Molecular cloning and characterization of form I antigen genes of *Shigella sonnei*

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Sau3AI-generated DNA fragments of the *Shigella sonnei* large plasmid encoding the form I antigen were cloned into *Escherichia coli* with cosmid vector pHSG262. One resulting plasmid, designated pJK1137, was studied further. Restriction endonuclease mapping and analysis of transposon Tn3 insertion mutants demonstrated that the form I antigen genes were located within a region of about 12.6 kb consisting of the two contiguous *HindIII* fragments of 1.26 kb and 12.4 kb. The results of complementation studies between Tn3 insertion mutants of pJK1137 and recombinant plasmids carrying different parts of the form I antigen genes indicated that the 12.6 kb DNA sequence contained at least four gene clusters, regions A, B, C and D. Analysis of radioactively labelled proteins in minicells demonstrated that the DNA sequence of about 12.6 kb coded for at least four specific proteins of 42, 23, 48 and 39 kDa. The former two were coded by region A, the latter two by region D.

Introduction

Diarrhoeal disease is a major cause of morbidity and mortality worldwide, especially in developing countries. *Shigella* remains one of the most important causes of diarrhoeal disease. Although the number of *Shigella* infections has been reduced over the past several decades, a considerable number of cases, most of them travellers returning from overseas, still occur annually in Japan. *Shigella sonnei*, one of the four species of this genus, is currently responsible for more than half of the shigellosis cases reported in Japan.

Bacteria of the genus *Shigella* produce, via the processes of penetration, multiplication and intercellular dissemination, an infection that is normally limited to the epithelial cells of the colon and that results in bloody and/or mucous diarrhoea. Previous genetic studies of *Shigella flexneri* have revealed that virulence in this species is multideterminant, with plasmid-encoded and widely separated chromosomal regions being required (Sansonetti *et al.*, 1983). In addition to the attributes responsible for epithelial cell penetration and multiplication, the somatic O antigen is also important for virulence in *Shigella* (Okamura *et al.*, 1983; Sansonetti *et al.*, 1981, 1983). Earlier studies on vaccination (DuPont *et al.*, 1969; Formal *et al.*, 1966; Mel *et al.*, 1965) indicated that protection against bacillary dysentery is

associated with O antigen specificity. Although it is known that the somatic O antigen is one of the virulence factors and induces a protective immune response in humans, it is still unclear how the O antigen participates in the pathogenesis of *Shigella* infection.

All virulent *Shigella sonnei* strains, unlike other *Shigella* species, comprise a single serotype which produces smooth colonies expressing a somatic antigen termed form I. This antigenic specificity corresponds to the O side chains of the lipopolysaccharide layer, which are composed of disaccharide repeating subunits containing two unusual amino sugars (Kenne *et al.*, 1980; Gamian & Romanowska, 1982). These smooth colonies are usually unstable and dissociate to rough-appearing colonies termed form II, which are uniformly avirulent. Unlike the majority of the species of the family *Enterobacteriaceae*, in which O antigen genes are located on the bacterial chromosome, in *S. sonnei* (Kopecko *et al.*, 1980) and in a strain of *Escherichia coli* O111 (Riley *et al.*, 1987) the complete O antigen genes are plasmid-borne. Previous reports have demonstrated that a large 120 MDa plasmid of *S. sonnei* is unique in that it is responsible not only for the ability to penetrate epithelial cells (Sansonetti *et al.*, 1981), but also for the expression of the form I antigen (Kopecko *et al.*, 1980).

In the present study, we cloned the form I antigen genes from a DNA library of the large plasmid of *S.*

sonnei and characterized these genes by transposon-insertion mutagenesis and complementation studies.

