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Small Virulence Plasmid of *Shigella dysenteriae* 1 Strain W30864 Encodes a 41,000-Dalton Protein Involved in Formation of Specific Lipopolysaccharide Side Chains of Serotype 1 Isolates

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A 6-megadalton plasmid, pHW400, of *Shigella dysenteriae* 1 strain W30864 was previously found to specify one or more functions for O-antigen production and bacterial virulence (H. Watanabe and K. N. Timmis, Infect. Immun. 43:391–396, 1984). The region of pHW401, a Tn*801*-tagged derivative of pHW400, responsible for O-antigen production has been localized by gene cloning and Tn*5* transposon mutagenesis. Analysis of lipopolysaccharide isolated from *S. dysenteriae* 1 bacteria carrying mutant plasmids revealed that the determinant for O-antigen synthesis, designated *rfp*, codes for a function involved in the formation of the O-polysaccharide side chain structure of lipopolysaccharide. Analysis of radioactively labeled proteins synthesized in minicells of *Escherichia coli* carrying mutant plasmids identified the product of the *rfp* gene as a 41,000-dalton protein. Southern hybridization with a DNA fragment carrying the *rfp* gene demonstrated that this determinant is present on 6-megadalton plasmids in other isolates of *S. dysenteriae* 1 but is not present at all in a variety of other *Shigella*, *E. coli*, and *Salmonella typhimurium* strains that were tested.

Shigella spp. and some strains of *Escherichia coli* cause bacillary dysentery, an acute but self-limiting colitis whose severity ranges from a mild inflammation to the formation of diffuse ulcerative lesions. The essential virulence features of dysentery-producing bacteria are their ability to penetrate, multiply within, and kill epithelial cells of the colon, to spread to and kill adjacent cells, and thereby to provoke an acute inflammatory reaction (11). Genetic studies on *Shigella flexneri* have revealed that virulence is multifactorial and requires genetic determinants located on a large plasmid and at least three widely separated regions of the bacterial chromosome. The 140-megadalton (Mdal) virulence plasmid encodes a function for bacterial penetration of epithelial cells (20); the histidine (*his*) region of the chromosome encodes O-antigen production (6); the xylose (*xyl*)-rhamnose (*rha*) region encodes a function for bacterial survival in the mucosa (8); and the purine E (*purE*) region specifies a function needed for the provocation of keratoconjunctivitis in the guinea pig eye (Serény test; 7). By means of the stepwise conjugal transfer of these genetic determinants from *S. flexneri*, Sansonetti et al. (18) were able to construct derivatives of an *E. coli* K-12 strain which exhibited the typical pathogenicity of *Shigella* spp. as measured in a variety of laboratory models.

The O-antigen, until recently suspected of being an essential virulence factor of *Shigella* spp. (17, 18), is specified by chromosomal genes in *S. flexneri* but by genes on a 120-Mdal plasmid in *Shigella sonnei* (15, 19). Recently, it was shown that a 6-Mdal plasmid, pHW400, of *Shigella dysenteriae* 1 strain W30864 (24) specifies one or more (but not all) lipopolysaccharide (LPS) biosynthesis functions. In this communication, we describe experiments that localized an LPS determinant, designated *rfp*, of plasmid pHW400, demonstrated it to be an essential virulence determinant, and identified its product as a 41,000-dalton protein. The *rfp* gene was shown to be present on similar sized plasmids in other *S. dysenteriae* 1 isolates but absent from the genetic information carried by other *Shigella* serotypes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. dysenteriae* 1 strains 47-80, 11-81, and W30864 and its O-antigen-negative derivative W30864-22, which lacks the 6-Mdal O-antigen plasmid pHW400, have been described previously (24). Other isolates of *Shigella* spp., *Salmonella typhimurium*, and *E. coli* used for the hybridization experiments were from our stocks. *E. coli* K-12 strain C600::Tn*5* (9) is a C600 (2) derivative which carries the kanamycin resistance transposon Tn*5* in its chromosome. P678-54 (1) is a standard minicell-producing strain of *E. coli* K-12. Plasmid pHW401 (24) is a derivative of plasmid pHW400 tagged with the ampicillin resistance transposon Tn*801*. Plasmid pACYC184 is a vector plasmid that encodes resistance to chloramphenicol and tetracycline and that contains a unique *Bam*HI cleavage site within its tetracycline resistance gene (4). Plasmid pACYC177 is a related vector plasmid that encodes resistance to kanamycin and ampicillin and that contains a unique *Pst*I cleavage site within its ampicillin resistance gene (4).

Media and culture conditions. Bacteria were routinely grown at 37°C in L-broth or on plates of L-agar or antibiotic medium no. 3 (Difco Laboratories, Detroit, Mich.) solidified with 1.5% agar (Difco). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 6 μg/ml. Sulphur-free Hershey salts medium (25), which was used in minicell experiments, contained 0.1 M Tris-hydrochloride (pH 7.4)–0.09 M NaCl–0.04 M KCl–0.02 M NH_4Cl–0.64 mM KH_2PO_4–0.1 mM $CaCl_2$–1 mM $MgCl_2$–0.2% (wt/vol) glucose.

Serological tests. The production of *S. dysenteriae* 1 O-antigen was determined by slide agglutination with *S. dysen-*

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teriae 1 type-specific antiserum preabsorbed with W30864-22 bacteria.

Virulence assay. The Serény test (21) was used to determine bacterial virulence. Twenty microliters (5×10^8 cells) of a dense bacterial suspension in phosphate-buffered saline was instilled into the conjunctival sac of a guinea pig, and the animal was observed daily for 6 days. A reaction was considered to be positive when the cornea became opaque and conjunctivitis developed within 3 days.

