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Plasmid Profile Analysis of a Salmonellosis Outbreak and Identification of a Restriction and Modification System

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After an outbreak of salmonellosis in humans caused by *Salmonella typhimurium* bacteriophage type 135, 62 isolates from human, animal, and water sources were retained for further analysis. Most of the isolates (92%) could be placed in one of five plasmid pattern groups, with a majority containing a common 60-kilobase plasmid and a smaller 3.8-kilobase-pair plasmid. This small plasmid, pIMVS1, was labeled with [^{32}P]phosphate and used as a probe in subsequent colony and Southern hybridization studies. We concluded that pIMVS1 from isolates obtained from humans was genetically different from plasmids of a similar size found in isolates from chickens. Studies to characterize pIMVS1 were undertaken to determine if it codes for known virulence factors. It did not appear to be associated with the formation of attachment pili or major outer membrane proteins. By using transposon mutagenesis techniques, Tn*3*(Ap^r) was inserted into pIMVS1, and the existence of a restriction and modification system was deduced.

Bacterial plasmids are known to confer a wide variety of phenotypic modifications and genetic flexibility upon their hosts by carrying genes that may code for toxin production, iron sequestration, adhesiveness, and serum resistance (15, 18). The existence of such plasmids in *Salmonella* species has been well documented, and comparative plasmid DNA analysis has been shown to be useful for tracing the source of outbreaks of salmonellosis (6, 8, 9, 13, 14, 16).

Salmonella typhimurium commonly contains a large virulence plasmid of 60 kilobase pairs (kb) (6, 8–10, 14). This plasmid may harbor additional antibiotic resistances as the result of transposon interruption but becomes unstable as an epidemiological marker because it is indispensable for virulence.

A salmonellosis outbreak of *S. typhimurium* bacteriophage type 135 occurred in the Sydney metropolitan area between January and March 1985. Isolates from various sources within the area, before, during, and after the outbreak, were retained for analysis. Our aims were to evaluate plasmid analysis as a means of identifying strains and to determine the distribution of particular plasmids other than the large 60-kb virulence plasmid to see if these might be associated with virulence factors.

MATERIALS AND METHODS

Bacterial strains. Bacterial isolates were received by the National Salmonella Reference Laboratory, Adelaide, South Australia, from a number of clinical laboratories within the Sydney metropolitan area. These isolates were subcultured onto nutrient agar slopes and serotyped by slide agglutination.

Phage typing of isolates obtained from humans was carried out by the Microbiology Diagnostic Unit at Melbourne University, and isolates from nonhumans were typed by the National Salmonella Reference Laboratory. All phage typing was performed by the method of Callow (7), using standard international sets of phages supplied by Public Health Laboratory Service, Colindale, London, United Kingdom.

Isolates were also tested for resistance to the antibiotics streptomycin, tetracycline, chloramphenicol, gentamicin, ampicillin, cefoxitin, cephalothin, sulfonamide, and trimethoprim by using a disk agar diffusion method.

The standard bacterial strains used for genetic studies were supplied by the University of Adelaide, Department of Microbiology. They were *Escherichia coli* K-12 C600 (F$^-$ *supE tonA1 thr leu*), C600 harboring the F-like R factor R1-19 (Cm^r Kan^r Neo^r Amp^r Str^r sulfonamide^r), DH1 (F$^-$ *gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44* λ$^-$), LE392 (F$^-$ *supF supE hsdR galK trpR metB thi*), and MC 1061.

Plasmid DNA isolation. Preliminary investigation of *Salmonella* isolates revealed that the most satisfactory results for plasmid extraction were obtained by using the method of Birnboim and Doly (3). The procedure was modified by allowing lysis to proceed for a further 10 min and by including purification steps in the extraction.

Proteins were removed by extractions with equal volumes of phenol and chloroform (1:1), followed by several extractions with water-saturated ether. Plasmid DNA was precipitated in 0.5 M sodium chloride and 66% filtered ethanol, pelleted, and suspended in 40 μl of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA).

A sample of this plasmid DNA (10 to 20 μl) was digested for further analysis with two restriction endonucleases, *Eco*RI and *Hin*dIII (Boehringer Mannheim Biochemicals), as recommended by the supplier. Restriction fragments were separated by electrophoresis on 0.8% agarose gels (Ultra Pure DNA grade; Bio-Rad Laboratories) by using 1× TBE electrophoresis buffer (0.09 M Tris hydrochloride 0.09 M boric acid, 0.008 M EDTA [pH 8.0]) run for 16 h at 40 mA. Gels were stained with ethidium bromide and photographed under UV transillumination by using Polaroid 667 negative film. For comparisons between gels, a molecular weight marker, lambda DNA digested with *Hin*dIII, was used (molecular weight marker 11; Boehringer Mannheim).

