JOURNAL OF BACTERIOLOGY, May 1976, p. 579-586

Vol. 126, No. 2
Printed in U.S.A.

Role of a Lipopolysaccharide Gene for Immunogenicity of the Enterobacterial Common Antigen

G. SCHMIDT,* D. MÄNNEL, H. MAYER, H. Y. WHANG, AND E. NETER

Max-Planck-Institut für Immunbiologie, D-78 Freiburg i.Br., Germany, and Departments of Pediatrics and Microbiology, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14222*

Received for publication 27 November 1975

It is known that only certain strains of the family of *Enterobacteriaceae*, notably rough (R) mutants with the type R1 or R4 core, evoke antibodies in high titers against the common enterobacterial antigen (CA) after immunization of rabbits with heated cell suspensions. The present investigation deals with genetic and immunochemical aspects of certain R1 and R4 mutants isolated from *Escherichia coli* O8 and various *Shigella* serotypes which, unexpectedly, do not induce CA antibody formation. Immunochemical and genetical (transduction and conjugation) experiments revealed that the rough phenotype of these special mutants was evoked by a mutation of a *pyrE*-linked *rfa* gene, called *rfaL*, which is involved in translocation of O-specific polysaccharides onto the lipopolysaccharide core. The transduction of the defective *rfaL* allele into appropriate rough recipients results in transductants which have simultaneously lost the ability to evoke CA antibodies. This finding suggests that a close connection exists between the function of the *rfaL* gene and the expression of CA immunogenicity in R1 and R4 mutants. One of the strains synthesized neither O-hapten nor CA, suggesting a mutation in a region equivalent to the *rfe* genes of *Salmonella*.

Since the discovery by means of the hemagglutination test of the enterobacterial common antigen (CA) by Kunin et al. (5), it has been known that, although almost all wild-type strains of *Enterobacteriaceae* produce this antigenic determinant, only a few, notably *Escherichia coli* O14, evoke CA antibodies in high titers in rabbits. This unique feature of *E. coli* O14 is particularly evident when heated cell suspensions or supernatants thereof are used for immunization. When viable cell suspensions are employed for immunization certain other strains elicit CA antibody formation, although to a lesser extent than *E. coli* O14 (24). In this presentation CA immunogenicity is defined as the ability of heated suspensions to evoke CA antibodies in the rabbit upon intravenous injection; CA antigenicity is defined as the capacity of the material to specifically inhibit hemagglutination of CA-modified erythrocytes by CA antibodies and to modify erythrocytes for agglutination by CA antibodies.

Investigations into the nature of immunogenicity revealed that with rough (R) mutants of *E. coli*, CA immunogenicity is closely related to the type of the lipopolysaccharide (LPS) core (11, 21). So far as the hitherto known LPS core types are concerned, the following information is relevant. In the LPS molecule the basal core oligosaccharide is the connecting link between the lipid part and the highly variable O-specific polysaccharides (7). The LPS core is less variable than the polysaccharide side chains and, so far as is known at this time, is identical in all *Salmonella* serotypes. In *E. coli* five different complete core regions, designated as R1 to R4 and K-12 (represented by *E. coli* K-12) have so far been identified (17-19, 21). It is of interest to note that the R1, R3, and R4 LPS core types have also been found in certain *Shigella* serotypes (12). So far as *E. coli* O14 is concerned, recent investigations with the O14 test strain and other strains typed as O14 have revealed that these CA-immunogenic strains do not represent smooth O serotypes; rather, they are encapsulated (K7 antigen) rough strains with the complete LPS core of the R4 type (21). Additional studies have revealed that rough mutants of *E. coli* with the complete R1 LPS core also provoke CA antibody formation, in contrast to the smooth parent strains and rough mutants with incomplete core (11, 24). On the other hand, rough mutants of *E. coli* which have the R2 or R3 core as well as rough mutants of *Salmonella* with the Ra core type fail to engender CA antibodies. These nonimmunogenic strains, however, have the CA determinant, as demonstrated by means of the hemagglutination and hemagglutination-inhibition tests. Thus, it became evident that, for CA to be

immunogenic among rough mutants, the LPS core types R1 or the serologically related R4 are essential. Recently, it was shown that *E. coli* K-12, a rough strain, also evoked CA antibodies (unpublished observation).

The present investigation is concerned with the CA immunogenicity of various *Shigella* and *E. coli* rough mutants with the R1 or R4 core, respectively, which, contrary to expectation, fail to evoke CA antibodies. It is shown that the lack of CA immunogenicity is due to defects in a gene involved in LPS synthesis.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study are listed in Table 1. The smooth (O8) *E. coli* Hfr59 strain used as donor transfers its chromosome in a counterclockwise direction with the histidine operon (*his*) as a leading locus (19). *E. coli* F947, used as recipient in P1 transductions, is a *pyrE*$^-$ mutant of *E. coli* F870, a rough derivative from *E. coli* O8. The strain F947 synthesizes a K-12 core as the result of the introduction of *rfa* genes from an *E. coli* K-12 Hfr donor, as described previously (17). The rough phenotype of F947 originates from a defective *rfb* region which blocks the synthesis of O8-specific polysaccharides. Additional markers of *E. coli* F947 include defects in histidine (*his*$^-$) and methionine (*met*$^-$) synthesis and resistance to streptomycin (*str*^{r}). The other strains listed in Table 1 represent rough mutants which are the subject of the present investigations.

