Identification and Characterization of a New Gene of *Escherichia coli* K-12 Involved in Outer Membrane Permeability

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ABSTRACT

Using a genetic selection for mutations which allow large maltodextrins to cross the outer membrane of *Escherichia coli* in the absence of the LamB maltoporin, we have obtained and characterized two mutations that define a new locus of *E. coli*. We have designated this locus *imp* for *i*ncreased *m*embrane *p*ermeability. Mapping studies show that the *imp* gene resides at approximately 1.2 min on the *E. coli* chromosome. The mutations alter the permeability of the outer membrane resulting in increased sensitivity to detergents, antibiotics and dyes. The mutations are nonreverting and codominant. Genetic analysis of the mutations suggest that the *imp* gene is an essential gene. We describe a general cloning strategy that can be used to clone both dominant and recessive alleles. Using this technique, we have cloned the wild-type and mutant *imp* alleles onto a low copy number plasmid.

THE outer membrane of *Escherichia coli* forms the first permeability barrier of the cell. All substances that enter the cell must cross this barrier. As such, it provides protection against a variety of agents including degradative enzymes, detergents, chemical agents and antibiotics. The structure of this membrane consists of lipopolysaccharide (LPS) (outer leaflet), phospholipids (predominantly in the inner leaflet), and integral as well as peripheral proteins [see NIKAIDO and VAARA (1985, 1987) for a review of the structure]. The permeability of the outer membrane can be altered by mutations as well as by treatment of cells with various chemical agents such as EDTA (LEIVE 1965), local anesthetics (LABEDAN 1988) and inhibitors of LPS biosynthesis (GOLDMAN *et al.* 1987). Mutations at a number of genetic loci alter the outer membrane's permeability. These include mutations affecting LPS structure (COLEMAN and DESPHANDE 1985), lipid biosynthesis (RAETZ and FOULDS 1977), the structure of the porins (BENSON, OCCI and SAMPSON 1988; MISRA and BENSON 1988a,b SAMSON and BENSON 1985), and mutations such as *acrA* (HENSON and WALKER 1982), *abs* (CLARK 1984) and *tolC* (WHITNEY 1971), as well as other colicin tolerant mutations (NAGEL DE ZWAIG and LURIA 1967; DAVIES and REEVES 1975) which result in increased permeability by unknown mechanisms.

We have described a genetic selection for mutants with increased outer membrane permeability (BENSON and DECLOUX 1985). This selection relies on the fact that in order for *E. coli* to grow on maltodextrins larger than maltotriose, a functional LamB maltoporin is required (WANDERSMAN, SCHWARTZ and FERENCI 1979). By starting with a LamB$^-$ strain and selecting for growth on a mixture of maltodextrins, we have identified several different classes of mutants (MISRA *et al.* 1987). We report here the further genetic and biochemical characterization of one of these classes of mutants which, in addition to conferring a Dex$^+$ phenotype, confers a dramatic increase in sensitivity to a variety of antibiotics, detergents and dyes. These mutations define a new locus of *E. coli* (*imp*) which appears to be an essential gene.

MATERIALS AND METHODS

Bacterial strains: Bacterial strains and plasmids used in this study are listed in Table 1.

Media and chemicals: Minimal (M63) and Luria (L) agar and broth media are as described in SILHAVY, BERMAN and ENQUIST (1984). Maltodextrins were purchased from Pfanstiehl Laboratories, Waukegan, Illinois, and purified by dialysis as described previously (BENSON, OCCI and SAMPSON 1988). Antibiotic sensitivity disks were purchased from Difco Laboratories, Detroit, Michigan, or BBL Microbiological Systems, Cockeysville, Maryland. [^{14}C]Maltose was purchased from Amersham Corp., Arlington Heights, Illinois. Novobiocin (Nov), erythromycin (Em), and kasugamycin (Ksg) were purchased from Sigma Chemical Corp., St. Louis, Missouri, and used at 50 μg/ml (Nov) and 25 μg/ml (Em and Ksg). Arginine and uracil were added to minimal media to a concentration of 33 μg/ml.

Genetic manipulations: Procedures involving F′ matings, Hfr mapping, P1 transductions and Tn*10* pools are described in SILHAVY, BERMAN and ENQUIST (1984) and MILLER (1972). The *ksgA*560::Tn*10* insertion was isolated from a Tn*10* pool prepared on MC4100. We capitalized upon the fact that insertions in the *ksgA* gene confer resistance to kasugamycin (Ksg^r) (ANDRESSON and DAVIES 1980). A P1 lysate prepared on a MC4100 Tn*10* pool was used to

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TABLE 1

Bacterial strains and plasmids

Strain or plasmid	Genotype	Source
Strains		
Jef8	HFR *thrA carB*8 *relA*1 *metB*1 *spoT*1	GIGOT *et al.* (1984)
SE14	F'104/ *thr*-1 *leuB*6 Δ(*gpt-proA*62) *recA*13 *argE*3 *thi*-1 *ara*-14 *lacY*1 *galK*2 *xyl*-7 *mtl*-1 *tsx*-33 *supE*44 *hisG*4 *rpsL*31	M. SCHMIDT
MC4100	F⁻ *araD*139 Δ(*argF-lac*)U1698 *rpsL*150 *relA* *flb*B5301 *ptsF*25 *deoC*1	CASADABAN (1976)
MC1000	F⁻ *araD*139 Δ(*araABC-leu*)7679 *galU* galK Δ(*lac*)X74 *rpsL thi*	M. CASADABAN
MCR106	MC4100 Δ*lamB*106	EMR and SILHAVY (1980)
DME553	MCR106 Δ*ompF*80	This study
ECB4623	*araD*139 galK Δ(*lac*)X74 *rpsL thi imp*-4213	This study
BAS803	F'104/MCR106 *thr*-43::Tn*10* *imp*-4213 *srl*517::Tn*5* *recA*56	This study
BAS849	MCR106 *imp*-4213	This study
BAS1329	MCR106 *zab*::Tn*10* Δ(*araABC-leu*)7679 *imp*-4213 *srl*517::Tn*5* *recA*56	This study
BAS1335	MCR106 *leu*::Tn*10* *imp*-208 *srl*517::Tn*5* *recA*56	This study
BAS1340	F'104/BAS1329	This study
BAS1347	F'104/BAS1335	This study
BAS1365	F'104-1/MCR106 *zab*::Tn*10* Δ(*araABC-leu*)7679 *imp*-4213 *recA*::Km	This study
BAS1362	MCR106 *imp*-4213 *srl*300::Tn*10* *recA*56	This study
BAS1363	MCR106 *srl*300::Tn*10* *recA*56	This study
RAM105	MCR106 Δ*ompF*80 *zei*06::Tn*10*	MISRA and BENSON (1988b)
RAM127	MCR106 Δ*opmF*80 *imp*-208	MISRA and BENSON (1988a)
Plasmids		
pRAM1005	Km^R, pSC101 replicon	This study
pSAB5402	Km^R, vector pRAM1005 based, cloned *imp* gene	This study
pSAB5420	Km^R, vector pRAM1005 based *imp*-4213	This study

transduce MCR106 to tetracycline resistance (Tc^r). Of approximately 2000 transductants, five were Ksg^r. To determine if these were *ksgA*::Tn*10* insertions, we checked linkage to the *carB* (1') and *leu* (2') genetic markers. In one case (*ksgA*560::Tn*10*) we could demonstrate that the Tn*10* determinant was linked to the *carB* and *leu* genes and showed a >90% linkage to the *imp* gene. The presence of the *carB* mutation was assayed by plating cells on glucose minimal plates plus and minus supplementation with arginine and uracil (GIGOT *et al.* 1980).