Methods

Bacterial strains and plasmids. *Shigella sonnei* form I strain HW383 harbouring pSS120 (Watanabe & Nakamura, 1985) was used as a source of form I DNA. A form II strain, *S. sonnei* HW506, was obtained by curing *S. sonnei* HW383 of pSS120. *Escherichia coli* strains HB101 (*hsdS20 recA13 ara proA lacY galK rpsL xyl mtl supE*) (Boyer & Roulland-Dussoix, 1969), JM109 (*recA endA gyrA thi hsdR17 supE relA1 Δ(lac-proAB)/F'[proA⁺B⁺ lacI^q lacZΔM15 traD]*) (Yanisch-Perron *et al.*, 1985) and K12 921 (Yoshimori *et al.*, 1972) were used as recipients in transformation. *E. coli* TH1219 (*F⁻ minB recA1 rpsL tsr tar*) (Harayama & Hazelbauer, 1982) is a minicell-producing strain of *E. coli* K12. DNAs of pUC18 (Yanisch-Perron *et al.*, 1985), pACYC184 (Chang & Cohen, 1978), pBR322 (Maniatis *et al.*, 1982) and cosmid pHSG262 (Brady *et al.*, 1984) were used for cloning. The plasmid pKDEC100 (kindly supplied by Dr H. Danbara, Kitasato Institute), used as the Tn3 transposon donor, is a thermosensitive-replication mutant of pHSG415 (Hashimoto-Gotoh *et al.*, 1981). Most of the other plasmids were prepared in this study.

Media and reagents. Bacteria were grown in L-broth (Lennox, 1955) or on plates of Antibiotic medium no. 3 (PAB; Difco) with 1.5% (w/v) agar. The following antibiotics were used: ampicillin (Ap), kanamycin (Km), chloramphenicol (Cm) and tetracycline (Tc). Restriction enzymes were purchased from Toyobo.

Preparation of antigen. For immunization of rabbits and preparation of the agglutination antigens, test strains were cultivated on PAB agar plates. The organisms were harvested from agar plates in 0.85% sterile saline. For use as vaccines, formalin (0.3%, v/v) killed cells were diluted in sterile saline to a density of approximately 1×10^9 organisms ml⁻¹. For use in the agglutination test, the formalin- or heat-killed cells were standardized in sterile saline to an OD₆₅₀ of 0.4.

Preparation of antisera. Young, male rabbits, each weighing 3 to 4 kg, were used. Intravenous injections of the vaccine were given twice weekly. The doses were 0.25, 0.5, 1.0 and 1.5 ml, respectively, for the first 2 weeks and 2.0 ml each for the last four doses. At 1 week after the last injection, blood was collected aseptically and serum was obtained.

Absorption of antisera. Formalin- or heat-treated packed cells (100 mg) were added to 1.0 ml of undiluted or 10-fold diluted antiserum. The antiserum/cell suspensions were mixed and incubated in a 37 °C water bath for 2 h, followed by overnight incubation at 4 °C. The absorbed antiserum was collected by centrifugation.

Slide agglutination test. For most of the antigenic analysis of form I and form II antigens, slide agglutination tests were done using the commercial form I and form II antigen-specific rabbit antisera (Denka-Seiken).

Quantitative agglutination test. Serial dilutions (0.5 ml) starting at a 1:10 dilution of serum were mixed in test tubes with 0.5 ml of a suspension of whole cells of *S. sonnei*. The tubes were kept at 37 °C for 2 h and at 4 °C overnight. Endpoints were taken as the reciprocal of the highest dilution of serum to give complete agglutination of the bacterial suspension by comparison with a negative saline control.

Construction of a gene library of *S. sonnei* form I plasmid DNA. The methods used for cosmid cloning were essentially as described by Maniatis *et al.* (1982). Form I plasmid DNA of the 120 MDa plasmid in *S. sonnei* HW383, prepared as described previously (Watanabe &

Nakamura, 1986), was partially digested with restriction endonuclease *Sau3AI*, and fragments in the size range 30 to 50 kb were isolated by sucrose density-gradient centrifugation. These fragments were ligated into the *Bam*HI site of cosmid vector pHSG262. Clones of *E. coli* HB101 harbouring cosmids containing insertions of form I antigen genes were examined by the slide agglutination test.

