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Expression of Lipopolysaccharide O Antigen in *Escherichia coli* K-12 Hybrids Containing Plasmid and Chromosomal Genes from *Shigella dysenteriae* 1

THOMAS L. HALE,[1]* PATRICIA GUERRY,[1]† ROBERT C. SEID, JR.,[1] CHRISTINE KAPFER,[1] MICHELE E. WINGFIELD,[1] CARL B. REAVES,[1] LOUIS S. BARON,[2] AND SAMUEL B. FORMAL[1]

Departments of Bacterial Diseases[1] and Bacterial Immunology,[2] Walter Reed Army Institute of Research, Washington, D.C. 20307

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The requirement for both plasmid and chromosomal genes in the biosynthesis of *Shigella dysenteriae* 1 lipopolysaccharide O antigen was demonstrated in *Escherichia coli-Shigella* hybrids. A 6-megadalton *S. dysenteriae* 1 plasmid, designated pWR23, was phenotypically tagged with the Tn*3* ampicillin-resistance transposon. The tagged plasmid, designated pWR24, was transferred by transformation or conjugal mobilization to a rough *E. coli* K-12 recipient. Although the resultant hybrids were agglutinated in *S. dysenteriae* 1 antiserum, they did not remove all of the anti-Shiga agglutinins in absorption experiments. Modified lipid A core structure was detected in these hybrids, but Shiga O antigen was not expressed. When the *his*$^+$ locus of the *S. dysenteriae* 1 chromosome was transferred by transduction to *E. coli* K-12 containing pWR24, complete Shiga O antigen was expressed. Lipopolysaccharide extracted from these hybrids was indistinguishable chemically, electrophoretically, and serologically from native *S. dysenteriae* 1 lipopolysaccharide.

Information concerning the location of genes which control the expression of the somatic antigens of shigellae is of importance for the construction of living oral vaccines against bacillary dysentery. Studies in the past have shown that transfer of the group and type antigens from *Shigella flexneri* 2a to *Escherichia coli* K-12 is associated with the *his*$^+$ and *pro*$^+$ chromosomal markers (4, 18). In the case of *Shigella sonnei*, however, the genes controlling the expression of the form I antigen are located on a 120-megadalton (Mdal) plasmid (10, 17, 19). Recently Watanabe and Timmis (23) have made the important observation that a 6-Mdal plasmid is involved in the expression of the somatic antigen of *Shigella dysenteriae* 1. Variants which have lost this plasmid do not express the antigen and are avirulent. Transfer of the plasmid back to the rough variant restores virulence and the ability to express *S. dysenteriae* 1 antigen. The purpose of this communication is to present evidence that the expression of the *S. dysenteriae* 1 somatic antigen by *E. coli* K-12 depends not only on the transfer of a 6-Mdal plasmid but also on a chromosomal region cotransducible with the *his*$^+$ marker from *S. dysenteriae* 1.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in genetic experiments are listed in Table 1. In addition to the strains in Table 1, strain 43A15, a wild-type strain of *S. dysenteriae* type 1 from the stock collection of Walter Reed Army Institute of Research, was used to raise standard Shiga rabbit antiserum.

Plasmid DNA isolation. Plasmid DNA for analysis by agarose gel electrophoresis was prepared by a modification of the method of Casse et al. (1). When used in bacterial transformations, plasmid DNA was prepared by the method of Clewell and Helinski (2). Plasmid DNA was examined in 0.7% agarose slab gels as described previously (10). Plasmid size was estimated from the electrophoretic mobility of reference plasmids contained in *E. coli* V517 (9).

Bacterial transformations. *E. coli* cells were transformed with plasmid DNA by the method of Kushner (11). Transformants were selected on tryptic soy agar supplemented with ampicillin (20 μg/ml).

Bacteriophage. Phage P1CMclr100 was obtained from J. L. Rosner (16). Lysates were prepared by thermal induction of appropriate lysogens in L broth at 40°C for 35 min, and these were titrated on *E. coli* strain 395-1.

Antisera. The antisera were prepared by inoculating rabbits with living organisms. Before use in slide agglutination tests, all antisera were absorbed with whole cells of *E. coli* K-12 strain 395-1.

