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Conjugation-Deficient Mutants of *Escherichia coli* Distinguish Classes of Functions of the Outer Membrane OmpA Protein

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Summary. Sixty-two *E. coli* mutants, selected as being deficient as recipients in F factor conjugation, are altered either in the amount or function of the outer membrane OmpA protein or in lipopolysaccharide structure. These two components may function together in conjugation, since the residual conjugation activity of a mutant lacking OmpA protein was unaffected by the additional presence of a lipopolysaccharide defect. Sixty of the strains carried mutations mapping to *ompA*, and these could be divided into classes depending on the amount of OmpA protein in their membranes. Representatives of these classes of mutant alleles failed to complement in diploids, indicating that they all affect the *ompA* structural gene and nearby sequences needed for its expression. The properties of these classes distinguish three groups of OmpA protein functions: 1) the structural function in the outer membrane in providing resistance to chelating agents and the hydrophobic antibiotic novobiocin, 2) the receptor functions in phage TuII* and K3 infection, and 3) the functions of binding cells together during conjugation, facilitating the uptake of receptor-bound colicin K or L, and allowing phage Ox2 to infect. Different cellular amounts or sites in OmpA protein are thus required for these three groups of functions.

Introduction

The outer membrane of *Escherichia coli* acts as a barrier to protect cells against toxic molecules in their environment including digestive enzymes and small hydrophobic molecules such as bile salts (Nikaido 1979). Two of the most abundant outer membrane proteins, the OmpA protein and the murein lipoprotein, appear to have overlapping functions in maintaining outer membrane structure. Thus, double mutants, lacking both proteins, grow as spherical rather than rod-shaped cells in which the murein and outer membrane layers may become separated from each other (Sonntag et al. 1978). Such structural defects appear to disrupt the barrier function of the outer membrane, so that the double mutants are sensitive to hydrophobic antibiotics and bile salts. They also require high concentrations of divalent cations for optimal growth (Sonntag et al. 1978). Less extreme structural defects are seen in mutants lacking either OmpA protein or murein lipoprotein alone (Foulds and Barrett 1973; Yem and Wu 1978; Suzuki et al. 1978). Pore function has been proposed for OmpA protein (Manning et al. 1977), but subsequent experiments have failed to substantiate this claim (Nikaido 1979). In addition to its structural role, the OmpA protein is required for a variety of interactions of cells with external agents, including infection by phages (K3, TuII* and Ox2), penetration of receptor-bound colicins (K and L), and tight binding of recipient to donor cells during F factor conjugation (Foulds and Barrett 1973; Davies and Reeves 1975; Manning et al. 1976; Henning et al. 1978; Achtman et al. 1978).

How are the various functions of OmpA protein related to each other? Many *ompA* mutants have been isolated after selections for insensitivity to phage or colicins. Most of these mutants lacked most or all OmpA protein and were therefore pleiotropically defective in OmpA protein functions (Foulds and Barrett 1973; Manning et al. 1976; Henning et al. 1978). In contrast, three out of five *ompA* mutants selected for loss of conjugation recipient ability retained OmpA protein in the membrane (Havekes and Hoekstra 1976; Havekes 1978). Properties of these mutants were examined by Achtman et al. (1978), who pointed out a correlation of colicin L insensitivity with conjugation deficiency.

To further investigate the relationship between different functions of OmpA protein, we have developed a simple direct selection for mutants unable to act as recipients in F factor conjugation. Sixty-two mutants fall into well-defined phenotypic classes which identify processes related to each other in their requirement for OmpA protein.

Materials and Methods

Media. LB and M9 media were made according to Miller (1972), except that M9 was supplemented with 6 mg ferric citrate per liter and 0.4% glucose. M9-glycerol contained 0.2% glycerol instead of 0.4% glucose. LA medium was LB solidified with 1.5% agar, L soft agar was LB solidified with 0.7% agar. Media supplement concentrations were: kanamycin and spectinomycin at 30 µg/ml, tetracycline at 15 µg/ml, streptomycin at 100 µg/ml and amino acids at 20 µg/ml. Phosphate buffer contained per liter: 7 g

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Table 1. Bacterial strains

Strain	Genotype	Source and reference
JC3272	*his trp lys tsx gal malA lacΔX74 strA*	Achtman (Achtman, 1975)
JC5484	F42 *lacI3/his trp ton tsx lacΔX74 spc*	Achtman (Achtman, 1975)
PL2	HfrH *thi*-1 *galE28 relA*1	Henning (Coleman and Leive, 1979)
KL725	F106/*thi-1 pyrD34 his-68 trp-45 recA1 galK35 mtl-2 xyl-7 malA118*	*E. coli* Genetic Stock Center (Low, 1972)
JE5513	Hfr Cavalli *lpp5508 man-1 pps*	Hirota (Suzuki et al., 1978)
JM15	*cysE50 lpcA*	*E. coli* Genetic Stock Center (Jones-Mortimer, 1968)
CC102	*his trp lys tsx galE28 malA lacΔX74 strA*	*galE* from PL2 to JC3272
CC104	F42 *lacI3 zzf-301*::Tn*5*/*his trp ton tsx galE28 lacΔX74 spc*	JC5484 with *galE* and *zzf-301*::Tn*5* (see text)
CC105	as CC104, *zzf-302*::Tn*10*	see text
CC107	F42 *lacI3 zzf-301*::Tn*5*/*his trp lys tsx galE28 malA lacΔX74 spc*	F42 transfer from CC104 to CC102
CC108	as CC102, *zcb-108*::Tn*5*	*ompA*-linked Tn*5* (see text)
CC109	as CC102, *zcb-109*::Tn*5 rpo-109*	*ompA*-linked Tn*5* (see text) and rifampicin resistance
CC205	as CC102, *ompA885*	conjugation-deficient mutant
CC223	as CC102, *rfa-223*	conjugation-deficient mutant
CC236	as CC102, *ompA886*	conjugation-deficient mutant
CC240	as CC102, *ompA887*	conjugation-deficient mutant
CC263	as CC102, *ompA888*	conjugation-deficient mutant
CC276	as JC3272, *ompA901*::Tn*5*	see text
CC277	as CC102, *ompA902*::Tn*5*	see text
CC617	as CC102, *zdg-102*::Tn*5*	*lpp*-linked Tn*5* (see text)
CC621	as JC3272, *ompA885 zcb-108*::Tn*5*	*ompA885* from CC205
CC622	as JC3272, *ompA886 zcb-108*::Tn*5*	*ompA886* from CC236
CC623	as JC3272, *ompA887 zcb-108*::Tn*5*	*ompA887* from CC240
CC624	as JC3272, *ompA888 zcb-108*::Tn*5*	*ompA888* from CC263
CC625	as JC3272, *rfa-223 zia-104*::Tn*5*	*rfa-223* from CC223
CC641	as CC223, *ompA901*::Tn*5*	*ompA901*::Tn*5* from CC276

