Identification and Antigenic Characterization of Virulence-Associated, Plasmid-Coded Proteins of *Shigella* spp. and Enteroinvasive *Escherichia coli*

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Seven plasmid-coded polypeptides, designated a through g, were identified by two-dimensional nonequilibrium pH gradient electrophoresis of radiolabeled extracts from minicells of virulent *Shigella flexneri* serotypes 2a and 5 and enteroinvasive *Escherichia coli* O143. These polypeptides were deemed to be products of 140-megadalton (MDa) virulence-associated plasmids because they were not synthesized in minicells which were not harboring a 140-MDa plasmid or in minicells which were carrying an F *lac* plasmid of the same incompatibility group. Synthesis of these polypeptides was repressed in minicells incubated at 30°C and in minicells isolated from a noninvasive opaque colonial variant, even though these strains harbored a 140-MDa plasmid. Enriched fractions of polypeptides b, c, and d were obtained from *S. flexneri* serotype 5 by preparative isoelectric focusing, and polyclonal rabbit antisera recognizing each polypeptide were raised. These antisera were able to detect cross-reacting plasmid-coded polypeptide antigens in *S. flexneri* serotype 3, *Shigella sonnei*, and enteroinvasive *E. coli* O143. In addition, Western blots of minicell extracts from *S. flexneri* serotype 5 or *E. coli* O143 indicated that plasmid-coded polypeptides a through d were recognized by convalescent antiserum from a monkey infected with *S. flexneri* serotype 2a.

The ability to invade the colonic epithelium is an essential virulence mechanism of shigellae and enteroinvasive *Escherichia coli*. Recently, the genetic basis of the invasive phenotype has been elucidated by the discovery of virulence-associated plasmids which are necessary for epithelial cell invasion (8, 10, 24, 25). Loss of these plasmids is accompanied by the loss of the invasive phenotype, and this phenotype is restored when a virulence-associated plasmid is reintroduced into plasmid-free avirulent variants (10, 22). Apparently these plasmids, which range from 120 to 140 megadaltons (MDa), encode one or more key enzymes or virulence determinants which are necessary for expression of the invasive phenotype. Therefore, plasmid-coded proteins expressed in anucleate minicells from invasive strains of *Shigella sonnei*, *Shigella flexneri*, or enteroinvasive *E. coli* have been studied. A similar complement of 15 to 20 radiolabeled, i.e., plasmid-coded, polypeptides can be demonstrated in minicells from each of these species, while 6 or less polypeptides are labeled in minicells from noninvasive strains (10). Thus, a specific subset of shigella proteins has been linked to genetic loci which are known to be necessary for epithelial cell invasion.

In the present study, this complex mixture of radiolabeled minicell polypeptides was studied by the O'Farrell et al. two-dimensional gel electrophoresis technique (20). This technique allows more detailed analysis than the one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure used previously (10, 29). A complement of seven plasmid-coded polypeptides was found to be unique to invasive strains of *S. flexneri* and *E. coli*. Expression of these polypeptides was partially

repressed at 30°C, which is a nonpermissive temperature for the invasive phenotype (16). In addition, a noninvasive opaque colonial variant of *S. flexneri* serotype 2a failed to express these polypeptides. Finally, Western blot analysis of various enteroinvasive species indicated that the virulence-associated plasmids of *S. flexneri*, *S. sonnei*, and *E. coli* express polypeptides which are very similar not only in terms of molecular mass and isoelectric point but also antigenically. Western blots with serum from a monkey which had recovered from shigella infection indicated that four of the virulence-associated polypeptides were dominant proteinaceous antigens. The ubiquity of these polypeptides among enteroinvasive strains and the strong immune response which they induce during an infection suggests that they may function as determinants of the invasive phenotype.

MATERIALS AND METHODS

Organisms. The bacterial strains used in this study are listed in Table 1. Minicell-producing mutants were isolated after mutagenesis of parental strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Gemski and Griffin (9). *Salmonella typhi* 643, a cystine and tryptophan auxotroph harboring a wild-type F *lac* plasmid, served as an F *lac* donor.

