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NOTES

Bacteriophage K20 Requires Both the OmpF Porin and Lipopolysaccharide for Receptor Function

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Mutations which prevent absorption of the bacteriophage K20 to *Escherichia coli* K-12 were selected by using an altered OmpF protein which confers the ability to grow on maltodextrin in the absence of the LamB maltoporin. The mutations map in the *rfa* gene cluster and alter the structure of the lipopolysaccharide core.

Bacteriophages are used as diagnostic tools for the identification and taxonomy of bacteria and can serve as model systems for the study of membrane receptors. One limitation in using bacteriophages to probe membrane structure is that the selection for bacteriophage-resistant mutants often yields mutants which prevent expression of the receptor. In certain cases, additional selections or screens have been used to identify mutants which produce and export mutant receptors (5, 10, 13). This approach has been useful in defining specific membrane protein domains and topology (10, 14, 17).

The OmpF outer membrane protein, encoded by the *ompF* gene, facilitates the diffusion of small molecules (<600 daltons) across the outer membrane and serves as the receptor for various bacteriophages, including the bacteriophage K20 (for a review, see reference 4).

Certain *ompF* mutations (3) allow *Escherichia coli* to grow on maltodextrins (Dex^+ phenotype) in the absence of the LamB maltoporin, which is normally required to allow these large substrates to enter the cell (18). Certain *ompF*(Dex) alleles increase the permeability of the outer membrane to various antibiotics, detergents, and dyes (3). We refer to this phenotype as hyperpermeability (Hyp).

Starting with the Dex^+ Hyp strain JAS100, K20-resistant ($K20^r$) Dex^+ mutants were isolated by plating 0.1 ml (ca. 5×10^8 cells) of an overnight culture on maltodextrin minimal agar spread with 10^9 K20 bacteriophages. $K20^r$ colonies were obtained at a frequency of approximately 2×10^{-6}. A total of 19 independent Dex^+ $K20^r$ mutants, obtained from individual cultures, were purified on dextrin medium and characterized. The isolates were of two classes. Class 1 mutants (four isolates) were $K20^r$ Dex^+ and no longer exhibited the Hyp phenotype. Class 2 mutants (15 isolates) were $K20^r$ Dex^+ Hyp and mucoid at 37°C on both glucose minimal agar and L agar medium but not on dextrin minimal agar. The mucoid phenotype was reduced at 42°C.

To determine whether the $K20^r$ phenotype resulted from an alteration in *ompF*, the *ompF* genes from the mutants were transduced into an Φ(*ompF-lacZ*) strain (PLB3260) by P1 transduction with a linked (ca. 50%) *zcb*::Tn*10* marker (Tc^r). The *lacZ* fusion allows us to easily identify transductants in which the *ompF* region in the recipient ($LacZ^+$) has been replaced with the *ompF* region from the donor ($LacZ^-$). All $LacZ^-$ transductants from the class 1 mutants were $K20^r$ Dex^- and nonhyperpermeable (wild type; Hyp^{wt}), indicating that the OmpF protein in these transductants either is not expressed or is nonfunctional. Since the original isolates were Dex^+, we conclude that the class 1 mutants must have carried a second mutation that conferred the Dex^+ phenotype. Nothing more was done with these isolates. The class 2 mutants yielded $LacZ^-$ $K20^s$ Dex^+ Hyp transductants. This phenotype is identical to that of the parental strain (JAS100) and suggested that the $K20^r$ phenotype in these mutants is not due to an alteration at *ompF*. Three isolates of this type, JAS2202, JAS2212, and JAS2217, were selected for further study (Table 1).