Culture media. As solid complete medium $D_{1,5}$-agar (16) with 0.3% glucose and 1.5% agar and as fluid medium Standard I broth (E. Merck, AG, Darmstadt, Germany) were used. For the selection of transductants and recombinants Davis minimal agar (6) with 0.3% glucose and, when necessary, supplemented with appropriate amino acids (20 μg/ml) was employed. For propagation of P1*kc* phage and transduction the following medium was used: tryptose (Difco Laboratories, Detroit, Mich.), 10 g; Difco yeast extract, 5 g; NaCl, 8 g; glucose, 1 g; and 1,000 ml of distilled water. This medium was supplemented with calcium chloride to 5×10^{-3} M. When used as solid medium 15 g of agar was added to 1,000 ml.

Transduction. For transduction experiments we used phage P1*kc*, a derivative of P1. Phage P1*kc* lysates were prepared on the respective donor strains, using the agar layer method described by Adams (1). The resulting phage lysates usually contained approximately 10^{10} plaque-forming units.

For transduction overnight broth cultures of the recipients were diluted 20-fold in broth with calcium chloride and incubated at 37 C in a shaking water bath for 4 h. Equal volumes of broth culture and P1*kc* lysate were mixed to give a multiplicity of nearly 1. After incubation at 37 C for 20 min, 0.1-ml samples were plated on appropriately supplemented selective agar and incubated for 48 h at 37 C. Transductants were transferred first onto selective medium and then streaked on complete agar for single-colony isolation. The purified transductants were tested for unselected markers.

Bacteriophages. The rough-specific phages U3, C21, and 6SR were used to determine the LPS core present in transductants. Phage U3 (23) lyses specifically cells with the *E. coli* K-12 core, whereas phages 6SR and C21 are active on rough strains with the R1 core (17, 19). The phages were propagated on their respective hosts as described previously (17).

Conjugation. Freshly grown suspensions of donor and recipient cells were mixed in broth in amounts to yield approximately 5×10^7 donor and 5×10^8 recipient cells. After a 2-h incubation at 37 C aliquots of 0.1 ml of suitable dilutions were plated on appropriately supplemented minimal agar containing 100 μg of streptomycin per ml for counterselection of the streptomycin-sensitive donor *E. coli* Hfr59. After incubation at 37 C for 48 h recombinants were first transferred onto the selection agar and then streaked on complete agar from which single colonies were isolated. The recombinants were screened by slide agglutination in 3.5% saline and in anti-*E. coli* O8 serum (diluted 1:20 in 0.2% saline to prevent spontaneous agglutination of rough mutants).

TABLE 1. *Bacterial strains*

Strain	Description[a]	Derived from	Reference
Escherichia coli			
Hfr59	Smooth, O8:K27$^-$	E56b(O8:K27)	19
F947	Rough, complete K-12 core *rfb*$^-$, *his*$^-$, *met*$^-$, *pyrE*$^-$, *str*^{r}	F870 (R)[b]	17
F470	Rough, complete R1 core, *rfb*$^-$	E56b(O8:K27)	19
F614	Rough, complete R1 core, *rfaL*	E56b(O8:K27)	20
S. dysenteriae			
F3126	Rough, complete R4 core	O-type 1 (F3127)	11
F3160	Rough, complete R1 core	O-type 3 (F3130)	This publication
F3161	Rough, complete R1 core	O-type 4 (F3131)	This publication
S. flexneri			
F3153	Rough, complete R1 core	O-type 6 (F3149)	This publication

[a] Gene symbols: *rfa* and *rfb*, genes participating in the biosynthesis of lipopolysaccharide; *his*, histidine; *met*, methionine; *pyr*, pyrimidine; *str*^{r}, resistance to streptomycin.

[b] F870, a rough mutant (*rfb*$^-$) of *E. coli* O8, synthesizes a K-12 LPS core, due to the introduction of *rfa* genes from the *E. coli* K-12 Hfr donor W1895 (17).

Isolation and chemical analyses of LPS. LPS of *Shigella* R mutants was isolated by the extraction procedure of Galanos et al. (3) with a mixture of phenol-chloroform-light petroleum ether. The aldoses were liberated by acid hydrolysis with 0.1 N HCl for 48 h at 100 C and converted into the alditol acetate derivatives (14) for gas-liquid chromatography. The samples were injected as chloroform solutions into a Varian aerograph (model 1520 B) fitted with a metal column (0.32 by 152 cm) at column temperatures of 165 to 175 C. Xylitol pentaacetate was used as reference compound for quantitation (19).