Recombinant DNA methods. Rapid isolation of plasmid DNA was performed by the method of Birnboim & Doly (1979). When necessary, plasmid DNA was further purified by ultracentrifugation in caesium chloride/ethidium bromide density gradients. Restriction-endonuclease-cleaved DNA was analysed by electrophoresis through horizontal 0.7% (w/v) agarose gels or vertical 3.5% (w/v) polyacrylamide gels in TBE buffer (89 mM-Tris base, 89 mM-boric acid, 2.5 mM-disodium EDTA, pH 8.3) or E buffer (40 mM-Tris, 2 mM-disodium EDTA pH 7.9) (Kado & Liu, 1981). Restriction fragments of DNAs of phages λ and φX174 were used as molecular markers. Restriction fragments of DNA were isolated from low-melting agarose gel (Sea plaque Agarose, FMC) as described by Weislander (1979). Ligation was performed using a Takara ligation kit at 16 °C for 1 h. Transformation with ligated DNA was carried out according to the procedure of Mandel & Higa (1970).

Tn3 insertion mutagenesis. *E. coli* HB101 harbouring both pJK1137 and pKDEC100 was preincubated in PAB containing both Ap (100 µg ml⁻¹) and Km (50 µg ml⁻¹) at 30 °C. The culture was spread on PAB agar plates containing both Ap (500 µg ml⁻¹) and Km (100 µg ml⁻¹) and incubated at 43 °C. The resulting colonies were purified three times on the same medium at 43 °C.

Analysis of lipopolysaccharide (LPS). LPS from whole-cell lysates was prepared by the procedure of Hitchcock & Brown (1983), and subjected to SDS-PAGE (12% acrylamide), using the buffer system of Laemmli (1970). LPS bands were visualized by silver staining (Hitchcock & Brown, 1983).

Analysis of plasmid-encoded proteins in minicells. Preparation and labelling of minicells were performed essentially as described previously (Kato *et al.*, 1984). The labelled proteins were subjected to SDS-PAGE and autoradiographed.

Results

Gene bank of the form I antigen genes

Sau3AI-generated DNA fragments of pSS120 were cloned into *E. coli* HB101 with cosmid vector pHSG262. Nine out of 260 clones harbouring recombinant cosmids were found to express form I antigen as determined by the slide agglutination test. Two of the form I-antigen-positive recombinant clones, designated HB101(pJK1137) and HB101(pJK1139), were selected for further study. Both these clones agglutinated with form I antiserum at the same level as *S. sonnei* HW383(pSS120). Stability of the recombinant plasmids was tested in *E. coli* HB101, after incubation in Penassay broth for about 15 generations at 37 °C without Km. pJK1137 was maintained in the host strain at a frequency of 70%, whereas pJK1139 was unstable, being maintained at a frequency of only 15%.

Quantitative agglutination test

S. sonnei HW383 and HW506(pJK1137), a transformant of HW506 with pJK1137, both of which agglutinated strongly with form I antiserum in the slide agglutination test, were used to immunize rabbits and antisera were obtained. The antisera were then used in reciprocal agglutinin absorption tests, with formalin- or heat-killed bacterial cells as antigens. The results suggested that the two strains shared an identical form I O antigen (Table 1). The polyclonal sera we used (even in the absorbed anti-HW383 sera) may have contained antibodies against the virulence-associated proteins encoded by the virulence plasmid. However, even with relatively high agglutination titres of serum, the virulence-associated proteins are not detected by agglutination tests (LaBrec *et al.*, 1964), and *Shigella* has no H antigens and few K antigens. We therefore conclude that we are looking mostly at the O antigens by the agglutination test.

Analysis of LPS by SDS-PAGE

To confirm the results of the agglutination tests, these isogenic strains were examined for LPS structure by SDS-PAGE. pJK1137 expressed in *E. coli* and *S. sonnei* similar patterns of O-specific or ladder bands in SDS-PAGE as those of the wild-type HW383 (Fig. 1 *a*, lane 4; Fig. 1 *b*, lanes 4 and 5). In contrast, the form II strain HW506 retained only the low-molecular-mass band that may represent all or part of the polysaccharide core unit conjugated to lipid A (Fig. 1 *b*, lane 6).