Detection of LPS. LPS was detected by the procedure of Hitchcock and Brown (13). In brief, pellets of bacteria (2×10^8 cells) were solubilized in 50 μl of lysing buffer containing 2% sodium dodecyl sulfate (SDS)–4% 2-mercaptoethanol–10% glycerol–1 M Tris-hydrochloride (pH 6.8)–0.001% bromophenol blue. Lysates were heated at 100°C for 10 min. Proteinase K (25 μg) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) dissolved in 10 μl of lysing buffer was then added to each boiled lysate, and incubation was carried out at 60°C for 60 min. LPS preparations were analyzed by electrophoresis in an SDS–12.5% polyacrylamide gel with the Laemmli buffer system (16). LPS bands were subsequently visualized by silver staining (13).

Analysis of protein expression in minicells. Minicells were purified from 250-ml L-broth cultures of plasmid-containing derivatives of strain P678-54 by differential centrifugation and two sucrose density gradient separations (10). The number of minicells in purified suspensions was determined by counting in a Petroff-Hausser chamber, and their degree of contamination was measured by plating dilutions on L-agar; minicell suspensions usually contained less than one vegetative cell per 10^6 minicells. Purified minicells were suspended in 1.5 ml of sulphur-free Hershey salts medium supplemented with 100 μg each of threonine and leucine per ml and 1 μg of vitamin B_1 per ml and starved for sulphur by incubation for 30 min at 37°C. They were then labeled with 20 μCi of [^{35}S]methionine per ml (1,200 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 1 h. Labeled minicells were harvested, suspended in 30 μl of 50 mM Tris-hydrochloride (pH 6.8)–10 mM $MgCl_2$ buffer, and frozen. Samples (10 μl) were suspended in SDS sample buffer (0.167 M Tris-hydrochloride (pH 6.8), 33% [vol/vol] glycerol, 3.33% [wt/vol] SDS, 0.5 M 2-mercaptoethanol, trace of bromophenol blue), boiled for 3 min, and loaded onto a 0.1% SDS–15 or 16.5% polyacrylamide gel. Electrophoresis was carried out at a constant current of 20 mA by the method of Laemmli (16). The following molecular weight markers were used: phosphorylase β (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,300).

After electrophoresis, the gel was fixed and stained in an aqueous solution of 45% methanol, 10% acetic acid, and 0.1% Coomassie brilliant blue for 1 h, followed by destaining in a solution of 20% methanol and 7% acetic acid. After washing the gel with H_2O, it was soaked in 0.5 M sodium salicylate for 30 min at room temperature and then dried without rinsing (3). Stained molecular-weight standards on dried gels were marked with radioactive ink, and the gels were exposed to Fuji X-ray film RXO-H at −80°C for 1 to 4 days.

Other procedures. Techniques for conventional genetic manipulations, the isolation of plasmid and total DNA, nick translation, transformation of plasmid DNA, cleavage of plasmids with restriction enzymes, agarose gel electrophoretic analysis of DNA fragments, Southern blotting and hybridization, mapping of restriction endonuclease sites, and gene cloning have all been described previously (22–24).

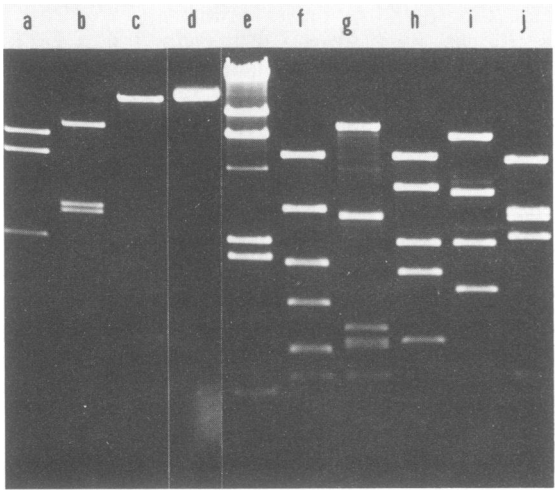

FIG. 1. Mapping of restriction endonuclease cleavage sites in plasmid pHW401. pHW401 DNA was cleaved with the indicated restriction endonucleases under conditions recommended by the suppliers, and the fragments thereby generated were analyzed by electrophoresis through a Tris-borate-buffered 0.8% agarose gel as described previously (24). Lanes: (a) *Bam*HI, (b) *Pst*I, (c) *Bgl*II, (d) *Sma*I, (e) *Hin*dIII-digested λ DNA size markers, (f) *Bam*HI-*Pst*I, (g) *Pst*I-*Bgl*II, (h) *Bgl*II-*Bam*HI, (i) *Sma*I-*Bam*HI, and (j) *Sma*I-*Pst*I. The map obtained from this data is shown in Fig. 2.

RESULTS

Localization of endonuclease cleavage sites on plasmid pHW401. Plasmid pHW401 is a Tn*801*-tagged derivative of the 6-Mdal O-antigen plasmid of *S. dysenteriae* 1 strain W30864 (24). To establish reference points for mapping of the LPS gene(s), we subjected pHW401 plasmid DNA to digestion with individual and pairs of restriction endonucleases, *Bam*HI, *Bgl*II, *Pst*I, and *Sma*I, and analyzed the fragments thereby generated by electrophoresis through agarose gels (Fig. 1). The information obtained permitted construction of the restriction map of pHW401 given in Fig. 2.

Cloning of fragments of pHW401 in pACYC177 and pACYC184. To locate approximately the O-antigen determinant, we cloned various *Bam*HI and *Pst*I fragments of pHW401 in the pACYC184 and pACYC177 vector plasmids and examined the ability of the hybrid plasmids thereby constructed to enable bacteria of strain W30864-22 to be agglutinated in *S. dysenteriae* 1-specific antiserum. Two hybrid plasmids, namely pHW427 and pHW485, were found to carry the O-antigen determinant of pHW401 (Fig. 2). Plasmid pHW427 carries pHW401 *Bam*HI fragment B-1, whereas plasmid pHW485 carries *Pst*I fragment P-2; the 3.1-kilobase (kb) fragment P-2 is entirely contained within fragment B-1 (Fig. 2).