Preparation of minicell lysates. Suspensions of minicells were prepared and radiolabeled as previously described (10) with the following modification: minicells were labeled in methionine assay-minimal glucose salts medium (without antibiotics) containing 100 μ Ci of [35 S]methionine (985 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. After 4 h of labeling, the minicells were washed three times in normal saline and solubilized at 25°C in an 8 M urea solution containing 2% octyl- β -D-glucopyranoside (Calbiochem-Behring, La Jolla, Calif.), 2% pH 3 to 10 Ampholine (LKB, Gaithersburg, Md.), and 5% 2-mercaptoethanol. After 30 min, approximately 90% of the 35 S-

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TABLE 1. Bacterial strains and plasmids

Strain	Serotype	Plasmid content	Invasive	Origin and comments
<i>Shigella flexneri</i>				
M25-8AMinI	1b	Small cryptic plasmids	—	Previous work (10); NTG ^a mutant of M25-8A (minicell producer)
M25-8AMinI(F lac)	1b	F lac plasmid + small cryptic plasmids	—	Present work (minicell producer)
2457T	2a	2 large + 2 small cryptic plasmids	+	Walter Reed Collection
2457TMinI	2a	Same as 2457T	+	Present work; NTG mutant of 2457T (minicell producer)
24570	2a	Same as 2457T	—	Previous work (13); spontaneous colonial variant of 2457T
J17B	3a	1 large + 1 small cryptic plasmid	+	Walter Reed Collection
M90T	5	1 large + 1 small cryptic plasmid	+	Walter Reed Collection
M90TMinI	5	Same as M90T	+	Previous work (10); NTG mutant of M90T (minicell producer)
WR6078 (M90TA2)	5	Small cryptic plasmids	—	Previous work (25); spontaneous colonial variant of M90T
M90TMinII-1	5	Small cryptic plasmids	—	Previous work (10); spontaneous loss of pWR110 from M90TMinII
<i>Shigella sonnei</i>				
53G	Form I	1 large + 1 small cryptic plasmid	+	Walter Reed Collection
53GII	Form II	Small cryptic plasmids	—	Previous work (24); spontaneous colonial variant of 53G
<i>Escherichia coli</i>				
4608-58	O142	1 large plasmid	+	Walter Reed Collection
4608-58MinVI	O143	Same as 4608-58	+	Previous work (10); NTG mutant of 4608-58 (minicell producer)
B2C	O6:H16	1 large plasmid	—	Enterotoxigenic isolate from Vietnam; Walter Reed Collection
<i>Salmonella typhi</i>				
643 (F lac)	9, 12	1 large plasmid	—	Previous work (8); donor of F lac

^a NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

labeled components were solubilized by this procedure, and remaining cellular debris was pelleted by centrifugation at $15,000 \times g$ for 5 min. Sample volume was adjusted to approximately 3×10^6 cpm/25 μ l by the addition of the same solubilizing solution described above, and samples were stored at -70°C for subsequent use in two-dimensional electrophoresis.

Preparation of minicell supernatants. Suspensions of minicells were prepared and radiolabeled as described above. After 4 h of labeling, the minicells were pelleted by centrifugation for 5 min at $20,000 \times g$. The supernatant was collected, and phenylmethylsulfonyl fluoride (Boehringer

GmbH, Mannheim, Federal Republic of Germany) was added at 5.0×10^{-4} M. The supernatant was then dialyzed for 24 h at 4°C against deionized water and lyophilized. The resulting residue was resuspended in the solubilizing solution described above and adjusted to approximately 3×10^6 cpm in 25 μ l. Samples were then stored at -70°C for subsequent use in two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis. Two-dimensional nonequilibrium pH gradient electrophoresis was performed on solubilized bacterial proteins essentially by the procedure of O'Farrell et al. (20). Proteins were resolved by isoelectric focusing in flatbed polyacrylamide gels consisting of 5%

acrylamide (cross-linked with bisacrylamide), 2% Triton X-100, 4.5% pH 3.5 to 10 Ampholine, 0.6% pH 5 to 7 Ampholine, and 8 M urea. Protein samples were added close to the anode, and constant power of 25 W was applied. After 2×10^3 Vh, a linear pH gradient of approximately 5.0 to 9.5 was established. When equilibrium isoelectric focusing was performed the samples were focused for 2×10^4 Vh. After the isoelectric focusing was terminated, individual sample tracts were excised and frozen at -70°C .

When the second dimension SDS-PAGE was performed, the sample tracts were thawed and equilibrated for 1 h in two changes of the SDS sample buffer described by O'Farrell (19). These polyacrylamide strips were then inserted between the glass plates of a vertical slab electrophoresis cell containing a 5% polyacrylamide stacking gel and a 13% running gel prepared as described by Laemmli (14). Since the lower edge of the inserted sample strip rested directly upon the surface of the stacking gel, the need for an agarose transition zone (19) between the first and second dimension gels was eliminated. Small agarose wells were sometimes made beyond the end of the sample strip so that ^{14}C -labeled molecular weight standards (New England Nuclear Corp.) or extracted bacterial proteins could be electrophoresed along with the electrofocused bacterial proteins (1). SDS-PAGE was performed at a constant current of 15 mA per gel until the buffer front had reached the bottom of the separating gel. Fixation and fluorography of dried gels were performed as previously described (10).

