BtuB (involved in vitamin B_{12} uptake) (14), Tsx (involved in nucleoside uptake) (12), and FepA (involved in ferric enterochelin uptake) (29). The data presented in this work, in conjunction with earlier work (4), demonstrate that FadL is also an outer membrane protein involved in the selective uptake of metabolically important compounds (long-chain fatty acids). The precise role of FadL as a component of the long-chain fatty acid transport system of *E. coli* is currently under study.

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