Bacteria grown on $D_{1,5}$-agar (16) and preextracted with phenol-chloroform-petroleum ether were subsequently treated with hot phenol-water for extraction of the phenol-chloroform-petroleum ether-insoluble O-specific hapten. The resulting aqueous phase contains, in addition to the bulk of ribonucleic acid, residual R LPS and, in certain instances, O-specific hapten and glucan. Ultracentrifugation (105,000 × *g*, 4 h) removes LPS, whereas ribonucleic acid and O-specific hapten remain in the supernatant fraction (L_1-GR, 12). The presence of the respective O-specific hapten was then examined by serological methods, e.g., by immunoelectrophoresis with the respective O serum.

Serological methods: detection of O-specific hapten. Agar gel electrophoresis was carried out by the micro-method of Scheidegger (15), using an electrophoresis chamber (Gelman Instrument Company, Ann Arbor, Mich.) and diethylbarbituric acid-sodium veronal-calcium lactate buffer (4) at 10 V/cm for 1 h. The L_1-GR lyophilisate was dissolved as a 1% solution in saline, and 5 μg was filled into the antigen well of the agar layer. Undiluted rabbit antiserum against the corresponding S form was placed into the antiserum trough. Precipitation arcs were read after incubation for 24 h at 4 C.

CA determination. The presence of CA was determined by the hemagglutination test as reported in detail previously (24, 25). Briefly, the strains were grown on 60 ml of brain veal agar in Kolle flasks for 18 h at 37 C, and the resulting growth was suspended in 25 ml of phosphate hemagglutination buffer (pH 7.3; Difco) per Kolle flask. The supernatant of the suspension, after heating at 100 C for 1 h and centrifugation at 23,500 × *g* for 20 min, was used for modification of erythrocytes. A 2.5% rabbit erythrocyte suspension was washed three times in phosphate hemagglutination buffer. The above antigen preparation was added to the sediment in amounts sufficient to restore the erythrocyte concentration of 2.5%. The antigen-erythrocyte mixture was incubated in a water bath at 37 C for 30 min, and the erythrocytes were washed to remove excess antigen. CA antiserum in twofold serial dilutions (0.2 ml) was mixed with an equal volume of antigenically modified rabbit erythrocytes. The mixtures were incubated in a water bath at 37 C for 30 min, and the resulting hemagglutination was read grossly after centrifugation at 1,300 × *g* for 2 min. Hemagglutination in titers similar to those obtained with a known CA antigen were considered as tentative evidence for the presence of CA. Confirmation was obtained in hemagglutination-inhibition tests, in which the supernatant of the culture under investigation was mixed with the CA antiserum, the latter in twofold serial dilutions. The mixtures were incubated for 30 min at 37 C, erythrocytes modified with the ethanol-soluble semipurified CA obtained from *S. typhimurium* were then added, and the hemagglutination test was completed as above. Reduction of the antibody titer by at least 75% was considered to be evidence of the presence of CA.

Immunization. To determine whether the strains under investigation evoked CA antibodies in rabbits, the following immunization schedule was used, as described previously (24). Groups of three New Zealand white rabbits were immunized intravenously with the heated (100 C, 1 h) bacterial suspension (approximately 10^9 cells/ml) harvested from a Kolle flask with brain veal agar in 25 ml of hemagglutination buffer. On days 0, 3, and 7, 1 ml of the suspension in a dilution of 1:100 was injected, and on day 10 1 ml of a dilution of 1:10 was injected. Blood samples were obtained prior to immunization and on days 7, 10, and 14 following initiation of immunization. The sera were kept frozen at −20 C until use.

Titration of CA antibodies. As described previously (24, 25), sera were titrated for the presence of CA antibodies in serial twofold dilutions and in amounts of 0.2 ml. To the serum samples were added equal amounts of erythrocytes modified by CA (ethanol-soluble fraction of *S. typhimurium*). The hemagglutination test was completed, and the specificity of the CA antibodies was confirmed by hemagglutination-inhibition tests as described above.

RESULTS

Characterization of rough mutants. The rough strains *E. coli* F470 and F614 (20) and *Shigella dysenteriae* F3126 (12) have been described previously. Their LPS cores belong to the R1 (F470, F614) and R4 (F3126) types indicated in Table 3. The other three *Shigella* R mutants listed in Table 1 were isolated as rough-looking colonies during cultivation of the respective smooth parent strains on nutrient agar. These mutants were purified by repeated isolation of single colonies. The purified isolants had the characteristics of typical R mutants, growing in fluid medium with a heavy sediment and agglutinating spontaneously in 3.5% saline solution. The core type of each of the three newly isolated *Shigella* rough mutants was identified by comparison with standard core type mutants using the same serologic methods (passive hemagglutination, serum absorption) as described previously (12). Thus, it could be shown that in passive hemagglutination tests LPS from F3160, F3161, and F3153 strongly reacted with a standard R1 antiserum and that the cells of these strains completely absorbed the hemagglutinating antibodies of antiserum prepared against the homologous R1 test strain.