Preparative isoelectric focusing (PIEF). Overnight cultures of *S. flexneri* M90T serotype 5 were grown at 37°C in 2 liters of Penassay broth (Difco Laboratories, Detroit, Mich.), collected by centrifugation, and washed twice in 1% (wt/vol) glycine. The bacterial pellet was suspended in a solubilization solution consisting of 2% octyl- β -D-glucopyranoside, 8 M urea, 5% 2-mercaptoethanol, and 50 μM phenylmethylsulfonyl fluoride. To this mixture a preparation of ^{35}S -labeled minicell extract (2×10^6 cpm) was added so that fractions containing plasmid-coded polypeptides could be identified. The entire mixture was incubated for 1 h at 37°C , and then 5.0 ml of pH 3.5 to 10 Ampholine, 0.5 ml of pH 5.0 to 7.0 Ampholine, and 40 ml of deionized water were added. This mixture was centrifuged at $3,000 \times g$ for 15 min. The supernatant, which contained solubilized shigella proteins, was collected and mixed with 5 g of Ultradex (LKB), and the total volume was adjusted to approximately 105 ml. This slurry was poured into the flatbed electrophoresis apparatus, and the excess water was removed by evaporation. Electrofocusing was performed for 16 h at a constant power of 8 W with electrode solutions consisting of 1 N NaOH at the cathode and 1 M H_3PO_4 at the anode. After electrofocusing, the pH range was determined with a surface electrode, and the slurry was divided with a stainless-steel grid into 30 fractions. The material in each fraction was collected, and the soluble protein was eluted with 5.0 ml of 1% octyl- β -D-glucopyranoside in 5 M urea. The eluates were monitored for ^{35}S activity and then were dialyzed extensively against 50 mM NH_4HCO_3 . Finally, all samples were analyzed by one-dimensional SDS-PAGE and fluorography to confirm the presence of one or more plasmid-coded polypeptides, and those fractions containing such proteins were used to immunize rabbits.

Immunization of rabbits. PIEF fractions containing plasmid-coded proteins were concentrated by lyophilization, solubilized in saline, and adjusted to a concentration of 2 mg of protein per ml. Rabbits were injected intradermally at several sites with a total of 1 mg of antigen mixed with

complete Freund adjuvant. After 6 weeks, the animals were boosted with 1 mg of the same antigen without adjuvant. The animals were bled 2 weeks after the booster injection. Sera were collected and absorbed with a sonicated membrane fraction from strain M90TA2. This membrane fraction, which had been treated with Triton X-100, was removed from the absorbed sera by centrifugation at $100,000 \times g$ for 1 h.

Monkey antiserum. As part of a previous study (6), a rhesus monkey was orally challenged with virulent *S. flexneri* 2457T serotype 2a. Symptoms of infection (diarrhea or dysentery) appeared after 1 day and persisted for 6 days. Treatment with ampicillin was commenced 4 days postchallenge. This monkey was bled before challenge and at 2 weeks postchallenge. A 1:200 dilution of postchallenge serum was used in some of the Western blots described below.

One-dimensional PAGE. Whole-cell extracts were prepared by heating bacteria at 100°C for 3 min in sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 12% glycerol, 0.01% bromphenol blue, and 0.005 mM phenylmethylsulfonyl fluoride in 0.005 M phosphate buffer at pH 7.2. SDS-PAGE was performed in gels consisting of 14% (wt/vol) acrylamide cross-linked with *N,N'*-diallyltartardiamide with a discontinuous buffer system (14).

Western blots. Immunoblots on bacterial polypeptides separated in a one-dimensional format were performed as previously described (4). Bound antibody was detected by ^{125}I -labeled protein A, and autoradiography was performed on Kodak Blue Brand film. This procedure was modified when radiolabeled minicell polypeptides were separated in two-dimensional gels and electrophoretically transferred to nitrocellulose. The location of plasmid-coded polypeptides, which were labeled with [^{35}S]methionine, was determined by autoradiography of dried nitrocellulose sheets. Binding of rabbit or monkey antibody to the same electroblots was subsequently analyzed by rehydrating the nitrocellulose sheets in Tris-saline (10 mM Tris hydrochloride, 0.95% NaCl, pH 7.4), blocking unbound sites with 2% casein in Tris-saline, and proceeding with antibody binding as previously described (4). After treatment with ^{125}I -protein A, the immunoblots were placed in cassettes with two blank film sheets separating the nitrocellulose sheet from an unexposed Kodak Blue Brand test film lying on an intensifying screen. Since the beta particles of ^{35}S were blocked by the blank films while the gamma rays of ^{125}I were able to penetrate these films, spots appearing on the test film represented only the polypeptides which were recognized by antibody.

