Evidence that His^{110} of the protein FadL in the outer membrane of *Escherichia coli* is involved in the binding and uptake of long-chain fatty acids: possible role of this residue in carboxylate binding

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The binding of exogenous fatty acids to the outer-membrane protein FadL of *Escherichia coli* is specific for long-chain fatty acids (C_{14}–C_{18}). Oleoyl alcohol [(*Z*)-9-octadecen-1-ol] and methyl oleate were unable to displace FadL-specific binding of [^{3}H]oleate ($C_{18:1}$), suggesting that the carboxylate of the long-chain fatty acid was required for binding. Therefore the binding of exogenous fatty acids to FadL is governed, in part, by the carboxy group of the long-chain fatty acid. Treatment of whole cells with 1 mM diethyl pyrocarbonate (DEPC) depressed binding by 43–73% over the range of oleate concentrations used (10–500 nM). On the basis of these results and the notion that histidine residues often play a role involving proton transfer and charge-pairing, the five histidine residues within FadL (His^{110}, His^{226}, His^{327}, His^{345} and His^{418}) were replaced by alanine using site-directed mutagenesis. Altered FadL proteins were correctly localized in the outer membrane at wild-type levels and retained the heat-modifiable property characteristic of the wild-type protein. Initial screening of these *fadL* mutants revealed that the replacement of His^{110} by Ala resulted in a decreased growth rate on minimal oleate/agar plates. The rates of long-chain fatty acid transport for Δ*fadL* strains harbouring each mutation on a plasmid, with the exception of *fadL*H110A, were the same, or nearly the same, as those for the wild-type. *fadL*H110A was also defective in binding, arguing that the functional effect of this mutation was at the level of long-chain-fatty-acid binding. Other mutants had levels of long-chain-fatty-acid binding that were either the same, or nearly the same, as those for the wild-type. On the basis of competition experiments, DEPC treatment and the analyses of the mutants, His^{110} may function in carboxylate binding.

INTRODUCTION

Fatty acids are essential components of cellular membranes and are important sources of metabolic energy. In *Escherichia coli*, the transport of long-chain fatty acids (C_{12}–C_{18}) into the cell occurs by a multicomponent process that results in their enzymic conversion into CoA thioesters prior to metabolic utilization. These compounds traverse the bacterial cell envelope by a high-affinity, saturable, energy-dependent process that requires the outer-membrane-bound fatty acid binding and transport protein FadL (product of the *fadL* gene) and the inner-membrane-associated acyl-CoA synthetase (product of the *fadD* gene). For the uptake of exogenous long-chain fatty acids, transport and activation are postulated to be linked in a manner such that the esterification of long-chain fatty acids provides the driving force for transport. The periplasmic protein Tsp (product of the *tsp* gene) appears to respond to the energized state of the cell and enhances the rate of transport [1]. The *fadL*, *fadD* and *tsp* genes have been cloned and sequenced and their respective gene products purified and characterized [1–3]. An oleate-binding protein distinct from FadL has been purified from total cell envelope that may represent the inner-membrane-bound component of the long-chain-fatty-acid transport system in *E. coli* [4,5].

The outer-membrane protein FadL is a central component of the long-chain-fatty-acid transport system. We have previously demonstrated that this protein contains two functional activities that can be distinguished: long-chain-fatty-acid binding and long-chain-fatty-acid transport [6]. FadL binds the long-chain fatty acid oleate ($C_{18:1}$) by a high-affinity process ($K_D = 2.0 \times 10^{-7}$M) and via an as-yet-undefined mechanism mediates the transport of these compounds across the outer membrane [7]. We hypothesize that FadL forms a specific channel for long-chain fatty acids that becomes accessible only upon ligand binding [2]. In order to understand how FadL facilitates long-chain-fatty-acid binding, we reasoned that the carboxy and acyl groups of the long-chain fatty acid may play different, but important, roles. We have previously demonstrated that the length of the acyl chain provides substrate specificity [7]. However, the role of the carboxy group in the binding of exogenous long-chain fatty acids had not been addressed. The goals of the present work were [1] to define whether the carboxylate of the long-chain fatty acid was required for FadL-specific binding and [2], if so, to define specific residues within FadL that could potentially interact with the carboxylate in the binding reaction.