Conversely, cells of the *E. coli* R1 prototype strain (F470) completely removed the antibodies against the three *Shigella* R mutants. The outcome of the serologic tests thus demonstrated the presence of an R1 LPS core in the rough *Shigella* strains F3160, F3161, and F3153.

The quantitative sugar composition of the core oligosaccharides of the various *Shigella* rough mutants was determined (Table 2). A comparison of the values for the different strains shown in Table 2 with those of the previously established R1 core type mutant F470 suggests the presence of a R1 core in F3160, F3161, and F3153. Thus, the results of chemical analyses are consistent with those of the serologic tests.

CA and its immunogenicity. None of the antisera produced against cells of the four rough *Shigella* mutants listed in Table 1 contain CA antibodies. Nevertheless, as shown by serologic determination analysis, the rough mutants, with the exception of *Shigella* F3160, can synthesize CA (Table 3). The lack of CA synthesis by F3160 explains the absence of CA immunogenicity. On the other hand, the nonimmunogenicity of CA in the *Shigella* R1 and R4 mutants (F3126, F3153, F3161) was unexpected, since certain *E. coli* mutants of these core types evoke CA antibodies in high titers. Recent observations, however, have revealed that *E. coli* R1 mutants may be non-immunogenic with regard to CA. Thus, the *E. coli* R1 mutants F470 and F614, both derived from wild-type *E. coli* E56b (O8), differ in their CA immunogenicity: F470 evokes CA antibodies and F614 does not (11). The genetical analysis of both these strains has shown that different genetic defects have led to the same rough phenotype, namely, rough mutants with the complete R1 core. In *E. coli* F470 the S/R mutation site is located within the *his*-linked *rfb* region, which controls the synthesis of the O-specific polysaccharides. The rough phenotype of F614 is caused by a defect of a *mtl*-linked gene (20), which is involved in the translocation of O-specific polysaccharides onto the complete core. This gene is apparently part of a cluster of different *rfa* genes responsible for core synthesis (20). Its equivalent in *Salmonella* is designated as *rfaL* (22). Defects in the *rfb* genes (block of O-chain synthesis) or in the *rfaL* gene result in the same rough phenotype, namely, rough mutants with a complete core. Mutants with a defective *rfaL* gene but otherwise intact *rf* genes accumulate O-specific chains (O-hapten), which can be detected by immunoelectrophoretic methods using O-specific antisera (see Materials and Methods). The *Shigella* R1 mu-

TABLE 2. *Sugar composition of LPS from Shigella R mutants*[a]

Strain	Molar ratios of neutral sugars (galactose = 2.0)				
	Galactose	Glucose	Heptose	KDO	GlcN
F3160	2.0	3.3	3.5	+	+
F3161	2.0	3.5	2.4	+	+
F3153	2.0	3.3	2.4	+	+
F470 R1 prototype	2.0	3.0	2.9	+	+

[a] GlcN is only present in the lipid A moiety, but not in the core oligosaccharide. Abbreviations: KDO, 2-Keto-3-deoxy-octonate; GlcN, D-glucosamine. Symbol: (+) present.

TABLE 3. *Characterization of different E. coli and Shigella rough mutants*

Strain	R core type	CA[a]	CA_{im}[b]	O hapten[c]	Genetic defect assumed
E. coli					
F470	R1	+	+	−	*rfb*
F614	R1	+	−	+	*rfaL*
S. dysenteriae					
F3126	R4	+	−	−	*rfaL*
F3160	R1	−	−	−	*rfe*
F3161	R1	+	−	+	*rfaL*
S. flexneri					
F3153	R1	+	−	+	*rfaL*

[a] Symbols: (+) Presence of enterobacterial common antigen (CA) as tested by passive hemagglutination; (−) absence of enterobacterial common antigen (CA) as tested by passive hemagglutination.

[b] CA_{im}, Immunogenic CA as tested by immunization of rabbits.

[c] Symbols: (+) Precipitation of aqueous solutions of L_1-GR fraction (O hapten) with specific O-antisera; (−) no precipitation. O hapten is uncharged in F614 but negatively charged in F3161 and F3153. This agrees with recent results of Dmitriev et al. (2).

tants, with the exception of F3160, can synthesize O-specific hapten (Table 3). They are considered, therefore, to have a defect in the translocation system for O-specific polysaccharides similar to that of *E. coli* F614.