Plasmid pHW401 contains two cleavage sites for the *Bgl*II enzyme, both of them located within the P-2 fragment. To confirm the approximate localization of the LPS determinant, the small *Bgl*II fragment from pHW401 was cloned in the *Bam*HI site in the tetracycline resistance determinant of pACYC184. The resulting hybrid plasmid, pHW486, carrying *Bgl*II fragment Bg-2 (Fig. 2), did not direct the synthesis of O-antigen in W30864-22 bacteria. To test whether the O-antigen determinant might be entirely located on a segment of the P-2 fragment to the right or left of the *Bgl*II fragment Bg-2, a *Bgl*II deletion derivative of pHW401, containing only

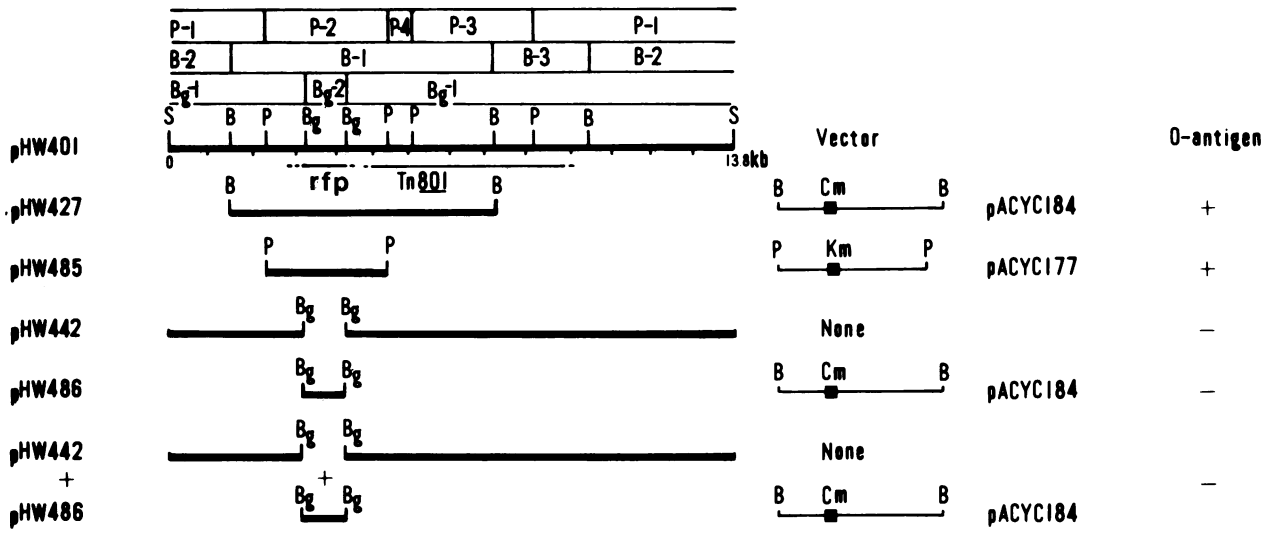

FIG. 2. Subcloning of fragments carrying O-Antigen determinant of pHW401. The upper part of the figure shows a restriction endonuclease cleavage map of plasmid pHW401. The unique *Sma*I site was assigned coordinates 0/13.8 kb. P, B, and Bg numbers indicate restriction endonuclease fragments generated by *Pst*I, *Bam*HI, and *Bgl*II, respectively. The fragments are numbered by size as they exist in the pHW401 plasmid. The segments of pHW401 present in the various hybrid plasmids are indicated by thick lines, and the vectors into which they have been cloned are shown on the right. O-antigen + or – indicates whether or not the hybrid plasmids cause W30864-22 bacteria to agglutinate in *S. dysenteriae* 1 type-specific antiserum preabsorbed with W30864-22 bacteria.

the large *Bgl*II fragment Bg-1 was constructed by digestion of pHW401 with *Bgl*II, self-ligation, and selection of transformed bacteria resistant to ampicillin. One plasmid of this type that was generated, pHW442, was found not to specify O-antigen production (Fig. 2). These results suggested either that one (or both) of the two *Bgl*II sites of pHW401 lies within the determinant(s) responsible for O-antigen production or that there are two or more O-antigen genes, one on *Bgl*II fragment B-2 and another on *Bgl*II fragment B-1, such that neither fragment alone permits O-antigen production. The latter possibility was ruled out by demonstrating that coexistence of the two plasmids, pHW442 and pHW486, also did not allow O-antigen production (Fig. 2).

Generation of O-antigen-negative insertion mutant derivatives of pHW401. To map precisely the O-antigen gene(s), to determine whether one or more genes are involved, and to enable identification of the product(s) of the O-antigen gene(s), we isolated and analyzed O-antigen-negative insertion mutants of plasmid pHW401. To obtain these, we introduced plasmid pHW401 into *E. coli* K-12 C600::Tn*5* by transformation. Five transformants were purified and cultured in L-broth for a further 20 generations. Plasmid DNA was then prepared from each clone and used to transform cells of strain C600. Of 114 kanamycin-resistant transformants obtained, 105 were also resistant to ampicillin, whereas 7 were sensitive. The fact that the latter class of Tn*5* derivative plasmids contained the Tn*5* element within the ampicillin resistance gene of Tn*801* was confirmed by restriction endonuclease cleavage analysis (the Tn*5* insertions in these seven mutant plasmids were located in *Bam*HI fragment B-3, the fragment containing the ampicillin resistance determinant of Tn*801*; data not shown).