Results of the present study provide evidence that is consistent with the proposal that the carboxy group of the long-chain fatty acid is required for FadL-specific binding. Furthermore, the results of the present study indicate that His^{110} of FadL either maintains a domain crucial for long-chain-fatty-acid binding or participates directly in the binding reaction by charge-pairing with the carboxylate of the long-chain fatty acid.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains used in these studies were derived from *E. coli* K12 and have been previously described [8–11]. Bacterial cultures were grown at 37 °C in a Lab Line gyratory shaker in Luria broth (LB) or tryptone broth (TB). When minimal medium

Abbreviations used: LB, Luria broth; TB, tryptone broth; DEPC, diethyl pyrocarbonate.

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was required, medium E or medium M9 supplemented with vitamin B_1 was used [12]. Carbon sources, sterilized separately, were added to final concentrations of 25 mM glucose, 25 mM potassium acetate, 5 mM decanoate, or 5 mM oleate. When oleate or decanoate were used as a carbon source, polyoxyethylene 20 cetyl ether (Brij 58) was added to a final concentration of 0.5%. As required, amino acids were added to a final concentration of 0.01%. When required to maintain plasmids, antibiotics were added to 100 μg/ml ampicillin and 40 μg/ml kanamycin. Growth of bacterial cultures was routinely monitored using a Klett–Summerson colorimeter equipped with a blue filter.

Long-chain fatty acid binding and transport

Long-chain-fatty-acid binding in the Δ*fadL fadD* strain LS6929 harbouring plasmids containing the mutant *fadL* alleles was determined as previously described [7]. Briefly, cells were grown to mid-exponential phase (5×10^6 cells/ml) in TB containing 100 μg/ml ampicillin. Following growth, cells were harvested by centrifugation, washed with 5 ml of binding buffer [10 mM potassium phosphate (pH 6.0)/10 mM NaCl] and resuspended in binding buffer containing 100 μg/ml chloramphenicol to a final cell density of 1×10^7 cells/ml. Cells were starved for a carbon source in the presence of chloramphenicol for 20 min at 37 °C, at which time they were diluted 1:1 into a reaction cocktail containing 20 nM, 200 nM or 1000 nM [^{3}H]oleate and 100 μg/ml chloramphenicol in binding buffer. The final protein concentrations in the reaction mixture ranged from 0.095 l to 0.125 mg/ml. The binding reaction was continued for 15 min at 37 °C, at which time the cells were pelleted by centrifugation, washed once with binding buffer and resuspended in the original volume of binding buffer. Two 100 μl samples of resuspended cells were taken and subjected to liquid-scintillation counting. Positive and negative controls were Δ*fadL fadD* strain LS6929 harbouring pN130 (*fadL*$^+$) or the plasmid vector pACYC177 respectively. All data were expressed as pmol of oleate bound/mg of cellular protein.

In experiments using diethyl pyrocarbonate (DEPC), cells were grown and prepared as described above. Prior to assay, cells were resuspended to a final cell density of 1×10^7 cells/ml in binding buffer containing 100 μg/ml chloramphenicol and 1 mM DEPC. Stock solutions of DEPC were prepared in ethanol [13]. Following a 20 min incubation at 37 °C in the absence of a carbon source, oleate binding was tested as described above.

Competition experiments were carried out using our standard long-chain-fatty-acid-binding procedures as previously described [7]. Cells were grown and prepared as described above and [^{3}H]oleate binding defined in the presence of unlabelled oleate, oleoyl alcohol [(*Z*)-9-octadecen-1-ol] or methyl oleate. [^{3}H]Oleate was held constant at 200 nM and competing ligands (oleate, oleoyl alcohol or methyl oleate) varied from 10 to 5000 nM. All data were expressed as a percentage of maximal oleate bound and plotted against the concentration of the competing ligand.