$Na_2HPO_4 \cdot 2H_2O$, 3 g KH_2PO_4, 4 g NaCl, 1 ml 1 M $MgSO_4$.

Bacteria and Phages. The genotypes of many of the *E. coli* K-12 derivatives used in this study are shown in Table 1. Strains CC201-CC263 are conjugation-deficient mutants of CC102 (see below).

Cultures were mutagenized with transposons Tn*5* or Tn*10* essentially according to the procedure of Shaw and Berg (1979). The strains carrying Tn*5* insertions into *ompA* (CC276 and CC277) were isolated after Tn*5* mutagenesis by selection for resistance to phage TuII*. Tn*5* insertions near *ompA* (*zcb-108*::Tn*5* and *zcb-109*::Tn*5*) were selected by phage P1 cotransduction with a mutant *ompA* allele conferring phage TuII*-resistance. The Tn*5* insertion linked to *rfa* (*zia-104*::Tn*5*) was selected by virtue of its P1 cotransduction with $cysE^+$ and *rfa-223* into JM15. The Tn*5* insertion linked to *lpp* (*zdg-102*::Tn*5*) was selected by its cotransduction with lpp^+, selected by EDTA resistance using an Lpp^-OmpA^- (Class A1) recipient and the medium conditions described in the lengend to Table 6. The lpp^- gene was transduced into other strains by its linkage to *zdg-102*::Tn*5*; kanamycin-resistant transductants were screened for the absence of murein lipoprotein by SDS-polyacrylamide gel electrophoresis. To isolate insertions into F42, efficient cotransfer of transposon-mediated antibiotic-resistance with the F42 Lac^+ marker was selected by screening on plates.

Phages were TuII* (from U. Henning), Ox2, U3, C21, K3, K3hl (from A. Pugsley) and P1*vir* and M12 (from M. Achtman).

Genetic Techniques. Phage P1 transductions, bacterial matings, and other techniques were done according to Miller (1972). Selection after transduction for transposon-mediated antibiotic resistance was generally done using appropriately supplemented LA.

Mutagenesis and Selection of Conjugation-Deficient Mutants. Ethylmethanesulfonate (EMS) mutagenesis was done essentially according to Miller (1972), with treatment of cells in phosphate buffer with 1% EMS for 60 min at 37° C. Diethyl Sulfate mutagenesis was done according to Roth (1970), with cells in LB treated 20 min, 37° C with 5% diethylsulfate. Outgrowth in LB after mutagenesis allowed 10^3 to 10^{11}-fold increase in cell number. Cultures were grown separately after mutagenesis to help ensure that mutants were of independent origin, and normally only one mutant from each culture was saved. Strain CC201 was a spontaneous mutant, strain CC202-CC222 were isolated after diethylsulfate treatment, and strains CC223-CC263 were isolated after EMS treatment.

Mutagen-treated cultures of the F^-GalE^- Strain CC102 growing exponentially in LB were mixed with LB cultures of the donor (CC104) carrying F42 *lacI3 zzf-301*::Tn*5* at a ratio of 1 recipient: 9 donor cells. Cultures were incubated for conjugation at 37° C for 2 h with shaking, followed by 1/10 dilution into M9-glycerol + 0.002% lactose. After shaking at 37° C for 7–8 h to kill recipient cells which had conjugated, dilutions were plated onto LA + streptomycin to select CC102-derived survivors. Colonies that formed were replica plated onto LA alone and LA + kanamycin. Those showing kanamycin-

Table 2. Classes of conjugation-deficient recipient mutants

Mutant class	Number mutants	Genetic linkage[a]	OmpA protein[b]	Conjugation efficiency[c]		Representative alleles
				Liquid	Filter	
Wild-type	–	–	present	(1.0)	(1.0)	–
A1	9	*ompA*	absent	3×10^{-3}	0.2	*ompA885*
A2	33	*ompA*	present	8×10^{-3}	0.7	*ompA886*
A3	18	*ompA*	reduced amount	0.3	0.9	*ompA887, ompA888*
B	2	*rfa*	present	1.5×10^{-2}	0.5	*rfa-223*

[a] Determined by phage P1 transduction using donors containing transposon Tn*5* near *ompA* or *rfa*. Linkage was determined for all strains except one class B mutant (CC204)

[b] Determined for all mutants by electrophoresis and phage K3hl sensitivity tests

[c] Expressed as proportion recombinants per male cell, with wild-type efficiency defined as 1.0. Values are means of two (filter matings) or four (liquid matings) determinations of strains JC3272 and CC621-CC625. Liquid conjugation efficiencies were similar for the representative alleles in the original genetic background. The donor strain was CC105 in these tests. The absolute conjugation efficiency for the parent strain under these conditions is 0.5–1.0 for liquid matings, 1.5–2.5 for filter matings

sensitivity were purified and checked for resistance to the male-specific phage M12 (Brinton et al. 1964). Greater than 90% of the strains surviving these selection steps were reproducibly conjugation-deficient. In the selections of strains CC223-CC263, colonies were additionally screened for phage TuII* sensitivity, with those showing complete resistance discarded.