F'104-1 was constructed by recombining the *thr* and *carB*8 mutations from Jef8 onto F'104 by a series of genetic crosses (data not shown). F'104-1 will complement a *leu*::Tn*10* insertion but does not complement the *carB*8 mutation. F'104-1 was then crossed into BAS1314 to yield strain BAS1361. We then introduced the *recA*111::Km insertion into this strain to generate strain BAS1365. The *recA*111::Km insertion was kindly provided by GREG PHILLIPS and is an insertion of a 1.4-kb fragment carrying the kanamycin determinant from pUC-4-K (Pharmacia Inc., Piscataway, New Jersey) into the *Pst*I site of the *recA* gene (G. PHILLIPS, personal communication).

Phenotypic tests: Detergent and antibiotic sensitivity tests were done as described previously (BENSON and DECLOUX 1985). Growth tests were done as described (MISRA and BENSON 1988a) except that dialyzed maltodextrin was used instead of defined size maltodextrins.

Biochemical tests: [^{14}C]Maltose uptake assays, preparations of whole-cell envelopes and SDS-polyacrylamide gel electrophoresis were done as described (MISRA and BENSON 1988a).

DNA methods: DNA methods are as described in MISRA and BENSON (1988b).

Construction of pRAM1005 and a chromosomal clone bank: A partial restriction map of pRAM1005 is shown in Figure 1. pRAM1005 has unique *Bam*HI and *Eco*RI cloning sites and a pSC101 replicon system. It was constructed in several steps from pRAM1001 (MISRA and BENSON 1988b) basically by inserting the *Hin*dIII-*Bam*HI fragment carrying the kanamycin resistance determinant from pNEO (P-L Biochemicals, Milwaukee, Wisconsin) between the *Hin*DIII and *Eco*RI sites of pRAM1001. Chromosomal DNA was prepared from RAM105, digested with *Bam*HI and then ligated into pRAM1005 which had been digested with *Bam*HI and treated with alkaline phosphatase. The ligated mixture was transformed into MC1000. Transformants were selected on L plates containing kanamycin. Approximately 10,000 transformants were pooled and plasmid DNA was extracted from this pool.

RESULTS

Isolation of the *imp*-4213 and *imp*-208 mutants: We have previously described a selection for obtaining mutations that affect outer membrane permeability. The selection is for growth on maltodextrins in the absence of the LamB maltoporin, and it yields pre-

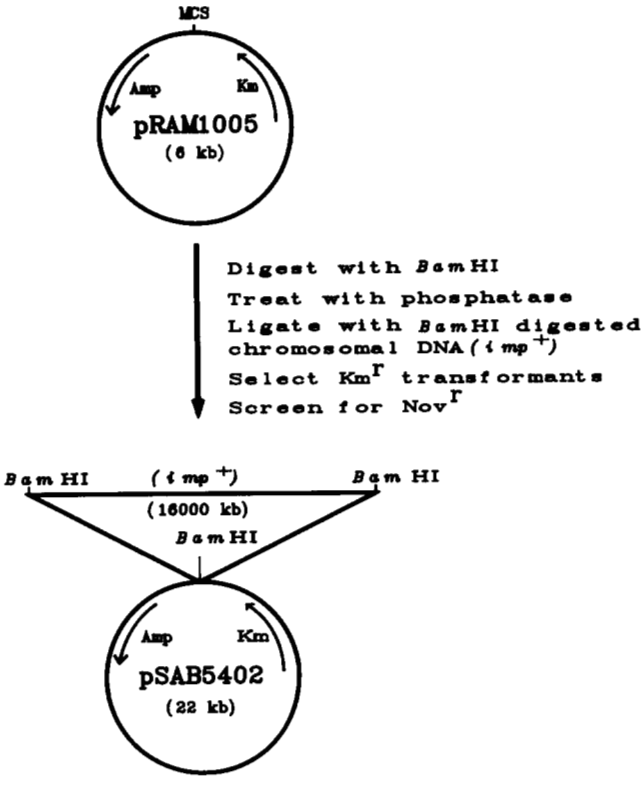

FIGURE 1.—Partial restriction maps of pRAM1005 and pSAB5402. The details for isolation of pSAB5402 are given in the text. The plasmids are not drawn to scale. The multicloning site (MCS) present in pRAM1005 has the following restriction sites; *Eco*RI-*Sal*I-*Kpn*I-*Sam*I-*Bam*HI. The chromosomal insert containing the *imp* gene was cloned into the *Bam*HI site. The sizes of the plasmids are approximate.

dominantly mutants with alterations in the major porins, OmpF or OmpC (MISRA *et al.* 1987). In addition to these types of mutations, we obtained two isolates, 4213 and 208, in which the Dex$^+$ mutation did not map to either porin gene and conferred sensitivity to MacConkey agar (Mac^s), Nov (Nov^s), and Em (Em^s). Isolate 4213 was found among 200 spontaneous independent Dex$^+$ mutants obtained from MCR106, and was the only mutant which did not contain an *ompF*(Dex) mutation. Isolate 208 was found among 70 spontaneous independent Dex$^+$ mutants obtained from RAM105. The exact frequency at which these isolates occurred is difficult to obtain since they appeared as colonies 4 to 6 days after plating. The estimated frequency of the *ompF*(Dex) and *ompC*(Dex) mutations are 5×10^{-9} (BENSON, OCCI and SAMPSON 1988) and 1×10^{-10} (MISRA and BENSON 1988b), respectively. On this basis, the frequency of the 4213 and 208 isolates would be less than 1×10^{-11}.

Phenotypic characterization of the *imp* mutants: To ensure that the phenotypes represented the effects of a single genetic alteration, we transferred the *imp* mutations by P1 transduction into strain MCR106 using a linked Tn*10* marker. Genetic studies (see below) showed that the *imp* mutations mapped to the

TABLE 2

Sensitivity to various compounds

Compound (concentration or amount)	Inhibition zone (mm)	
	MCR106*imp*$^+$	BAS849(*imp*-4213)
Chemicals, Dyes and Detergents		
$CuSO_4$ (1.19 mg)	11	14
Deoxycholate (1500 μg)	0	13
Triton X-100 (750 μg)	0	8
SDS (750 μg)	0	12
Eosin Y (5%)	0	7
Antibiotics		
Tetracyclines		
Chlorotetracycline (30 μg)	14	20
Oxytetracycline (30 μg)	15	16
Tetracycline (5 μg)	9	13
Betalactams		
Ampicillin (50 μg)	14	27
Carbenicillin (100 μg)	22	29
Cephalothin (30 μg)	13	15
Penicillin G (10 units)	0	0
Others		
Bacitracin (10 units)	0	22
Chloramphenicol (5 μg)	7	13
Erythromycin (15 μg)	0	19
Naladixic Acid (5 μg)	11	17
Novobiocin (30 μg)	0	22
Rifampin (15 μg)	8	26
Trimethaprim (5 μg)	17	26

Sensitivity is expressed as the size (mm) of the zone of growth inhibition from the center of the disc. The size of the disc is 6 mm.