Long-chain-fatty-acid transport was measured as previously described [9], except that all plasmid-bearing cells (in the Δ*fadL* strain PN114) were grown in medium TB containing 100 μg/ml ampicillin, 5 mM oleate and 0.5% Brij 58 to mid-exponential phase (5×10^6 cells/ml). Following growth, cells were harvested by centrifugation, washed once with minimal media M9 containing 0.5% Brij 58 (M9-Brij) and resuspended in M9 Brij containing 100 μg/ml chloramphenicol (M9-Brij-Cm). Prior to assay, cells were starved for a carbon source for 15 min at 37 °C. For assay, 1 ml of starved cells was diluted 1:1 into a reaction cocktail containing 200 μM [^{3}H]oleate in M9-Brij-Cm at 37 °C. The final protein concentrations in the reaction mixture ranged from 0.095 to 0.125 mg/ml. Duplicate samples (100 μl) were filtered through a Gellman GN-6 membrane prewetted with M9-Brij at $t = 0$ and $t = 2$ min after the initiation of the reaction and washed with 3×5 ml of M9-Brij. The filters were air-dried and radioactivity was measured by liquid-scintillation counting. The results were expressed in pmol of oleate transported/min per mg of cellular protein.

Protein concentrations were measured in cell extracts after sonication by the method of Bradford [14] using BSA as a standard. All long-chain-fatty-acid binding and transport data represent the average results for at least three independent experiments and were subjected to analysis of variance using StatView (Abacus Concepts).

General recombinant DNA methods

Restriction, ligation, and transformation procedures have been previously described [15]. Oligonucleotides (17–24-mers; for mutagenesis and sequencing) were synthesized on a Pharmacia–LKB Gene Assembler Plus and purified by phenol/chloroform extraction and ethanol precipitation. Plasmid DNA containing the different site-directed mutations was sequenced using the chain-termination method of Sanger et al. [16] using [α-^{35}S]dATP and Sequenase (v 2.0; United States Biochemical Corp.). Synthetic oligonucleotides (17-and 21-mers) complementary to various regions of the *fadL* gene were used as primers [17].

Generation of site-directed mutations in *fadL*

Site-directed mutations in the *fadL* gene were generated using the Altered Sites mutagenesis system of Promega as previously described [9]. Sequences of the mutagenic oligonucleotides used are summarized in Figure 2(a) below. Once the desired mutation was confirmed, the *Bam*HI fragment containing the *fadL* gene was gel-purified and subcloned into pACYC177. These final plasmid constructions are illustrated in Figure 2(b) below and, upon transformation into the appropriate host strains, were analysed as detailed below.

Protein characterization

Western blots of cell extracts from PN114 harbouring the mutant plasmids were performed as previously described [6]. Total cellular protein (25–100 μg total) or isolated outer-membrane protein (purified as detailed in [18]) was subjected to SDS/PAGE [19] and electrophoretically transferred on to nitrocellulose membranes (0.45 μm pore size; Bio-Rad) overnight at room temperature. FadL and altered FadL proteins were detected using rabbit polyclonal anti-FadL serum and goat anti-rabbit IgG–peroxidase.

To determine whether the modified FadL encoded by each of the *fadL* alleles resulting in histidine replacement by alanine retained the characteristic heat-modifiable property of FadL (M_r of 43000 when heated in SDS at 100 °C and an M_r of 33000 when heated at 40 °C in the presence of SDS), outer-membrane proteins from the different mutants were incubated at 40 or 100 °C for 10 min in the presence of SDS prior to separation by SDS/PAGE [19]. Western-blotting analyses were carried out as described above.

Chemicals

[^{3}H]Oleate and [α-^{35}S]dATP were purchased from New England Nuclear. Enzymes for DNA sequencing (Sequenase) were ob-

tained from United States Biochemicals, and enzymes for routine DNA manipulations were obtained from Bethesda Research Laboratories, Pharmacia–LKB, United States Biochemicals or New England Biolabs. DEPC was obtained from Sigma. Chemicals for the synthesis of *fadL*-specific and mutagenic oligonucleotides were purchased from Pharmacia–LKB. Goat anti-rabbit IgG–peroxidase conjugate was obtained from Cappel and Bio-Rad. Antibiotics and other supplements for bacterial growth were obtained from Difco and Sigma. All other chemicals were obtained from standard suppliers and were of reagent grade. The amino acid residues in FadL are numbered to include the 27-amino-acid-residue signal peptide [17].