To map the determinant for $K20^r$, the three JAS strains were mated with a series of Hfr strains as described by Wanner (19). We first removed the *zcb*::Tn*10* as described below. The Hfr mapping (data not shown) indicated that the mutations mapped in the 78-to-90-min region of the *E. coli* chromosome. To further define the location of the K20 resistance determinant, we tested linkage to a number of known markers in this region by P1 cotransduction. The alterations all map to the *rfa* gene cluster (Fig. 1). The linkages to *mtl*, *zia*::Tn*10*, and *pyrE* were 16, 39, and 28%, respectively (Fig. 1). The linkages and gene order were determined by a series of three- and four-factor crosses, a representative sample of which is given in Table 2. We have tentatively designated the mutations as *rfa-2202*, *rfa-2212*, and *rfa-2217*.

The *rfa* gene cluster encodes enzymes involved in the biosynthesis of lipopolysaccharide (LPS) (1, 6). To determine whether the mutations alter the LPS structure, we extracted LPS by using a modification of the procedures described by Morrison and Leive (11). Cultures (5 ml) were grown overnight in L broth at 37°C. The cells were collected by centrifugation, washed with an equal volume of 4°C water, and suspended in 0.4 ml of ice-cold 0.85% (wt/vol) NaCl. The cells were then transferred to an Eppendorf tube, an equal volume of water-saturated phenol was added, and the mixture was incubated at 65°C for 15 min. The mixture was chilled on ice (5 min), and the phases were separated by centrifugation for 2 min in a Microfuge (Beckman Instruments, Inc.). The aqueous phase was extracted twice with 2 volumes of cold butanol by rocking the mixture for 15 min at

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TABLE 1. Bacterial strains used

Strain	Genotype and source or derivation (reference)
MC4100	F^- *araD139* Δ(*argF-lac*)*U169 rpsL150 relA flbB5301 ptsF25 deoC1*; laboratory strain (3)
MCR106	MC4100 Δ(*lamB*)*106* (3)
PLB3255	MC4100 Δ(*lamB*)*106 ompF3255* (3)
PLB3260	MCR106 Φ(*ompF'-lacZ*$^+$)*16-13* (3)
JAS100	MC4100 Δ(*lamB*)*106 ompF3255 zcb*::Tn*10*; *zcb*::Tn*10* was introduced into PLB3255 by P1 transduction
JAS2202	JAS100 *rfa-2202*; this study
JAS2212	JAS100 *rfa-2212*; this study
JAS2217	JAS100 *rfa-2217*; this study
JAS2502	MCR106 *rfa-2202*; the *ompF* wild-type allele was introduced into JAS2202 as described in the text
JAS2512	MCR106 *rfa-2212*; the *ompF* wild-type allele was introduced into JAS2212 as described in the text
JAS2517	MCR106 *rfa-2217*; the *ompF* wild-type allele was introduced into JAS2217 as described in the text
JAS2602	MCR106 Φ(*ompF'-lacZ*$^+$)*16-13 pyrD34 rfa-2202*; the *ompF* and *pyrD* alleles were introduced into JAS2202 as described in the text
JAS2612	MCR106 Φ(*ompF'-lacZ*$^+$)*16-13 pyrD34 rfa-2212*; the *ompF* and *pyrD* alleles were introduced into JAS2212 as described in the text
JAS2617	MCR106 Φ(*ompF'-lacZ*$^+$)*16-13 pyrD34 rfa-2217*; the *ompF* and *pyrD* alleles were introduced into JAS2217 as described in the text
JAS4602	MCR106 *zia*::Tn*10 pyrE60 mtl rfa-2202*; the *pyrE60* and *mtl* mutations were introduced into JAS2502 by P1 transduction from strains RK4900 and RK1041 obtained from C. Schnaitman (2)
JAS4603	MCR106 *zia*::Tn*10 pyrE60 mtl*; the *pyrE60* and *mtl* mutations were introduced into MCR106 by P1 transduction from strains RK4900 and RK1041 obtained from C. Schnaitman (2)

4°C, followed by treatment with a 1:100 dilution of RNase and DNase (10 μg/ml, 100 mM $MgSO_4$) for 5 min at 37°C and with protease K (25 ng/ml) for 1 h at 65°C. The mixture was then extracted a final time with butanol, and the aqueous phase was dried in a Speedvac (Savant Instruments, Inc., Hicksville, N.Y.) and suspended in 100 μl of water. Samples (20 to 30 μl) were then electrophoresed and silver stained as described by Hitchcock and Brown (8). Figure 2 shows the results of one such gel. The mutants all had LPS which appeared to be defective in the core sugars. This finding supports the genetic observation that the $K20^r$ phenotype resulted from *rfa* mutations.