Gel electrophoresis of LPS. The Westphal and Jann procedure (24) was used to extract lipopolysaccharides (LPS), and 2 μg of LPS from each strain was loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gels prepared by the method of Laemmli (12). LPS was visualized by a silver-staining procedure (22). Electrophoretic-transfer (Western) blotting was performed essentially as described by Peppler (15). Nitrocellulose dot blotting was performed by spotting a 10% SDS lysate of either a bacterial-cell pellet or a cultural supernatant on nitrocellulose paper and developing by the standard Western blot procedure (15).

Sugar analysis of LPS. A partition chromatography system with a cationic-exchange resin column in the Li^+ form and 90% ethanol as the mobile phase was used for analysis of neutral monosaccharides in hydrolyzed LPS samples (21).

RESULTS

Phenotypic tagging and transfer of pWR24 to *E. coli* K-12 strain 395-1. A 6-Mdal plasmid of *S. dysenteriae* 1, designated pWR23, was tagged with the ampicillin-resistance transposon Tn*3*. *E. coli* HU679 (F'*ts114lac*::Tn*3*), which served as the Tn*3* donor, was mated with *S. dysenteriae* 1 strain

* Corresponding author.
† Present address: Naval Medical Research Institute, Bethesda, MD 20814.

TABLE 1. Bacterial strains and plasmids

Species and strain	Relevant characteristics[a]	Plasmids
Escherichia coli		
395-1[b]	*lac proA2 leu argE3 his-4 rfbB*	
HU679	*trp leu thi* Ap	F'*ts114lac*::Tn*3*
J53	*pro met* Tc	R386
Shigella dysenteriae 1		
1617	*met thr ser trp nad*	Two large plasmids and pWR23
JVA105-5	*met thr ser trp nad*	Two large plasmids and a 6-Mdal plasmid

[a] *pro*, *leu*, *arg*, *his*, *trp*, *thi*, *met*, *thr*, *ser*, and *nad* are mutations imposing requirements for proline, leucine, arginine, histidine, tryptophane, thiamine, methionine, threonine, serine, and nicotinic acid, respectively; *lac* is lactose nonutilizing; Ap and Tc are plasmid-borne resistance to ampicillin and tetracycline.

[b] *E. coli* 395-1 is a nalidixic acid-resistant variant of K-12 strain WB1133.

1617 (Fig. 1, lane A), and strains with the transposon inserted in pWR23 were isolated as described by Watanabe and Timmis (23). One of these strains (7558-1-2-4 [Fig. 1, lane B]) with a tagged plasmid designated pWR24 was chosen as a plasmid donor. Since the Ap marker in strain 7558-1-2-4 was not self-transmissible, it was mobilized with pR386 carrying the Tc marker. The latter plasmid was first transferred from *E. coli* J53 to strain 7558-1-2-4 by conjugation and selection on medium containing tetracycline. The resultant strain, 7591-3-2, was mated with *E. coli* 395-1, and transconjugant EC100 was selected on medium containing ampicillin. This transconjugant harbored plasmids pWR24 and pR386. The Ap marker was also transferred to strain 395-1 by transformation with plasmid DNA from strain 7558-1-2-4 and selection on medium containing ampicillin. The resulting transformant, designated EC101, harbored only plasmid pWR24. *E. coli* hybrids which had acquired pWR24 by transformation (see Table 3) or by mobilization (data not shown) were agglutinated in antiserum against *S. dysenteriae* 1 strain 43A15 which had been absorbed with strain 395-1 to remove cross-reacting *E. coli* agglutinins. When the Shiga antiserum prepared against strain 43A15 was absorbed with both *E. coli* K-12 strain 395-1 and transformant strain EC101, it no longer agglutinated transconjugant strain EC100, but it did continue to agglutinate the wild-type *S. dysenteriae* 1 strain 1617.

Transfer of chromosomal markers to *E. coli* K-12(pWR24) strains by conjugation. The above experiments indicated that transfer of pWR24 to *E. coli* K-12 did not result in the full expression of *S. dysenteriae* 1 surface antigens; therefore, the role of chromosomal genes was investigated. In preliminary experiments, the *S. dysenteriae* 1 *his*$^+$ region was transferred by conjugation from Hfr strain JVA70-105 to *E. coli* K-12 strain 395-1. These recombinants failed to react in *S. dysenteriae* 1 antiserum which had been absorbed with strain 395-1. Lysates and cultural supernatants of these strains also failed to react with the absorbed strain 43A15 antiserum in a nitrocellulose dot blot assay. Apparently, no *S. dysenteriae* 1 O antigen was synthesized by *E. coli* transconjugants which had incorporated the His$^+$ chromosomal marker. However, conjugal mobilization of pWR24 into these strains resulted in transconjugants which reacted in the dot blot assay and agglutination tests just as did a wild-type *S. dysenteriae* 1 strain (data not shown).