1.2′ region of the *E. coli* chromosome and were linked to known Tn*10* markers in this region. In over 50 independent transductions involving the mutant *imp* alleles the pleiotropic effects conferred by the alterations, *i.e.*, antibiotic sensitivity, detergent sensitivity and the Dex$^+$ phenotype always moved as a single unit. This suggests that the various phenotypes of the *imp*-4213 and *imp*-208 mutations are caused by single genetic alterations.

The sensitivity profile of BAS849 (*imp*-4213) is identical to that of the original isolate 4213. The increased permeability extends to a diverse set of compounds including detergents, metals, dyes, and numerous antibiotics (Table 2). However, we did not detect any increase in sensitivity to the aminoglycosides; amikacin, gentamycin, kanamycin and neomycin or to polymyxin, clindamycin, or EDTA. For the detergents; deoxycholate and SDS, and large antibiotics such as, erythromycin, novobiocin, bacitracin, and rifampin the increase in sensitivity is dramatic. To quantify the degree of change we determined by a twofold dilution method the minimum inhibitory concentrations (MICs) for rifampin (Rif), novobiocin, erythromycin, ampicillin (Amp) and chloroamphenicol (Cm). The MIC values for BAS849 are: Rif, <0.5 μg/ml; Nov, 2 μg/ml; Em, <0.5 μg/ml; Amp, <0.5 μg/ml; and Cm, 2 μg/ml. The MIC values for the parent strain MCR106 are: Rif, >64 μg/ml; Nov,

>128 μg/ml; Em, >128 μg/ml; Amp, 2 μg/ml; and Cm, 6 μg/ml. Thus, the relative change in sensitivity to the large hydrophobic antibiotics (Nov, Em, Rif) can be as large as two orders of magnitude. In contrast, the increase in sensitivity to Amp and Cm, which predominately cross the outer membrane via the porins, is only 4- to 6-fold.

To determine if the general structure of the outer membrane was disrupted, we assayed sensitivity to the following bacteriophages: λ, K20, Hy2, SS4, Φ80, Tu2, Tu4, T6, P1, and U3. In no case did we detect a difference in sensitivity as compared to the control strain MCR106. The *imp*-208 allele results in nearly identical phenotypes when introduced into MCR106.

Genetic characterization of the *imp*-4213 mutation: Our initial genetic mapping indicated that the mutation present in isolate 4213 mapped to neither *ompF* nor *ompC*. To determine the map position of the mutation that conferred the Dex$^+$ and increased permeability phenotypes, we did Hfr mapping using Hfr donor strains BW6164, BW6169, BW6175 (WANNER 1986) and HfrG6 (HOFNUNG, HATIFIELD and SCHWARTZ 1974). The results indicated that the *imp* mutation was near the *thr* gene at 0′ on the chromosome (data not shown). To further define the map location, we did two-, three-, and four-factor crosses using the generalized transducing phage P1^{vir}. A sample of the mapping results are shown in Table 3 and the cotransduction linkage of the *imp* gene to adjacent loci is given in Figure 2. We noted that the linkage of the *imp*-4213 and wild-type alleles to flanking markers was not the same. The linkage of the *imp*$^+$ allele is always higher than the linkage of the *imp*-4213 allele to the same markers, *ksgA* and *leu* (Figure 2). Since the *ksgA* and *leu* genes lie on opposite sides of the *imp* gene, we interpret this to mean there is a selective bias against the mutant *imp* alleles and for acquisition of the *imp*$^+$ allele.

The gene order, based on the mapping studies, is *carB-ksgA-imp-ara-leu* and we estimate the map position of *imp* to be at approximately 1.2′ on the *E. coli* chromosome. The position of the *zab*4292::Tn*5* and *zab*::Tn*10* are shown for informational purposes.

The *imp*-208 allele maps to the same position as the *imp*-4213 mutation. To determine if we could get recombination between the two mutant alleles, we crossed each mutation against the other by selecting the adjacent *ksgA*560::Tn*10* or *leu*$^+$ marker. We did not detect any wild-type recombinants among the >900 transductants screened. This suggests that the two mutations are either very close to each other, identical alterations, or overlapping deletions.

Diploid and reversion analysis of the *imp* alleles: To determine if the *imp*-4213 and *imp*-208 mutations are dominant or recessive, we constructed strains BAS1340 (*imp*-4213/F′104 *imp*$^+$) and BAS1347 (*imp*-208/F′104 *imp*$^+$). The mutant Dex$^+$ and Mac^s phenotypes are recessive while the Nov^s and Em^s phenotypes are dominant (Table 4). We noted that if the strain was *rec*A$^+$ wild-type (*imp*$^+$) Nov^r Mac^r Dex$^-$ colonies quickly accumulated in the culture. These colonies presumably arise by recombinational loss (via homogenization) of the mutant allele to wild type. As with the mapping studies, this suggests that there is a selective bias against the mutant allele.

The detergent and drug sensitivities conferred by the *imp* mutations provided a convenient means to select for revertants. We did this three ways; selection for Mac^r, Nov^r and Em^r colonies. Spontaneous Mac^r colonies occur at a frequency of approximately 2 × 10^{-6}. Of 20 spontaneous and 2-amino-purine induced Mac^r revertants tested, all were pseudorevertants. In each we were able to rescue the *imp*-4213 allele from these strains (D. WOODWORTH, unpublished data). Selection for the spontaneous Nov^r or Em^r revertants at 37° give neither true revertants nor pseudorevertants. However, from mutagenized cultures it is pos-

TABLE 3

Three- and four-factor mapping data

Cross	Genetic Markers *carB*	*ksgA*::Tn*10*	*imp*	*leu*	No. of Isolates
A. Donor	−	Tc^r	4312	+	
Recipient	+	Tc^s	wt	−	
	−	Tc^r	wt	−	23
	+	Tc^r	wt	−	48
	+	Tc^r	wt	+	4
	−	Tc^r	wt	+	6
	+	Tc^r	4213	+	35
	+	Tc^r	4213	−	20
	−	Tc^r	4213	+	8
	−	Tc^r	4213	−	14
				Total	158
B. Donor	−	Tc^r	4313		
Recipient	+	Tc^s	wt		
	+	Tc^r	4213		60
	+	Tc^r	wt		25
	−	Tc^r	4213		33
	−	Tc^r	wt		4
	−	Tc^r	4213		5
				Total	127
C. Donor		Tc^r	4313	+	
Recipient		Tc^s	wt	−	
		Tc^r	wt	−	27
		Tc^r	4213	−	13
		Tc^r	4213	+	17
		Tc^r	wt	+	2
				Total	59

Transductions were done as described in MATERIALS AND METHODS. In all cases, the selection was for Tc^r transductants. The *imp* alleles were scored by sensitivity to Nov and Em as well as the Dex$^+$ phenotype, wt means wild type, 4213 means the *imp*-4312 confered sensitivities. The *carB* and *leu* alleles were scored by the appropriate auxotropy, − = auxotropic, + = prototropic.