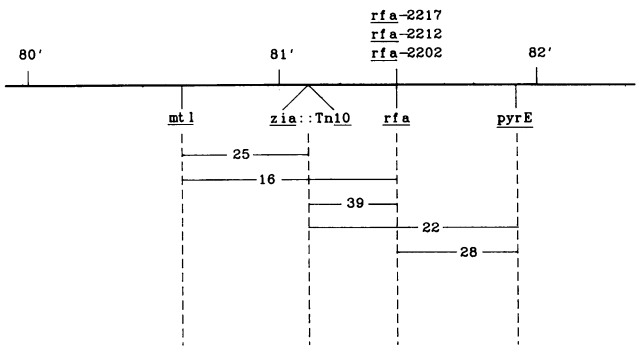

FIG. 1. Map locations of the $K20^r$ mutations. The map positions of the designated genes are from Bachmann (1). The P1 linkage for the various genes was determined by three-factor crosses as described in the text.

TABLE 2. Three-factor mapping data[a]

Cross	Unselected markers[b]				No. of isolates	Frequency (%)
	mtl	*zia*::Tn*10*	*rfa*	*pyrE*		
JAS4602 (donor) × MC4100 (recipient)[c]		D	R	R	175	80
		D	D	R	28	13
		D	D	D	13	6
		D	R	D	3	1
	D	D	R		112	51
	R	D	R		66	30
	D	D	R		23	11
	R	D	D		18	8
JAS4603 (donor) × JAS2502 (recipient)[d]		D	D	D	34	40
		D	D	R	28	30
		D	R	R	27	29
		D	R	D	1	1
	R	D	D		37	40
	D	D	D		28	30
	D	D	R		15	17
	R	D	R		12	13

[a] P1 transductions were done as described in reference 16. Tetracycline-resistant transductants were selected and purified on L agar containing 25 μg of tetracycline per ml. Individual colonies were then tested by replica plating to the minimal agar containing mannitol to check the *mtl* marker, to glucose minimal agar with and without uracil to check the *pyrE* marker, and by cross-streaking over K20 to check for bacteriophage resistance (*rfa* marker). D, Donor; R, recipient.

[b] The unselected markers were *pyrE* and *rfa* or *rfa* and *mtl*. *zia*::Tn*10* was the selected marker in all cases.

[c] 219 colonies were tested.

[d] 90 colonies were tested.

To determine whether the mutations were specific for the *ompF3255* allele, the *ompF3255* allele was replaced with an *ompF-lacZ* fusion and a *ompF*$^+$ allele. We first introduced an *ompF-lacZ* fusion and a linked *pyrD* mutation by select-

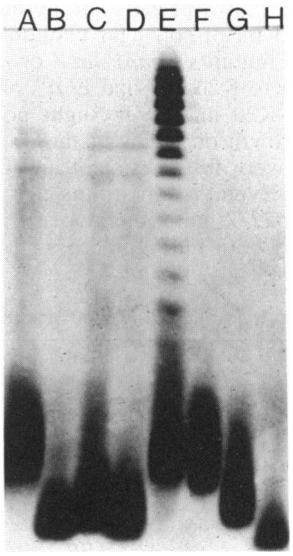

FIG. 2. Silver-stained LPS from the $K20^r$ mutants. LPS was prepared, electrophoresed, and silver stained as described in the text. The LPS in the various lanes are as follows: A, MCR106; B, JAS2202; C, JAS2212; and D, JAS2217. Lanes E through H contain purified LPS purchased from List Biological Labortories, Campbell, Calif., as follows: E, LPS from a smooth *E. coli* strain (K235); F, *E. coli* K-12 mm294 (complete core LPS); G, *E. coli* K-12 D31m4 (Re-type LPS); and H, *E. coli* K-12 J5 (Rc-type LPS).