The simultaneous lack of O-hapten and of CA in *Shigella* F3160 may be the result of a defect in a gene region which is equivalent to the *ilv*-linked *rfe* gene cluster found in *Salmonella* (8). These *rfe* genes participate in CA synthesis and are required also for the production of O-specific chains in certain serotypes of *Salmonella* (9, 10). The characteristics of the different rough mutants are presented in Table 3.

In the R4 mutant F3126 O-specific hapten could not be demonstrated. We assume, therefore, that this strain either carries a defect additional to a *rfaL* mutation (e.g., an *rfb* defect) or hitherto unknown mutation(s).

Genetic experiments. Transduction. The results summarized in Table 3 suggest that the inability to evoke CA antibodies of *E. coli* F614 as well as of F3153 (R1) and F3161 (R1) appears to be closely connected with a certain mutation in the LPS synthesis presumably due to a defective gene termed *rfaL* according to nomenclature of *Salmonella* genetics (22).

In unpublished experiments it was shown that, as in *Salmonella*, in *E. coli* and in *Shigella* as well, core (*rfa*) genes are cotransducible with *pyrE* by the transducing phage P1*kc*. This suggested that, analogous to *Salmonella*, a cluster of *rfa* genes presumably harboring the *rfaL* gene is located in this region. The general plan of our transduction experiments was to transfer the *pyrE*-linked *rfa* cluster of the above-mentioned R mutants into appropriate recipients. Examination of the transductants should elucidate whether they have obtained the S/R mutation site (presumably *rfaL*) of the donors and simultaneously have become nonimmunogenic with regard to CA.

The above-mentioned strains were susceptible to phage P1*kc* and thus suitable for transductions. *E. coli* F470 and the smooth parents of F3153 and F3161, *Shigella flexneri* type 6 (F3149) and *Shigella dysenteriae* type 4 (F3131), respectively, were also P1*kc* sensitive and were included in the transduction experiments for control purposes as donors with intact *rfa* genes.

The transducing phage P1*kc* was propagated on each of these strains. As recipient the rough *E. coli* F947 (*rfb*$^-$, *pyrE*$^-$) strain which has the K-12 core (see Materials and Methods) was used. From mixtures of each P1 lysate and the recipient F947 *pyrE*$^+$ transductants were selected on appropriate selective media. After single-colony purification the transductants were tested for unselected markers. Their core type (R1 or K-12) was inferred from their sensitivity patterns to appropriate phages. Thus, *pyrE*$^+$ transductants, which inherited the *rfa* genes for R1 core synthesis, should be sensitive to phages C21 and 6SR but resistant to the K-12-specific phage U3. Conversely, the K-12 core should be detected by the sensitivity of the transductants to phage U3 and resistance to phages C21 and 6SR (17). The results of the transduction experiments with each of the six donors are presented in Table 4. According to the phage reaction patterns a considerable portion of the transductants synthesize the R1 core in consequence of cotransduced donor *rfa* genes. All transductants had retained the auxotrophic markers for histidine and methionine.

LPS extracted from the R1-like transductants F2812 and F2688 were used in passive hemagglutination tests with R1 and K-12 antisera. For comparison the results with LPS of R1 strains and the K-12 recipient are included (Table 5). The results of the hemagglutination tests showed that the R1 transductants derived from smooth and rough *Shigella* donors, respectively, synthesize LPS which are serologically identical with that of the prototype R1 mutant *E. coli* F470. The serological results were confirmed by quantitative sugar analyses of LPS isolated from different R1 transductants.

CA immunogenicity of transductants. Selected *pyrE*$^+$ transductants, having received *rfa* genes from different donors for the R1 core synthesis or still having the recipient K-12 core, were used for immunization of rabbits. The antisera were tested for the presence of CA antibodies. The results given in Table 6 show that those transductants which received the

TABLE 4. *Inheritance of rfa genes of different donors (R1) in P1 transduction of pyrE*$^+$ *to recipient E. coli F947 (K-12)*[a]

pyrE$^+$ selected from donor	Total no. of transductants tested	No. of transductants with core		Transfer frequency of donor *rfa* genes (%)
		K-12	R1	
F470	123	71	52	42
F614	56	31	25	45
F3131	50	41	9	18
F3161	48	35	13	27
F3149	49	39	10	20
F3153	50	38	12	24

[a] Sensitivity to phages 6SR and C21 indicates the presence of the R1 core resulting from a transfer of the donor *rfa* genes. Sensitivity to phage U3 was taken as evidence for the presence of the recipient K-12 core.

unselected *rfa* genes of *E. coli* F614 (F1027, F1028), *Shigella* F3153 (F2868), or *Shigella* F3161 (F2688) do not evoke CA antibodies. This indicates that the non-immunogenicity of CA of these strains is indeed closely connected with a mutation within the *pyrE*-linked *rfa* cluster. The transductant F2689 retained its CA immunogenicity because *rfa* genes of the non-immunogenic donor (F3161) were not cotransduced with *pyrE*.

To ascertain whether this mutation concerns the *rfaL* allele in the respective transductants, we performed conjugation experiments with an appropriate *E. coli* Hrf donor.