Plasmid DNA isolated from each of the remaining 105 ampicillin- and kanamycin-resistant transformants was introduced into the W30864-22 strain by transformation. One kanamycin- and ampicillin-resistant transformant from each DNA sample was selected and tested for O-antigen production by agglutination with *S. dysenteriae* 1 antiserum. Eight transformants were identified as being O-antigen negative. Plasmid DNA was then prepared in quantity from the corresponding C600 transformants and characterized by cleavage analysis with various restriction endonucleases. None of the insertion mutants retained the original *Bgl*II fragment Bg-2 of pHW401 (Fig. 3); i.e., the insertions were all located within this fragment (digestion patterns of only four of the eight mutant plasmids are shown in Fig. 3A; the remainder exhibited *Bgl*II digestion patterns similar to that of pHW453 or pHW457; data not shown). Note that mutant plasmid pHW452 (Fig. 3A, lane c) contained one more *Bgl*II cleavage site than the other insertion mutant plasmids; this, perhaps, was caused by the insertion of Tn*5*.

Plasmid DNAs from some of the O-antigen-positive insertion mutant plasmids were also characterized. Three mutant plasmids, pHW456, pHW470, and pHW478, were found to contain the Tn*5* element inside of the *Pst*I-fragment P-2 but outside of the *Bgl*II fragment Bg-2 (Fig. 3B). Thus, from the results obtained by cloning fragments of pHW401 and by generating insertion mutants of this plasmid, the determinant(s) for O-antigen production were localized to a region less than 2 kb in length and centered on *Bgl*II fragment Bg-2.

Analysis of LPS production by O-antigen-positive and -negative strains. The O-antigen of *Shigella* spp. consists of the repeating sugar units of the LPS side chain (14). To confirm the results of the agglutination tests, the production of LPS in mutant strains was examined by SDS-polyacrylamide gel electrophoresis. Whole-cell lysates of W30864, W30864-22, and plasmid-containing derivatives of the latter were subjected to SDS-polyacrylamide gel electrophoresis, followed by visualization of LPS bands by silver staining, as described above. As can be seen in Fig. 4, the O-antigen-positive strain W30864 and the derivatives of W30864-22 carrying pHW401, pHW470, and pHW478 (Fig. 4, lanes a, c, f, and g), respectively, gave regularly spaced banding patterns representing LPS molecules having increasing numbers of side chain repeat units, which are characteristic of a smooth LPS phenotype (12). In contrast, the O-antigen-negative strain W30864-22 and its derivatives carrying pHW457 and pHW452 (Fig. 4, lanes b, d, and e, respective-

