ANTIMICROBIAL SUSCEPTIBILITY

A spontaneous 99-kb chromosomal deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis in *Shigella flexneri* 2a

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A Tn5-generated mutant (strain S2430) of *Shigella flexneri* 2a (strain YSH6000) exhibited attenuated virulence and, in addition to the Tn5 insertion in the *SalI* K fragment of its virulence plasmid, had a 99-kb deletion within its chromosome. Unlike its wild-type parent, strain S2430 was susceptible to ampicillin, streptomycin, tetracycline and chloramphenicol. An independent multi-antibiotic susceptible variant of strain YSH6000 had a similar deletion. Southern blot analysis of pulsed field electrophoresis gels enabled the sizing of this deletion and its mapping to a region of the chromosome on *NotI* fragment D bounded by the *S. flexneri* homologues of *ompA* and *pyrC*. Hybridisation experiments with a probe specific to the multi-antibiotic resistance region indicated that this large deletion was responsible for antibiotic susceptibility. Both strain S2430 and a derivative of the antibiotic-susceptible variant, with a Tn5 insertion in its *SalI* K fragment, exhibited an equal reduction in contact haemolysis compared with the Tn5-bearing derivative of strain YSH6000. However, strain S2430 alone clearly displayed delayed plaque forming ability in LLC-MK2 monolayers, suggesting that the two examples of this deletion may not be identical.

Introduction

Shigella spp., predominantly human pathogens, are the causative agents of bacillary dysentery. Disease follows ingestion of virulent organisms which invade the colonic epithelial cells, multiply intracellularly and spread to adjacent cells. The resulting inflammation and ulceration of the mucosa account for the symptoms of pyrexia, abdominal colic and bloody diarrhoea [1].

Many proteins play a role in this multi-step process of pathogenesis. The majority are encoded by a large 100–140-MDa plasmid present in all virulent strains of *Shigella* spp. and enteroinvasive *Escherichia coli* [2]. The antigens IpaBCD are essential for primary invasion [3, 4] which occurs *via* a process of bacteria-directed phagocytosis [5]. The IcsB [6] and VacJ [7] proteins are critical for intercellular spread and the many *mxi* and *spa* gene products are required for normal surface expression and secretion of Ipa proteins [8–10]. VirG, a 116-kDa surface-exposed protein, facilitates intracellular and intercellular move-

ment *via* the deposition of a trail of F-actin [11]. VirK regulates the post-transcriptional expression of VirG [12]; VirB [13] and VirF [14] are transcriptional activators involved in switching on the virulence phenotype.

Besides these plasmid-borne determinants, several chromosomal loci have been identified. The *virR* (*hns*) gene is responsible for temperature-dependent expression [15] and the *ompB* locus (*envZ* and *ompR*) governs osmoregulation of the many virulence genes [16]. Other critical determinants of virulence include the lipopolysaccharide biosynthetic genes [17], the iron siderophore aerobactin genes [18], the superoxide dismutase gene [19] and *vacB* which is involved in the post-transcriptional regulation of expression of IpaBCD and VirG [20].

Apart from the many virulence-associated determinants, another aspect of *Shigella* spp. of major clinical significance is the high prevalence of antibiotic multi-resistant strains. Most of these strains harbour easily mobilisable resistance plasmids [21, 22], but strains exhibiting non-transferable, possibly chromosomally located resistance genes have been identified [23]. However, no molecular studies have been performed to elucidate the basis of chromosomal resistance.

This paper describes the further characterisation of a Tn5-generated mutant of *S. flexneri* 2a strain YSH6000. The mutant, S2430, had been shown previously to exhibit reduced contact haemolysis (CH), delayed plaque forming ability in LLC-MK2 cell monolayers and a delayed ability to provoke keratoconjunctivitis in mice [24]. These workers had attributed this attenuation of virulence to a Tn5-mediated disruption of a presumptive chromosomal virulence-associated locus, *vacD*. The present study examined the Tn5 insertion in strain S2430, the reduction in contact haemolytic activity (CHA) in relation to a spontaneous, 99-kb deletion from the chromosome and loss of resistance to the antibiotics chloramphenicol, streptomycin, tetracycline and ampicillin, to elucidate the origins of chromosomally encoded antibiotic multi-resistance in *Shigella* spp.