RESULTS

Competition of FadL-specific [^{3}H]oleate binding by oleate, oleoyl alcohol and methyl oleate

Displacement studies of FadL-bound [^{3}H]oleate by unlabelled oleate, oleoyl alcohol and methyl oleate were performed to determine whether the carboxylate of the long-chain fatty acid was involved in this binding reaction. Figure 1 shows that FadL-bound [^{3}H]oleate could be displaced by the native ligand, a finding in agreement with our previous results [7]. Methyl oleate and oleoyl alcohol could only displace 15 and 30% respectively of [^{3}H]oleate bound to FadL. These results are complicated by the fact that the precise concentration of free ligand is unknown. Unlabled oleate could displace nearly all the bound [^{3}H]oleate in a concentration-dependent manner (even at concentrations of oleate where micelles are present). In the absence of an oleate-binding activity, mixed micelles of oleoyl alcohol or methyl oleate and [^{3}H]oleate are predicted, especially at higher concentrations of competitor. In the presence of whole cells containing FadL, [^{3}H]oleate became bound and could not be displaced by either oleoyl alcohol and methyl oleate. While the low solubility of oleoyl alcohol and methyl oleate in aqueous conditions may have contributed to the inability of these compounds to displace the bound [^{3}H]oleate, the data are consistent with the proposal that binding requires a free carboxylate group. Therefore these results support the hypothesis that there is an interaction between the carboxylate of the long-chain fatty acid and a specific residue(s) within FadL to promote binding.

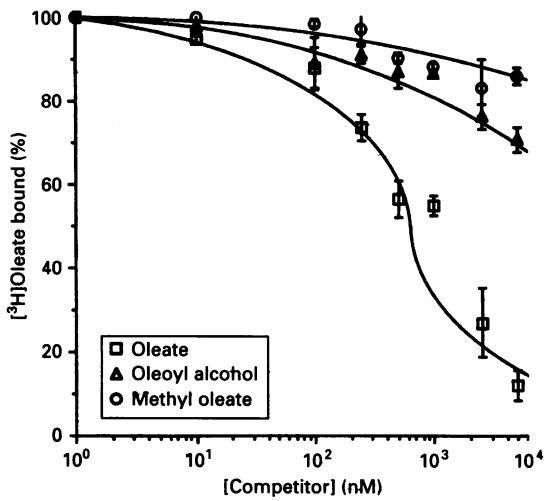

Figure 1 Displacement of FadL-specific [^{3}H]oleate binding by oleate, oleoyl alcohol and methyl oleate

Cells were incubated in a reaction cocktail containing 200 nM [^{3}H]oleate and increasing concentrations of competitor as described in the Materials and methods section. The non-specific (background) oleate binding measured in the Δ*fadL5 fadD fadR* strain LS6929 was subtracted from the binding measured in the *fadD fadR* strain LS6928 to give FadL-specific oleate binding. The values given are percentages of maximal binding (230 ± 21 pmol/mg of protein being equivalent to 100% binding). Error bars indicate the S.E.M. from four independent experiments.

Table 1 FadL-specific oleate binding following treatment with DEPC

Cells were incubated without (−) or with (+) 1 mM DEPC (DEPC) prior to assay and binding was measured at oleate concentrations of 10 nM, 100 nM and 500 nM as described in the Materials and methods section. The non-specific (background) oleate binding, measured in the Δ*fadL5 fadD fadR* strain LS6929, was subtracted from the binding measured in the *fadD fadR* strain LS6928 to give FadL-specific oleate binding.

		Oleate binding (±S.E.M., $n = 4$) (pmol/mg of protein)		
DEPC	[Oleate] (nM) ...	10	100	500
−		5.50 (0.87)	76.00 (14.66)	425.059 (118.44)
+		1.50 (1.19)	43.25 (15.12)	145.25 (79.44)

DEPC treatment of whole cells and long-chain-fatty-acid binding

As a first step to define residues within FadL that may be involved in ligand binding, we chose to modify surface-exposed histidine residues within FadL to *N*-carbethoxyhistidine derivatives using DEPC. Following chemical modification, the levels of oleate binding were defined as detailed in the Materials and methods section. Treatment of whole cells with DEPC reduced the levels of oleate binding by 43–73% when compared with cells not treated with DEPC (Table 1). These results argue that a surface-exposed histidine residue within FadL may be required for long-chain-fatty-acid binding. We interpret these results with caution, as DEPC modification of whole cells followed by a functional assay (oleate binding) represents a complex system at best for definitive analyses. Nonetheless, chemical modification as a tool to address the roles of surface-exposed residues requires that the integrity of the outer membrane remain intact. While our results may have resulted in a overall loss of activity of FadL given this complexity, they are consistent with the possible involvement of a surface-exposed histidine residue in the overall phenomenon of oleate binding. If this is the case, then a surface-exposed histidine residue within FadL may participate in the electrostatic interaction with carboxy group of the long-chain fatty acid predicted by the displacement studies.