To determine whether chromosomal regions other than the *his*$^+$ region would also complement pWR24 and allow Shiga O antigen expression, the *pro*$^+$, *arg*$^+$, *leu*$^+$, or *leu*$^+$ *arg*$^+$ chromosomal regions were transferred to EC101 (containing pWR24) by conjugal mating with *S. dysenteriae* 1 Hfr strain JVA70-105. In serological tests, these recombinants were identical to strains harboring only the plasmid. One of these strains, 7589-2-1 (*leu*$^+$ *arg*$^+$, pWR24), was remated with *S. dysenteriae* 1 Hfr strain JVA70-105, and selections were made for histidine independence. Of 10 His$^+$ hybrids, 9 agglutinated in antiserum against strain 43A15 which had been absorbed with plasmid-containing strain EC101. These strains were indistinguishable from wild-type *S. dysenteriae* 1 strain 1617 in serological tests, and they absorbed all agglutinins for strain 1617 from the antiserum against *S. dysenteriae* 1 strain 43A15. In addition, an antiserum prepared against one of these strains, 7592-2D-1, agglutinated *S. dysenteriae* 1 strain 1617. Absorption of this serum with strain 1617 and *E. coli* K-12 strain 395-1 removed all agglutinins for 7592-2D-1 (data not shown).

Transfer of the His$^+$ trait by transduction. The conjugation experiments described above indicated that, in addition to pWR24, a chromosomal region associated with the *his*$^+$ locus was necessary for expression of *S. dysenteriae* 1 somatic antigen. To show that this locus is closely linked to the *his*$^+$ region and that no disparate chromosomal regions were involved, the *Shigella his*$^+$ locus was transferred to an *E. coli* K-12 strain by transduction.

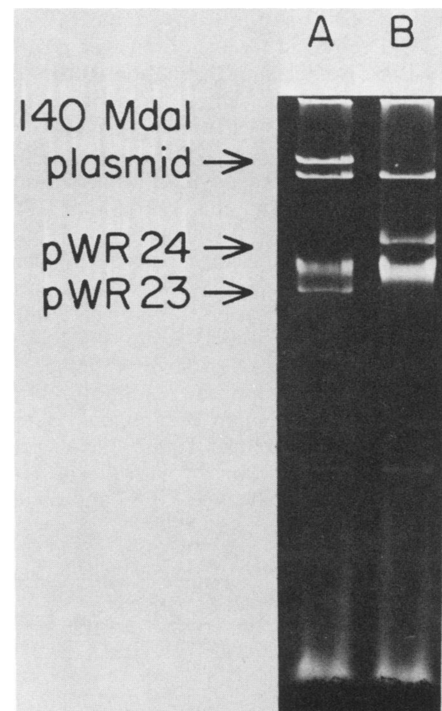

FIG. 1. Agarose gel electrophoresis of plasmid DNA extracted from *S. dysenteriae* 1. (A) Wild-type strain 1617 with the 140-Mdal virulence-associated plasmid and the 6-Mdal pWR23 identified; (B) strain 7558-1-2-4 with pWR24 (pWR23 tagged with Tn*3*) identified. The latter strain lost the 140-Mdal plasmid upon incubation at 42°C (23), but it retained the smooth LPS phenotype and subsequently served as a donor of pWR24.

TABLE 2. Description of *E. coli* K-12 hybrid strains

Strain	Expt no.	Genotype	Relevant phenotype
EC100	7600-2-1	pWR24,[a] R386[a]	Ap, Tc
EC101	7586-6-2	pWR24[b]	Ap
EC102	7600-2-1T	pWR24,[a] R386[a] *his*$^{+c}$	Ap, Tc, His$^+$
EC103	7630-1	pWR24[b] *his*$^{+c}$	Ap, His$^+$

[a] Plasmids acquired by conjugal mobilization.
[b] Plasmid acquired by transformation.
[c] Chromosomal region acquired by transduction.