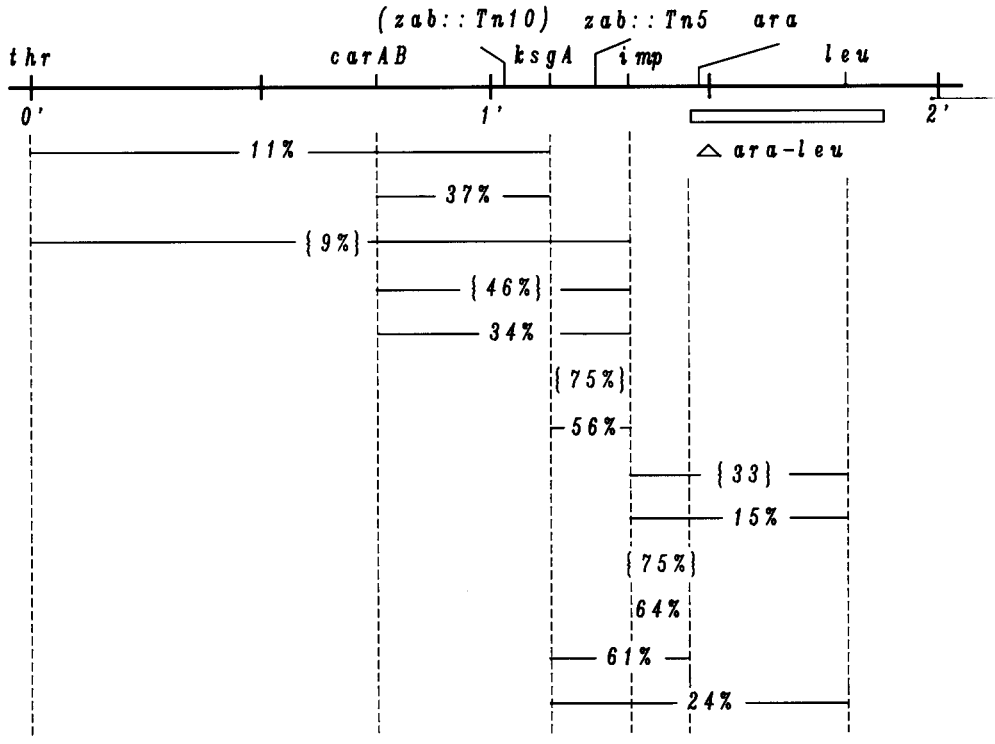

FIGURE 2.—Linkage map the *E. coli* chromosomal region surrounding the *imp* gene. Linkages were determined by two factor P1 cotransductions. Numbers in brackets { } represent the frequency for the wild-type *imp* allele. All other numbers were determined using the *imp*-4213 allele. In all cases, the numbers represent the average of several independent transduction experiments.

TABLE 4

Diploid analysis of the *imp* alleles

Strain	Phenotypes			
	Dex	Mac	Nov	Em
MCR106 (wild type)	−	R	R	R
BAS1329 (*imp*-4213 *zab*::Tn*10*)	+	S	S	S
BAS1340 (*imp*-4213 *zab*::Tn*10*/F′104*imp*$^+$)	−	R	S	S
BAS1335 (*imp*-208 *leu*::Tn*10*)	+	S	S	S
BAS1347 (*imp*-208 *leu*::Tn*10*/F′104*imp*$^+$)	−	R	S	S

All strains except MCR106 are *recA56*. Phenotypes were tested as described in MATERIALS AND METHODS. S = sensitive, R = resistant, − = no growth, + = good growth after 36 hr.

TABLE 5

Reversion analysis of the *imp* alleles

Strain	Reversion on L-Nov plates	Reversion on L-Tc-Nov plates
BAS1329 (*imp*-4213 *zab*::Tn*10*)	>4 × 10^{-9}	ND
BAS1340 (*imp*-4213 *zab*::Tn*10*/F′104*imp*$^+$)	5.4 × 10^{-2}	4.6 × 10^{-5}
BAS1335 (*imp*-208 *leu*::Tn*10*)	>6 × 10^{-9}	ND
BAS1347 (*imp*-208 *leu*::Tn*10*/F′104*imp*$^+$)	4.6 × 10^{-5}	5.4 × 10^{-2}

All strains are *recA56*. L-Nov = Luria medium with 50 m/ml novobiocin, L-Tc-Nov = Luria medium with 25 μ/ml tetracycline plus 50 μ/ml novobiocin. ND = not done.

sible to obtain rare Nov^r and Em^r pseudorevertants (R. FORTIER, personal communication).

The *imp* gene appears to be essential: We noted that the diploid strains BAS1340 and BAS1347, unlike the haploid strain from which they were derived, segregated Nov^r colonies at high frequency. Both strains yield Nov^r colonies at frequencies as high as 10^{-2} (Table 5) and in all cases the Em^s phenotype is simultaneously lost. One explanation for this unusual finding is that these colonies arise by inactivation of the mutant chromosomal *imp* allele. Many of the Nov^r colonies were also Tc^s. In fact, we were able to detect Tc^s colonies without imposing the Nov^r selection by simple replica plate techniques (data not shown).

When we maintained selection for Tc^r the frequency of Nov^r colonies was reduced several orders of magnitude (Table 5). One explanation for these findings is that the Nov^r colonies arise by deletion events and that these events are stimulated by the adjacent Tn*10*. To test this, we constructed strain BAS1365, as described in MATERIALS AND METHODS. This strain carries F′104-1 which has the *carB*8 allele and thus allows us to detect loss of the chromosomal *carB*$^+$ allele as shown in Figure 3. Strain BAS1365 yields Nov^r Tc^s CarB$^-$ colonies at a frequency of 10^{-3} to 10^{-5} thus showing that the Nov^r colonies can arise by deletions which remove both the Tn*10* and the *carB* gene.

Two observations suggest that it is the *imp* mutation, and not some unusual property of the Tn*10*, that results in the apparent deletion events. Strains which are diploid for the wild type *imp* allele do not yield