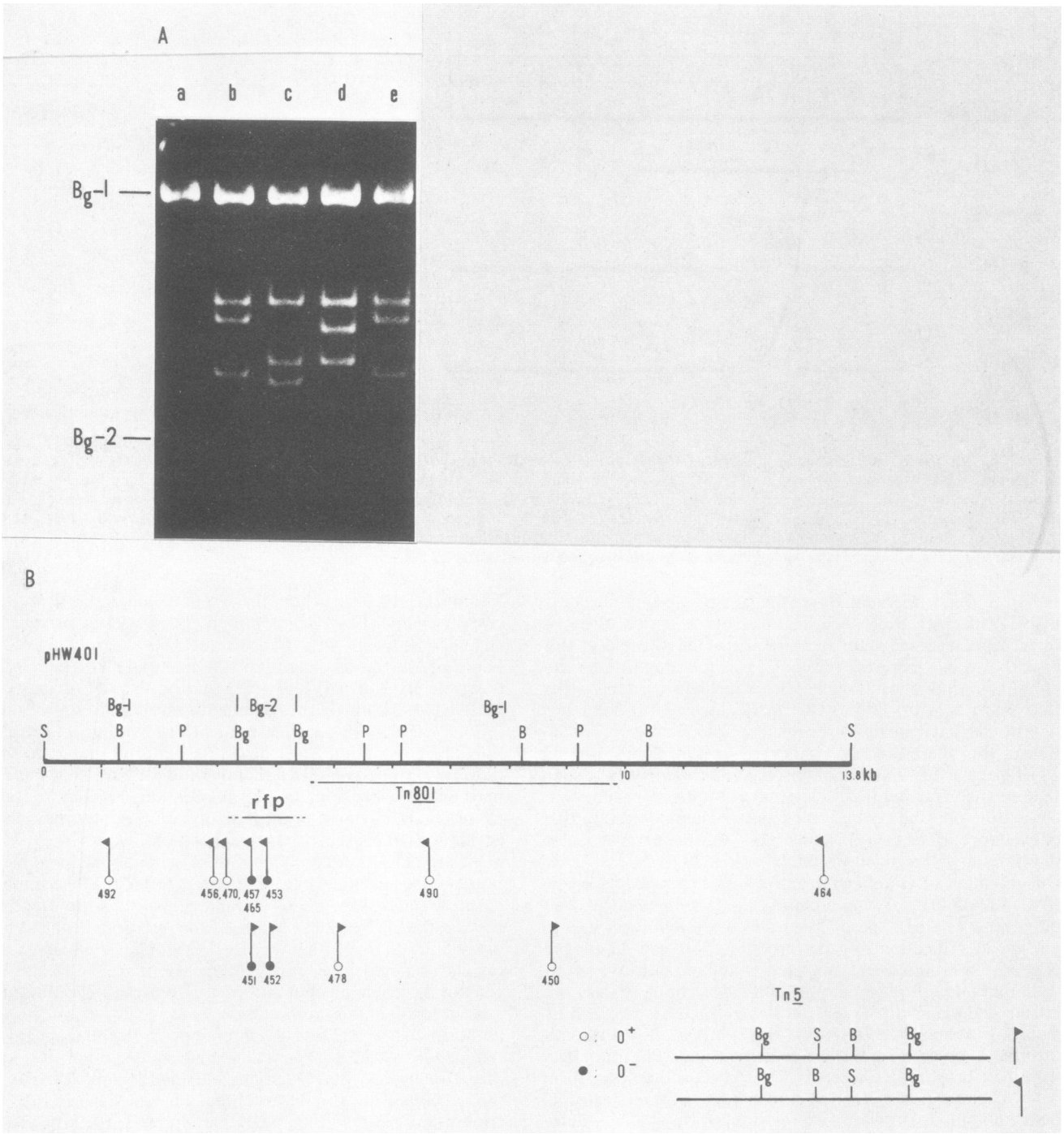

FIG. 3. Localization of O-antigen determinant of pHW401 by Tn*5* transposon mutagenesis. Transposon mutant derivatives of pHW401 were generated as described in the text. The position and direction of Tn*5* elements in mutant plasmids were determined by restriction endonuclease cleavage analysis. (A) *Bgl*II-digested plasmids separated by electrophoresis in 0.8% agarose. Lanes: (a) pHW401 *rfp*$^+$; (b, c, d, and e) pHW451, pHW452, pHW453, and pHW457, respectively (all *rfp*$^-$). (B) Locations of Tn*5* elements in mutant plasmids: the map of pHW401 is that shown in Fig. 2. Insertions which abolished *S. dysenteriae* 1 O-antigen production (*rfp*$^-$) are indicated by filled circles, whereas insertions that did not abolish O-antigen production are indicated by open circles. Filled triangles indicate the orientation of inserted Tn*5* elements with respect to the map of pHW401 as drawn.

ly) gave only the diffuse low-molecular-weight band that represents all or part of the polysaccharide core unit conjugated to lipid A (12). The remaining O-antigen-negative derivatives of W30864-22 carrying Tn*5* insertion mutant derivatives of pHW401 gave similar patterns (data not shown). Thus, the pHW401 plasmid specifies one or more functions necessary for production of the complete LPS structure.

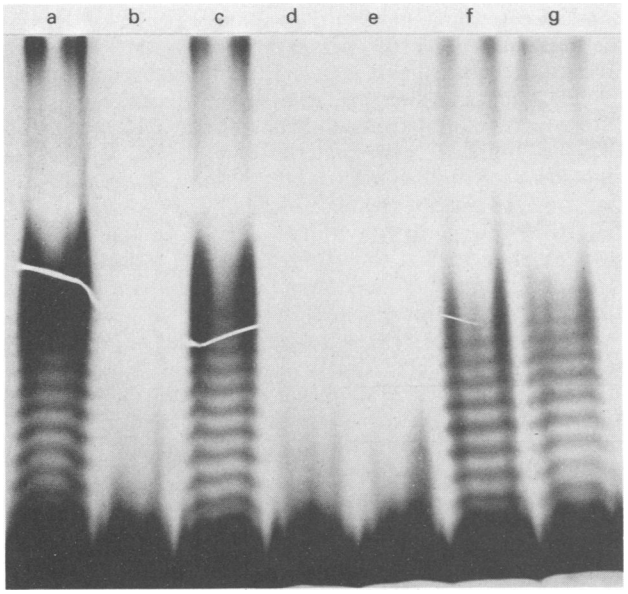

FIG. 4. LPS banding patterns of *S. dysenteriae* 1 bacteria carrying mutant plasmids. Deproteinized bacterial lysates were subjected to SDS-polyacrylamide gel electrophoresis, and LPS bands were revealed by silver staining as described in the text. Lanes: (a) *S. dysenteriae* 1 strain W30864, (b) derivative W30864-22, (c) W30864-22 (pHW401), (d) W30864-22 (pHW457), (e) W30864-22 (pHW452), (f) W30864-22 (pHW470), and (g) W30864-22 (pHW478).

Identification of the product of a pHW401 O-antigen gene. Minicells of plasmid-carrying derivatives of the P678-54 strain were purified and labeled with [^{35}S]methionine, and the radioactive proteins were analyzed by electrophoresis through an SDS-polyacrylamide gel followed by autoradiography. Minicells containing the pHW401 plasmid synthesized a protein (labeled rfp) having an M_r of ca. 41,000 (Fig. 5, lanes a and g), which was not synthesized by bacteria carrying plasmid pHW442, which lacked *Bgl*II fragment Bg-2, and which failed to permit the production of LPS (lanes b and h). Similarly, Tn*5* insertion mutant plasmids that failed to permit the synthesis of O-antigen also failed to direct the synthesis of the protein of M_r 41,000 (lanes c and f), whereas a Tn*5* mutant plasmid that permitted LPS synthesis also directed the synthesis of the 41,000 protein (lane e). Similar results were obtained for other Tn*5* mutant plasmids. The pACYC177 hybrid plasmid, pHW485, containing the *Pst*I fragment P-2 of pHW401, also permitted the synthesis of the 41,000-dalton polypeptide in minicells (lane d). These results strongly suggest that the product of the (an) O-antigen determinant of plasmid pHW401 is the 41,000 polypeptide. The determinant of this polypeptide has been designated *rfp* (*rf*, to be consistent with the LPS determinants of *S. typhimurium*, and *p* for plasmid).

Correlation between virulence and an active *rfp* gene. W30864-22 bacteria containing plasmid pHW485, a pACYC177 hybrid carrying the 3.1-kb P-2 fragment of plasmid pHW401, are fully virulent in the Serény test (guinea pig keratoconjunctivitis model); thus, the P-2 fragment con-