RESULTS

Identification of plasmid-coded polypeptides associated with the enteroinvasive phenotype. Minicell suspensions from invasive *S. flexneri* M90TMinI serotype 5 were incubated with [^{35}S]methionine and solubilized in a nonionic detergent, and the polypeptides were separated by a two-dimensional electrophoresis system. A complement of up to 40 polypeptides was discernible in fluorograms (Fig. 1); however, four of these polypeptides, which are marked by asterisks, were also labeled in minicells from a syngeneic strain which lacked the 140-MDa plasmid (10). A complement of seven polypeptides, designated a through g, were always expressed in minicells from invasive organisms, and these seven polypeptides were usually the most heavily labeled minicell components in enteroinvasive strains (see Fig. 4A and 6A and C). It can also be seen in Fig. 1 and 6A that there is an additional heavily radiolabeled polypeptide, marked with an arrow in Fig. 1, which has a molecular mass of 37

FIG. 1. Two-dimensional electrophoretic analysis of [^{35}S]methionine-labeled plasmid-coded polypeptides extracted from invasive *S. flexneri* M90TMinI minicells. Radiolabeled products were detected on X-ray film exposed for 48 h. Polypeptides designated a through g were radiolabeled in minicells containing a 140-MDa plasmid, while the four polypeptides marked with asterisks are labeled in M90TMinI-1 minicells which lack the 140-MDa plasmid (10). The polypeptide marked with an arrow is discussed in the text. Std, Standards (K, 10^3 Da).

kDa and an isoelectric point of 6.4. These parameters are shared by the major outer membrane protein of *S. flexneri* (data not shown). Since the major outer membrane protein is a chromosomal product, the origin of the polypeptide synthesized in minicells is uncertain. When this polypeptide was found to be weakly labeled in shigella minicells containing a plasmid which did not confer the invasive phenotype (see Fig. 3) and was not found in an enteroinvasive *E. coli* strain (see Fig. 6B), it was provisionally excluded from the list of polypeptides which are unique to the virulence plasmid.

The isoelectric points and molecular masses of these virulence-associated polypeptides are shown in Table 2. The isoelectric points of polypeptides b, c, and d are approximations because these polypeptides exhibited multiple isoelectric species. Since the polypeptides were not heated in the presence of urea, these species were probably not carbamylation products (2). Multiple isoelectric species are characteristic of plasmid-coded gene products expressed in minicells, and such multiple species usually yield identical peptide maps (17). The isoelectric point of polypeptides e, f, and g was probably higher than the stated 9.4, since these polypeptides were lost upon equilibrium isoelectric focusing. The isoelectric point of polypeptides a through d was not changed under the latter electrofocusing conditions.

Synthesis of polypeptides a and b was not detected when strain M90TMinI was grown at 30°C and labeled at 30°C (Fig. 2). These are nonpermissive conditions with respect to the invasive phenotype (16). Expression of polypeptides c through g was also repressed at 30°C, but these polypeptides were sometimes weakly detectable if films were exposed for up to 14 days (Fig. 2). Labeling of the four background polypeptides normally synthesized in M90TMinI-1 minicells lacking the 140-MDa plasmid (10) continued at

30°C, indicating that methionine transport still occurs at this temperature. All seven virulence-associated polypeptides (a through g) were expressed if M90TMinI was grown at 30°C but labeled at 37°C for 4 h. These virulence-associated polypeptides were detected in enteroinvasive strains of *S. flexneri* serotype 2a (2457TMinI) and *E. coli* O143 (4608-58MinVI) but not in a spontaneously arising avirulent serotype 2a strain which has an opaque colonial morphology (24570MinI) (Table 2). The latter strain retains a copy of the 140-MDa plasmid, but it is apparently unable to express key determinants of invasion (13).