To transduce these *his*$^+$-linked *S. dysenteriae* 1 genes, *E. coli* K-12 transconjugant strain 7592-2D-1, which had inherited the His$^+$ characteristic from *S. dysenteriae* 1, was lysogenized with the thermoinducible mutant phage P1CMclr100 isolated by Rosner (16). A lysate of 10^9 particles per ml was prepared by thermal induction of this lysogen, and this was used to transduce the *his*$^+$ genes to pWR24-containing strains EC101. Over 90% of the His$^+$ transductants agglutinated in the strain 43A15 serum which had been adsorbed with pWR24-containing strain EC101. Two of these transductants were chosen for serological analysis. Strain EC103 (derived from EC101) had acquired both pWR24 and *his*$^+$ by transduction. The other strain (EC102) was derived from EC100, so it had acquired pWR24 and pR386 by conjugal mobilization and *his*$^+$ by transduction (see Table 2). The agglutination reactions of EC102 and EC103 were identical, so only EC103 is shown in Table 3. These hybrids were serologically indistinguishable from wild-type *S. dysenteriae* 1 strain 1617. In addition, an antiserum prepared against strain EC103 agglutinated strain 1617. Absorption of this serum with strains 1617 and *E. coli* K-12 strain 395-1 removed all agglutinins for EC103.

Analysis of LPS. Bacterial agglutination studies described above indicated that some Shiga antigenic determinants were expressed on the surface of *E. coli* hybrids harboring pWR24. To determine if these were somatic antigens, LPS was extracted and separated on SDS-polyacrylamide gels. The single silver-stained band characteristic of LPS from *E. coli* K-12 is shown in Fig. 2A, lane 1 (7). This band is composed of lipid A-core oligosaccharide molecules, and antibody raised against strain 395-1 was bound to this band in an immunoblot or Western blot assay (data not shown). When pWR24 was transferred to strain 395-1 by conjugal mobilization, a slightly heavier silver-staining band was apparent in LPS extracted from the resultant hybrid EC100. This band (Fig. 2A, lane 2) carried antigenic determinants which were unique to the transformant because antiserum raised against EC100 and absorbed with *E. coli* K-12 strain 395-1 reacted only with the heavier LPS band on a Western

TABLE 3. Slide agglutination reactions of parental and hybrid strains in absorbed antisera

Strain	Antisera[a]: 43A15 (395-1)	EC101 (395-1)	EC103 (395-1)	43A15 (395-1 and EC101)	43A15 (395-1 and EC103)
395-1	−	−	−	−	−
EC101	+	+	+	−	−
EC103	+	−	+	+	−
1617	+	−	+	+	−

[a] Eliciting antigen (absorbing antigen[s]).

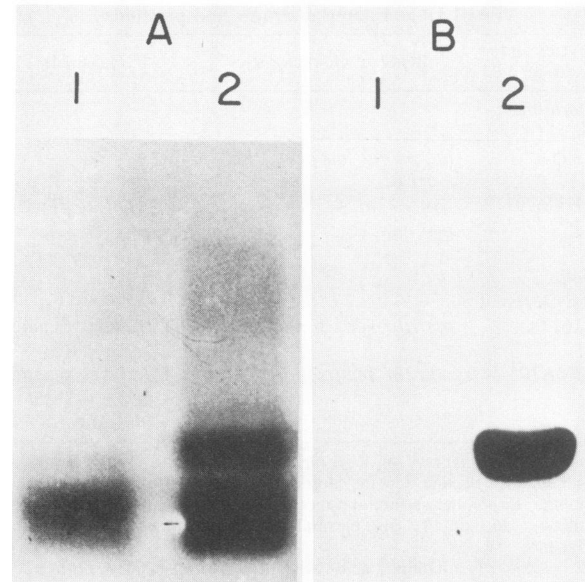

FIG. 2. SDS-polyacrylamide gel electrophoresis of LPS extracted from *E. coli* K-12 strain 395-1 (lane 1) or *E. coli* K-12 hybrid strain EC100 with pWR24 (lane 2). (A) Silver-stained gel; (B) Western blot with anti-EC100 absorbed with whole cells of strain 395-1. Only the heavier band of EC100 LPS was recognized by the latter antiserum.

blot (Fig. 2B, lane 2). This absorbed antiserum did not bind to any LPS determinants expressed by EC102 or *S. dysenteriae* 1 strain 1617 (data not shown). Quantitative sugar analysis showed that only the core sugars glucose, galactose, and heptose were present in LPS from strain EC100 (Fig. 3B), and the relative proportions of these sugars were similar to those seen in the *E. coli* K-12 parent (Fig. 3A).