For some selections (mutants CC201-CC222), M9-glycerol + 0.002% lactose was replaced by Vogel-Bonner medium (Roth 1970) lacking glucose and containing 0.2% glycerol and 0.002% lactose.

Measurement of Conjugation Efficiency. To measure the conjugation efficiency of a recipient strain in liquid, an exponentially growing culture of the F^- strain to be tested was mixed with 1/10 volume of an exponentially growing culture of an F′ factor-containing strain. After 30 min incubation with shaking at 37° C, mixtures were cooled on ice and dilutions plated to determine the number of recombinants formed and the number of donor cells present. The ratio of recombinants to donor cells was defined as the efficiency of conjugation for different recipient strains, normalized to a parental strain value of 1.0 (see Table 2). CC104 and CC105 were used as donor strains, and CC102-derived strains as recipients. Recombinants were selected by their resistance to both streptomycin and kanamycin or streptomycin and tetracycline, and donors by their resistance to spectinomycin. Filter conjugations were performed using the same conditions except that after mixing donor and recipient cells, 2 ml volumes were filtered onto cellulose ester filter disks (diameter 2.5 cm, pore diameter 0.45 μm), placed onto prewarmed LA plates for 30 min, 37° C and removed to cold LA plates until being dispersed by vortex mixing in 2 ml ice cold LB, diluted and plated.

Phage and Colicin Sensitivity. Initial screens of conjugation mutants for phage TuII* sensitivity were done on LA by cross-streaking phage against single bacterial colonies, and scored after overnight incubation at 37° C. Quantitative tests were done by spotting phage dilutions onto LA plates previously overlayered with LA soft agar containing $2–5 \times 10^8$ cells/ml. Phage plaques were counted after overnight incubation at 37° C.

Colicin sensitivity was screened routinely after overnight incubation by measuring the growth inhibition of strains cross-streaked against a colicin-producing strain previously grown overnight on LA, chloroform-killed and overlayered with LA soft agar (Davies and Reeves 1975). Colicin sensitivity was quantitatively determined following the method of Davies and Reeves (1975). Colicin was prepared from exponentially-growing cultures treated with 500 ng mitomycin C per ml for 2.5 h. Cells were concentrated 40-fold into phosphate buffer after sedimentation (5 min at $4{,}000 \times g$) and disrupted in a French pressure cell (20,000 lb/in^2). Fast-sedimenting material was removed by subsequent centrifugations at $3{,}000 \times g$ for 5 min and $50{,}000 \times g$ for 30 min, with the final supernatant used as the colicin preparation. The source of colicin L was JF246 (Foulds and Barrett 1973); that for colicin K was *E. coli* K-12 W3110 carrying ColK.K235 (Pugsley 1981). Serial 2-fold dilutions of colicin preparations were spotted onto LA plates overlayered with L soft agar containing cells to be tested, as for phage sensitivity testing. The titer of colicin causing complete clearing in the lawn of cells after overnight incubation at 37° C was determined as a measure of the colicin sensitivity of the strain.

Genetic Linkage. Mutations were tested for their linkage to *ompA* by phage P1 transduction from donor strains (CC108 or CC109) carrying Tn*5* linked to *ompA* (greater than 50% cotransduced). Kanamycin-resistant transductants were purified and tested for their conjugation efficiency in liquid (and sometimes also for colicin K sensitivity). Of the two strains carrying mutations not linked to *ompA* in this test, one (carrying *rfa-223*) was shown to be linked in transduction to *rfa* using the transposon *zia-104*::Tn*5*.

Electrophoretic Analysis of Outer Membrane Proteins. Outer membrane fractions were prepared from 22 ml cultures of strains growing with shaking in LB, 37°C, at densities of $1–2 \times 10^9$ cells/ml. Cultures were centrifuged (10 min at $7{,}700 \times g$), and the cell pellets resuspended in 1 ml 10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, followed by lysis in a French pressure cell (20,000 lb/in^2). Whole membranes were sedimented from lysates at $48{,}000 \times g$ for 60 min. Pellets were resuspended in 1 ml 2% Triton X-100, 10 mM Tris HCl, pH 8.0, 5 mM $MgCl_2$, and extracted by incuba-

tion at 37° C for 15 min (Schnaitman 1971). The pellets from a further centrifugation (48,000 × *g*, 60 min) were resuspended in 0.1 ml electrophoresis sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli 1970). In some cases, proteins were analysed in polyacrylamide gels containing SDS and 8 M urea (Pugsley and Schnaitman 1979).

Quantitation of Outer Membrane Proteins. SDS-polyacrylamide and SDS-urea polyacrylamide gels of 1% Triton X-100-extracted membrane fractions were photographed after Coomasie Blue staining. The negatives were scanned using a Joyce-Lobel Chromoscan MK11 Double-Beam Recording Densitometer, and the amounts of protein in the OmpA protein and OmpC + OmpF protein bands were determined by comparisons with known amounts of bovine serum albumin and lysozyme electrophoresed in parallel. Total cell protein in the extracts from which the membrane fractions were prepared was determined by Lowry assay of the detergent-soluble fractions from each preparation, corrected for the missing outer membrane protein (15% of the total).

Selection of Phage-Resistant Mutants. Mutants resistant to phage TuII* were selected by mixing 0.1 ml of cells in late exponential or stationary phase growth with 10^7–10^8 phage, followed by spreading on LA. Resistant mutant colonies appeared after overnight incubation at 37° C.