Materials and methods

Bacterial strains, media and growth conditions

The wild-type strain *S. flexneri* 2a YSH6000 [25], the antibiotic-susceptible derivative YSH6000T [12] and the virulence-plasmid-free derivative YSH6200 [14] have been described previously. Strain S2430 was constructed by Okada *et al.* [24]. The Tn5-bearing strains K6000 and K6000T were constructed by P1 transduction [18], with S2430 passaged lysate, of strains YSH6000 and YSH6000T, respectively. *E. coli* K-12 strain DH5 α was used for cloning. Strains were grown routinely at 37°C in LB media with the addition of kanamycin 50 mg/L when necessary. Antibiotic susceptibility was assayed by streaking individual clones on to LB agar supplemented with chloramphenicol 40 mg/L, tetracycline 25 mg/L, streptomycin 25 mg/L or ampicillin 100 mg/L.

Molecular biological techniques

Genomic DNA was isolated by the small scale preparation method of Ausubel *et al.* [26]. Plasmid DNA was isolated by a modification of the alkaline lysis method [27]. The 230-kb virulence plasmid was purified as described by Sasakawa *et al.* [25]. Standard cloning procedures with the plasmid vectors pUC18/19 or their trimethoprim-resistant derivatives pUC18/19-Tp [28] were employed. The 3.3-kb native plasmid of strain YSH6000 [25] and the 6.5-kb mini-replicon derived from pMYSH6000 [29] were used as low copy number vectors. Transformation of *E. coli* was achieved following electroporation with a BioRad Gene Pulser. Nucleotide sequencing was performed by the dideoxy chain termination method [30].

Preparation of high mol.-wt (HMW) genomic DNA and pulsed field gel electrophoresis (PFGE)

HMW DNA was prepared as described by Smith and Cantor [31] with the exclusion of the detergent Brij-58

from the lysis solution. Low melting temperature (LMT) agarose plugs (100 μ l) containing *c.* 2 μ g of DNA were pre-equilibrated with digestion buffer before incubation for 16 h at 37°C with 10 U of *NotI* in a 250- μ l reaction mixture (50 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 100 mM NaCl; 1 mM dithioerythritol, DTE; bovine serum albumin, BSA 100 mg/L). *NotI*/*SfiI* double digests were performed as a two-stage procedure with a 4-h 50°C digestion with 10 U of *SfiI* in reaction buffer (10 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 50 mM NaCl; 1 mM DTE; BSA 100 mg/L) followed by incubation for 16 h at 37°C with 10 U of *NotI* as described earlier. After digestion, the agarose plugs were incubated in ES (0.5 M EDTA, pH 9.5; lauroyl sarcosine 1%) at 50°C for 2 h and equilibrated in TE (10 mM Tris, pH 8.0; 1 mM EDTA) at room temperature before PFGE in a CHEF BioRad system in agarose 1% gels and a running buffer consisting of 0.5 $\times$ TBE (44.5 mM Tris; 44.5 mM boric acid; 1 mM EDTA). Gels were electrophoresed at 180 V for 24 h with pulse times of 20–25 s, at 14°C.

Southern hybridisation

After electrophoresis, DNA was transferred on to positively-charged nylon membranes (Boehringer Mannheim) by a vacuum blotting apparatus (TE80 TransVac, Hoefer) and a protocol involving three consecutive 10–20-min steps in depurination (0.25 M HCl), denaturation (1.5 M NaCl; 0.5 M NaOH) and neutralisation (1 M Tris, pH 7.0; 1.5 M NaCl) solutions. This was followed by 1–2-h transfer with 20 $\times$ SSC (3 M NaCl; 0.3 M sodium citrate). Membranes were then baked at 80°C for 2 h and hybridisation was performed over 16 h under conditions of high stringency. Probes used in this study were labelled with digoxigenin with protocols described by the manufacturer (Boehringer Mannheim). Besides those previously described, they consisted of the 1.5-kb *BglII*/*HpaI* fragment of Tn5 (IS50), the 1.0-kb *BglII*/*SmaI* fragment of Tn5 (kanamycin resistance gene), the 2.9-kb *DraI*/*HpaI* fragment of pSBA61 (this study, DNA flanking transposon in strain S2430), the 1.2-kb *EcoRI*/*HindIII* fragment of pTU500 (*ompA*) [32], the 1.6-kb *PstI* fragment of pLF4 (*ompF*) [33] and the 1.6-kb *EcoRI* fragment of pBHM107 (*pyrC*) [34]. The *NotI* fragment D of YSH6000 was purified by Agarose treatment from LMT gel after PFGE. The purified fragment was digested with *Sau3AI* before a standard random primed labelling reaction. Chemiluminescent detection with Lumigen PPD (Boehringer Mannheim) was used routinely. When required, the membranes were stripped by the protocol provided by Boehringer Mannheim.