These data indicate that acquisition of pWR24 by *E. coli* K-12 was associated with additions to the K-12 core which made it heavier and antigenically unique. The modifications were not chemically characteristic of O antigen repeat units of *S. dysenteriae* 1 side chains, and LPS extracted from hybrid EC100 did not bind antibodies evoked by *S. dysenteriae* 1 strain 43A15 (Fig. 4B, lane 1). Apparently agglutinins in Shiga antiserum did not recognize somatic antigens on hybrids such as EC100 and EC101. However, they may have recognized plasmid-coded outer membrane proteins (8).

When the *his*$^+$ chromosomal region was transduced from *S. dysenteriae* 1 to strain EC100, the resulting *E. coli* hybrid (EC102) expressed electrophoretically heterogeneous LPS components. LPS extracted from EC102 was resolved into 18 silver-staining bands representing lipid A-core oligosaccharide molecules and lipid A-core molecules with up to 17 O-side chain repeat units affixed (Fig. 4A, lane 3). A predilection for polysaccharides containing 9 to 15 repeat units was observed. Quantitative sugar analysis revealed a preponderance of rhamnose and galactose in LPS extracted from strain EC102 (Fig. 3C). The electrophoretic profile (Fig. 4A, lane 2) and sugar analysis (data not shown) of LPS extracted from *S. dysenteriae* 1 strain 1617 were very similar to that of EC102. Western blot analysis indicated that the O-side chain components of the latter hybrid were recognized by antiserum against *S. dysenteriae* 1 strain 43A15 which had been absorbed with *E. coli* K-12 strain 395-1 (Fig. 4B, lane 3). Data obtained with hybrid strains EC100 and EC102 were duplicated in experiments with transformant EC101 (containing pWR24) and with hybrid EC103, containing the

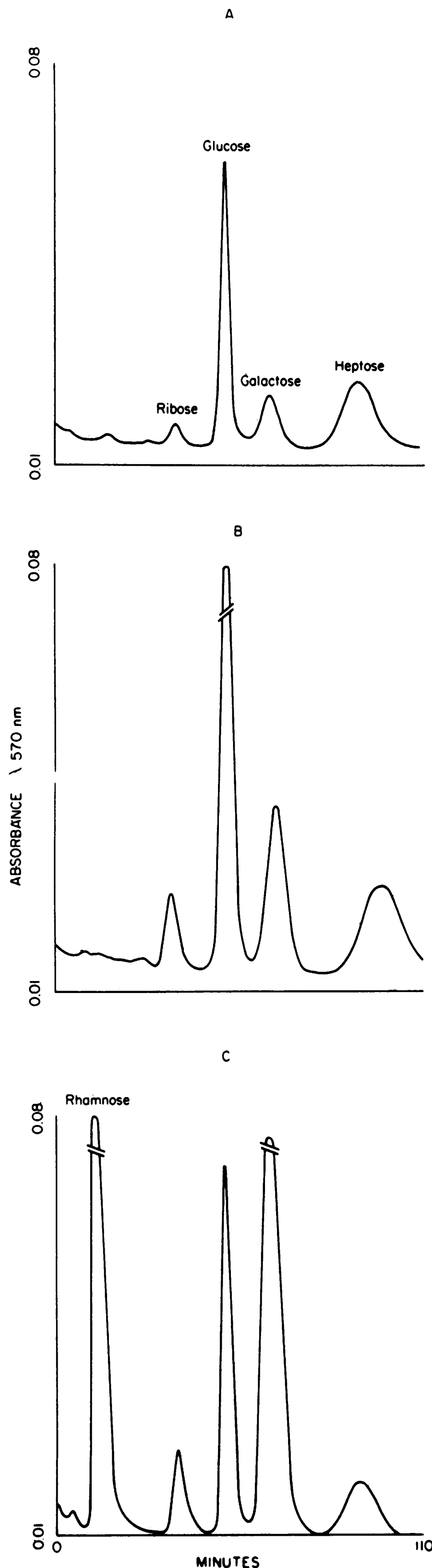

FIG. 3. Chromatogram of LPS hydrolysates from (A) *E. coli* K-12 strain 395-1, (B) *E. coli* K-12 hybrid strain EC100, and (C) *E. coli* K-12 hybrid strain EC102. Separated monosaccharide peaks were identified by chromatography of authentic standards as described earlier (14).