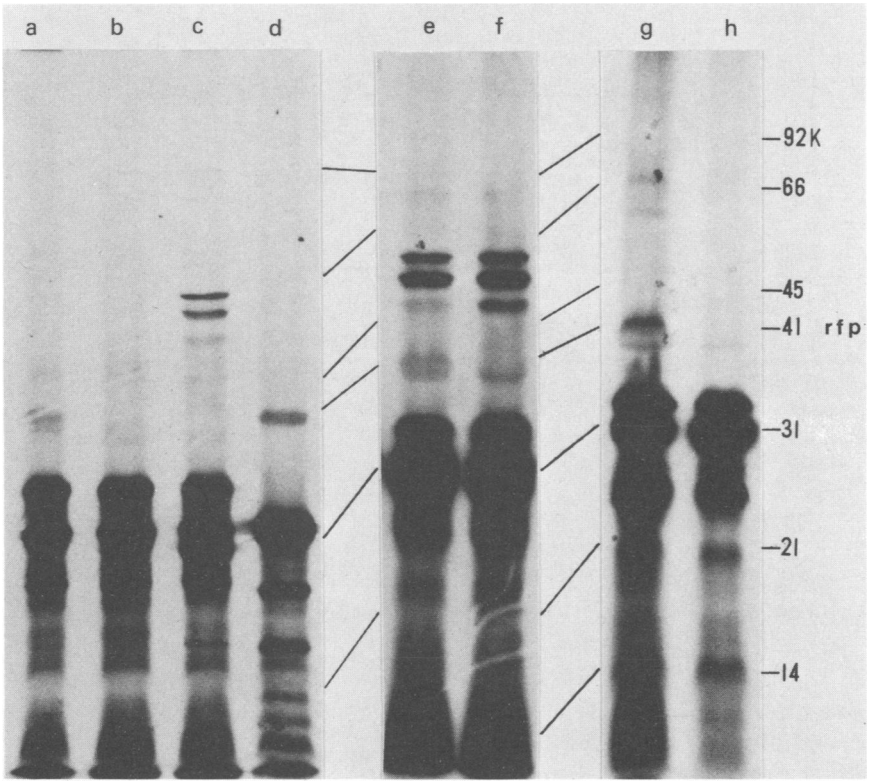

FIG. 5. Identification of the product of the *rfp* gene. *E. coli* P678-54 minicells were prepared and labeled and the minicell lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography, as described in the text. The molecular weights of the protein standards used are shown on the right. Polyacrylamide gels of 15% (lanes a to d) and 16.5% (lanes e to h) were used. The minicells contained the following plasmids: (lanes a and g) pHW401 (*rfp*$^+$), (lanes b and h) pHW442 (*rfp*$^-$), which is a pHW401 derivative lacking the small *Bgl*II fragment Bg-2; (lanes c, e, and f) pHW452 (*rfp*$^-$), pHW470 (*rfp*$^+$), and pHW457 (*rfp*$^-$), respectively, which are Tn*5* transposon mutant plasmids; and (d) pHW485 (*rfp*$^+$), a pACYC177 hybrid plasmid containing the *Pst*I P-2 fragment of pHW401.

TABLE 1. Correlation between O-antigen production and virulence in *S. dysenteriae* 1

Strain	O-antigen[a]	Serény test (no. of positive/ total)[b]
W30864	+	4/4
W30864-22	−	0/4
W30864-22 (pHW401)	+	4/4
W30864-22 (pHW451)	−	0/3
W30864-22 (pHW452)	−	0/3
W30864-22 (pHW453)	−	0/3
W30864-22 (pHW457)	−	0/3
W30864-22 (pHW456)	+	3/3
W30864-22 (pHW470)	+	3/3
W30864-22 (pHW442)	−	0/3
W30864-22 (pHW485)	+	3/3

[a] Determined by LPS analysis as described in the text.

[b] A total of three or four W30864-22 transformants for each plasmid indicated were tested. A few O-antigen-negative strains provoked a slight clouding of the eye 5 to 6 days after the challenge, as described previously (24).

tains all determinants of virulence and LPS biosynthesis of the pHW400/pHW401 plasmids (Table 1). Moreover, W30864-22 bacteria carrying Tn*5* transposon mutant derivative plasmids, in which the Tn*5* element was located in the P-2 fragment, showed an absolute correlation of virulence with LPS biosynthesis: thus, bacteria carrying *rfp*$^+$ mutant plasmids pHW456 and pHW470 were virulent, whereas bacteria carrying *rfp*$^-$ mutant plasmids pHW451, pHW452, pHW453, and pHW457 were nonvirulent (Table 1). These results strongly suggest that the *rfp* gene on pHW400 is also the virulence determinant and that no additional virulence determinants exist on this plasmid.

The *rfp* gene is present on 6-Mdal plasmids of other isolates of *S. dysenteriae* 1. To examine the prevalence of the *rfp* gene in other isolates of *S. dysenteriae* 1, and in various isolates of *Shigella* spp., *Salmonella typhimurium*, and *E. coli*, we carried out Southern blotting and hybridization of plasmid DNA and of total DNA obtained from these strains, using as a probe radioactively labeled isolated Bg-2 fragment from plasmid pHW401. As can be seen in Figs. 6 and 7, the *rfp*