Restriction cleavage analysis of the recombinant plasmids harbouring form I antigen genes

Two of the recombinant plasmids described above were analysed with the restriction enzymes *EcoRI*, *SalI*, *HindIII* and *BglII*. Both of the plasmids, after cleavage with *HindIII*, yielded a common 12.4 kb fragment, which consisted of *BglII* fragments of 5.3, 1.66, 0.68, 0.66, and 0.72 kb, and *HindIII*–*BglII* fragments of 2.6 and 0.78 kb (Fig. 2). On the basis of the above results, we speculated that the 12.4 kb *HindIII* fragment might contain all the genes encoding form I antigen. Therefore, the 12.4 kb *HindIII* fragment of pJK1137 was cloned into the *HindIII* site of pBR322, and the resulting recombinant plasmid pIP35 was used to transform strains HB101 and HW506. However, the transformed strains did not direct the synthesis of active form I antigen (Fig. 2). To define the whole region of form I antigen genes on the recombinant plasmid, pJK1137 DNA was subjected to detailed restriction cleavage analysis. The restriction endonuclease cleavage map of pJK1137 is shown in Fig. 2.

Table 1. Reciprocal agglutinin absorption test

The results were the same regardless of whether the antigens were formalin- or heat-killed.

Antiserum prepared against	Absorption with	Agglutinin titre against	
		HW383	HW506 (pJK1137)
HW383	–	1280	1280
	HW506 (pJK1137)	< 20	< 20
HW506 (pJK1137)	–	640	640
	HW383	< 20	< 20

–, Unabsorbed.

Isolation of form I antigen-negative Tn3 insertion mutants of pJK1137

Using a thermosensitive-replication mutant of pKDEC100 carrying transposon Tn3, we constructed Tn3 insertion mutants of pJK1137. The insertion sites of Tn3 were assigned to each of the *HindIII* fragments after cleavage analysis with *HindIII*. Of 60 form I antigen-negative Tn3 insertion mutants obtained, 58 contained Tn3 molecules within a 12.4 kb *HindIII* fragment, the remaining two within the 1.26 kb *HindIII* fragment (Figs 2 and 3). The Tn3 insertions into *HindIII* fragments of 17.7, 6.0 and 1.26 kb were identified as being form I antigen-positive. We could not detect form I antigen-positive strains with Tn3 inserted into the 12.4 kb *HindIII* fragment. The results indicated that form I antigen genes were located at least on the contiguous *HindIII* fragments of 1.26 and 12.4 kb (Figs 2 and 3). The precise positions of Tn3 insertions in plasmid pJK1137 were determined on some of the plasmids with or without form I antigen production by calculating the sizes of *SalI*–*BamHI* and *HindIII*–*BamHI* fragments of the respective plasmids. *SalI* and *HindIII* have no cleavage site on Tn3, and *BamHI* has only one cleavage site on Tn3 and no cleavage site on pJK1137. Some of the Tn3 insertions, which are indicated as pIP, are shown in Fig. 3. Of 30 form I antigen-negative plasmids, 22 had Tn3 insertions in the regions between the Tn3 insertion site of pIP2 and that of pIP4, and one into the same site as pIP1. On the other hand, one of the form I antigen-positive plasmids, designated pIP30, had Tn3 inserted about 0.2 kb from one end of the cosmid vector DNA (Fig. 3). Another form I antigen-positive plasmid, designated pIP31, had Tn3 inserted about 1.6 kb from one end of the 12.4 kb *HindIII* fragment (Fig. 3). These results indicated that the region indispensable for biosynthesis of form I antigen is located on the region of about 14.2 kb between the Tn3 insertion sites of pIP30 and pIP31 (Fig. 3).