TABLE 3. Sensitivity to erythromycin and novobiocin[a]

Strain	*ompF* allele	Zone of growth inhibition (mm) with:	
		Erythromycin (15 mg)	Novobiocin (30 mg)
MC4100	*ompF*$^+$	0	0
PLB3260	Φ(*ompF-lacZ*)	0	0
JAS2502	*ompF*$^+$	11	9
JAS2512	*ompF*$^+$	10	15
JAS2517	*ompF*$^+$	10	9
JAS2602	Φ(*ompF-lacZ*)	12	13
JAS2612	Φ(*ompF-lacZ*)	10	17
JAS2617	Φ(*ompF-lacZ*)	10	10

[a] Sensitivity was tested as described previously (3).

ing Lac$^+$ P1 transductants and screening for a PyrD$^-$ Tc^s (loss of the *zcb*::Tn*10*) phenotype. The *ompF* wild-type allele was then introduced by cotransduction with a *pyrD*$^+$ allele. The *rfa* mutations are not allele specific, since the *ompF*$^+$ *rfa* strains are K20^r and mucoid.

rfa mutants have increased sensitivity to hydrophobic antibiotics such as novobiocin and erythromycin (2, 6). We tested whether the *rfa-2212*, *rfa-2212*, and *rfa-2217* derivatives which carry either an *ompF*$^+$ gene or an *ompF-lacZ* fusion also exhibit this phenotype. The *rfa* mutations we isolated increased sensitivity to these antibiotics (Table 3) and to detergents (data not shown). They did not alter the sensitivity of the cells to β-lactams, chloramphenicol, or aminoglycoside antibiotics.

To test whether the mutations affected other components of the outer membrane, we tested sensitivity to a number of bacteriophages (Table 4). Each of the mutants showed a reduced efficiency of plating (EOP) for a number of bacteriophages, including bacteriophage λ (data not shown). The sensitivity to the various bacteriophages was determined by two methods: by spot testing 100-fold dilutions of the test bacteriophage on a lawn of cells and by directly plating 10^7 and 10^3 PFU in 3 ml of soft agar containing 10^8 cells. We noted that *ompF* mutants (*ompF-lacZ* or *ompF*::Tn*5*) were partially resistant to K20 and had EOPs of 10^{-4} to 10^{-5}. In the plating test, K20 made very light pock-type areas of decreased cell growth on lawns of these cells. In contrast, the *rfa* mutants were fully resistant and did not show the pock phenotype. Even though strains carrying the *rfa-2202*, *rfa-2212*, and *rfa-2217* mutations appear resistant to bacteriophage P1 *vir* by both EOP and cross-streaking tests, they will function as either donors or recipients in P1 transduction, although at a reduced efficiency.

TABLE 4. Resistance to various bacteriophages[a]

Phage	Receptor	Resistance of strain:			
		MCR106	JAS2502	JAS2512	JAS2517
K20	OmpF-LPS	S	R	R	R
Hy2	OmpC	S	SR	SR	SR
SS4	OmpC	S	SR	SR	SR
λh^{80}	TonA	S	S	S	S
Ox2	OmpA-LPS	S	PR	PR	PR
Tu2*	OmpA-LPS	S	SR	SR	SR
T4	OmpC-LPS	S	R	R	R
T6	Tsx	S	SR	SR	SR
P1	LPS	S	R	R	R
U3	LPS	S	R	R	R

[a] Sensitivity to the various bacteriophages was tested as described in the text. The scoring is as described by Hancock and Reeves (7). R, Resistant (EOP < 10^{-7}); PR, partially resistant (EOP ≤ 10^{-2}); SR, slightly resistant (EOP > 10^{-2}); S, sensitive (EOP = 1).