Cell envelope preparations. Cell envelopes were prepared

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by the small-scale method by using overnight shaking cultures (10 ml) as described previously by Manning et al. (12).

Pili preparations were prepared by heat treatment of cells by using the method of Stirm et al. (21). Cells were grown on solid medium by the method of Smyth (19).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 11 to 20% polyacrylamide gradient gels by using a modified procedure of Lugtenberg et al. (11) as described by Achtman et al. (1).

Transformation. Transformation using plasmid DNA was performed by using $CaCl_2$-treated cells as described by Brown et al. (5).

Transposon mutagenesis. Plasmid R1-19 harboring the transposon Tn*3* was transferred by filter mating from *E. coli* K-12 C600 (R1-19) to an *S. typhimurium* strain containing pIMVS1 (R. Morona, personal communication). Samples (10 ml) of overnight culture of donor and recipient were harvested, and each was mixed in 0.3 ml of nutrient broth and spread on a nitrocellulose filter (0.45-μm pore size; Gelman Sciences, Inc.). The filter was placed on a nutrient agar plate and incubated for 4 h at 37°C.

The filter was then removed, and the cells were suspended in minimal salts and plated on minimal agar containing ampicillin to select for the transposon phenotype.

Crude plasmid DNA was prepared from 12 independent transconjugants and used to transform *E. coli* K-12 DH1. Transformants were selected on nutrient agar containing ampicillin (25 mg/liter). These transformants were purified, and their plasmid DNA was extracted and subjected to restriction analysis to localize the site(s) of Tn*3* insertion.

RESULTS

Plasmid DNA isolation. Plasmid DNA was extracted from a total of 62 isolates of *S. typhimurium* phage type 135 from a variety of sources in the affected outbreak area. These included bovine and avian isolates as well as isolates from raw meat, water, and humans. The plasmid DNA from each strain was separated by electrophoresis on agarose gels, and the plasmid profiles were compared. Approximately 80% of the strains contained plasmids, and of these, 92% could be placed in one of five plasmid pattern groups. Nearly 60% of these belonged to a single group which contained a common 60-kb plasmid and a smaller 3.8-kb plasmid (Fig. 1, left panel). Interestingly, this small plasmid, designated pIMVS1, occurred mainly within strains isolated during the 3-month outbreak period from January through March 1985. We concluded that this pattern was common to all isolates involved in the outbreak and that the others were sporadic nonepidemic patterns occurring normally in that locality.

The majority of isolates containing the large and small plasmid pattern were of human origin, but a similar profile was also seen in isolates from chicken and water samples from the outbreak area. The plasmids of these isolates were further analyzed by digestion with the restriction endonucleases *Eco*RI and *Hin*dIII and separation of the fragments by gel electrophoresis. Digest patterns showed that isolates from chickens were different from isolates from humans with similar plasmid profiles. No *Hin*dIII site was detected in the small plasmid present in the isolates from chickens. Thus, the small plasmid present in the isolates from humans was not the same as the small plasmid contained in the isolates from chickens.

Southern hybridization. Southern transfer techniques (20) were used to examine whether the 3.8-kb plasmids were identical in all samples. Plasmid DNA from isolates was transferred onto filters and hybridized by using a probe labeled with [^{32}P]phosphate prepared from purified pIMVS1 extracted from an isolate obtained from a human (Fig. 1, right panel). This probe hybridized to all isolates from humans and most from water but not to any of the isolates from chickens. Thus, pIMVS1 in strains from humans was genetically different from the plasmid DNA of similar size seen in isolates from chickens. Plasmid pIMVS1 appeared to be stable in the environment and could be isolated from environmental water samples collected 3 months after the outbreak.

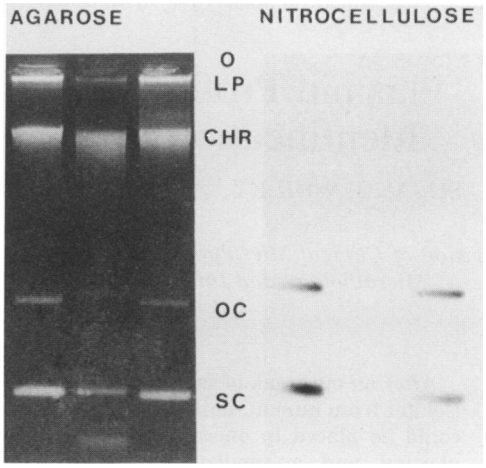

FIG. 1. Southern hybridization analysis of plasmids by using pIMVS1 as a probe. Lanes: 1 and 3, isolates from humans; 2, isolates from chickens. O, origin; CHR, chromosomal DNA; OS, open circular plasmid DNA; SC, supercoiled plasmid DNA. The left panel shows the plasmid pattern after agarose gel electrophoresis and staining with ethidium bromide under UV light. The right panel shows the autoradiograph of the same gel after transfer to nitrocellulose and hybridization with ^{32}P-labeled pIMVS1.