Fig. 1. SDS-PAGE patterns of the LPS of form I antigen-positive and -negative strains. Whole-cell lysates treated with proteinase K were subjected to SDS-PAGE and detected by silver staining. Lanes: (a) 1, *Salmonella typhimurium* LT-2 as a standard; 2, *E. coli* HB101(pIP6); 3, HB101(pIP36); 4, HB101(pJK1137); 5, HB101. (b) 1, LT-2; 2, *S. sonnei* HW506(pIP6); 3, HW506(pIP36); 4, HW506(pJK1137); 5, *S. sonnei* HW383; 6, HW506.

Fig. 2. Restriction cleavage map of pJK1137 and subcloning of form I antigen genes. The upper part of the figure shows the restriction map of pJK1137. The cosmid vector pHSG262 is shown by the open box. The following enzymes were used: H, *Hind*III; B, *Bgl*II; Bm, *Bam*HI; E, *Eco*RI; S, *Sal*I; Sm, *Sma*I. The unique *Bam*HI site was only assigned onto the transposon Tn3. A 12.4 kb *Hind*III fragment and a 17.8 kb *Sma*I fragment of pJK1137 were cloned into the vector plasmids pBR322 and pUC18 respectively. Insertion of transposon Tn3 into the 6.0 kb *Hind*III fragment (plasmid pIP31) of pJK1137 (Fig. 3) did not affect the form I antigen-positive phenotype (open circle). A *Sal*I-*Bam*HI fragment of 20.7 kb prepared from pIP31 was also cloned into the vector pACYC184. The minimum size of form I antigen regions estimated in this study is shown at the bottom by a thick bar. +, Form I antigen-positive; -, form I antigen-negative.

Subcloning of essential genes for the synthesis of *S. sonnei* form I antigen

As a first step in subcloning the form I antigen genes, a 17.8 kb *Sma*I fragment of pJK1137, which contained the complete form I antigen region along with a 1.2 kb

portion of cosmid vector pHSG262 DNA, was cloned into the *Sma*I site of the plasmid vector pUC18. The recombinant plasmid, designated pIP36, produced the form I antigen in the *E. coli* HB101 and *S. sonnei* HW506 (Fig. 1a, b, lanes 3; Fig. 2). A second recombinant plasmid, designated pIP37, was a pACYC184-ligated

Fig. 3. The physical location of Tn3 insertions on the three contiguous *Hind*III fragments of pJK1137, and the recombinant plasmids prepared for the complementation studies. Plasmids marked with open circles and solid circles were positive and negative for form I antigen, respectively. The other symbols are as in the legend to Fig. 2. The *Sal*I–*Bam*HI fragments of Tn3 insertion mutants of pJK1137 were cloned into the *Sal*I–*Bam*HI site of pACYC184. The *Sma*I–*Bam*HI fragments of Tn3 insertion mutants of pJK1137 were cloned into the *Hinc*II–*Bam*HI site of pACYC184. *Hind*III–*Bam*HI fragments of pIP5 and pIP4 were also cloned into the *Hind*III–*Bam*HI site of pACYC184, and the resulting plasmids were designated pIP22 and pIP23 respectively. The region of Tn3 insertion sites of pIP30, pIP1 and pIP2 is enlarged.

recombinant of the 20.7 kb *Sal*I–*Bam*HI fragment prepared from the form I antigen-positive plasmid pIP31. Strains HB101 and HW506 harbouring pIP37 also produced the form I antigen (Fig. 2). We then tried to determine whether the 6.0 kb *Hind*III fragment is necessary for expression of the form I antigen. The plasmids pIP22 and pIP23 (Fig. 3), in which the *Bam*HI–*Hind*III fragment of pIP5 and pIP4 had been cloned into the vector pACYC184 respectively, were separately introduced into *E. coli* K12 921 carrying a resident plasmid pIP17 or pIP18 (Fig. 3) by selection for resistances to Ap, Cm and Km. In this experiment, neither of the plasmids contained the 6.0 kb *Hind*III fragment. The *E. coli* strain harbouring both pIP22 and pIP17 (or pIP18) directed the synthesis of form I antigen. Similar results were obtained with the strain harbouring both pIP23 and pIP17 (or pIP18). These results showed that the 6.0 kb *Hind*III fragment is not essential for form I antigen production. We concluded that the essential genes for form I antigen synthesis are located on the region of about 12.6 kb in length and ranged in the two contiguous *Hind*III fragments of 1.26 kb and 12.4 kb (Fig. 2).