Virulence phenotype assays

The (Congo red binding) Pcr phenotype was assessed by streaking different strains on to two halves of LB

agar plates supplemented with Congo red 0.01%. The plates were streaked in duplicate and incubated overnight at both 37°C and 42°C, after which the Pcr phenotype was scored by direct comparison between the two strains on the same plate.

The CH assay was based on the method of Sansonetti *et al.* [35]. Bacteria were grown for 3 h at 37°C in trypticase soy broth with or without kanamycin 50 mg/L. Cultures were centrifuged at 4000 *g*, washed with PBS (137 mM NaCl; 6.5 mM K₂HPO₄; 2.5 mM KH₂PO₄; pH 7.2) and concentrated *c.* 40-fold. Varying dilutions of this bacterial concentrate in PBS were used in the CH assay and appropriate dilutions were also plated to obtain accurate bacterial counts. The bacterial dilutions were each pre-mixed with an equal volume of freshly washed sheep erythrocytes suspended in PBS at a concentration of *c.* 1×10^{10} /ml. One hundred μ l of the bacteria/erythrocyte mixtures were dispensed into each well of a 96-well flat-bottom microtitration plate. The plates were centrifuged at 1500 *g* for 15 min at room temperature before incubation for 1 h at 37°C. The bacteria/erythrocyte pellets were then resuspended with 150 μ l of fresh PBS/well. The microtitration plates were centrifuged once again before removal of 100 μ l of supernate from each well. Haemolysis was assayed by measuring the absorbance of the supernate at 570 nm.

The focus plaque assay (Fpa) was performed as described by Sasakawa *et al.* [36]. Confluent LLC-MK2 monolayers established in 96-well tissue culture trays were infected with the various bacterial strains at a multiplicity of infection of *c.* 5×10^3 . The monolayers were observed with low power light microscopy over a 72-h period. Photographs were taken at 24 and 40 h post-infection to document progression of plaques.

Results

Mapping of the Tn5 insertion in S2430

A 7.6-kb *EcoRV* fragment containing the entire Tn5 and 1.9 kb of flanking shigella DNA was cloned into pUC18 by selecting for kanamycin resistance, generating the plasmid pSBA61. The flanking regions were partially sequenced and appropriate primers were synthesised and used in a PCR reaction to generate the 1.9-kb fragment from the wild-type strain YSH6000 DNA. This PCR fragment was cloned and its restriction digest profile was identical to that of a 1.9-kb segment of DNA bounded by *EcoRV* sites on the *SalI* K fragment of the virulence plasmid pMYSH6000 [12]. Furthermore, the *SalI* profile of the virulence plasmid of strain S2430 was identical to that of strain YSH6000 except that it lacked the wild-type 7.8-kb *SalI* fragment and, instead, had two additional fragments of 7.3 kb and 6.2 kb, consistent with the insertion of a 5.7-kb

Tn5 element, bearing a unique *SalI* site, into the *SalI* K fragment of strain S2430. Southern blots with both IS50 and kanamycin resistance gene probes confirmed that this was the only copy of Tn5 in strain S2430.

Cloning of three antibiotic resistance determinants

Conjugation studies had shown that strain S2430, unlike the parent strain YSH6000, had become susceptible to tetracycline 25 mg/L, ampicillin 100 mg/L, chloramphenicol 40 mg/L and streptomycin 25 mg/L. Three of the wild-type antibiotic resistance determinants were cloned to characterise this deletion. DNA from strain YSH6000 was digested with *EcoRI*, *HindIII* or *PstI*, and ligated to appropriately prepared pUC18 vectors. Transformants were selected on the basis of chloramphenicol, streptomycin or tetracycline resistance. An 8.9-kb *HindIII* fragment resulting in chloramphenicol resistance, and a 2.2-kb *HindIII* fragment conferring streptomycin resistance were isolated. When the 8.9-kb fragment was cloned into low-copy number plasmids, tetracycline resistance also was expressed.