Construction and phenotypic characterizations of *fadL* His-to-Ala mutants

To test the proposal that a specific histidine residue within FadL is required for long-chain-fatty-acid binding, each of the five histidine residues (His^{110}, His^{226}, His^{327}, His^{345} and His^{418}) were replaced by alanine to define their individual contribution to FadL function. Alanine was chosen for these substitutions as this residue is not likely to alter protein structure and lacks a charge [20]. Following the generation of each of these *fadL* mutations, the physiochemical parameters of the modified FadL proteins were compared with those defined for the wild-type (membrane

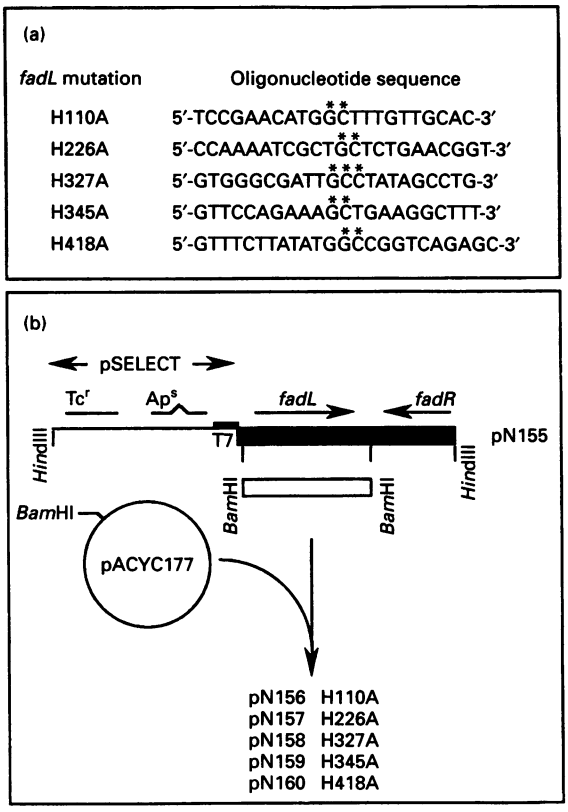

Figure 2 Construction and nomenclature of histidine-to-alanine substitutions in the *fadL* gene

(**a**) The nucleotide sequences of each of the mutagenic oligonucleotides (changes are noted by the asterisks) used to generate the collection of His-to-Ala substitutions in FadL. (**b**) The wild-type *fadL* gene linked to the *fadR* gene was cloned from pN132 into pSELECT to generate the phagemid pN155. The *fadR* gene was included to provide stability as otherwise *fadL* is toxic in high-copy plasmids. Five different mutagenic oligonucleotides were used to generate the *fadL* mutations H110A, H226A, H327A, H345A and H418A. Following mutagenesis and conformation of the desired changes, *Bam*HI fragments containing the desired changes were subcloned from the pN155 derivatives into pACYC177 to generate the recombinant plasmids pN156–pN160.

localization, levels within the outer membrane and ability to facilitate oleate binding and transport). Figure 2 illustrates the construction and designation of these site-directed *fadL* mutations. Following verification of the nucleotide change(s) in these *fadL* mutants, they were subcloned as *Bam*HI cassettes from the pN155 derivatives into pACYC177 [21] for analysis. The plasmid vector pACYC177 was ideally suited for these studies, owing to the moderate copy number [21]. Each of these constructs was transformed into the Δ*fadL fadR* strain LS6164 and stippled to minimal oleate plates. Growth was monitored at 37 °C over 96 h. Transformants harbouring the wild-type *fadL* gene (in plasmid pN130) had visible growth between 12 and 24 h. This was also the case for transformants harbouring the *fadL*H226A and *fadL*H418A mutations, indicating that neither of these replacements had any effect on FadL function. Replacements of His^{327} and His^{345} by alanine resulted in a decreased growth rate on oleate plates; growth was visible between 24 and 48 h. The mutant containing the *fadL* allele *fadL*H110A was observed to grow on oleate between 72 and 96 h. These data indicated that alanine replacements at His^{110}, His^{327}, and His^{345} were phenotypically distinct from the wild-type and suggested alterations in either FadL function or structure. Of particular interest to us was