Transfer of *F lac* to M25-8AminI and detection of metabolically radiolabeled minicell polypeptides. The 120-MDa plasmid of *S. sonnei* (24) and the 140-MDa plasmid of *S. flexneri* (25) exhibit some incompatibility with the FI reference plasmid R386, suggesting that they belong to the FI incompatibility group. To identify the complement of polypeptides which would be expressed in minicells carrying a typical FI plasmid, we studied expression of *F lac*, a classic FI reference plasmid, in an *S. flexneri* background. The *F lac* plasmid was transferred from *Salmonella typhi* 643 to *S. flexneri* M25-8AminI by overnight mating of broth cultures. Transconjugants were plated on minimal medium (13) with 0.2% lactose as a sole carbon source, and colonies from this medium were subcultured on MacConkey medium to confirm the presence of *F lac*. One of these lactose-fermenting colonies, designated M25-8AminI(*F lac*), was chosen for analysis of plasmid-coded polypeptides. Radiolabeled minicells from this strain contained approximately 30 de novo-synthesized polypeptides which were detectable in fluorograms of two-dimensional gels (Fig. 3). The heavily labeled polypeptide marked with an asterisk was labeled in M25-8AminI controls lacking a large plasmid (10). Viru-

TABLE 2. Virulence-associated polypeptides metabolically radiolabeled with [³⁵S]methionine in minicells

Polypeptide designation	Apparent pI ^a	Molecular mass (kDa)	Presence in minicell-producing bacterial strains					
			M90TMinI	M90TMinI ^b	M90TMinII-1 ^c	2457TMinI	24570MinI ^d	4608-58MinVI
a	6.8	78	+	—	—	+	—	+
b	7.0	62	+	—	—	+	—	+
c	7.2	43	+	+ ^e	—	+	—	+
d	6.3	38	+	+ ^e	—	+	—	+
e	9.3	53	+	—	—	+	—	+
f	9.4	25	+	+ ^e	—	+	—	+
g	9.3	20	+	+ ^e	—	+	—	+

^a Isoelectric point could be influenced by nonequilibrium electrofocusing conditions or by noncovalent associations maintained in the presence of nonionic detergent and urea.

^b M90TMinI grown and radiolabeled at 30°C, a nonpermissive temperature for the invasive phenotype (16).

^c Avirulent mutant which has lost a 140-MDa plasmid (10).

^d Avirulent opaque colonial variant (13).

^e Weakly expressed at 30°C.

lence-associated polypeptides a through g, which were prominently labeled in minicells from enteroinvasive strain M90TMinI (Fig. 1), were not labeled in these noninvasive minicells. The weakly labeled polypeptide marked with an arrow in Fig. 3 has the same apparent molecular weight and isoelectric point as the polypeptide marked with an arrow in Fig. 1.

Enrichment of virulence-associated polypeptides by PIEF. Virulent cultures of *S. flexneri* M90T were extracted with nonionic detergent and urea, and these extracts were subsequently mixed with radiolabeled extracts from M90TMinI minicells. These solubilized proteins were separated in PIEF

beds with a final pH gradient of 4.5 to 9.0. Fractions were collected, and those which contained the radioactive tracer proteins were analyzed by SDS-PAGE and fluorography. Polypeptide b was found in fractions with a pH of 5.6 to 5.8, polypeptide c was found at pH 7.1 to 7.7, and polypeptide d was found at pH 6.2 to 6.5. The apparent isoelectric point of polypeptides c and d corresponded to that seen in analytical two-dimensional gels (Table 2), while polypeptide b was somewhat more acidic than expected. Coomassie blue-stained SDS-PAGE gels showed that each of these PIEF fractions contained several unlabeled polypeptide species in addition to a specific plasmid-coded polypeptide (data not

FIG. 2. Two-dimensional electrophoretic analysis of [³⁵S]methionine-labeled plasmid-coded polypeptides extracted from *S. flexneri* M90TMinI minicells which were grown at 30°C and labeled at 30°C. The standard (Std) well on the right shows the same extract separated in a one-dimensional format. The four polypeptides marked with asterisks in Fig. 1 are the predominant plasmid-coded products radiolabeled in this extract. Weakly labeled polypeptides c and d were only detected on X-ray film which was exposed for 14 days. K, 10³ Da.

FIG. 3. Two-dimensional electrophoretic analysis of [^{35}S]methionine-labeled plasmid-coded polypeptides extracted from noninvasive M25-8AMinI(F *lac*) minicells. The circles emphasize the absence of seven polypeptides which were labeled in minicells from invasive M90TMinI shown in Fig. 1. The arrow marks a polypeptide of the same apparent isoelectric point and electrophoretic mobility as the polypeptide which was marked with an arrow in Fig. 1. Numbers on right show positions of molecular size standards (K, 10^3 Da).

shown). Polypeptides a, e, f, and g were not recovered from any PIEF fraction.

Western blots with rabbit antiserum against PIEF fractions. Rabbit antiserum was raised against PIEF fractions containing plasmid-coded polypeptides b, c, or d. The sera were extensively absorbed with *S. flexneri* M90TA2, an avirulent M90T strain which has lost the 140-MDa plasmid, and they were then tested by the Western blot technique for the presence of antibody against polypeptides from an extract of M90TMinI minicells.