Southern blot analysis with probes linked to resistance determinants

The 8.9-kb *HindIII* fragment encoding both chloramphenicol and tetracycline resistance was labelled and used in a Southern blot experiment to probe the nature of the genetic alteration in strain S2430. This yielded a multi-band background profile consistent with the presence of repeat elements on the chromosome of both YSH6000 and its mutant S2430. However, there was no specific 8.9-kb *HindIII* hybridising band in strain S2430, indicating a deletion linked to the resistance determinants in this strain. To clarify these results, a 0.2-kb *HindIII/HindII* fragment derived from the 8.9-kb *HindIII* fragment was used in a Southern blot to probe genomic DNA from strains YSH6000, S2430 and YSH6000T, a spontaneous variant of strain YSH6000 known to be susceptible to all four antibiotics [12]. The 0.2-kb probe hybridised with the predicted restriction fragments in strain YSH6000 while the corresponding fragments in strains S2430 and YSH6000T were absent (Fig. 1), thus confirming that a deletion linked to the resistance determinants had occurred in both strains S2430 and YSH6000T. The additional bands seen in Fig. 1A correspond to the small native plasmids in these strains and appear to be the result of non-specific hybridisation. Southern blot hybridisation with a 1.7-kb *HindIII/HindII* fragment derived from the opposite end of the 8.9-kb *HindIII* fragment (Fig. 1B) showed that this region of DNA also had been deleted in strains S2430 and YSH6000T, leading to the conclusion that a minimum of 8.9 kb of DNA had been deleted in these strains (results not shown).

Fig. 1. (A) Southern hybridisation analysis with a 0.2-kb *Hind*III/*Hind*II probe derived from the 8.9-kb *Hind*III fragment of strain YSH6000 encoding both chloramphenicol and tetracycline resistance. Lane 1, strain YSH6000T DNA digested with *Hind*III; 2, strain YSH6000 DNA digested with *Hind*III; 3, strain S2430 DNA digested with *Hind*III; 4, strain YSH6000T DNA digested with *Hind*III/*Pst*I; 5, strain YSH6000 DNA digested with *Hind*III/*Pst*I; 6, strain S2430 DNA digested with *Hind*III/*Pst*I. Arrows indicate the 8.9-kb and 4.0-kb hybridising bands in strain YSH6000. The sizes in kb of λ *Hind*III markers are shown on the left. (B) Restriction map of the 8.9-kb *Hind*III fragment described above. The shaded box on the right indicates the 0.2-kb *Hind*III/*Hind*II probe used; the double-headed arrows indicate the predicted size of the two hybridising fragments in the Southern blot shown in (A). The dashed line represents the 1.7-kb *Hind*III/*Hind*II probe used in a subsequent Southern blot experiment. H and P indicate cleavage sites for *Hind*III and *Pst*I, respectively.

PFGE analysis of *Not*I-digested genomic DNA

DNA from strains YSH6000, S2430 and YSH6000T was digested with *Not*I and analysed by PFGE, thereby confirming that the *Not*I profile of strain S2430 was altered as previously reported [24, 37], with the 323-kb *Not*I fragment D absent and in its place a new 224-kb fragment (Fig. 2A). Okada *et al.* [37] thought that this alteration was caused by Tn5 insertion into *Not*I fragment D, resulting in the cleavage of the 323-kb fragment into 224-kb and 99-kb fragments. Observation of ethidium bromide-stained PFGEs run in the present study could not eliminate completely the possibility of the existence of an additional 99-kb fragment in strain S2430, resulting from the presence of several normal bands in that size range. Strain YSH6000T exhibited a similar alteration of its *Not*I profile; however, careful examination of the 224-kb fragment in strain S2430 revealed it to be a doublet, which was not present in strain YSH6000T (Fig. 2A). The additional band in strain S2430 was caused by the linearisation of its Tn5-bearing 230-kb plasmid resulting from the cleavage of *Not*I sites contained within Tn5. Southern blots probed

with *Not*I fragment D from strain YSH6000 showed a multi-band profile, consistent with the presence of repeat elements within *Not*I fragment D. The probe hybridised strongly to the 323-kb band in strain YSH6000 and the 224-kb fragment in both strains S2430 and YSH6000T, but there was no additional 99-kb band derived from *Not*I fragment D in either of the latter two strains (Fig. 2B), proving conclusively that *Not*I fragment D in both strains S2430 and YSH6000T had undergone a large 99-kb deletion. This finding is consistent with the findings of the subsequent analysis with *Not*I D-specific probes. The additional strongly hybridising bands seen in lanes 2 and 4 (Fig. 2B) may result from hybridisation with circular pMYSH6000 co-migrating with corresponding chromosomal *Not*I fragments of strains YSH6000 and YSH6000T, respectively.