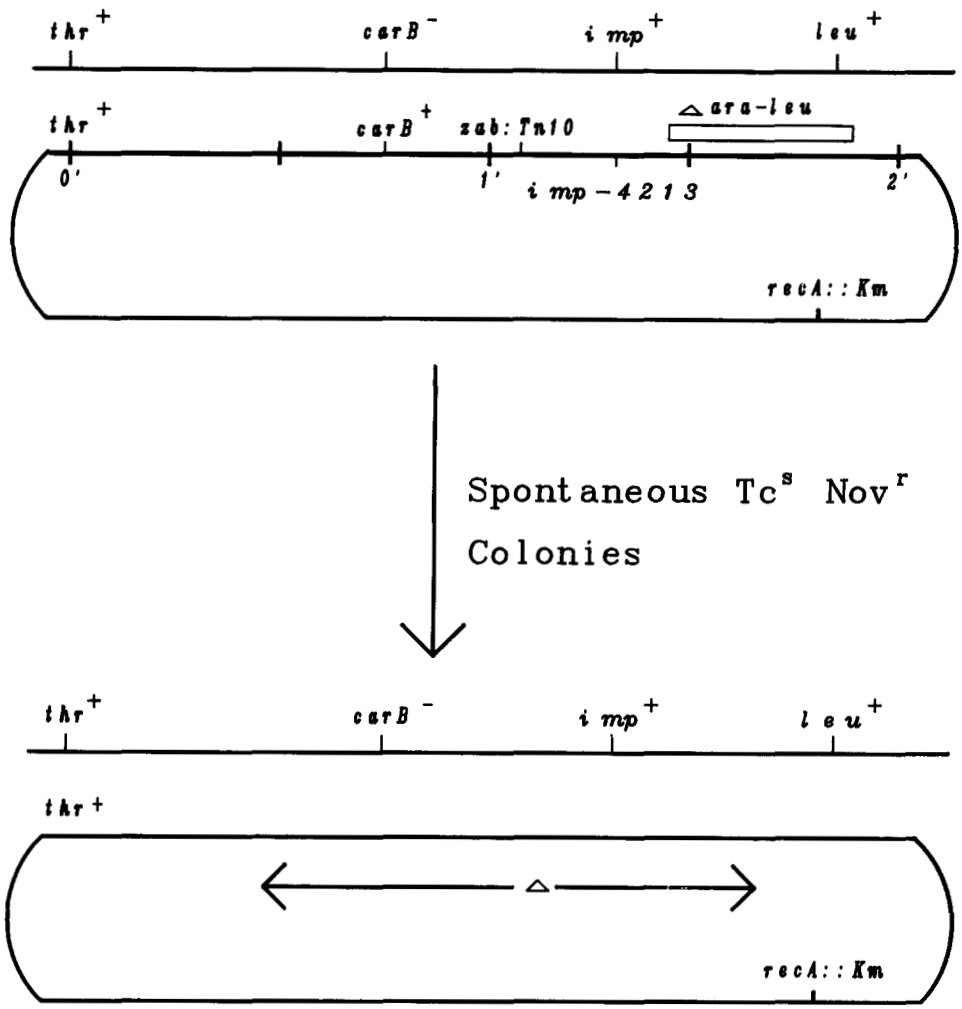

FIGURE 3.—Spontaneous inactivation of the mutant *imp* allele by a deletion event. The rationale for and results of the depicted experiment are described in the text. The straight lines show the relevant genes present on F'104-1, and below it, the oval depicts the relevant genes present on the chromosome. The phenotype of the starting diploid strain represented in the upper part of the figure is Km^r Nov^s Em^s Tc^r and CarB$^+$. The lower part of the figure shows the predicted structure after the deletion event. The phenotype of the resulting diploid deletion strains shown in the lower part of the diagram is Km^r Nov^r Em^r Tc^s and CarB$^-$.

Tc^s colonies, and two different Tn*10*s (*zab*::Tn*10* and *leu*::Tn*10*) both stimulate the frequency of Nov^r colonies (Table 5). However, not all close by Tn*10*s do so. Strain BAS803 (*thr*::Tn*10* *imp*-4213/F'104 *imp*$^+$) does not yield Tc^s colonies, and the frequency of the Nov^r "revertants" is several orders of magnitude less than that seen with similar diploids carrying the *zab*::Tn*10* or the *leu*::Tn*10*.

These observations, coupled with the fact that haploid strains do not revert nor yield Tc^s colonies regardless of the *imp* or Tn*10* alleles they carry, suggest that the *imp* gene is essential and would explain why we see inactivate of the mutant *imp* allele only in the diploids.

Porin independence of the *imp* phenotypes: To determine if the phenotypes conferred by the *imp* mutations operated by altering the properties of the OmpF and OmpC porins, we constructed strains lacking one or both porins. Strains which lack one or both porins retain all of the *imp* associated phenotypes *i.e.*, Nov^s, Em^s, Mac^s Dex$^+$ (data not shown). Thus, the sensitivities conferred by the *imp* mutation do not require the presence of the porins. It was noted that on minimal media (maltodextrins or glucose) the porin minus strains regardless of the *imp* allele they carried had a reduced growth rate. This slower growth phenotype is indicative of a porin-deficient phenotype.

Biochemical analysis of the *imp* mutants: As a crude assay of outer membrane structure, we analyzed the protein profile of whole cell envelopes and soluble fractions prepared from strains carrying the *imp*-4213, *imp*-208, and *imp*$^+$ alleles. We could detect no difference in the protein profiles on SDS-PAGE gels in any of the fractions (data not shown).

To quantitate the changes in outer membrane permeability due to the *imp* mutations, we did growth tests and [^{14}C]maltose uptake assays. In complex media strain BAS849(*imp*-4213) and the *imp*$^+$ parent MCR106 have nearly identical growth rates (Fig. 4A). Growth tests using 10 mM maltodextrin as the carbon source show that the presence of the *imp* mutation increases the growth rate (Figure 4B). At higher concentrations of maltodextrins (16 mM) the two strains have identical growth rates (data not shown). Both *imp* mutations increase the rate at which labelled maltose is taken up by the cell (Figure 5). This is best

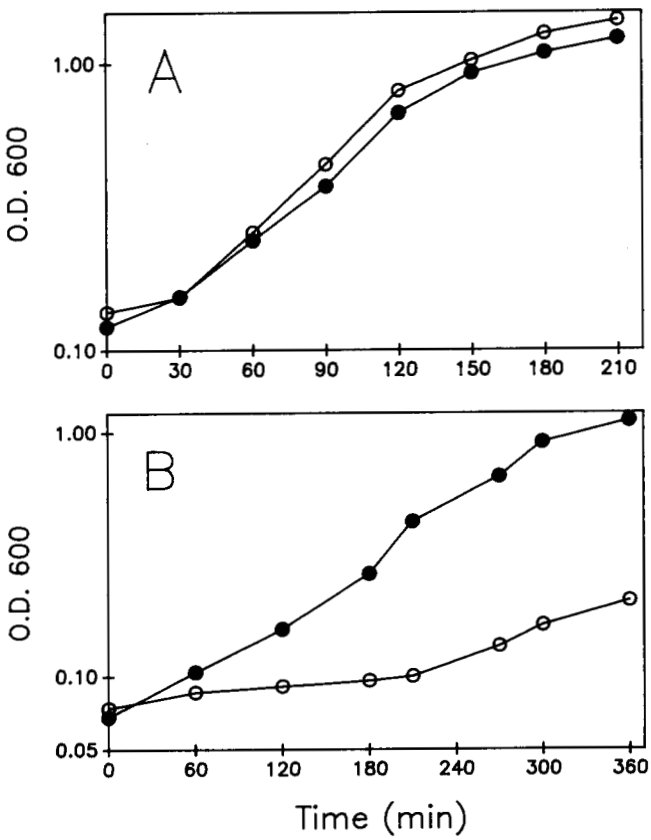

FIGURE 4.—Growth test of *imp*$^+$ *imp*-4312 strains. (A) Results of growth tests in LB. (B) Results of growth test in 10 mM maltodextrin. MCR106 (*imp*$^+$) = open circles BAS849 (*imp*-4213) = filled circles.