Complementation studies between Tn3 insertion mutants of pJK1137 and recombinant plasmids carrying different parts of the form I antigen genes

The related gene clusters of form I antigen synthesis were estimated by complementation studies between Tn3 insertion mutants of pJK1137 and recombinant plasmids carrying different parts of the form I antigen genes. As a first step, we obtained the recombinant plasmids pIP11–pIP18 by isolating through electrophoresis in a low-melting agarose gel the *Sal*I–*Bam*HI fragments of the eight different Tn3 insertion mutants (pIP1–pIP8) of pJK1137 and ligating them into the *Sal*I–*Bam*HI site of vector pACYC184 (Fig. 3). By the same procedure, we also obtained the recombinant plasmids pIP21, pIP24, pIP25, pIP27 and pIP28, in which *Sal*I–*Bam*HI fragments of pIP1, pIP4, pIP5, pIP7 and pIP8, respectively, were cloned into the vector pACYC184 (Fig. 3). Complementation studies were carried out on *S. sonnei* HW506 (form II). Strains of HW506 independently harbouring one of eight different Tn3 insertion mutants of pJK1137 were super-infected with each of the recombinant plasmids by selection for resistances to Ap,

Fig. 4. Summary of gene clusters for biosynthesis of form I antigen derived from the results presented in this study. The approximate boundaries of the regions A, B, C and D were determined by the complementation studies (Table 2). The sizes of the proteins encoded by genes in regions A and D are indicated. Proteins encoded by regions C and D were not identified in this study. The symbols are as in the legend to Fig. 3.

Table 2. Results of complementation tests between *Tn3* insertion mutants of *pJK1137* and plasmids carrying different parts of the form I antigen genes

Super-infected plasmid	pJK1137::Tn3 in HW506							
	pIP1	pIP2	pIP3	pIP4	pIP5	pIP6	pIP7	pIP8
pIP11	—	—	—	—	—	—	—	—
pIP12	—	—	—	—	—	—	—	—
pIP13	—	—	—	—	—	—	—	—
pIP14	—	—	—	—	—	—	—	—
pIP15	—	—	—	—	—	—	—	—
pIP16	+	+	+	+	+	—	—	—
pIP17	+	+	+	+	+	+	—	—
pIP18	+	+	+	+	+	+	+	—
pIP28	—	—	—	—	—	—	—	—
pIP27	—	—	—	—	—	—	—	±
pIP24	—	—	—	—	—	+	+	+
pIP21	—	—	—	—	—	+	+	+

+, Agglutination; ±, weak agglutination; —, no agglutination.

Km and Cm, and transformants thus obtained were then examined for form I antigen production. The results of the complementation tests are summarized in Table 2. When each of the recombinant plasmids, pIP11, pIP12, pIP13, pIP14 and pIP15, was independently introduced into HW506 harbouring one of the *Tn3* insertion mutants, none of the transformants could synthesize active form I antigen. pIP16 restored the synthesis of form I antigen of HW506 harbouring the *Tn3* insertion mutant plasmids pIP1, pIP2, pIP3, pIP4, or pIP5. Plasmids pIP17 and pIP18 complemented pIP6 and pIP7, respectively. Similar results were obtained with complementation tests between recombinant plasmids pIP21–pIP28 and the *Tn3* insertion mutants (Table 2). The results of our complementation studies suggested that the form I antigen genes within the two contiguous

HindIII fragments of 1.26 kb and 12.4 kb are composed of at least four gene clusters (Fig. 4).