Mapping of the deletion on the *S. flexneri* 2a chromosome

The *Not*I PFGE profiles of strains YSH6000, S2430 and YSH6000T showed that the deletion was within

Fig. 2. (A) PFGE profile of *NotI*-digested DNA of *Shigella* strains. Lane 1, λ concatamers; 2, strain YSH6000 DNA digested with *NotI*; 3, strain S2430 DNA digested with *NotI*; 4, strain YSH6000T DNA digested with *NotI*; 5, digoxigenin-labelled λ *HindIII* markers. (B) Southern hybridisation analysis of the gel shown in (A) with the *NotI* fragment D from strain YSH6000 as a probe. Double arrows indicate the positions and sizes of *NotI* fragment D from strain YSH6000 (323 kb) and the corresponding truncated fragment in strains S2430 and YSH6000T (224 kb). The positions of standard DNA size markers are shown on the left.

chromosomal *NotI* fragment D. Okada *et al.* [37] demonstrated that the *E. coli* genes *ompF*, *ompA* and *pyrC* hybridised specifically with *NotI* fragment D of strain YSH6000. To further localise the deletion in strains S2430 and YSH6000T, a strategy involving double-digestion with *NotI* and *SfiI*, followed by resolution on PFGE and hybridisation with the three *NotI* D-specific probes identified by Okada *et al.* [37] was utilised. The results confirmed the presence of *ompF*, *ompA* and *pyrC* homologues on the 323-kb *NotI* D fragment of strain YSH6000 and showed that none of the genes was lost following the deletion in strain S2430 or YSH6000T (Fig. 3A–C). In strains S2430 and YSH6000T, all three genes were detected on a 166-kb fragment flanked by *NotI* or *SfiI* sites (Fig. 3A–C; lane 2, S2430; lane 3, YSH6000T). Hybridisation of YSH6000 genome with the probes resulted in two bands in each case. With *ompF* and *ompA*, 155-kb and

265-kb bands were seen (Fig. 3A and B; lane 1), while *pyrC* hybridised with 110-kb and 265-kb bands (Fig. 3C; lane 1). This dual-band pattern appeared to be the result of an additional *SfiI* site in *NotI* fragment D of strain YSH6000 that was less susceptible to digestion. Complete digestion would have resulted in only the smaller of the two bands in each case. This proposal is supported by the fact that the sum of the two smaller fragment sizes (110 kb, 155 kb) appeared equal to the 265-kb product of partial digestion that hybridised with each of the three probes. The absence of this additional *SfiI* site in strains S2430 and YSH6000T indicated that the site lay within the deleted region. Therefore, by mapping this site in strain YSH6000 it was possible to locate more precisely the deletion. As *pyrC* hybridised with a 110-kb fragment and both *ompA* and *ompF* hybridised with a 155-kb fragment, it was concluded that the deletion occurred between the *ompA* and *pyrC*

Fig. 3. Southern hybridisation analysis of *NotI*- and *NotI/SfiI*-digested DNA resolved by PFGE. The same membrane was stripped and re-probed with the following probes: **A**, *ompF*; **B**, *ompA*; **C**, *pyrC*; **D**, 0.2-kb *HindIII/HindII* fragment linked to the chloramphenicol and tetracycline resistance determinants. Lane 1, strain YSH6000 DNA digested with *NotI/SfiI*; 2, strain S2430 DNA digested with *NotI/SfiI*; 3, strain YSH6000T DNA digested with *NotI/SfiI*; 4, strain YSH6000 DNA digested with *NotI*, plus digoxigenin-labelled λ *HindIII* markers; 5, strain S2430 DNA digested with *NotI*; 6, strain YSH6000T DNA digested with *NotI*. Arrows to the right of (A) and (B) indicate the size of the various hybridising fragments in both of these panels. Arrows to the right of (C) and (D) indicate the size of all hybridising fragments seen in (C) and of the three fragments seen in (D). Arrowheads within panels highlight the fainter hybridising bands. The positions of some of the known *NotI* fragments of strain YSH6000 [37] and of the larger λ *HindIII* fragments, are shown on the left.

homologues of strain YSH6000 (Fig. 6). A further hybridisation experiment probing with the 0.2-kb *HindIII/HindII* fragment linked to the resistance determinants confirmed that the deletion resulting in multiple antibiotic susceptibility was the same as that in *NotI* fragment D (Fig. 3D), and allowed the mapping of this resistance region to the *pyrC* side of the additional *SfiI* site.

Virulence phenotype assays

To clarify the effect of the Tn5 insertion in strain S2430, strains of YSH6000 and YSH6000T, K6000 and K6000T, respectively, carrying Tn5 in a position identical to that in strain S2430 were constructed by P1 transduction. Hybridisation with a probe derived from DNA flanking the Tn5 in strain S2430 confirmed

the correct genotype in these transductants. Strains YSH6000, S2430 and YSH6000T were observed during this study to vary in their ability to bind Congo red (Pcr⁺). All three strains were Pcr⁺; however, it was possible to score the strains based on the intensity of Congo red binding. Strain YSH6000 was the darkest, with strain YSH6000T being intermediate and strain S2430 clearly exhibiting the weakest Pcr phenotype. This result was mirrored by strains K6000 and K6000T, each exhibiting an ability to bind Congo red identical to that of its parent.