Results

1. Selection of Conjugation-Deficient Mutants

Mutants of *E. coli* deficient as recipients of F factor DNA were isolated by a direct two-step selection, as detailed in *Materials and Methods*. The first step relied on the fact that galactose epimerase-deficient ($GalE^-$) mutants are poisoned by lactose only if they are also able to ferment lactose (Malamy 1966). Mutagenized cultures of F^-Lac^- $GalE^-$ cells were mixed with an excess of cells carrying an F*lac* for conjugation, followed by exposure to lactose and selection of recipient-derived cells by streptomycin resistance. Cells accepting the F*lac* were poisoned by lactose. The F*lac* factor also carried an insertion of the kanamycin resistance transposon Tn*5*. Therefore, as a second step of selection, colonies grown from lactose-resistant cells were screened for kanamycin sensitivity by replica plating. Kanamycin-sensitive cells were checked for resistance to the male-specific phage M12 (Brinton et al. 1964). Each step of the selection provided up to about 1000-fold enrichment for strongly conjugation-deficient mutants (not shown).

2. Classes of Conjugation-Deficient Mutants

Sixty-two conjugation-deficient mutants were independently isolated. Each mutant was analyzed for the genetic map location of its lesion (Table 2), its conjugation efficiency (Table 2), outer membrane protein composition (Fig. 1) and phage sensitivity pattern (Table 4). Most of the mutations (60/62) were linked to the *ompA* locus ("class A" mutants). The remaining two mutants appeared to have altered lipopolysaccharide (see below), and the lesion in one was shown to be linked to the *rfa* locus ("class B" mutants). The *ompA* mutants could be subdivided (classes A1-A3) based on the amount of OmpA protein

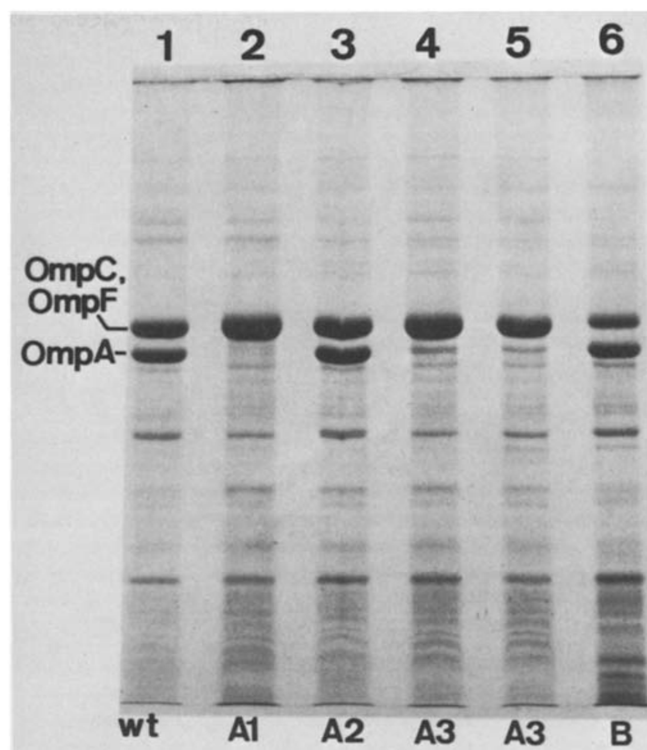

Fig. 1. Outer membrane proteins of different classes of conjugation-deficient mutants. The proteins present in outer membrane fractions of representative mutants were analyzed by SDS-polyacrylamide gel electrophoresis: lane 1, JC3272; lane 2, CC621; lane 3, CC622; lane 4, CC623; lane 5, CC624; lane 6, CC625

in their outer membranes, as assessed by SDS-polyacrylamide gel electrophoresis of outer membrane fractions and sensitivity to phage K3hl, a host-range mutant of phage K3 which can infect cells with electrophoretically undetectable levels of OmpA protein (Manning et al. 1976).

Class A1 mutants lack detectable amounts of OmpA polypeptide in outer membrane fractions (Fig. 1) and are resistant to phage K3hl (Table 4). Two *ompA* mutations generated by transposon Tn*5* insertion fall into class A1 (not shown), as do nonsense mutations isolated earlier (Henning et al. 1978). Class A2 mutants have a normal amount of OmpA polypeptide in their outer membranes, and are sensitive to phage K3hl. Class A3 *ompA* mutants shown detectable, but reduced amounts of OmpA protein in their outer membranes, and are at least partially sensitive to phage K3hl.

The amounts of abundant outer membrane proteins relative to total cell protein for representative mutants are shown in Table 3. Class A1 mutants lack detectable amounts of OmpA protein ($<5\%$ the wild-type value), whereas the two A2 mutants show 50–100% of the wild-type amount. Class A3 mutants are heterogeneous, ranging from those without electrophoretically detectable OmpA protein ($<5\%$ the wild-type amount for CC201 and CC209) to about 15% the wild-type amount (CC215). The class B mutant examined showed about 50% of the normal amount of OmpA protein, and an even lower amount of OmpC and OmpF proteins, a pattern frequently observed in mutants with altered lipopolysaccharide (Lugtenberg et al. 1976).

Table 3. Quantitation of outer membrane proteins in conjugation-deficient mutants[a]

Mutant	Class	OmpA protein	OmpC + OmpF proteins	OmpA / OmpC + OmpF protein ratio
CC102	wild-type	0.039 (100%)	0.044	0.89
CC205	A1	<0.002 (<5%)	0.055	<0.04
CC277	A1	<0.002 (<5%)	0.025	<0.08
CC236	A2	0.039 (100%)	0.040	0.98
CC253	A2	0.025 (64%)	0.032	0.76
CC201	A3	<0.002 (<5%)	0.050	<0.04
CC209	A3	<0.002 (<5%)	0.042	<0.05
CC215	A3	0.006 (15%)	0.067	0.09
CC240	A3	0.003 (8%)	0.031	0.10
CC250	A3	0.004 (10%)	0.046	0.09
CC263	A3	0.004 (10%)	0.051	0.08
CC223	B	0.018 (46%)	0.014	1.30