The results obtained with antiserum raised against the PIEF fraction containing polypeptide b are shown in Fig. 4. Panel A shows plasmid-coded polypeptides which were metabolically labeled with [^{35}S]methionine. These polypeptides were separated in a two-dimensional format, electroblotted to nitrocellulose paper, and visualized by autoradiography. The same electroblot was then exposed to rabbit antiserum and subsequently to ^{125}I -protein A. Autoradiography of this Western blot (shown in panel B) revealed the location of all polypeptides which were recognized by rabbit immunoglobulin G in addition to the ^{35}S -labeled plasmid-coded polypeptides seen in panel A. Finally, the ^{35}S emissions from the latter polypeptides were blocked by multiple sheets of X-ray film, and only the polypeptides which had bound immunoglobulin G were demonstrated by autoradiography with an intensifying screen (panel C). By alignment of the latter spots with those seen in panels B and A, it was apparent that antibody in this serum, which had been absorbed with a shigella strain lacking the 140-MDa plasmid, recognized plasmid-coded polypeptide b.

Two agarose wells were cast on the end of the two-dimensional gel shown in Fig. 4. Unlabeled SDS extracts of

S. flexneri M90T and M90TA2 were loaded into these wells and electrophoresed along with the electrofocused polypeptides from M90TMinI. Panels B and C show that the rabbit antiserum strongly reacted with a band in the tract which contained the virulent M90T extract, and this band had the same molecular mass as the polypeptide identified as b in the two-dimensional format. It should also be noted from panel C that five additional polypeptides were recognized by this antiserum, but since these polypeptides were also recognized in the extract of M90TA2, they probably represent epitopes on chromosomal products which were not exposed in the membrane of the avirulent bacteria used in the absorption procedure. One of these chromosomal polypeptides had a molecular mass which was similar to that of plasmid-coded polypeptide b. Fortunately, this polypeptide had a more acidic isoelectric point than the plasmid-coded species. Although this chromosomal polypeptide was radiolabeled in the lane which contained the extract from avirulent strain M90TA2, comparatively little antibody was bound, so the absence of plasmid-coded polypeptide b from this extract was easily discernible.

Rabbit antisera raised against PIEF fractions containing plasmid-coded polypeptides c or d were also analyzed as described above, and in each instance the absorbed antisera specifically recognized the appropriate polypeptide on Western blots (data not shown). Each of these antisera also recognized a few other protein antigens; however, antibody against the latter polypeptides reacted with extracts from both virulent and avirulent strains. Presumably these polypeptides are encoded by genes on the chromosome and are not exposed on the surface of the M90TA2 cells which were used to remove antibody against chromosomal pro-

FIG. 4. Electrophoretogram of [^{35}S]methionine-labeled plasmid-coded polypeptides extracted from invasive *S. flexneri* M90TMinI minicells and separated in a two-dimensional format. (A) ^{35}S -labeled polypeptides detected by autoradiography; (B) the same electrophoretogram after incubation with rabbit antiserum raised against a PIEF fraction from M90T containing polypeptide b. Antibody specifically bound to electroblotted antigens was detected with ^{125}I -protein A (Western blot). Both plasmid-coded and antigenic polypeptides were visualized by autoradiography. Panel C shows the same Western blot with ^{35}S emissions blocked by layers of film and ^{125}I emissions enhanced by an intensifying screen. Only antigenic polypeptides are seen in this autoradiograph. On the right side of the electrophoretogram are lanes containing unlabeled whole-cell extracts from virulent M90T (left) or avirulent M90TA2 (right).

teins. The availability of rabbit antisera recognizing three different virulence-associated polypeptides encoded by the 140-MDa plasmid of *S. flexneri* serotype 5 allowed the use of these antisera as probes to search for similar polypeptides in other enteroinvasive strains.

Figure 5 shows a composite of three Western blots of whole-cell SDS extracts from *S. flexneri* serotype 3 (lane 1), *S. flexneri* serotype 5 (lanes 2 and 3), *S. sonnei* (lanes 4 and 5), and *E. coli* (lanes 6, 7, and 8). The rabbit antisera which were used in these blots were raised against antigens of *S.*

FIG. 5. Western blots of whole-cell extracts from the following strains (lanes): 1, *S. flexneri* serotype 3 (J17B); 2, *S. flexneri* serotype 5 (M90T); 3, *S. flexneri* serotype 5 (M90TA2); 4, *S. sonnei* form I (53G); 5, *S. sonnei* form II (53GII); 6, *E. coli* O143 (4608-58); 7, *E. coli* K-12 (395-1); 8, *E. coli* O6:H16 (B2C). Symbols: +, virulent cultures which are able to invade HeLa cells; -, avirulent, noninvasive strains which have lost either a 120- or a 140-MDa plasmid. This figure is a composite of three individual Western blots of the same set of extracts which were incubated with rabbit antisera recognizing plasmid-coded polypeptide b, c, or d. Only the region of the gel containing the polypeptide of interest is shown. The antigenic band migrating below polypeptide d is one of the major outer membrane proteins.