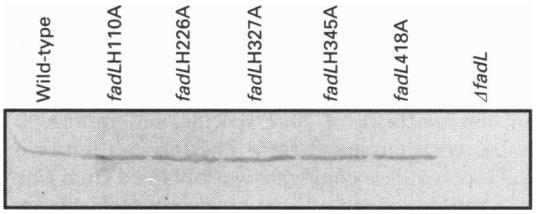

Figure 3 Western blot of total proteins from the *fadL* His-to-Ala substitutions

This represents a characteristic Western blot of total cellular protein (25 μg) from the Δ*fadL* strain PN114 carrying the plasmids harbouring the indicated *fadL* mutation probed with anti-FadL serum.

the markedly reduced growth rate of the *fadL*H110A mutant on solid oleate minimal media.

Characterization of modified FadL protein levels and protein localization

The levels of these different modified FadL proteins were determined in whole cells and in isolated outer membranes. Figure 3 is a representative Western blot of proteins from whole cells probed with anti-FadL serum. In both whole cells and in outer membranes, the levels of these modified FadL proteins were analysed using an image analysis system and shown to be equivalent to wild-type FadL. Furthermore, each of these modified FadL proteins retained the characteristic heat-modifiable property of FadL (when heated at 100 °C in the presence of SDS the M_r was 43000, whereas when the protein was heated at 40 °C, the M_r was 33000). These data imply that all five modified FadL proteins were intact and that the data described above concerning differences in the phenotypes of these *fadL* mutants on minimal plates containing oleate were likely due to specific alterations in the ability of these proteins to bind and/or transport long-chain fatty acids at wild-type levels.

Long-chain-fatty-acid transport profiles of the *fadL* mutants

Oleate transport was measured in the Δ*fadL fadR* strain PN114 harbouring the plasmids containing the individual *fadL* point mutations (Table 2). The levels of transport for strains harbouring the mutations *fadL*H226A, *fadL*H327A, *fadLH*345A, and *fadL*H418A were essentially the same as those of the wild-type ($P = 0.134$, $P = 0.584$, $P = 0.881$ and $P = 0.689$ respectively). In contrast, the His^{110}- to-Ala substitution resulted in levels of oleate transport that were significantly reduced when compared with the wild-type ($P = 0.007$). These results indicated that His^{110} was playing a crucial role in FadL function, either at the level of long-chain-fatty-acid binding or transport.

Long-chain-fatty-acid binding profiles of the *fadL* mutants

To characterize further this collection of *fadL* mutants, oleate binding was measured in the Δ*fadL fadD* strain LS6929 harbouring plasmids containing this collection of *fadL* mutations. The *fadL* mutation H110A resulted in lowering the levels of long-chain-fatty-acid binding, indicating that the functional defect in this mutant was at the level of binding (Table 2). No significant

Table 2 Oleate binding and transport properties of the histidine-to-alanine substitutions within FadL

The binding experiments were done, at the oleate concentrations indicated, on strain LS6929 (Δ*fadL5 fadD fadR*) harbouring the different *fadL* mutations as described in the Materials and methods section. The data presented for both the binding and transport experiments represent means for three independent experiments. The transport experiments were done as described in the Materials and methods section, in a reaction mixture containing 100 μM [^{3}H]oleate, in the Δ*fadL* strain PN114 harbouring plasmids containing the different *fadL* mutations. The nomenclature of the *fadL* mutations is described in Figure 2.