[a] Expressed as µg outer membrane protein per µg total cell protein, as determined by densitomitry. For each mutant, the table presents mean values of 5–10 determinations, in most cases from two independent outer membrane preparations. The value of OmpA protein for CC102 corresponds to 9×10^4 copies/cell, but is a minimum value because it does not account for losses of protein during membrane preparation and electrophoresis

Table 4. Phage sensitivities of conjugation-deficient mutants[a]

Mutant class	Phage K3	Phage K3hl	Phage TuII*	Phage Ox2	Phage U3[b]
wild-type	sensitive	sensitive	sensitive	sensitive	sensitive
A1	not sensitive (9/9)	not sensitive (9/9)	not sensitive (9/9)	not sensitive (3/3)	sensitive (1/1)
A2	sensitive (18/18)	sensitive (18/18)	sensitive (33/33)	not sensitive (33/33)	sensitive (1/1)
A3	sensitive (12/13)[c]	sensitive (9/10)[c]	sensitive (14/18)[c] or part. sens. (3/18)[c]	not sensitive (8/18)[d] or part. sens. (9/18)[d]	sensitive (2/2)
B	sensitive (1/1)	sensitive (1/1)	sensitive (2/2)	part. sens. (2/2)	not sensitive (1/1)

[a] Determined by spot tests of strains CC201-CC263. Mutant strains judged to be sensitive to a phage showed an efficiency of plating (e.o.p.) 0.1–1.0 relative to wild-type, not sensitive showed an e.o.p. $\leqq 10^{-6}$, and partially sensitive showed an e.o.p. 10^{-4}–10^{-2}. Together with each result is given the number of mutants showing the stated behavior of those tested, e.g., sensitive (14/18) means 18 strains were tested and 14 had an e.o.p. of 0.1–1.0

[b] Data shown for mutant alleles in a $galE^+$ genetic background (CC621-CC625)

[c] One exceptional strain, CC209, was partially sensitive to phage K3hl (e.o.p. 10^{-2}) and not sensitive to phages K3 and TuII* (e.o.p. $<10^{-6}$)

[d] One exceptional strain, CC215, showed very turbid plaques at e.o.p. 0.1

The conjugation efficiencies of representative mutants in liquid increased in the order A1 < A2 < B < A3 (Table 2). Representatives of all classes conjugated more efficiently on filters than in liquid, as previously observed (Table 2, Havekes and Hoekstra 1976; Achtman et al. 1978).

3. Phage Sensitivity of Conjugation-Deficient Mutants

Phages TuII* and K3 require OmpA protein to infect *E. coli*, and phage K3hl is an extended host-range mutant of phage K3 (Manning et al. 1976; Henning et al. 1978). Class A1 mutants were resistant to all three phages; while class A2 and B mutants were fully sensitive. Most class A3 mutants were also fully sensitive to all three phages, although a few exceptional strains (including CC201 and CC209) showed partial or full resistance (Table 4).

Manning et al. (1976) reported that mutants lacking OmpA protein are resistant to phage Ox2. Table 4 shows that, in contrast to phage TuII* and K3, all classes of conjugation-deficient mutants showed increased resistance to this phage, including the class B lipopolysaccharide mutants. The decrease in the efficiency of phage Ox2 plating of different mutant classes paralleled their decrease in conjugation efficiency (compare Tables 2 and 4).

Phage U3 is unable to infect many mutants of *E. coli* K-12 with altered lipopolysaccharide (Watson and Paigen, 1971). Of the mutants isolated, only those of class B showed an increased resistance to phage U3 over the parent strain (Table 4).

4. Colicin Sensitivity of Conjugation-Deficient Mutants

Mutants lacking OmpA protein adsorb, but are not killed by colicins K and L (Foulds and Barrett 1973; Davies and

Table 5. Colicin sensitivities of representative conjugation-deficient mutants

Mutant class	Allele[a]	Colicin sensitivity[b]	
		Colicin L	Colicin K
wild-type	–	sensitive (1)	sensitive (1)
A1	*ompA885*	not sensitive (125)	not sensitive (>250)
A2	*ompA886*	partially sensitive (8)	not sensitive (>250)
A3	*ompA887*, *ompA888*	partially sensitive (16)	partially sensitive (16)
B	*rfa-223*	not sensitive (125)	more sensitive (0.25)

[a] Results for CC205, CC223, CC240 and CC263 are shown. Strains CC621-CC625 gave quantitatively similar results. All other conjugation-deficient mutants were tested for colicin sensitivity by cross-streaking, with results within a particular mutant class similar to those for the representative strains shown

[b] Determined by spot tests using colicin preparations. The values in parentheses refer to the relative concentration of colicin needed to inhibit growth of the strain tested on LA expressed in arbitrary units

Reeves 1975). All classes of *ompA* mutants (A1-A3) showed decreased sensitivity to both colicins K and L, although the patterns of sensitivity differed for the two colicins (Table 5). For example, class A2 and class A3 mutants were about equally sensitive to colicin L, but A3 mutants were much more sensitive to colicin K than were A2 mutants. For these mutants, colicin K insensitivity paralleled conjugation deficiency more closely than did colicin L insensitivity. In contrast, class B lipopolysaccharide mutants were insensitive to colicin L but more sensitive to colicin K than wild-type.

A screen for colicin K sensitivity was included in some selections in an attempt to isolate conjugation-deficient *ompA* mutants remaining sensitive to colicin K. However, four strains so isolated were all class A3 mutants showing only partial colicin K sensitivity.