Strain S2430 was reported to have reduced CHA [24]. In this study its CHA was compared with that of strains K6000 and K6000T to clarify the independent effects of the Tn5 and of the chromosomal deletion on CH phenotype (Fig. 4A). The three strains were grown in trypticase soy broth with kanamycin. A separate comparison between strains YSH6000, YSH6000T and YSH6200, a virulence-plasmid-free derivative of strain YSH6000, grown in antibiotic-free broth was also performed (Fig. 4B). Strain YSH6200 did not induce any haemolysis. Within the range of (1×10^9)–(5×10^9) cfu/ml, both strains S2430 and K6000T exhibited significantly lower levels of haemolysis than strain K6000. Strains S2430 and K6000T displayed similar values of CH throughout this range. At 3×10^9 cfu/ml the CHA of strains S2430 and K6000T, expressed as a percentage of that of strain K6000, was 46% and 47% respectively. Similarly, the CHA of strain YSH6000T, expressed as a percentage of that of strain YSH6000, measured at the same bacterial concentration was 48%, supporting the

conclusion that the attenuation in this phenotype was wholly attributable to the deletions in strains S2430 and YSH6000T.

The Fpa measures the ability of the bacteria to invade an initial cell in the LLC-MK2 monolayer, resulting in a focus, and the subsequent intercellular spread leading to plaque formation. Strain S2430 has been reported to exhibit a delayed Fpa⁺ phenotype [24]. Results of a typical Fpa assay are shown in Fig. 5. Panels on the left show the extent of plaque formation at 24 h; those on the right show plaques at 40 h post-infection. Under low power light microscopy plaque formation resulting from the destruction of contiguous cells in the monolayer was apparent as dark granular areas. Strain YSH6200 (Fig. 5K and L) did not infect the monolayer, while both strains YSH6000 (Fig. 5G and H) and K6000 (Fig. 5A and B) showed normal progression with significant plaque formation after 24 h. Strains YSH6000T (Fig. 5I and J) and K6000T (Fig. 5E and F) exhibited an Fpa phenotype indistinguishable from that of strain YSH6000. However, strain S2430 (Fig. 5C and D) clearly exhibited a delay in its Fpa phenotype, requiring > 48 h to form plaques similar to those produced at 24 h by the wild-type strain.

Discussion

A large 99-kb spontaneous chromosomal deletion was identified in two independent strains of *S. flexneri* 2a. No data are available on the frequency of this deletion

Fig. 4. Contact haemolytic activity of *Shigella* strains: **A**, K6000 (□), K6000T (■) and S2430 (△) were grown in trypticase soy broth containing kanamycin 50 mg/L; **B**, YSH6000(+), YSH6000T (○) and YSH6200 (▲) were grown in trypticase soy broth alone. All strains were harvested and concentrated after incubation for 3 h at 37°C. Contact haemolytic activity was measured by recording the $A_{570\text{nm}}$ of the supernate after incubation for 1 h with sheep erythrocytes. Absorbance measurements and bacterial counts shown represent the means from four replicates. The data shown reflect the results of duplicate experiments.

event in *S. flexneri*, although comparable genetic alterations in *E. coli* and *Yersinia pestis* occur at frequencies of 10^{-5} – 10^{-3} [38]. The deletion was mapped to a region of *NotI* fragment D, bounded by the *S. flexneri* homologues of *ompA* and *pyrC*. Okada *et al.* [37] had shown previously that the *serS* and the *ptsG* genes hybridised respectively with *NotI* fragments K and O, which flank fragment D. Given this, and the similarity in genetic organisation between *E. coli* K-12 and *S. flexneri* 2a [37], a direct comparison between the *E. coli* linkage map and of the corresponding region of the *S. flexneri* 2a chromosome was attempted (Fig. 6). The spacing between *ompF* and *pyrC* in *E. coli* is 120 kb [39, 40], while in *S. flexneri* 2a strain YSH6000 the postulated approximate spacing is 220 kb but is 'restored' to 120 kb in the two spontaneous deletants. This coincidence of sizes suggests that strain YSH6000 evolved after the insertion of a large element into this region, whilst strain YSH6000T may be representative of an ancestral form of the wild-type strain YSH6000 (Fig. 6).