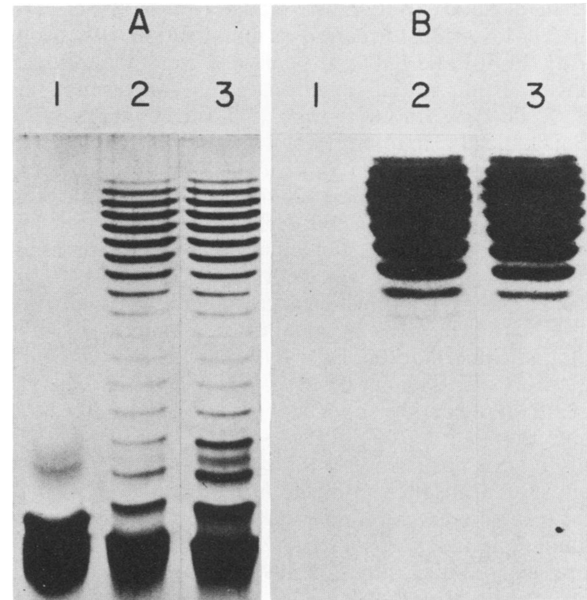

FIG. 4. SDS-polyacrylamide gel electrophoresis of LPS from *E. coli* K-12 hybrid strain EC100 (lane 1), from *S. dysenteriae* 1 strain 1617 (lane 2), and from EC102 with *S. dysenteriae* 1 his^+ chromosomal locus plus pWR24 (lane 3). (A) Silver-stained gel; (B) Western blot with anti-43A15 (wild-type *S. dysenteriae* 1) absorbed with *E. coli* strain 395-1.

transformed pWR24 and the transduced his^+ region from *S. dysenteriae* 1 strain 1617 (data not shown).

DISCUSSION

Recently Watanabe and Timmis (23) have shown that genes located on a 6-Mdal plasmid are necessary for the expression of somatic antigen in *S. dysenteriae* 1. Loss of this plasmid upon cultivation at 42°C is associated with lack of O antigen expression and decreased virulence. These characteristics are restored in transformants which have acquired a 9.2-Mdal derivative plasmid (pHW401) consisting of the 6-Mdal plasmid and the Tn*801* ampicillin-resistance transposon. Transformation of *E. coli* HB101 with pHW401 results in clones which weakly agglutinate in *S. dysenteriae* 1 antiserum. These data indicate that Shiga antigen(s) expressed in *E. coli* transformants are either qualitatively or quantitatively different from those expressed in authentic *S. dysenteriae* 1 strains. Therefore, we have further investigated the expression of O antigen in *E. coli* K-12 containing the small *S. dysenteriae* 1 plasmid tagged with the Tn*3* ampicillin-resistance transposon (pWR24).

E. coli K-12 strain 395-1 was used as a recipient because we have used it in this capacity previously (4, 18), and it is the recipient in an *E. coli*-*S. flexneri* hybrid vaccine which we have recently constructed (5). Unfortunately, strain 395-1 is a substrain of strain W12 which has a defect in the *rfbB* locus (14). This defect prevents synthesis of TDP-rhamnose, an intermediate in the construction of rhamnose-containing O-side chains. Initial experiments indicated that transfer of pWR24 to *E. coli* K-12 strains which did not have an *rfbB* defect (J53, RC711, and W1895) did not result in the expression of *S. dysenteriae* 1 O-side chains. Therefore, the inability of pWR24 to promote full expression of O antigen in strain 395-1 was not due solely to the inability of the recipient to synthesize rhamnose intermediates.