5. EDTA and Novobiocin Sensitivity of Conjugation-Deficient Mutants

Some mutants lacking OmpA protein show increased sensitivity to the chelating agent EDTA and the hydrophobic antibiotic novobiocin (Foulds and Barrett 1973). These properties were examined for a number of conjugation-deficient mutants with and without murein lipoprotein (Table 6). In an *lpp*$^+$ genetic background, only mutants of class A1 showed a significant decrease in the efficiency of plating on EDTA agar (to about 0.1). In an *lpp*$^-$ genetic background, the plating efficiency of class A1 mutants was further reduced to $<10^{-6}$, and class A3 mutants also showed a low plating efficiency ($<10^{-6}$–10^{-3}). Class A2 and B mutants plated with about the same efficiency as wild-type on EDTA agar. Sensitivity to novobiocin paralleled that to EDTA for the class A mutants. In contrast, the class B mutant (in an *lpp*$^+$ or *lpp*$^-$ genetic background) plated with low efficiency on novobiocin-containing agar, a property associated with lipopolysaccharide defects (Roantree et al. 1977).

Table 6. EDTA and novobiocin sensitivities of conjugation-deficient mutants lacking murein lipoprotein

Strain	Efficiency of plating[a]		Number of mutant alleles tested
	+EDTA	+Novobiocin	
wild-type	0.9	0.8	–
lpp	0.8	0.9	1
ompA (class A1)	0.1	0.1–1.0	4
ompA (class A2)	0.7	0.7	2
ompA (class A3)	0.8	0.1–1.0	4
rfa (class B)	0.7	$<10^{-6}$	1
lpp-ompA (class A1)	$<10^{-6}$	$<10^{-5}$–10^{-3}	2
lpp-ompA (class A2)	0.9	0.8	12
lpp-ompA (class A3)	$<10^{-6}$–10^{-3}	$<10^{-5}$–10^{-3}	4
lpp-rfa (class B)	0.4	$<10^{-6}$	1

[a] Values for efficiency of plating are given relative to LA. Cells growing exponentially were diluted through LB and plated onto agar containing M9 medium supplemented with 1 mM EDTA and required amino acids and lacking $MgSO_4$ and $CaCl_2$, or LA supplemented with 30 µg novobiocin per ml, and scored after 1–2 days at 37° C. The "wild-type" strain is CC617. All other mutations tested are in the identical genetic background, except the *ompA* and *rfa* single mutants which are in a CC102 genetic background. Mutant alleles tested include all of those listed as representative in Table 2. Lpp$^-$ strains carry the deletion *lpp5508*

Table 7. Tests of complementation between classes of OmpA mutants

Colicin K concentration required for growth inhibition (Arbitrary units)[a]

ompA allele on chromosome	*ompA* allele on episome		
	no episome	A1(*ompA901*::Tn*5*)	A2(*ompA886*)
OmpA$^+$	(1.0)	–	0.5
A1	>125	>125	>125
A2	>125	>125	>125
A3	32	25	1–5

[a] Data are presented for RecA$^-$ derivatives of CC204, CC236, CC240 and CC263. Four additional RecA$^+$ derivatives from each *ompA* mutant class containing the episome with the *ompA886* allele were also tested with results analogous to those presented. Complementation for diploid strains carrying the *ompA886* allele on the episome (F106) was also examined using phage Ox2 sensitivity with results in agreement with those shown

6. Complementation Analysis of ompA Mutations

Complementation between different *ompA* mutant alleles was examined by measuring sensitivity to colicin K and phage Ox2. The presence of an episome carrying a class A1 mutant *ompA* allele generated by transposon Tn*5* insertion did not increase the colicin K sensitivities of class A1, A2 or A3 mutant strains (Table 7). Similarly, no increases

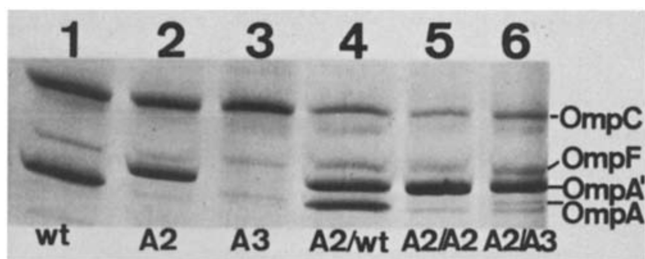

Fig. 2. Outer membrane proteins of *ompA* diploid strains. Outer membrane proteins of different mutants and mutant diploid strains were analyzed by SDS-urea-polyacrylamide gel electrophoresis. The region of the gel containing OmpA protein is shown: lane 1, *ompA*$^+$; lane 2, *ompA886*; lane 3, *ompA887*; lane 4, F′*ompA886*/*ompA*$^+$; lane 5, F′*ompA886*/*ompA886*; lane 6, F′*ompA886*/*ompA887*. Strains are *recA*$^-$ derivatives of CC102, CC236 or CC240 alone (lanes 1–3), or containing F106 *ompA886 zcb-108*::Tn*5* derived from KL725 (lanes 4–6)

in colicin K or phage Ox2 sensitivities were observed when an A2 allele was present in an A1 or A2 mutant background. However, when the A2 allele was present in an A3 mutant background there were increases in colicin K and phage Ox2 sensitivities, although generally not to the level of wild-type. Is this true genetic complementation? To test this, we took advantage of the finding (U. Hinz, unpublished results) that class A2 mutant OmpA protein migrates more slowly than wild-type protein in SDS-urea gel electrophoresis. This is shown in Fig. 2, in which the normal protein in lane 1 (labeled "OmpA") can be clearly distinguished from that of the class A2 mutant in lane 2 (labeled "OmpA′"). The reduced amount of OmpA protein in the class A3 mutant migrates at the normal position (lane 3). Lane 4 shows that both forms of OmpA protein are expressed in an A2/wild-type diploid cell, and lane 5 that only OmpA′ is present in an A2/A2 diploid. Lane 6 shows that an A2/A3 diploid strain does not make a normal amount of protein migrating at the "OmpA" position, indicating a lack of genetic complementation between the class A2 and A3 alleles. The increase in colicin K and phage Ox2 sensitivities in A2/A3 diploids over the parental single mutants shown in Table 7 therefore appears to be due to the presence of the products of the class A2 and A3 mutant genes in the same cell.