There have been several reports regarding the insertion of large elements, frequently plasmids, into the genome of bacteria [41]. Some of these plasmids have been shown to exist either as chromosomal or extra-chromosomal elements [42, 43]. Strain YSH6000 itself harbours a 230-kb virulence plasmid that is able to insert spontaneously into the *metB* gene on the chromosome resulting in loss of virulence. Virulence is restored following spontaneous excision of the plasmid [44]. Further presumptive evidence supporting the insertion hypothesis is the presence of two serine tRNA genes within the corresponding region of the *E. coli* genome [39, 45]. tRNA genes have been shown to serve frequently as targets for plasmid or phage insertion. Such insertions occur through homologous recombination between specific chromosomal and plasmid sequences, often resulting in the reconstitution of the target gene [41, 46]. Recently, Blum *et al.* [47] described the spontaneous deletion of 'islands of pathogenicity' in *E. coli*. The deleted regions of 70 kb and 190 kb that encode haemolysins and P-related fimbrial genes have been mapped precisely to tRNA genes. The two tRNA species are produced by the parent strain but neither is synthesised by the spontaneous double deletants. Sequence data across the point of excision confirmed a disruption of the tRNA genes [47], while Leong *et al.* [48], by ϕ 80 mediated site-specific inversion, demonstrated the

Fig. 5. Low power light microscopy showing plaque formation in LLC-MK2 cell monolayers infected with different strains of *S. flexneri* 2a. (A) and (B), strain K6000; (C) and (D), strain S2430; (E) and (F), strain K6000T; (G) and (H), strain YSH6000; (I) and (J), strain YSH6000T; (K) and (L), strain YSH6200. Panels on the left show typical plaque formation at 24 h; panels on the right reflect plaque formation at 40 h. The bar in panel (L) represents 400 μ m.

Fig. 6. Comparison of the genetic map of *E. coli* K-12 W3110 [39, 40] and of the hypothetical map of *S. flexneri* 2a YSH6000 between *serS* and *ptsG*. In *S. flexneri* 2a YSH6000 *serS* and *ptsG* are known to lie within *NotI* fragments K and O respectively, while *ompF*, *ompA* and *pyrC* lie on *NotI* fragment D [37]. The precise placement of these genes on the YSH6000 map remains speculative. To clarify the postulated gene alignment between these bacterial species, the 99-kb span of DNA deleted in strains S2430 and YSH6000T is shown as a lower hatched bar. The exact location of the deletion and of the resistance region (ACST) contained within remains to be determined. *SfiI* (S) sites shown have not been mapped and their placement as shown is in order to allow a schematic representation of the model. N, indicates *NotI* sites while the asterisk highlights the *SfiI* site that is partially resistant to digestion. The arrows at the bottom of the figure indicate the various *NotI* fragments.

occurrence of single base-pair substitutions, deletions and insertions during these homologous recombination events. Furthermore, Durand *et al.* [49] have reported that a mutation of *vacC*, a gene encoding tRNA-guanine transglycosylase, results in an altered virulence phenotype in *S. flexneri*, supporting the possibility that the mutation of a tRNA gene may affect virulence in *Shigella* spp.

The insertion of Tn5 in the virulence plasmid of strain S2430 was shown to have no effect on the virulence phenotype of that strain. The two P1 transductants, strains K6000 and K6000T, each had a virulence phenotype identical to that of its parent. On the other hand, this study demonstrated that the 99-kb deletion occurring in both strains S2430 and YSH6000T resulted in an attenuation of CHA and of Congo red binding. The correlation between the ability to bind Congo red and virulence in *S. flexneri* has been well established [50]. Others have shown that a reduction in CHA is associated with decreased primary invasion and impaired escape from the endocytic vacuole [3, 35]. However, despite the reduced CHA of strain YSH6000T, unlike strain S2430 it did not exhibit a delayed Fpa phenotype. This finding suggests that the reduction in CHA of strain S2430 is unrelated to its activity in the Fpa assay. Unlike previous studies in which attenuated strains exhibited a reduction in CHA of > 90% [3, 35], both strains S2430 and YSH6000T still exhibited *c.* 50% of wild-type CHA. This level of residual CHA was presumably sufficient to promote normal plaque-forming ability. A similar phenomenon was described by High *et al.* [3] in which a complemented *S. flexneri* mutant exhibiting only a

partial restoration of its haemolytic phenotype was restored to full invasive capacity in the HeLa cell assay. Nevertheless, the strong link between CHA and virulence in *Shigella* spp., as established by others [3, 35], supports our conclusion that the 99-kb chromosomal deletion results in the loss of a virulence-associated attribute.