flexneri serotype 5, but they recognized similar epitopes in all of the enteroinvasive strains tested. These epitopes were not detected in strains which did not harbor a virulence-associated plasmid. Thus, Western blot analysis suggests that antigenically similar plasmid-coded polypeptides are expressed by all enteroinvasive pathogens.

Western blots with convalescent serum from a monkey which had been infected with *S. flexneri* serotype 2a. Minicell suspensions from *S. flexneri* M90TMinI serotype 5 or enteroinvasive *E. coli* 4608-58MinVI were incubated with [^{35}S]methionine, the solubilized polypeptides were separated in a two-dimensional format and blotted to nitrocellulose, and radiolabeled components were visualized by autoradiography. Plasmid-coded polypeptides a through g were radiolabeled in minicells from both enteroinvasive species (Fig. 6A and C). These electroblots were then reacted with serum from a monkey which had been infected with *S. flexneri* serotype 2a, and antibody recognizing bacterial components was tagged with ^{125}I -labeled protein A. Emissions from ^{35}S were blocked with X-ray film, and autoradiography showed only ^{125}I -labeled spots. By overlaying the film which had been exposed to ^{35}S on the film which had been exposed to ^{125}I and matching the spots, it was concluded that four of the seven virulence-associated polypeptides were recognized by this convalescent antiserum (Fig. 6B and D). Prechallenge monkey serum did not recognize any of these polypeptides (data not shown).

DISCUSSION

Surveys of shigella and enteroinvasive *E. coli* strains from diverse geographical and historical origins have confirmed the ubiquity of large virulence-associated plasmids in invasive enteric pathogens (12, 21, 27). In addition, mobilization of the 140-MDa plasmid from *S. flexneri* serotype 5 into *E. coli* K-12 can confer the invasive phenotype on the latter normally noninvasive recipient strain (23). Since these investigations indicate that some of the determinants of the invasive phenotype are plasmid-coded, characterization of plasmid products is a high priority. Preliminary studies indicated that at least 10 polypeptides ranging from 12 kDa to 62 kDa (molecular mass) are always synthesized in minicells containing virulence-associated plasmids (10). However, it

FIG. 6. Polypeptides from minicells of *S. flexneri* M90TMinI (A and B) or *E. coli* 4608-58MinVI (C and D). Virulence-associated polypeptides are designated a through g. (A and C) Autoradiographs of electroblots showing polypeptides intrinsically labeled with [³⁵S]methionine; (B and D) Western blots of the same nitrocellulose sheets treated with convalescent antiserum from a rhesus monkey which had been infected with *S. flexneri* serotype 2a. A lane of unlabeled vegetative-cell extract from M90T was included in the gel which was electroblotted for panels A and B, and polypeptides a through d are seen in a one-dimensional track on the right side of panel B.

would not be expected that all plasmid-coded polypeptides are directly involved in the invasion process, even though they may be involved in necessary support functions. For example, six polypeptides are necessary for maintenance and replication of FI plasmids (18, 26). Since the virulence-associated plasmids of *S. sonnei* and *S. flexneri* segregate at a 50% rate when an FI reference plasmid is present in these species (24, 25), the native plasmids would be expected to express some FI-type regulatory polypeptides. However, these polypeptides do not in and of themselves constitute a functional determinant for the invasive phenotype because shigellae which harbor FI plasmids such as the *F lac* plasmid do not invade mammalian cells (24).

Identification of the polypeptides which would be encoded by an FI incompatibility group plasmid which was not associated with the invasive phenotype was a prerequisite for identification of products which are unique to the viru-

lence-associated plasmids. Therefore, the *F lac* plasmid was transferred to an *S. flexneri* serotype 1 minicell-producing strain which did not harbor the 140-MDa plasmid. As expected, this plasmid did not restore expression of the invasive phenotype. Metabolically radiolabeled minicell polypeptides were separated in a two-dimensional electrophoresis format and visualized by fluorography. About 30 radiolabeled polypeptides were detected by this method (Fig. 3). A similar group of 30 polypeptides was radiolabeled in invasive strains of *S. flexneri* serotypes 2a and 5, and enteroinvasive *E. coli*, but an additional complement of seven polypeptides was also labeled (Fig. 1). Since the latter virulence-associated polypeptides were usually heavily labeled (Fig. 4A and 6A) they may be coupled to a strong promoter(s). Under the conditions of limited RNA polymerase existing in minicells, such differential expression of plasmid products is expected (5). The seven virulence-

associated polypeptides were labeled in minicells from invasive strains of *S. flexneri* and *E. coli* but not in an avirulent colonial variant, and labeling was also repressed in organisms grown at 30°C. The molecular weights of six of the virulence-associated plasmid products which were identified in two-dimensional gels corresponded roughly to those calculated for virulence-associated polypeptides previously seen in one-dimensional gels (10).