7. Properties of Class B Mutants

Both class B mutants showed properties characteristic of lipopolysaccharide mutants, including preferential loss of OmpC and OmpF proteins from the outer membrane, increased sensitivity to novobiocin and rifampicin, and resistance to phage U3 (Tables 2, 3 and 6). The defect of one of them (CC223) was shown to map to the *rfa* locus (Table 2). Both mutant strains were sensitive to phages C21, P1 and T4, implying that their lipopolysaccharides still contain at least one heptose residue (Franklin 1969; Lindberg 1977).

8. Mating Efficiency of Class A1-Class B Double Mutants

Do OmpA protein and lipopolysaccharide act independently or jointly in conjugation? To test this, the conjugation efficiencies of a class A1 *ompA* mutant, a class B lipopolysaccharide mutant, and a strain carrying both mutations were compared (Table 8). The conjugation efficiency of the double mutant was reproducibly similar to that of the *ompA*$^-$ mutant alone, arguing that the two mutated functions act jointly rather than independently of each other. This result cannot be explained simply by the 50% decrease in the amount of OmpA protein in the outer membrane of the class B mutant, since class A3 mutants having less than 10% the normal amount of OmpA protein show much smaller conjugation deficiencies than the class B mutants (Table 2).

Table 8. Mating efficiencies of single and double conjugation-deficient mutants

Strain	Defect	Recombinant frequency[a]
CC102	none	(1.0)
CC276	OmpA protein[b]	2.5×10^{-3}
CC223	lipopolysaccharide	9.5×10^{-3}
CC641	OmpA protein + lipopolysaccharide	3.8×10^{-3}

[a] Expressed as proportion recombinants per male cell. Mean values for three independent tests are shown. The donor strain was CC105.

[b] Class A1, generated by transposon insertion (*ompA901*::Tn*5*)

Discussion

This paper describes the isolation and properties of 62 mutants selected for their inability to act as efficient F factor recipients in conjugation in liquid. Sixty of the mutants were found to be altered in the amount or function of OmpA protein and to map to the *ompA* locus, while the remaining two have altered lipopolysaccharide. The *ompA* mutants were subgrouped depending on the amount of OmpA protein detected in their outer membranes: class A1 mutants lacked detectable OmpA protein, class A2 mutants had a normal or nearly normal amount of OmpA protein, and class A3 mutants had detectable, but highly reduced amounts of OmpA protein. Representatives of the three classes of mutations failed to complement each other in diploid strains, indicating that the mutations fall within a single structural gene and neighboring sequences needed in *cis* for its expression. Class A1 and A3 mutant lesions thus appear to decrease the transcription, translation or normal membrane insertion of OmpA protein. Class A2 mutants appear to carry *ompA* missense mutations and to assemble an altered protein unable to function in conjugation. This interpretation is further supported by the finding (U. Hinz, unpublished results, and Fig. 2) that OmpA protein from class A2 mutants migrates differently from normal protein in SDS-urea polyacrylamide gel electrophoresis.

A double mutant with a class A1 OmpA defect and altered lipopolysaccharide conjugated no less efficiently than the OmpA mutant alone (Table 8), indicating that OmpA protein and lipopolysaccharide do not function independently in conjugation. The simplest interpretation of this result is that the direct interaction of OmpA protein with lipopolysaccharide is needed for recipient function in conjugation. This interpretation is supported by earlier results showing that OmpA protein and lipopolysaccharide

interact strongly *in vitro*, and that both molecules must be present for optimal inhibition of conjugation *in vitro* (Schweizer and Henning 1977; Schweizer et al. 1978).

These results show that there is a conjugation pathway that functions in recipient cells devoid of OmpA protein or with altered lipopolysaccharide. This pathway presumably functions more efficiently on a surface than in liquid (Table 2). The finding by Sanderson et al. (1981) that *Salmonella typhimurium* cells containing lipopolysaccharide with 0 side chains conjugate much more efficiently on filters than in liquid suggests that this OmpA protein-independent pathway may also be active in these strains. It is not known whether in this pathway some other cellular fuction substitutes for OmpA protein or whether the normal conjugation process simply operates at a lower efficiency in the absense of OmpA protein function.

The phenotypes of the classes of mutants distinguish the functions of OmpA protein into three groups. The first group consists of EDTA and novobiocin resistance when murein lipoprotein is absent, and presumably corresponds to the structural role of OmpA protein in the outer membrane (Sonntag et al. 1978). This may be the primary physiological function of OmpA protein. Class A1 and class A3 mutants are sensitive to these compounds, whereas class A2 mutants remain resistant (Table 6). Resistance is thus correlated with the amount of OmpA protein present in the membrane, and not with its ability to function in conjugation. Resistance to EDTA or novobiocin can be used as a simple positive genetic selection for the presence of either OmpA protein or murein lipoprotein (Manoil, unpublished observations).

The second group of functions consists of receptor activity toward phages TuII*, K3 and K3hl, which is defective only in conjugation mutants which lack all or nearly all OmpA protein (class A1 and a few class A3). The sites in OmpA protein altered in class A2 mutants are thus not required for its receptor function for these phages. A few *ompA* mutants with complementary properties, being resistant to phage K3 or TuII* but able to conjugate efficiently, have also been described (Manning et al. 1976; Henning et al. 1978). These results indicate that different sites of OmpA protein function in conjugation and reception of these phages.