Identification in minicells of proteins determined by form I antigen genes

The recombinant plasmids carrying different parts of the form I antigen regions were used to transform the minicell-producing strain TH1219 to determine the proteins encoded by the recombinant plasmids. Extracts of [³⁵S] labelled minicells were subjected to SDS-PAGE and autoradiographed. Two plasmids, pJK1137 and pIP37, which comprised the entire form I antigen genes, synthesized four specific proteins of apparent molecular mass 48, 42, 39, and 23 kDa (Fig. 5). The recombinant plasmid pIP35, which contained the 12.4 kb *HindIII* fragment in the vector pBR322, also synthesized the four proteins of the same sizes as those of pJK1137 and pIP37. From the results of the autoradiographs of the SDS-PAGE, we could not find any intrinsic difference between the proteins encoded by the plasmids pJK1137 and pIP35, although the former was form I antigen-positive, while the latter was form I antigen-negative. The plasmids pIP13, pIP14 and pIP15, comprising defective A region (Figs 3 and 4), did not direct the synthesis of any specific proteins, whereas the plasmid pIP16, carrying only intact A region (Figs 3 and 4), directed the synthesis of two proteins of 42 kDa and 23 kDa. On the other hand, plasmid pIP27, which contained an intact D region (Figs 3 and 4) directed synthesis of the proteins of 48 kDa and 39 kDa (data not shown). We could not identify precisely the products encoded by the B and C regions.

Discussion

The DNA fragments we have cloned appear to be essential and sufficient for full expression of *S. sonnei* form I antigen. Results of the reciprocal agglutinin absorption test and analysis of LPS by SDS-PAGE suggested that the DNA fragments we cloned in the vector plasmid expressed as much form I antigen as did the wild-type strain. However, detailed chemical analysis of the outer-membrane LPS of these strains should be done and is now under way. The present studies revealed that, in the *S. sonnei* large plasmid, there are several genes that are required for form I antigen production. The results also suggested that these genes are organized into at least four gene clusters (or operons). For unknown reasons it was found more suitable to use a *S. sonnei* form II strain than to use *E. coli* HB101 as a host strain for the plasmids in the complementation studies. The fact that the form I antigen consists of at least four disaccharide

Fig. 5. Identification of the proteins encoded by form I antigen genes. *E. coli* TH1219 minicells containing the plasmid indicated were incubated with [35 S]methionine (1000 Ci mmol $^{-1}$; 37 Tbq mmol $^{-1}$) for 15 min and the labelled proteins were separated on SDS-polyacrylamide gels (12% acrylamide) and autoradiographed. Proteins encoded by form I antigen genes are marked with arrows, indicating their apparent molecular masses. The minicells contained the following plasmids: cosmid vector pHSG262 (lane 1), pBR322 (lane 2), pACYC184 (lane 3), pIP35 (lane 4), pJK1137 (lane 5), pIP18 (lane 6), pIP37 (lane 7), pIP17 (lane 8), pIP16 (lane 9), pIP15 (lane 10), pIP14 (lane 11), and pIP13 (lane 12). The following molecular mass markers (Bio-Rad), indicated on the right, were used: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa).

repeating units (Gamian & Romanowska, 1982) suggested that the form I antigen genes are complex and consist of a number of biosynthetic and regulatory genes. The minicell analysis showed that there are four specific polypeptides encoded by form I antigen genes, two by the A region and another two by the D region. We could not, however, detect any polypeptide bands specific for the B and C regions. This may be attributed partly to the relatively large sizes of the form I gene fragments tested for minicell analysis. We are now doing DNA sequencing studies on these fragments of form I antigen genes.

It is documented that persons who recover from *Shigella* infections appear to have serotype-specific immunity (DuPont *et al.*, 1969; Mel *et al.*, 1965). However, it is still unclear what kind of immunity to O antigen is protective against *Shigella* infection, although serum antibodies or secretory antibodies produced at mucosal surfaces may be important. The results presented here may also provide prospects for a new and effective *Shigella* vaccine.

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