Conjugation experiments. The above-mentioned transductants and their parental recipient *E. coli* F947 possess a defective *rfb* region and consequently cannot produce O-specific polysaccharides. Therefore, the introduction of the *his*-linked *rfb* region of a smooth donor into transductants with intact *rfaL* genes should result in smooth hybrids with the donor's O specificity. In case the recipients have mutated *rfaL* genes, the resulting hybrids should retain the rough phenotype.

As donor we used *E. coli* Hfr59 (O8) and used as recipients different transductants with the R1 core, including those which are assumed to have intact *rfaL* genes, e.g., CA immunogenic transductants derived from donors *E. coli* F470, *Shigella* F3131, and *Shigella* F3149, respectively.

From each mating mixture of the donor with different recipients, 50 *his*$^+$*str*^{r} recombinants were selected on minimal agar supplemented with methionine and streptomycin. After repeated single-colony purification the recombinants of the crosses with the CA non-immunogenic recipient strains F1027, F2688, and F2868 were not able to express *E. coli* O8 specificity, although they can synthesize O8-specific hapten genic recipient strains F1027, F2688, and F2868 were not able to express *E. coli* 08 specificity, although they can synthesize 08-specific hapten demonstrable by agar precipitation methods, as evident from the study of some of the recombinants. Thus, it is assumed that the three abovementioned recipients, and consequently the *his*$^+$ hybrids thereof, possess a defective *rfaL* allele previously transferred by transduction from *E. coli* F614 and *Shigella* F3161 and F3153, respectively, into *E. coli* F947. On the other hand, most of the recombinants of the crosses with the CA-immunogenic transductants F1041, F2812, and F2867 showed O8 specificity in consequence of the introduction of the *his*-linked *rfb* regions. Therefore, these recipients must have intact *rfaL* genes.

The interpretation of the results of the genetic experiments leads to the suggestion that a mutation of the *rfaL* gene is the cause for non-

TABLE 5. *Passive hemagglutination of erythrocytes coated with different R LPS*

R LPS source	Reciprocal titers in antisera[a]	
	R1	K-12
Parents		
F470 (R1)	2,560	<20
F3161 (R1)	5,120	<20
F947 (K-12)	<20	640
Transductants		
F2812 (R1)[b]	5,120	<20
F2688 (R1)[c]	2,560	<20

[a] CA antibodies absorbed previously with *Salmonella greenside*.

[b] *rfa* genes are from *S. dysenteriae* type 4 F3131 (S).

[c] *rfa* genes are from *S. dysenteriae* F3161 (R).

TABLE 6. *CA antibody titer of antisera against different pyrE*$^+$ *transductants and the recipient F947 (K-12)*

Strain	Antiserum against		Reciprocal CA antibody titer
	Donor *rfa* genes (R1)[a]	Genes from donor	
Transductants			
F1041	+	F470 *E. coli* O8 (*rfaL*$^+$)	2,560
F1027	+	F614 *E. coli* O8 (*rfaL*$^-$)	<10
F1028	+	F614 *E. coli* O8 (*rfaL*$^-$)	<10
F2812	+	F3131 *S. dysenteriae* type 4 (*rfaL*$^+$)	1,280
F2813	−	F3131 *S. dysenteriae* type 4 (*rfaL*$^+$)	640
F2688	+	F3161 *S. dysenteriae* type 4 (*rfaL*$^-$)	<10
F2689	−	F3161 *S. dysenteriae* type 4 (*rfaL*$^-$)	320
F2867	+	F3149 *S. dysenteriae* type 6 (*rfaL*$^+$)	640
F2868	+	F3153 *S. dysenteriae* type 6 (*rfaL*$^-$)	<10
Recipient			
F947	−	−	160

[a] Symbols: (+) present; (−) absent.

TABLE 7. *Serological analysis of his⁺ recombinants from crosses between E. coli Hfr59 (O8) and different recipients*

Recipients			No. of *his⁺* recombinants with phenotype[b]	
Strain no.	CA_{im}[a]	Transductant from donor	Smooth $O8^+$	Rough $O8^-$
F1027	−	F614	0	50
F1041	+	F470	46	4
F2688	−	F3161	0	50
F2812	+	F3131	48	2
F2868	−	F3153	0	50
F2867	+	F3149	45	5

[a] Immunogenic CA. Symbols: (+) injection of heated cells into rabbits does evoke CA antibodies; (−) injection of heated cells into rabbits does not evoke CA antibodies.

[b] Smooth $O8^+$ is agglutinable in O8 antiserum and non-agglutinable in 3.5% saline. Rough $O8^-$ is non-agglutinable in O8 antiserum and agglutinable in 3.5% saline.

immunogenicity of the CA in cells of *E. coli* F614, *Shigella* F3161, and *Shigella* F3153. The genetic defect of *Shigella* F3126 could not be mapped because the transducing phage P1 could not be grown on this mutant. Further experiments are needed to prove whether in this strain a similar relation exists between S/R mutation and non-immunogenicity of CA.