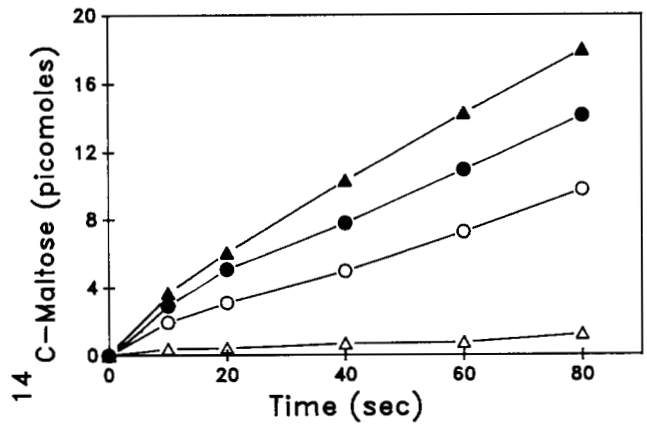

FIGURE 5.—Rate of [^{14}C]maltose uptake of the *imp* mutants. Uptake experiments were done as described in MATERIALS AND METHODS. RAM105 (OmpF$^-$ OmpC$^+$ *imp*$^+$), open triangles; MCR106 (OmpF$^+$ OmpC$^+$ *imp*$^+$), open circles; BAS849 (OmpF$^+$ OmpC$^+$ *imp*-4213), filled circles; and RAM127 (OmpF$^-$ OmpC$^+$ *imp*-208), filled triangles.

seen when strain DME553 (LamB$^-$ OmpF$^-$ OmpC$^+$ *imp*$^+$) is compared to strain RAM127 (LamB$^-$ OmpF$^-$ OmpC$^+$ *imp*-208). Strain MCR106 (LamB$^-$ OmpF$^+$ OmpC$^+$ *imp*$^+$) has a higher basal rate of uptake as compared to DME553 due to the presence of the OmpF porin, which is a functionally larger porin than OmpC (NIKAIDO and VAARA 1987; MISRA and BENSON 1988a). In all cases, the rate of uptake conferred by the *imp* mutations is only 10 to 15% of that observed with LamB$^+$ strains (data not shown).

Cloning of the *imp* gene onto a low copy number plasmid: A clone bank was constructed using the low copy number vector pRAM1005 as described in MATERIALS AND METHODS.To identify the clone which carried a plasmid encoding the *imp* gene, we made use of the fact that the *imp*-4213 mutation is rapidly lost by recombinational correction (see above). Plasmid DNA from the *Bam*HI clone bank was transformed into strain ECB4623. This strain is derived from MC1000 which has increased transformation efficiency with plasmid DNA. It was necessary to use this background because the *imp* mutations reduce transformation efficiency by 30- to 50-fold (data not shown). Out of approximately 2500 Km^r transformants screened, six were resistant (grew on) to MacConkey agar and segregated Nov^r colonies at high frequency. The plasmids from these colonies had identical *Bam*HI restriction patterns (data not shown). One plasmid, pSAB5402, was chosen for further analysis. A partial restriction map of plasmid pSAB5402 is given in Figure 1. This plasmid carries an insert of approximately 16 kb. To prove that this plasmid carried the *imp* gene, we recombined the *imp*-4213 mutation onto the plasmid as follows. We introduced pSAB5402 into BAS849 (*imp*-4213), extracted plasmid DNA and transformed this DNA back into BAS849 selecting the plasmid encoded Km^r determinant and then screened for transformants which were Dex$^+$ and Mac^s (diploid for the *imp*-4213 allele). Recall that the Dex$^+$ and Mac^s phenotypes are recessive and thus only if the *imp*-4213 allele is present on the plasmid would we expected these phenotypes in the transformants. Among 350 Km^r transformants, we obtained six colonies which were Dex$^+$ and Mac^s. Further analysis of the plasmid (pSAB5420) from one of these clones showed it had a restriction pattern identical to that of pSAB5402, and when introduced into BAS1363(*imp*$^+$) it conferred a Nov^s Dex$^+$ phenotype (Table 6). This proved physically and genetically that the plasmid carried the *imp*-4213 allele. The fact it has a restriction map identical to pSAB5402 suggests the *imp*-4213 mutation does not result from any gross rearrangement of DNA. We estimated we could detect deletions or insertions of 200 bp or more.

The codominance of the *imp* mutations suggest that they result in production of an altered gene product, and the phenotypes seen with the F′ diploids reflect the contributions of the mutant and wild-type gene products. Thus, we reasoned that if the gene dosage for one of the alleles is increased this should result in a stronger representation of the phenotype conferred by that gene product. This is indeed the case (Table 6). When the *imp*$^+$ allele is present in six copies

TABLE 6

Effects of the mutant and wild-type *imp* gene on a low copy plasmid

Strain	*imp* genotype		Disc sensitivity zones					Growth on maltodextrin
	Chromosome	Plasmid	SDS	DEO	Eos	Nov	Em	
BAS1362	*imp*-4213	None	10	14	10	19	15	+
BAS1362 pSAB5402	*imp*-4213	*imp*$^+$	0	0	0	10	(15)	−/+
BAS1362 pSAB5402	*imp*-4213	*imp*-4213	13	13	10	21	21	++
BAS1363	*imp*$^+$	None	0	0	0	0	(8)	-
BAS1363 pSAB5402	*imp*$^+$	*imp*$^+$	0	0	0	0	(14)	-
BAS1363 pSAB5420	*imp*$^+$	*imp*-4213	10	0	0	15	(18)	+

The numbers indicate the size (mm) of the killing zone. Number given in ( ) indicate a zone of growth inhibition, not killing. + = growth, − = no growth, −/+ = poor growth in 36 hr. SDS = sodium dodecyl sulfate, DEO = deoxycholate, Eos = eosin Y.

(BAS1362/pSAB5402) detergent, Nov^s, and the Dex$^+$ phenotypes are reduced relative to the haploid strain BAS1362. It appears that the presence of the *imp*$^+$ allele on the plasmid may itself alter outer membrane permeability slightly. We observe a weak Dex$^+$ phenotype with BAS1362/pSAB5402 (six copies of *imp*$^+$) whereas when the wild type gene is provided in single copy on F'104 the diploid is Dex$^-$. In addition, BAS1362/pSAB5402 shows a slight increase in Em^s compared to BAS1362 (Table 6). When *imp*-4213 is present on the plasmid (BAS1363/pSAB5402) there is an increase in sensitivity to SDS, Nov and the Dex$^+$ phenotype (Table 6). The presence of a single *imp*$^+$ gene is sufficient to eliminate sensitivity to deoxycholate and reduce Nov^s (Table 6). When the *imp*-4213 allele is present on both the plasmid and the chromosome (BAS1362/pSAB5420) the phenotypes conferred by the mutation are accentuated (Table 6).

DISCUSSION

We have utilized a genetic selection to gain insights into the barrier functions of the outer membrane of *E. coli*. The selection is based upon the uptake of a large sugar, maltodextrin, and yields several classes of mutants at unequal frequencies (MISRA *et al.* 1987; BENSON, OCCI and SAMPSON 1988). One rare class of mutations, *imp*, results in sensitivity to a variety of unrelated compounds including detergents, antibiotics and dyes. The two *imp* mutations characterized in this study are both nonreverting codominant mutations that define a new genetic locus which appears to be an essential gene that affects outer membrane permeability.

The *imp* mutants appeared as Dex$^+$ colonies several days after plating and, therefore, it is difficult to accurately assign a frequency for these mutations. It is likely, the mutations occurred after imposition of the selection. As such, it is possible that the selection itself may have influenced the type of mutants we obtained. Recent work by CAIRNS, OVERBAUGH and MILLER (1988) suggest that in nonlethal selection the selection can influence the type of mutation obtained.