The possibility that a further unrelated mutation in strain S2430 was responsible for the delayed Fpa phenotype is highly improbable, given that three independent genetic events would have had to coincide. Furthermore, as the *SalI* profiles of the virulence plasmid from strains S2430 and YSH6000 differed only with respect to *SalI* fragment K, an unrecognised deletion in the plasmid is unlikely. A more likely hypothesis is that this phenotypic difference is due to a small difference in the extent of the chromosomal deletion between these strains or the possibility that the deletion in strain YSH6000T is precise, whilst that in strain S2430 is imprecise, resulting in a mutated target gene. Confirmation of this hypothesis must await the precise identification of the endpoints of these deletion events.

Small deletions in virulence loci have been shown in several bacterial genera [38, 51]. However, besides *E. coli* [47] and now *S. flexneri*, the only other bacterial species known to us that has been shown to exhibit a large spontaneous chromosomal deletion resulting in an attenuation of virulence phenotype is *Y. pestis* [52, 53]. Fetherston *et al.* [52] and Lucier and Brubaker [53] have measured the deletion in *Y. pestis* at 102 kb. The *Y. pestis* deletant exhibited an alteration

of the Pcr phenotype, but unlike the *S. flexneri* strains, in which there was only a reduction of dye binding, the *Y. pestis* deletant displayed a Pcr⁻ phenotype [52]. Besides Congo red binding, the deleted *Y. pestis* DNA encodes iron-regulated proteins, some of which probably form an iron transport system required for growth at 37°C in iron-deficient media [54]. Lawlor and Payne [55] have described deletions in the chromosome of *S. flexneri* involving the aerobactin genes, although these deletions appear to be of variable size and are not well defined. The 99-kb deletion in *S. flexneri* 2a is unlikely to involve these genes as strain S2430 grew well in iron-depleted medium (data not shown) and the genes probably map to a different region of the chromosome [56].

Antibiotic multi-resistance in bacteria, especially *Shigella* spp., is frequently associated with plasmids, but chromosomally encoded resistance has also been described. Chromosomal antibiotic-multi resistance loci have been identified and cloned in *E. coli* [57, 58] and *Pseudomonas aeruginosa* [59]. Sequence and functional analysis of the locus in *E. coli* showed it to consist of an operon of three genes, *marRAB*, that exert a regulatory effect on several distant genes including *ompF* [57]. Resistance probably arises from an alteration in transport of a broad range of drugs across the bacterial membrane.

A *mar* locus, highly homologous to that in *E. coli*, has been identified in other members of the family Enterobacteriaceae including *Shigella* spp. [60]. However, the deletion in strain YSH6000 is unlikely to involve the equivalent locus for the following reasons. Firstly, strain YSH6000 exhibits high resistance to ampicillin, streptomycin, tetracycline and chloramphenicol whilst remaining susceptible to low concentrations of nalidixic acid, unlike the resistance pattern mediated by the *E. coli mar* locus. Secondly, the ability to clone the chloramphenicol and tetracycline resistance determinants on a fragment separate from the streptomycin resistance determinant strongly argues against an *E. coli*-type locus in which a single operon is responsible for all resistances. Indeed, recent work in this laboratory suggests the presence of β -lactamase and chloramphenicol acetyl transferase genes within the deleted region. Thirdly, the deletion in strain YSH6000 corresponds to position 22 min on the *E. coli* chromosome, whilst the *E. coli mar* locus has been mapped to 34 min [58]. Finally, the likelihood of IS1 elements being associated with the resistance region identified in this study points to a similarity with resistance determinants associated with resistance plasmids [61], such as the IncFII plasmids, R1 and R100. In these plasmids the resistance determinants are flanked by two directly repeated copies of IS1 and arise from a series of intercalated transposable elements carrying multiple individual resistance genes [62]. Further ongoing characterisation of this *S. flexneri* antibiotic-multi resistance region

should clarify the issue. A similar phenomenon involving the loss of an antibiotic-multi resistance region has been reported in *Staphylococcus aureus*, in which Inglis *et al.* [63] described the PFGE characterisation of methicillin-resistant and methicillin-sensitive strains. They found that the sensitive strains exhibited profiles similar to laboratory variants that have lost *c.* 40–70 kb of DNA carrying multiple resistance determinants clustered around the methicillin resistance gene [63].

Numerous epidemiological studies have consistently highlighted the prevalence and variety of antibiotic-multi resistance plasmids in *Shigella* spp. [21–23]. The four-drug resistance pattern of strain YSH6000 is common amongst clinical isolates [21]. The possibility that strain YSH6000 arose after the acquisition of an R-plasmid that subsequently integrated and stabilised within the genome, possibly as a result of incompatibility with another IncFII plasmid, the 230-kb virulence factor [64], remains an intriguing possibility.

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