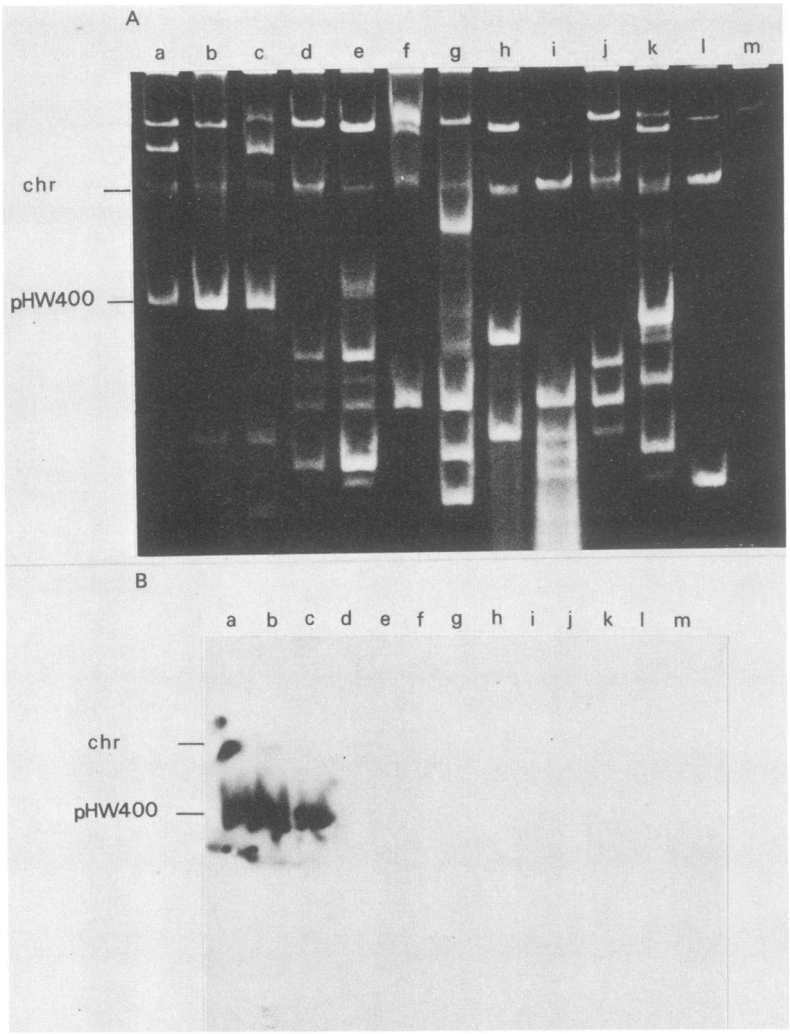

FIG. 6. Presence of the *rfp* gene on plasmids of *Shigella* spp. Plasmid DNAs prepared from various strains were fractionated on 0.8% agarose gels. Before the transfer of DNA to nitrocellulose filters (23), gels were soaked in 0.2 N HCl. Hybridization was carried out at 68°C for 24 h in a solution containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS–10× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll)–heat-denatured calf thymus DNA (50 μg/ml)–radioactive probe DNA (5×10^5 cpm/ml) (purified *Bgl*II fragment Bg-2 of plasmid pHW401), labeled by nick translation in the presence of ^{32}P-labeled dCTP (23). After hybridization, the filters were washed in a solution containing 0.1× SSC–0.1% SDS at 68°C for 24 h, dried, and exposed to Fuji X-ray film RXO-H at −80°C for 1 to 6 days. chr, Chromosomal and linear DNAs. Lanes: (a) *S. dysenteriae* 1 strain W30864, (b) *S. dysenteriae* 1 strain 47-80, (c) *S. dysenteriae* 1 strain 11-81, (d) *S. dysenteriae* 2 strain 259, (e) *S. dysenteriae* 5 strain 329, (f) *S. dysenteriae* 6 strain 440, (g) *S. dysenteriae* 9 strain 334, (h) *S. dysenteriae* 10 strain 314, (i) *S. flexneri* 1b strain 313, (j) *S. flexneri* 2a strain 283, (k) *S. sonnei* strain 262, (l) *S. boydii* 1 strain 328, and (m) *S. dysenteriae* 4 strain 333. (A) Agarose gel stained with ethidium bromide; (B) autoradiogram of hybridized filter.