When plasmid pWR24 was transferred to *E. coli* K-12 strain 395-1 by transformation or mobilization, the resultant hybrids, EC101 and E100, respectively, were phenotypically identical. Both strains agglutinated in antiserum raised against wild-type *S. dysenteriae* 1, but they could not absorb all Shiga-specific antibody from this serum. Western blot analysis indicated that the *Shigella* agglutinins did not recognize hybrid LPS determinants. Nonetheless, analysis of LPS extracted from either EC100 or EC101 showed that molecules of slightly different molecular mass were expressed by both of these hybrids and that these molecules carried antigenic determinants which were unique to rough *E. coli* hybrids harboring pWR24. Quantitative analysis of neutral sugars indicated that this LPS was chemically similar to the lipid A-core oligosaccharide of the *E. coli* K-12 parent. Thus, the LPS modification induced by these plasmids did not involve the addition of Shiga O-specific side chains. When rough hybrids harboring pWR24 were converted to smooth strains after transduction of the *his*$^+$ region from *S. dysenteriae* 1, the lipid A-core molecule was further modified, and the unique antigenic specificity was lost.

A possibly similar situation has been reported in the LPS phase transition of *S. sonnei* (6). In this case, the core of rough *S. sonnei* phase II organisms is modified when O-specific side chains are added. The modifications involve the loss of a pyrophosphorylethanolamine group and the addition of a terminal α-D-glucosaminyl residue. By analogy, it could be speculated that one of the functions of genes on pWR24 is to prepare the LPS core to accept *S. dysenteriae* 1 O-specific side chains and that the addition of side chains accompanies further core modifications. If these modifications involved amino groups, as is the case in *S. sonnei* phase transitions, they would not have been detected by the sugar analysis used in the current study.

Dmitriev et al. (3) have concluded that *S. dysenteriae* 1 O-specific polysaccharide side chains consist of repeating tetrasaccharide units of L-rhamnose, D-galactose, and *N*-acetyl-D-glucosamine in a 2:1:1 ratio. Therefore, quantitative sugar analysis of smooth *S. dysenteriae* 1 LPS would be expected to reveal a large proportion of rhamnose and galactose. *E. coli* hybrid strains EC102 and EC103 (containing pWR24 and the *S. dysenteriae* 1 *his*$^+$ chromosomal region) both yielded these two sugars in large quantities compared with the quantities of glucose and heptose core sugars. It should be noted that *N*-acetyl-D-glucosamine could not be quantitated by the methods used and that mannose cannot be differentiated from glucose by the chromatographic procedure. Nonetheless, the biochemical data supported the contention that the 18 silver-staining bands resolved by polyacrylamide gel electrophoresis of LPS extracted from strains EC102 and *S. dysenteriae* 1 1617 represent lipid A-core molecules with up to 17 identical O-side chain repeat units. Serological and immunoblot analysis also supported this contention.

Intergroup crosses of *Salmonella* spp. (13) and *Shigella* spp. (4) have shown that nearly all of the specific information necessary for O-side chain synthesis is contained in the *rfb* cluster near *his*. This cluster includes the previously mentioned *rfbB* locus, galactose-1-*p*-transferase, and probably other glycosyl transferases which determine the particular conformation of the O repeat unit. However, a dot blot of an *E. coli* hybrid containing the *S. dysenteriae* 1 *his*$^+$ region revealed no Shiga O-side chain biosynthesis. This indicated that the 6-Mdal plasmid was necessary not only for core modification but also for the synthesis of O-side chain monomers.

It is now apparent that the organization of genetic information necessary for synthesis of complete LPS differs in various *Shigella* species. In the current report, it was shown that both the 6-Mdal plasmid and the *his*$^+$ chromosomal region are necessary to induce expression of *S. dysenteriae* 1 somatic antigen in *E. coli*. A 120-Mdal plasmid is sufficient to transfer the expression of form I somatic antigen of *S. sonnei* to *E. coli* K-12 (unpublished data). In contrast to the plasmid involvement discussed above, synthesis of *S. flexneri* 2a group and type somatic antigens in *E. coli* K-12 is solely dependent on chromosomal determinants in the *his*$^+$ and *pro*$^+$ regions (18, 20). Location of genes necessary for the synthesis of *Shigella* somatic antigens will allow construction of *Shigella-E. coli* hybrids expressing these determinants, and it is hoped that such hybrids will prove useful as live oral vaccines which will evoke immunity against the various *Shigella* serotypes.

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