TABLE 5. Bacteriophage absorption assay[a]

Strain	Relevant genotype	Reduction in bacteriophage titer[b]	
		K20	P1 *vir*
MC4100	*ompF*$^+$ *rfa*$^+$	10–25	40–50
PLB3260	Φ(*ompF-lacZ*) *rfa*$^+$	0	ND
JAS100	*ompF3255 rfa*$^+$	10–25	15–20
JAS2502	*ompF*$^+$ *rfa-2202*	0	3–5
JAS2512	*ompF*$^+$ *rfa-2212*	0	ND
JAS2517	*ompF*$^+$ *rfa-2217*	0	ND

[a] Bacteriophage absorption tests were done as described in the text.
[b] The amount of reduction in bacteriophage titer is given as a multiplication factor; i.e., 10 indicates a 10-fold reduction in titer. ND, Not determined.

To determine whether the *rfa* mutations blocked K20 absorption, we assayed K20 absorption by combining 10^9 cells from a mid-log-phase culture with 10^8 PFU of the bacteriophage and allowing absorption to occur for 30 min at 37°C in 10 mM $MgSO_4$. The mixture was then diluted threefold with L broth (16), and the cells were removed by centrifugation for 1 min in a Microfuge. The titers of supernatant were then determined for PFU on a lawn of strain MC4100. Parallel tubes without cells were used as controls. The mutants were defective in the absorption of K20 and had reduced ability to absorb P1 *vir* (Table 5), which is consistent with the results shown in Table 4.

The selection procedure we used yields two classes of K20^r mutants. One class of mutants carries two mutations, one mutation that blocks expression of the OmpF3255 porin conferring a K20^r Hyp^{wt} Dex$^-$ phenotype and a second mutation which confers a Dex$^+$ phenotype. We believe that this class of mutants was obtained because the phenotypes conferred by the *ompF3255* allele are lost at a high frequency (10^{-5}), yielding K20^r Dex$^-$ Hyp^{wt} strains (S. Benson, unpublished data). Consequently, K20^r Dex$^-$ Hyp^{wt} cells will be present in the initial inoculum. These mutants are not killed by K20, and thus progeny can emerge as K20^r Hyp^{wt} Dex$^+$ mutants in the selection. We do not know why this type of mutant was obtained at such a high frequency (4 of 19 mutants). The fact that we repeatedly isolated this class of mutants underscores our lack of knowledge of the complexities of the events that occur under various lethal and nonlethal selection conditions.

The class 2 mutations confer resistance to K20 because of an alteration in LPS structure. This contention is supported by the genetic location of the alterations, biochemical analysis, and the observation that the lesions result in pleiotropic effects on bacteriophage and drug sensitivities and cause the cell to have a mucoid phenotype. This mucoidy is reduced at high temperature and eliminated by the addition of a *cpsB-lacZ* fusion mutation (data not shown). Since the mutants retain their Dex$^+$ phenotype, the *rfa* mutation does not block *ompF* expression. The level of OmpF protein in these mutants is slightly reduced when assayed by either immunoprecipitation or protein staining of the cell envelope (data not shown). These results, in combination with the EOP data (Table 4) and phage absorption studies (Table 5), suggest that K20 requires both the OmpF protein and LPS as its receptor. A number of other bacteriophages have similar receptor requirements (12, 15). The facts that the mutants can function as either donors or recipients in P1 transductions and that they show a intermediate level of P1 absorption (Table 4) suggest that the alteration does not completely

remove the LPS core oligosaccharides. The mutants appear to have an Rd-type LPS (6). This finding suggests that the phage recognizes the terminal sugar moieties of the LPS core. This selection procedure may be useful for the isolation of additional LPS mutants.

We do not know why we failed to isolate the desired *ompF* mutations. Similar approaches with outer membrane proteins have been fruitful (5, 9, 17). One explanation is that the target size of the *rfa* gene cluster is very large compared with the regions of the *ompF* gene where specific missense mutations with the selected phenotypes can be isolated. An alternatively, explanation is that the domain of the protein involved in the Dex^+ phenotype is also part of the bacteriophage receptor domain.

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