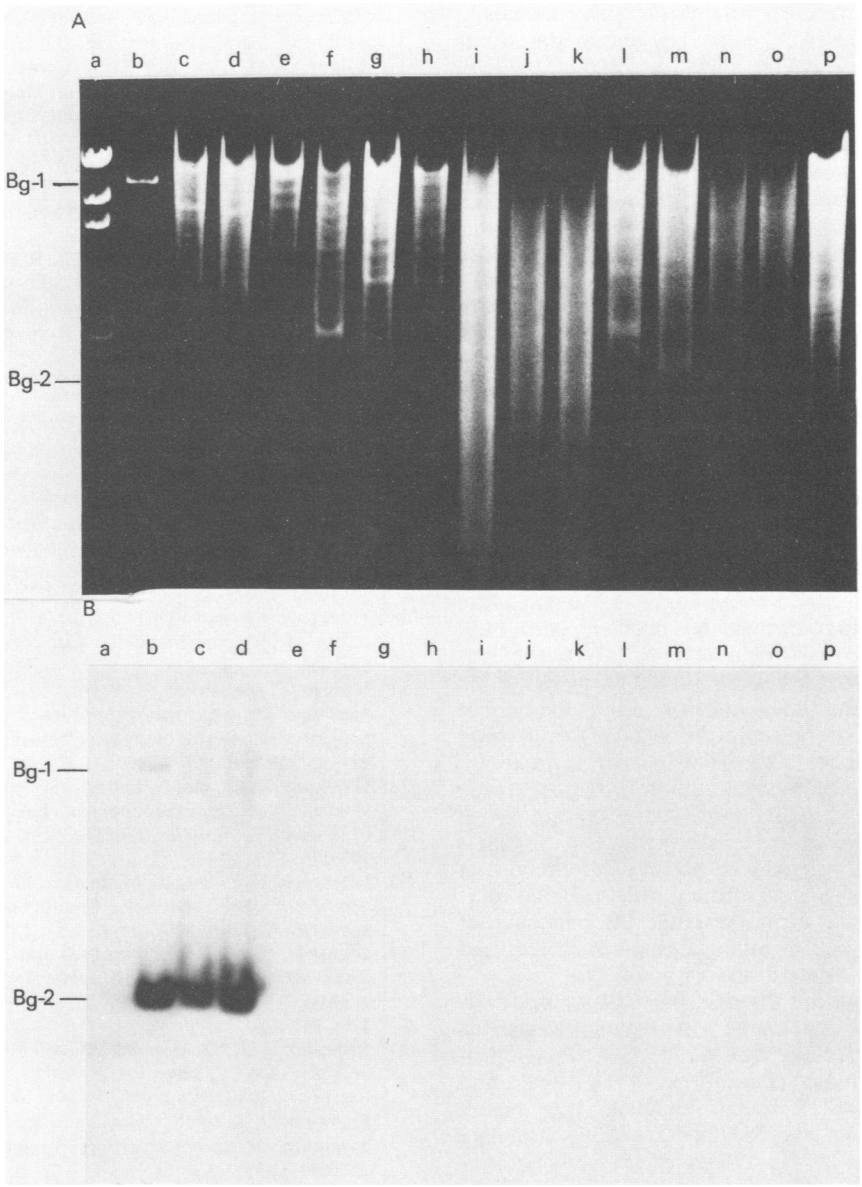

FIG. 7. Presence of the *rfp* gene in total DNA of various *Shigella*, *E. coli*, and *Salmonella* strains. Total DNAs were prepared from various strains and cleaved with the *Bgl*II restriction endonuclease. The fragments generated were fractionated by electrophoresis through a 0.8% agarose gel and transferred to a nitrocellulose filter. The filter was used for hybridization with [^{32}P]dCTP-labeled *Bgl*II fragment Bg-2 DNA of plasmid pHW401 as described in the legend to Fig. 6. Bg-1 and Bg-2 show the positions of the fragments of pHW401 generated by cleavage with the *Bgl*II restriction endonuclease. Lanes: (a) *Hin*dIII-digested λ DNA, (b) purified pHW401 DNA, (c) *S. dysenteriae* 1 strain W30864, (d) *S. dysenteriae* 1 strain 47-80, (e) *S. dysenteriae* 2 strain 259, (f) *S. dysenteriae* 5 strain 329, (g) *S. dysenteriae* 6 strain 440, (h) *S. dysenteriae* 9 strain 334, (i) *S. dysenteriae* 10 strain 314, (j) *S. flexneri* 1b strain 313, (k) *S. flexneri* 2a strain 283, (l) *S. sonnei* strain 262, (m) *S. boydii* 1 strain 328, (n) *E. coli* O14:K7, (o) *E. coli* K-12 C600, and (p) *Salmonella typhimurium* LT2 JB564. (A) Agarose gel stained with ethidium bromide; (B) autoradiogram of hybridized filter.

gene probe detected homologous sequences in 6-MdaI plasmids in two other *S. dysenteriae* 1 strains but not in plasmids of *S. dysenteriae* isolates of serotypes 2, 4, 5, 6, 9, and 10, *S. sonnei*, *S. flexneri* 1b and 2a, and *Shigella boydii* 1, nor in total DNA of these strains, nor in that of *Salmonella typhimurium* LT2 or *E. coli* O14:K7. The homology detected in the *S. dysenteriae* 1 strains was localized to *Bgl*II-generated fragments identical in size to that of the probe fragment. These results strongly suggest that the *rfp* gene specifies a function that is specific for the biosynthesis of the *S. dysenteriae* 1 O-antigen.

DISCUSSION

The pHW400 (pHW401) plasmid of *S. dysenteriae* 1 strain W30864 carries one or more determinants for O-antigen production and bacterial virulence (24). *S. dysenteriae* 1 derivatives lacking pHW400 but containing insertion mutant derivatives of pHW401 that no longer specify O-antigen production are not virulent; O-antigen production is thus an essential component of bacterial virulence. In this series of experiments, we have used cloning and deletion and insertion mutagenesis of the pHW401 plasmid to localize a gene,

designated *rfp*, for O-antigen production to a DNA segment centered on the small *Bgl*II fragment Bg-2 and to identify its product as a polypeptide having an M_r of 41,000. *S. dysenteriae* 1 W30864-22 bacteria that are *rfp*$^-$, and that therefore fail to synthesize the 41,000 protein, fail to produce LPS having the typical O-antigen side chains and are avirulent. The *rfp* gene appears to be specific to *S. dysenteriae* strains belonging to serotype 1 and therefore presumably encodes a function specific to the biosynthesis of the O-antigen of this serotype. The *rfp* gene was located on 6-Mdal plasmids in two other *S. dysenteriae* 1 isolates examined.

The side chain of *S. dysenteriae* 1 LPS (5) consists of repeated units of [α-L-rhamnose(1-3)-α-L-rhamnose-(1-2)-α-D-galactose(1-3)-α-D-*N*-acetyl-D-glucosamine]. The role of the *rfp* gene product could presumably be in formation of the side chain repeat unit, polymerization of the repeat units to form the complete side chain, or ligation of the completed side chain to the LPS core. Biochemical studies are now in progress to identify which of these possibilities is correct.

All information relating to O-antigen production and virulence that is specified by pHW400 is located on the 3.1-kb *Pst*I fragment P-2. Considering the locations of Tn*5* insertions in this region that either inactivate or fail to inactivate the *rfp* gene, and the size of *rfp*, which is probably ca. 1.1 kb, there are two regions that might encode further LPS or virulence functions. These are (i) the region to the left of the insertion in pHW456 (0.5 kb) and (ii) the region to the right of *rfp*, defined as the region between the insertion in plasmid pHW470 and the beginning of the Tn*801* element, minus *rfp* (1.5 kb − 1.1 kb = 0.4 kb), which could in principle encode two small polypeptides. A system of overlapping genes would provide additional coding capacity. Thus, although it seems probable that the *rfp* gene is the only determinant of LPS biosynthesis and virulence of the 6-Mdal plasmid of *S. dysenteriae* 1, our results do not exclude the existence of others. Further subcloning or mutagenesis will be required to obtain definitive information on this point.

Shigellosis is an important disease, particularly in developing countries, and the development of efficacious vaccines is highly desirable, especially against *S. dysenteriae* serotypes which cause the most severe forms of the disease and which result in the most frequent sequellae. It is highly probable that efficacious vaccines will need to stimulate local immunity in the large bowel against the somatic antigens of shigellae. The cloning of the *rfp* gene, the determinant of an essential O-antigen biosynthesis function, is an important step towards the construction of a noninvasive, nonpathogenic, O-antigen-positive, live anti-*S. dysenteriae* 1 vaccine strain.

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