DISCUSSION

It has been shown recently that CA antibodies in high titers are evoked in rabbits by rough mutants having the R1 or R4 LPS core (11, 24), even when heated suspensions are used for immunization. It was, therefore, an unexpected finding that one *E. coli* R1 mutant (F614) as well as various *Shigella* R1 and R4 mutants did not stimulate the formation of CA antibodies after injection of heated cell suspensions. Previous genetic investigations have shown that the S/R mutation of *E. coli* F614 concerns a gene of the *rfa* cluster (20). Since R1 strains like *E. coli* F470, which are defective in the *his*-linked *rfb* regions, are immunogenic with regard to CA, it was postulated that the kind of S/R mutation leading to the R1 phenotype might be of importance for the expression of CA immunogenicity. Therefore, a more detailed investigation of the above-mentioned *Shigella* rough mutants was carried out to determine the nature of the S/R mutation and its possible relationship to CA immunogenicity.

The results of the genetic experiments confirmed earlier findings (20) with *E. coli* F614; namely, that its rough phenotype is caused by mutation of an *rfa* gene involved in the translocation of O-specific chains onto the core. As shown here, the defective allelic region can be cotransduced with *pyrE* together with *rfa* genes determining the R1 core synthesis.

To our knowledge there is presently no information on gene loci in *Shigella* comparable to the *rfa* genes in *Salmonella* (22) and *E. coli* (20). The transduction experiments described here with smooth and rough *Shigella* strains have demonstrated that in *Shigella*, too, the core synthesis is directed by a cluster of genes (*rfa*) closely linked to *pyrE*. It is remarkable that the frequency of cotransduced *rfa* genes from *Shigella* is lower than from *E. coli* (Table 4). This might suggest that the *pyrE* gene and the *rfa* cluster are closer linked in *E. coli* than in *Shigella*.

The results of transduction and conjugation experiments with two CA non-immunogenic *Shigella* rough mutants (F3153 and F3161) suggest that in these strains, as in *E. coli* F614, the translocation of O-chains onto the core is blocked by a mutation of a *pyrE*-linked *rfa* gene. This gene should be called *rfaL* according to the designation of the corresponding *Salmonella* gene equivalent (22).

Moreover, genetic transfer experiments have revealed that there exists a close connection between CA immunogenicity of the bacterial cells and the function of the *rfaL* gene. This relationship becomes clear considering recent findings to the effect that CA in CA-immunogenic strains is associated with the LPS molecule (13). The association between CA and LPS accounts for CA immunogenicity of the respective strains. It is conceivable that the *rfaL* gene product, which is involved in the translocation of O and T1 chains onto the core (22), also participates in the enzymatic transfer of CA onto the LPS core of CA-immunogenic strains.

The participation of LPS genes in CA synthesis (*rfe;* 9, 10) and in the expression of its immunogenicity (*rfaL*) suggests that sugars are the main constituents of this antigen. This assumption was recently corroborated by the finding that CA is a heteropolymer of D-glucosamine and D-mannosamine uronic acid, partly esterified by palmitic acid (D. Männel and H. Mayer, manuscript in preparation).

The results of the present investigation suggest that CA antibodies will be elicited by those rough mutants which are blocked in O-chain synthesis and which have a complete core (like R1, R4, or K-12) capable of accepting the CA determinant. In contrast, rough mutants with the complete R2, R3, or *Salmonella* Ra core, which are also defective in O-chain synthesis (rfb^-), fail to evoke CA antibodies when heated cell suspensions are used for immunization

(11). One may, therefore, assume that these core types are not suitable for appropriate association with CA. Interestingly, these core types contain a common characteristic constituent, namely, *N*-acetyl-D-glucosamine, in their core oligosaccharides (7, 18, 19) and differ in this respect from the other known core types such as R1, R4, and K-12 which lack this sugar (19, 21). Possibly, the absence of glucosamine in the core oligosaccharide plays an important role for its acceptor properties for CA and thus for CA immunogenicity of whole cells.

ACKNOWLEDGMENTS

We are greatly indebted to B. A. D. Stocker, Stanford, Calif., for supplying us with phage P1*kc* and S. Hofmann, Robert-Koch-Institut, D-1000 Berlin, for supplying us with the *Shigella* type strains.

The excellent technical assistance of U. Fischer, A. Gutmann, and B. Straub is gratefully acknowledged.

ADDENDUM IN PROOF

With regard to the mapping of *Shigella rfa* genes we became aware of a recent study by C. Godard and E. Hannecart-Pokorni (Arch. Int. Phys. Biochem. 83:20–21, 1975) in which the R mutation site affecting the LPS core synthesis of an *S. flexneri* rough mutant was determined to be located near the *mtl* region. This is in accordance with our observations that *Shigella rfa* genes are closely linked to *pyrE*, which in turn is situated near the *mtl* genes.