We believe the mutations are not simple missence or null mutations based on their rarity and nonreverting and codominate nature, and suggest they result from similar or identical genetic events resulting in a gene product with partial or an acquired function(s). This explanation would account for the low frequency at which we obtained these mutations, and the fact that we are unable to detect recombination between the two mutations.

The *imp* mutations could be specific base pair substitutions, deletions or insertions. The first possibility is unlikely since we are unable to obtain true revertants either spontaneously or following treatment with 2-AP, NTG, or EMS (data not shown). We favor the possibility that the mutations result from specific deletion events since we should have been able to detect exact excision of an insertion in the reversion test. Analysis of the *imp*-4213 mutation on pSAB5420 indicates that if the mutation is a deletion, it is small (<200 bp) and thus could reside within a coding region or fuse two adjacent ones.

Mapping of the mutations places the *imp* gene at approximately 1.2 min on the *E. coli* chromosome between *ksgA* and *ara*. The gene order was obtained by four-factor mapping and is consistent with the two-factor cotransduction frequencies (Table 3, Figure 2). However, due to the high linkage between the *ksgA*, *zab*4292::Tn*5*, *zab*::Tn*10*, and the *imp* gene and the selective bias for the wild type *imp* allele, the relative position and suggested gene order should be viewed as tentative until confirmed by physical analysis of the cloned gene. There are no genes in this region of the chromosome that are known to affect outer membrane functions. Thus, the map position does not give insight into the nature of the *imp* gene product or its physiological role in the cell. It does show that the mutations define a new locus.

The mapping studies, and the fact that strains diploid for this region segregate Nov^r colonies at high frequency, suggest there is a bias against the mutant allele. The basis for the selection against the mutant *imp* alleles is not known. The fact that the haploid strains, which carry a single copy of the mutant *imp* gene, do not show any significant growth disadvantage in complex media and have a growth advantage in maltodextrin further clouds the issue. One possibility is that the disadvantage imposed by the *imp* mutation is not during the exponential growth phase but mediates its effects during some other phase of the

growth cycle, or in viability during stationary phase.

The appearance of the Nov^r colonies with the diploid strains is most easily explained by inactivation of the mutant *imp* allele. This suggestion is further supported by the fact such colonies lose all of the *imp* associated phenotypes (Dex$^+$ Nov^s Em^s Mac^s). This is in contrast to pseudorevertants obtained from haploid strains which lose only some of the *imp* associated sensitivities. Several factors may contribute to the apparent high frequency at which diploid strains segregate Nov^r colonies. These include a strong selection for loss of the mutant allele, stimulation of deletions from the adjacent Tn*10*s transposons (KLECKNER, REICHARDT and BOTSTEIN 1979) and the technical difficulty of starting with a population of cells that does not already contain preexisting Nov^r colonies. At the suggested frequency (10^{-2} to 10^{-4}) even small innoculums of cells will contain preexisting Nov^r cells.

Three lines of evidence suggest that the *imp* gene is essential. Both *imp* mutations are codominant and thus are not simple null mutations. Diploid strains inactivate the mutant allele at high frequency and this eliminates all of the increased permeability phenotypes; whereas, haploid strains are unable to do this, and direct selection for reversal of the increased permeability phenotypes yields only pseudorevertants. Lastly, attempts to obtain amber mutations, gene fusions to, or transposons in the *imp* gene have been unsuccessful.

Diploid and reversion analysis studies show that certain phenotypes conferred by the *imp* alterations can be separated, *i.e.*, Dex$^+$ and Mac^s from Nov^s and Em^s. One explanation for this is that the *imp* alteration effects two different barrier functions of the outer membrane, one in a recessive fashion and one in a dominant fashion. We favor the alternative simpler explanation that the various phenotypes reflect different levels of increased permeability within a single barrier function. At present it is not possible to know which, if either, of these alternatives are correct.

Phenotypic analysis of the *imp* mutants demonstrates the outer membrane barrier functions have been altered for a large number of compounds (Table 2). Disc sensitivity tests show that the *imp* mutations increases sensitivity to many but not all antibiotics. MIC determinations show that there is a greater than 100-fold increase in sensitivity to certain hydrophobic antibiotics which normally cross the outer membrane poorly but there is only a small increase in sensitivity to antibiotics which predominately cross the outer membrane via the porins. Interestingly, the general pattern of sensitivities is similar to that conferred by small in-frame deletions within the porin genes (MISRA and BENSON 1988b; BENSON, OCCI and SAMPSON 1988). We have suggested that such porin mutations confer these phenotype by altering the membrane structure (MISRA and BENSON 1988b; BENSON, OCCI and SAMPSON 1988). Whether the *imp* mutations cause a similar alteration, or impart an analogous phenotype via a different mechanism is not known. The porin independence of the *imp* phenotypes indicates that the porins are not required for the increased phenotype. Since the *imp* mutants exhibit reduced growth in the absence of both porins, this suggests that the *imp* mutations do not result in production of a new porin type protein consistent with the fact we see no alteration in peptide profile of the envelope. In addition, this suggests that diffusion of certain substances across the outer membrane is still limiting growth in the *imp* porin minus mutants.

One explanation for the observed sensitivities and Dex$^+$ phenotype is that some general property of the outer membrane has been altered. However since the mutants retain sensitivity to all the bacteriophages tested and do not show any significant reduction in growth rate, the defect appears not to result in any major disruption of the structure. We are unable to find a specific outer membrane biochemical alteration in the *imp* mutants. One explanation may be that the *imp* mutations alter a minor component of the outer membrane and our present assays are not sensitive enough. In the case of *tolC* mutants, which also have an increased permeability phenotype, cloning and overexpression of the *tolC* gene product (a minor outer membrane protein) were required in order to biochemically detect the alteration (MORONA, MANNING and REEVES 1983).

The fact that we can detect no change in sensitivity to LPS requiring bacteriophages, to EDTA or polymyxin suggests that LPS structure is not dramatically altered. This is further supported by the silver straining of the LPS from the *imp*-208 mutant which gives a pattern identical to the parental strain (data not shown). However, we cannot preclude the possibility that the *imp* mutations alter the LPS in a subtle fashion not detected by these assays. Recent work by **Roque** *et al.* (1988) on the hyperpermeable *abs* mutation has shown that alterations in polycationic binding of the LPS can result in a hyperpermeable phenotype. At present, we do not know if the *imp* mutations alter this property of the LPS.

The suggestion that the *imp* gene encodes an essential cellular function implies that this gene is involved in a fundamental cellular process. At present, the only known component of the outer membrane essential for cell viability is lipid A, the fatty acid component of LPS (RAETZ 1987). The possibility that lipid metabolism is altered in the *imp* mutants is an attractive one, but at this time we do not know if this is the case.

We have cloned the *imp*$^+$ gene onto a low copy number plasmid and used plasmid-chromosomal recombination to obtain the *imp*-4213 allele. The clon-

ing strategy is a general one and allows cloning of both dominant or recessive alleles. In our case, the wild type allele (Nov^r Mac^r) is recessive to the mutant Nov^s phenotype, and dominant for the Mac^r phenotype. We were able to identify the correct clone by screening for the dominant, Mac^r, plasmid determined phenotype or for recombinational loss of the chromosomal Nov^s phenotype. Most importantly, the system allowed us to demonstrate directly that we had cloned the correct gene by showing that the mutant *imp* alleles could be recombined onto the plasmid genome. This eliminated the possibility that the observed phenotype resulted form overexpression of a unrelated cloned gene. The plasmids were then used to further demonstrate the codominant nature of the mutations and show that gene dosage of the mutant and wild-type alleles correlated with the degree of the *imp* conferred phenotypes.