		Oleate binding (±S.E.M.) (pmol/mg of protein)			Oleate transport (±S.E.M.) (pmol/min per mg of protein)
fadL mutation	[Oleate] ...	10 nM	100 nM	500 nM	100μM
H110A		6.5 (0.1)	59.4 (3.6)	197.0 (15.6)	297.7 (67.4)
H226A		10.9 (0.4)	130.3 (9.9)	506.3 (6.4)	522.0 (44.3)
H327A		13.1 (1.0)	141.0 (15.2)	646.7 (14.7)	555.3 (165.7)
H345A		15.1 (1.4)	179.0 (10.6)	634.0 (39.7)	655.0 (105.8)
H418A		10.9 (0.7)	93.5 (1.6)	350.7 (24.4)	655.2 (11.6)
Wild-type		18.4 (3.3)	165.7 (7.7)	579.5 (38.7)	678.0 (58.9)
Δ*fadL5*		5.5 (0.3)	68.7 (5.7)	194.3 (8.6)	44.7 (13.1)

Table 3 Displacement of FadL-specific [^{3}H]oleate binding by unlabeled oleate, oleoyl alcohol and methyl oleate in His-to-Ala substitutions*

			Percentage of [^{3}H]oleate bound to FadL following displacement ($n = 3$)					
		Competing ligand ...	Oleate		Oleoyl alcohol		Methyl oleate	
fadL Mutation†	Plasmid	Concn. (nM) ...	500	5000	500	5000	500	5000
Wild-type	pN130		54‡	24	85	63	86	81
H110A	pN156		–§	–	–	–	–	–
H226A	pN157		59	24	83	63	85	81
H327A	pN158		60	24	82	62	86	76
H345A	pN159		54	25	78	64	85	79
H418A	pN160		54	23	77	58	87	81

* These studies were done on strain LS6929 (Δ*fadL5 fadD fadR*) harbouring the different plasmid constructs as described in the Materials and methods section.

† Nomenclature of the *fadL* mutations is described in Figure 2.

‡ Values are given in % [^{3}H] oleate bound to FadL following displacement; 100% FadL-specific binding of [^{3}H]oleate was 188.1 pmol/mg of protein ± 24.9 for the wild-type; 210.2 ± 19.7 for H226A; 214.8 ± 22.4 for H327A; 192.4 ± 28.5 for H345A; and 212.7 ± 24.3 for H418A.

§ The values of binding by *fadL*H110A were just above background and thus displacement profiles could not be determined.

differences in the levels of oleate binding were observed for the mutants *fadL*H226A, *fadL*H327A, and *fadL*H345A and the wild-type strain ($P > 0.50$). These data strongly argue that the histidine residues located at positions 226, 327 and 345 are not crucial for FadL function. Interestingly the level of oleate binding observed in *fadL*H418A was reduced when compared with the wild-type strain, but had transport levels that were equivalent to that of the wild-type. The most significant finding from these analyses was that His^{110} appears to play an essential role in facilitating long-chain-fatty-acid binding to FadL.

The FadL-specific oleate binding properties for each of these *fadL* mutants were further investigated by displacement experiments using two concentrations of competitor (500 and 5000 nM oleate, oleoyl alcohol or methyl oleate) while holding the concentration of [^{3}H]oleate at 200 nM. As oleate binding was near background in the strain harbouring the *fadL*H110A mutation, we were unable to show whether oleoyl alcohol or methyl oleate could displace the bound [^{3}H]oleate, as would be expected (Table 3). Each of the other four mutants had binding and displacement characteristics that were essentially wild-type, indicating that these four His-to-Ala substitutions were 'silent' with respect to the binding function associated with FadL.

DISCUSSION

The outer-membrane protein FadL is a central component of the long-chain-fatty-acid transport system of *E. coli*. FadL is required for both the binding and transport of exogenous long-chain fatty acids. The binding reaction associated with FadL occurs by a high-affinity process that is specific for long-chain fatty acids (i.e. C_{14}–C_{18}; $K_D = 2.0 \times 10^{-7}$M oleate) [7,22]. This outer-membrane protein also mediates the transport of these compounds across the outer membrane of the bacterial cell via a process that initially requires ligand binding [17]. Given the amphipathic nature of long-chain fatty acids, we postulated that the carboxy and acyl groups may interact with FadL during the binding reaction. Our previous work had clearly shown that binding specificity is a function of acyl chain length [7]. We chose to investigate the role of the carboxy group of the long-chain fatty acid by