LITERATURE CITED

1. **Adams, M. H.** 1959. Bacteriophages, p. 461–469. Interscience Publishers, Inc., New York.
2. **Dmitriev, B. A., L. V. Backinowsky, V. L. Lvov, N. K. Kochetkov, and I. L. Hofman.** 1975. Somatic antigen of *Shigella dysenteriae* type 3. Structural features of specific polysaccharide chain. Eur. J. Biochem. 50:539–547.
3. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for extraction of R lipopolysaccharides. Eur. J. Biochem. 9:245–249.
4. **Hirschfeld, J.** 1960. Immunoelectrophoresis procedure and application to the study of group-specific variations in sera. Sci. Tools (LKB, Stockholm) 7:18–25.
5. **Kunin, C. M., M. V. Beard, and N. E. Halmagyi.** 1962. Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other *Enterobacteriaceae*. Proc. Soc. Exp. Biol. Med. 111:160–166.
6. **Lederberg, J.** 1950. Isolation and characterization of biochemical mutants of bacteria. Methods Med. Res. 3:5–22.
7. **Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido.** 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145–224. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 4. Academic Press Inc., New York.
8. **Mäkelä, P. H., M. Jahkola, and O. Lüderitz.** 1970. A new gene cluster *rfe* concerned with the biosynthesis of *Salmonella* lipopolysaccharide. J. Gen. Microbiol. 60:91–106.
9. **Mäkelä, P. H., and H. Mayer.** 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis in *Salmonella* groups B and C_1. J. Bacteriol. 119:765–770.
10. **Mäkelä, P. H., H. Mayer, H. Y. Whang, and E. Neter.** 1974. Participation of lipopolysaccharide genes in determination of the enterobacterial common antigen: analysis of R mutants of *Salmonella minnesota*. J. Bacteriol. 119:760–764.
11. **Mayer, H., and G. Schmidt.** 1971. Hämagglutinine gegen ein gemeinsames Enterobakteriaceen-Antigen in *E. coli* R1-Antiseren. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 216:299–313.
12. **Mayer, H., and G. Schmidt.** 1973. The occurrence of three different lipopolysaccharide cores in *Shigella* and their relationship to known enterobacterial core types. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 224:345–354.
13. **Mayer, H., G. Schmidt, H. Y. Whang, and E. Neter.** 1972. Biochemical basis of the immunogenicity of the common enterobacterial antigen. Infect. Immun. 6:540–544.
14. **Sawardeker, J. S., H. J. Sloneker, and A. Jeans.** 1967. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Anal. Chem. 37:1602–1604.
15. **Scheidegger, J. J.** 1955. Une micro-méthode de l'immuno-electrophorèse. Int. Arch. Allergy Appl. Immunol. 7:103–110.
16. **Schlecht, S., and O. Westphal.** 1966. Wachstum und Lipopolysaccharid-Gehalt von Salmonellen bei Züchtung auf Agarnährböden. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 200:241–259.
17. **Schmidt, G.** 1973. Genetical studies on the lipopolysaccharide structure of *Escherichia coli* K-12. J. Gen. Microbiol. 77:151–160.
18. **Schmidt, G., I. Fromme, and H. Mayer.** 1970. Immunochemical studies on core lipopolysaccharides of *Enterobacteriaceae* of different genera. Eur. J. Biochem. 14:357–366.
19. **Schmidt, G., B. Jann, and K. Jann.** 1969. Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Different core regions in the lipopolysaccharides of O group 8. Eur. J. Biochem. 10:501–509.
20. **Schmidt, G., B. Jann, and K. Jann.** 1970. Studies on R mutants with an incomplete core, derived from *Escherichia coli* O8:K27. Eur. J. Biochem. 16:382–392.
21. **Schmidt, G., B. Jann, and K. Jann.** 1974. Genetic and immunochemical studies on *Escherichia coli* O14:K7:H^-. Eur. J. Biochem. 42:303–309.
22. **Stocker, B. A. D., and P. H. Mäkelä.** 1971. Genetical aspects of biosynthesis and structure of *Salmonella* lipopolysaccharide, p. 396–433. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 4. Academic Press Inc., New York.
23. **Watson, G., and K. Paigen.** 1971. Isolation and characterization of an *Escherichia coli* bacteriophage requiring cell wall galactose. J. Virol. 8:669–674.
24. **Whang, H. Y., H. Mayer, G. Schmidt, and E. Neter.** 1972. Immunogenicity of the common enterobacterial antigen produced by smooth and rough strains. Infect. Immun. 6:533–539.
25. **Whang, H. Y., and E. Neter.** 1967. Further studies on effect of endotoxin on antibody response of rabbit to common antigen of *Enterobacteriaceae*. J. Immunol. 98:948–957.