The extremely basic isoelectric point of a subset of plasmid-coded products designated e, f, and g suggests that they may be regulatory proteins which would bind to DNA. Alternatively, they could function as transport or anchorage proteins analogous to two proteins made by the K88 and K99 gene clusters (F. R. Mooi, E. Roosendaal, and F. K. deGraaf, Abstr. FEMS Meet. Mol. Biol. Microbiol. Pathol. 1985, Lulea, Sweden). It could also be speculated that protein e is actually a dimer which is stable in the nonionic detergent and urea used in isoelectric focusing but is partially disaggregated into f and g subunits by the SDS used in the second dimension of the electrophoresis procedure. Finally, it should be noted that all plasmid-coded polypeptides necessary for the invasive phenotype may not be detected by metabolically radiolabeling minicells. For example, the labeling of polypeptide a was very weak in the minicell preparation shown in Fig. 4A, and other polypeptides with a catalytic function might be missed entirely when using the radiolabeling technique. In addition, large plasmid-coded polypeptides might not enter the relatively high percentage polyacrylamide gels used in these studies. Nonetheless, these techniques have proved sensitive enough to identify seven virulence-associated proteins present in amounts which are below the detection levels of the protein stains used in previous studies (29).

Recently a 37-kilobase (kb) virulence-associated region of the 140-MDa plasmid of *S. flexneri* has been identified (15). Insertion mutagenesis and cosmid cloning show that this 37-kb region, which contains three *EcoRI* fragments of 7.6, 11.5, and 17 kb, carries all the plasmid loci which are necessary for the invasion of HeLa cells. Western blot analysis of an invasive cosmid clone confirmed that polypeptides a through d are encoded by this 37-kb region (15). Since no antisera against polypeptides e, f, and g are available, it is not known if they are also encoded by the cloned fragment. Even if it is assumed that polypeptide e is a separate species rather than a dimer of f and g, only approximately 8 kb of information would be required to encode all the virulence-associated products thus far identified.

The base sequence in the 17-kb *EcoRI* fragment from the 140-MDa plasmid is so highly conserved that this fragment can serve as a DNA hybridization probe to identify all virulent shigella isolates (3). The implication of this homogeneity is that a set of attributes which allows an enteric organism to induce its own endocytic uptake by epithelial cells occurs infrequently in nature and that once established, the genetic basis of this set of attributes is extremely stable. Thus, it would be expected that antibody which recognizes a component(s) expressed by plasmid virulence genes would cross-react with all enteroinvasive *Shigella* and *E. coli* strains. Western blots with rabbit antiserum raised against individual plasmid-coded polypeptides (Fig. 5), or using serum from a monkey which had been infected with shigellae (Fig. 6C and D), indicated that antibody against plasmid-coded polypeptides a through d cross-reacts with similar plasmid-coded polypeptides in enteroinvasive strains of many serotypes.

Polypeptides a through d from *S. flexneri* serotype 5 were the predominant proteinaceous antigens recognized by antiserum from a monkey which had been challenged with serotype 2 (Fig. 6B). A similar complement of antibody was found in serum from humans which had been naturally infected with *S. flexneri* serotype 2a or *S. sonnei*, and since these sera reacted strongly with intact bacteria in an enzyme-linked immunosorbent assay, at least some of the antigenic polypeptides were expressed on the bacterial surface (E. V. Oaks, T. L. Hale, and S. B. Formal, manuscript in preparation). Apparently these polypeptides, which are quantitatively minor components of the bacterial cell, are the primary constituents which allow the primate immune system to differentiate invasive shigellae from the endogenous intestinal flora. This remarkable finding, when considered in the context of the ubiquity of these polypeptides among enteroinvasive pathogens, suggests that they may be components of a hypothetical protein determinant of the invasive phenotype (11). Since we believe that such a virulence determinant should have the characteristics of a receptor which would bind to ligands on the host-cell plasma membrane and induce the uptake of enteroinvasive pathogens, future studies will test these polypeptides for receptor function.

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