employing displacement of FadL-bound [^{3}H]oleate with unlabelled oleate, oleoyl alcohol and methyl oleate. These results demonstrated that neither oleoyl alcohol or methyl oleate were able effectively to displace FadL-specific binding of [^{3}H]oleate. Despite the low solubility of these compounds under aqueous conditions, these data are consistent with the involvement of the carboxy group of the long-chain fatty acid in the binding reaction. Possible residues within FadL that could potentially interact with the carboxylate include arginine, lysine, and histidine. As a first step to analyse the contribution of these residues to FadL function, whole cells were treated with DEPC prior to assay to assess whether histidine residues within FadL were involved in the binding phenomenon.Treatment with DEPC reduced the levels of oleate binding significantly, indicating that a surface-exposed histidine residue within FadL was a likely candidate for the electrostatic interaction with the long-chain fatty acid predicted by the displacement studies.

The histidine residues within FadL were individually replaced by alanine residues so that their individual contributions to FadL activity could be assessed. Our first indication concerning the individual roles of these residues came from complementation experiments using the Δ*fadL* strain LS6164. While all of the His-to-Ala mutants in *fadL* were able to grow on minimal agar plates containing 5 mM oleate, only *fadL*H110A had a significantly reduced growth rate (appearing on minimal oleate plates only after 72 h of incubation at 37 °C). These data indicated that either *fadL*H110A resulted in an altered FadL protein that was not properly localized, was present at reduced levels or was defective in the binding and/or transport of the long-chain fatty acid oleate. While we have no physical data showing that the altered FadL protein from *fadL*H110A is conformationally wild-type, we suggest that this protein is in the wild-type conformation because it was correctly localized to the outer membrane at wild-type levels and maintained the characteristic heat-modifiable property of the wild-type protein.

The long-chain-fatty-acid binding and transport profiles defined for each of these His-to-Ala substitutions in FadL defined the defect that resulted in the reduced growth rate of *fadL*H110A. With the exception of *fadL*H110A, all site-directed constructs were wild-type or nearly wild-type with respect to long-chain-fatty-acid transport. Only *fadL*H110A had lowered binding and transport levels, arguing that His^{110} is involved in the binding of exogenous long-chain fatty acid. If His^{110} interacts directly with the carboxy group of the long-chain fatty acid, then this residue is likely to be exposed at the outer surface of the outer membrane. This interpretation agrees with the observations from the displacement experiments, which demonstrated that long-chain ligands lacking a carboxylate group were unable to compete for the binding of oleate. Therefore it seems plausible that there is a specific interaction between His^{110} and the carboxylate of the long-chain fatty acid to promote binding. Caution must be used in interpreting these results, however, as His^{110} may be involved in maintaining a structural domain of FadL required for long-chain fatty acid binding as opposed to a direct interaction with the carboxylate of the long-chain fatty acid. At the present time we cannot distinguish between these possibilities by our available technologies. As His^{110} is N-terminal proximal, these data support our previous suggestion that elements within the N-terminus of FadL are required for long-chain-fatty-acid binding [6].

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Our previous data demonstrating that oleate binding does not change significantly between pH 4.0 and 8.0 [7] are more consistent with the involvement of a surface-exposed arginine or lysine residue, as opposed to a histidine residue, in the binding reaction. While the results on the role of His^{110} in long-chain-fatty-acid binding presented here are reasonably clear, it is apparent that additional studies are required to assess the definitive role of this residue to FadL function. Additionally, it is equally important to assess the possible role(s) of surface-exposed arginine and lysine residues in FadL function.

The precise mechanism that allows FadL to bind specifically and transport long-chain fatty acids across the outer membrane is just starting to be defined. Our current data suggest that both the carboxylate and acyl chain of the long-chain fatty acid are essential for binding, predicting that both hydrophobic and charged amino acid residues within FadL participate in this process. The information presented here establishes a possible role for His^{110} in long-chain-fatty-acid binding. In particular, this residue may be involved in the binding of the carboxylate of the long-chain fatty acid. Our current work directed at defining the fatty-acid-binding domain of FadL using protein modification and affinity-labelled ligands should lead to a more thorough understanding of how this protein specifically binds long-chain fatty acids, and in particular establish the precise role of His^{110} in this process.

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