At present, we do not understand the molecular basis for the phenotypes observed in the *imp* mutants, nor the mechanisms by which the mutations cause the increased permeability. Thus, at this time, we cannot rule out the possibility that the effect on the outer membrane permeability may be indirect and that the *imp* mutations alter a process more directly related to some other aspect of the envelope structure such as the cell wall or the inner membrane. Nonetheless, we believe that these mutants define a new essential gene of *E. coli* and provide new tools for the genetic analysis of the complexities of the outer membrane. Specifically, we are now able to do localized mutagenesis to obtain additional *imp* mutations, and use the *imp* conferred phenotypes to develop selections for pseudorevertants which suppress one or more of the sensitivities conferred by the mutations. Such pseudorevertants may in their own right provide novel insights into how the outer membrane specifically excludes hydrophobic compounds from the cell, as well as to how the *imp* mutations alter outer membrane permeability.

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LITERATURE CITED

ANDRESSON, O. S., and J. E. DAVIES, 1980 Organization and Restriction Enzyme Clevage Map of the *ksgA-pdxA* region of the *Escherichia coli* chromosome. Mol. Gen. Genet. **179:** 211–216.

BENSON, S. A., and DECLOUX, 1985 Isolation and characterization of outer membrane permeability mutants in *Escherichia coli* K-12. J. Bacteriol. **161:** 361–367.

BENSON, S. A., J. L. OCCI and B. A. SAMPSON, 1988 Mutations that alter the pore function of the OmpF porin of *Escherichia coli* K12. J. Mol. Biol. **204:** 961–970.

CAIRNS, J., J. OVERBAUGH and S. MILLER, 1988 The origins of mutants . Nature **335:** 142–145.

CASADABAN, M. J., 1976 Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:** 541–555.

CLARK, D., 1984 Novel antibiotic hypersensitive mutants of *Escherichia coli* genetic mapping and chemical characterization. FEMS Microbiol. Lett. **21:** 189–195.

COLEMAN, W. G., JR., and K. DESHPANDE, 1985 New *cysE-pyrE*-linked *rfa* mutation in *Escherichia coli* K-12 that result in a heptoseless lipopolysaccharide. J. Bacteriol. **161:** 1209–1214.

DAVIES, J. K., and P. REEVES, 1975 Genetics of resistance to colcins in *Escherichia coli* K-12: cross-resistance among colicins of group A. J. Bacteriol. **123:** 102–117.

EMR, S., and T. J. SILHAVY, 1980 Mutations affecting localization of *Escherichia coli* outer membrane protein, the bacteriophage lambda receptor. J. Mol. Biol. **141:** 63–90.

GIGOT, D., M. CRABEEL, A. FELLER, D. CHARLIER, W. LISSENS, N. GLANSDORFF and A. PIERARD, 1980 Patterns of Polarity in the *Escherichia coli carAB* gene cluster. J. Bacteriol. **143:** 914–920.

GOLDMAN, R., W. KOHLBRENNER, P. LARTEY and A. PERNET, 1957 Antibacterial agents specifically inhibiting lipopolysaccharide synthesis. Nature **329:** 162–164.

HENSON, J. M., and J. R. WALKER, 1982 Genetic analysis of *arcA* and *lir* mutations of *Escherichia coli*. J. Bacteriol. **152:** 1301–1302.

HOFNUNG, M., D. HATFIELD and M. SCHWARTZ, 1974 *malB* region of *Escherichia coli* K-12: characterization of new mutants. J. Bacteriol. **117:** 40–47.

KLECKNER, N., K. REICHARDT and D. BOTSTEIN, 1979 Inversions and deletions of the *Salmonella* chromosome generated by the translocatable tetracycline element Tn*10*. J. Mol. Biol. **127:** 89–115.

LABEDAN, B., 1988 Increase in permeability of *Escherichia coli* outer membrane by local anesthetics and penetration of antibiotics. Antimicrob. Agents Chemother. **32:** 153–155.

LEIVE, L., 1965 Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. **21:** 290–296.

MILLER, J. H., 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

MISRA, M., and S. A. BENSON, 1988a Isolation and characterization of OmpC porin mutants with altered Pore properties. J. Bacteriol. **170:** 528–533.

MISRA, M., and S. A. BENSON, 1988b Genetic identification of the pore domain of the OmpC porin of *Escherichia coli* K-12. J. Bacteriol. **170:** 3611–3617.

MISRA, R., B. A. SAMPSON, J. L. OCCI and S. A. BENSON, 1987 Identification of mutants with altered outer membrane permeability, pp. 436–442, in *Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function*, edited by P. ACTOR, L. DANA-MOORE, M. HIGGINS, M. SALTON and G. D. SCHOCKMAN. Am. Soc. Microbiol., Washington, DC.

MORONA, R., P. A. MANNING and P. REEVES, 1983 Identification and characterization of the TolC protein, an outer membrane protein from *Escherichia coli*. J. Bacteriol. **153:** 693–699.

NAGEL DE ZWAIG, R., and S. E. LURIA, 1967 Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J. Bacteriol. **94:** 1112–1123.

NIKAIDO, H., and M. VAARA, 1985 Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:** 1–32.

NIKAIDO, H., and M. VAARA, 1987 Outer membrane, pp. 7–22, in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, edited by F. NEIDHARDT, J. INGRAHAM, K. B. BROOKS, B. MAGASANIK, M. SCHAECHTER and H. E. UMBARGER. American Society for Microbiology, Washington, D.C.

RAETZ, C. R. H., 1987 Structure and biosynthesis of lipid A in *Escherichia coli*, pp. 498–503 in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, edited by F. NEIDHARDT, J. INGRAHAM, K. B. BROOKS, B. MAGASANIK, M. SCHAECHTER and H. E. UMBARGER. American Society for Microbiology, Washington, D.C.

RAETZ, C. R. H., and J. FOULDS, 1977 Envelope composition and antibiotic hypersensitivity of *Escherichia coli* mutants defective in phosphatidylserine synthetase. J. Biol. Chem. **252:** 5911–5915.

ROCQUE, W. J., S. W. FESIK, A. HAUG and E. J. MCGROARTY, 1988 Polycation binding to isolated lipopolysaccharide from antibiotic-hypersusceptible mutant strains of *Escherichia coli*. Antimicrob. Agents Chemother. **32:** 308–313.

SAMPSON, B. A., and S. A. BENSON, 1987 *Escherichia coli* strains with defined mutations which alter cellular permeability. J. Ind. Microbiol. **1:** 335–340.

SILHAVY, T. J., M. L. BERMAN and L. W. ENQUIST, 1984 *Experiments with Gene Fusions*. Cold Spring Harbor, N.Y.

WANDERSMAN, C., M. SCHWARTZ and T. FERENCI, 1979 *Escherichia coli* mutants impaired in maltodextrin transport. J. Bacteriol. **140:** 1–3.

WANNER, B., 1986 Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. J. Mol. Biol. **191:** 39–58.

WHITNEY, E. N., 1971 The *tolC* locus in *Escherichia coli* K-12. Genetics **67:** 39–45.

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