The third group of functions is defective in all three classes of *ompA* mutants, and includes conjugation, colicin K and L uptake, and the ability to support phage Ox2 infection. Class A2 mutants are thus altered in sites of the protein directly required for each of these processes. Three conjugation-deficient mutants containing normal amounts of OmpA protein isolated by a different selection (Havekes 1978) are similar to those of the A2 class, being insensitive to colicins K and L but sensitive to phage K3 (Achtman et al. 1978). Most or all sites of the protein required for conjugation may thus also be needed for receptor-bound colicin K and L penetration and phage Ox2 infection. These sites might occur at domains of OmpA protein where it interacts with lipopolysaccharide, since class B lipopolysaccharide mutants are altered in the same group of functions. It is not known whether conjugation, colicin K and L penetration, and phage Ox2 infection share a binding site on the surface of OmpA protein which is altered in class A2 mutants, or whether there is some more complex function of OmpA protein that is affected. In either case, the role of OmpA protein in these processes is distinguished from its structural function in the outer membrane, which appears to be normal in class A2 mutants (Table 7).

Further genetic and biochemical analysis should reveal how many different sites in OmpA protein are affected in the A2 class of mutants, as well as identify which amino acids are altered. This, together with the analogous approach using mutants altered in other OmpA protein functions, should help to provide a detailed understanding of how the different functions of this protein are related to its structure.

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References

Achtman M (1975) Mating aggregates in *Escherichia coli* conjugation. J Bacteriol 123:505–515

Achtman M, Schwuchow S, Helmuth R, Morelli G, Manning P (1978) Cell-cell interactions in conjugating *Escherichia coli*: Con^- mutants and stabilization of mating aggregates. Mol Gen Genet 164:171–183

Brinton CC, Gemski P, Carnahan J (1964) A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K-12 and its role in chromosome transfer. Proc Natl Acad Sci USA 52:776–783

Coleman WC, Leive L (1979) Two mutations which affect the barrier function of the *Escherichia coli* K-12 outer membrane. J Bacteriol 139:899–910

Davies JK, Reeves P (1975) Genetics of resistance to colicins in *Escherichia coli* K-12: Cross-resistance among colicins of group A. J Bacteriol 123:102–117

Foulds J, Barrett C (1973) Characterization of *Escherichia coli* mutants tolerant to bacteriocin JF246: Two new classes of tolerant mutants. J Bacteriol 116:885–892

Franklin NC (1969) Mutation in *galU* gene of *E. coli* blocks phage P1 infection. Virology 38:189–191

Havekes L (1978) Conjugation in *Escherichia coli* K12: Studies on the role of the recipient cell. PhD Dissertation, Univ of Utrecht

Havekes L, Hoekstra WPM (1976) Characterization of an *Escherichia coli* K-12 F^-Con^- mutant. J Bacteriol 126:593–600

Henning U, Sonntag I, Hindennach I (1978) Mutants (*ompA*) affecting a major outer membrane protein of *Escherichia coli* K12. Eur J Biochem 92:491–498

Jones-Mortimer MC (1968) Positive control of sulphate reduction in *Escherichia coli* K12. Biochem J 110:589–595

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685

Lindberg AA (1977) Bacterial surface carbohydrates and bacteriophage adsorption. In: Sutherland IW (ed) Surface Carbohydrates of the Prokaryotic Cell. Academic Press, New York, pp 289–356

Low KB (1972) *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol Rev 36:587–607

Lugtenberg B, Peters R, Bernheimer H, Berendsen W (1976) Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Mol Gen Genet 147:251–262

Malamy M (1966) Frameshift mutations in the lactose operon of *E. coli*. Cold Spring Harb Symp Quant Biol 31:189–201

Manning P, Pugsley AP, Reeves P (1977) Defective growth functions in mutants of *Escherichia coli* K12 lacking a major outer membrane protein. J Mol Biol 116:285–300

Manning P, Puspurs A, Reeves P (1976) Outer membrane of

Escherichia coli K-12: Isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3. J Bacteriol 127:1080–1084

Miller J (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Lab, Cold Spring Harbor, NY

Nikaido H (1979) Nonspecific transport through the outer membrane. In: Inouye (ed) Bacterial Outer Membranes. Wiley and Sons, New York, pp 361–407

Pugsley AP (1981) Transcriptional regulation of colicin Ib synthesis. Mol Gen Genet 183:522–527

Pugsley AP, Schnaitman CA (1979) Factors affecting the electrophoretic mobility of the major outer membrane proteins of *Escherichia coli* in polyacrylamide gels. Biochim Biophys Acta 581:163–178

Roantree RJ, Kno T-T, MacPhee D (1977) The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium*. J Gen Microbiol 103:223–234

Roth JR (1970) Genetic techniques in studies of bacterial metabolism. Methods Enzymol 17:3–35

Sanderson KE, Janzer J, Head J (1981) Influence of lipopolysaccharide and protein in the cell envelope on recipient capacity in conjugation of *Salmonella typhimurium*. J Bacteriol 148:283–293

Schnaitman CA (1971) Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J Bacteriol 108:545–552

Schweizer M, Henning U (1977) Action of a major outer membrane protein in conjugation of *Escherichia coli* K-12. J Bacteriol 129:1651–1652

Schweizer M, Hindennach I, Henning U (1978) Major proteins of the *Escherichia coli* outer cell envelope membrane. Interaction of protein II* with lipopolysaccharide. Eur J Biochem 82:211–217

Shaw KJ, Berg CM (1979) *Escherichia coli* K-12 auxotrophs induced by insertion of the transposable element Tn*5*. Genetics 92:741–747

Sonntag I, Schwarz H, Hirota Y, Henning U (1978) Cell envelope and shape of *Escherichia coli:* Multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J Bacteriol 136:280–285

Suzuki H, Nishimura Y, Yasuda S, Nishimura A, Yamada M, Hirota Y (1978) Murein-lipoprotein of *Escherichia coli:* A protein involved in the stabilization of bacterial cell envelope. Mol Gen Genet 167:1–9

Watson G, Paigen K (1971) Isolation and characterization of an *Escherichia coli* bacteriophage requiring cell wall galactose. J Virol 8:669–674

Yem D, Wu HC (1978) Physiological characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein. J Bacteriol 133:1419–1426

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