Analysis of pIMVS1. Since pIMVS1 was detected mainly in isolates of *S. typhimurium* phage type 135 obtained from humans, it was examined further to determine if it was associated with any recognized virulence factors. Restriction endonuclease cleavage analysis failed to locate sites for the following enzymes on pIMVS1: *Bam*HI, *Pst*I, *Sal*I, *Sst*I, and *Xho*I. However, pIMVS1 did contain single cleavage sites for *Hin*dIII and *Bgl*II. These sites were used in an attempt to clone pIMVS1 into various conventional vectors, including pBR322, pUC18, pHC79, and pSUP205 and into various *E. coli* K12 derivatives (DH1, MC1061, and LE392). We could readily demonstrate efficient ligation, but we did not obtain any transformants. We concluded that cloning via these sites was lethal to the host.

Transposon mutagenesis. Transposon mutagenesis, a method by which certain genes may be labeled and directly selected for, was used on pIMVS1. *E. coli* harboring pIMVS1 was mutagenized with transposon Tn*3* (Amp^r), and the DNA was transformed into strain DH1. After selection on ampicillin plates, 12 transformants were picked, purified, and checked for the presence of recombinant plasmids. Interestingly, two of the transformants contained a plasmid corresponding to pIMVS1 but not containing Tn*3*. These transformants could have occurred either by precise excision of the transposon after transformation or by cotransformation of pIMVS1 with chromosomal DNA containing Tn*3*. These transformants proved to be useful controls for com-

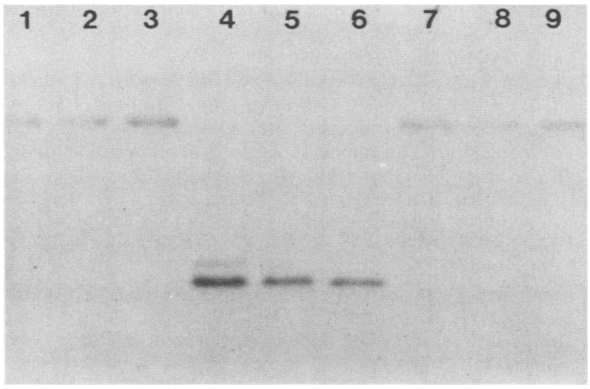

FIG. 2. Autoradiography after Southern DNA transfer of the plasmids introduced into *E. coli* K-12 and identified by using pIMVS1 as a probe. Lanes: 1 to 3 and 7 to 9, plasmid DNA from Tn*3* insertion mutants; 4 to 6, pIMVS1 which had been cotransformed and which did not contain Tn*3*.

parison with the Tn*3* insertion mutants of pIMVS1. The results were confirmed by Southern hybridization (Fig. 2). Precise localization for the sites of insertion of Tn*3* was determined by double digestions with restriction endonucleases *Pst*I-*Hin*dIII and *Pst*I-*Bgl*II. The results provided a map (Fig. 3) showing the probable sites of Tn*3* insertion.

Properties of pIMVS1. *E. coli* K-12 cells harboring pIMVS1 or pIMVS1::Tn*3* derivatives and *S. typhimurium* containing pIMVS1 were analyzed for various properties, including changes in the cell envelope proteins and pili, toxin production, and antibiotic resistances, all of which are commonly plasmid encoded (6, 8, 10). pIMVS1 was not associated with any of these virulence factors (results not shown).

Small plasmids may also be responsible for DNA modification and restriction (4). The possible existence of such a system in pIMVS1 was investigated by using bacteriophage lambda. This method was used by Arber (2) as reviewed by Boyer (4). The phage stock initially used was propagated on a plasmidless strain of *E. coli* K-12. The presence of pIMVS1 had a dramatic effect on the ability of the bacteriophage to plaque on the strain, reducing its plaquing efficiency by 10- to 100-fold. However, no effect was detected with any of the pIMVS1::Tn*3* derivatives, suggesting that Tn*3* was inactivating the function responsible for this effect on plaque formation.

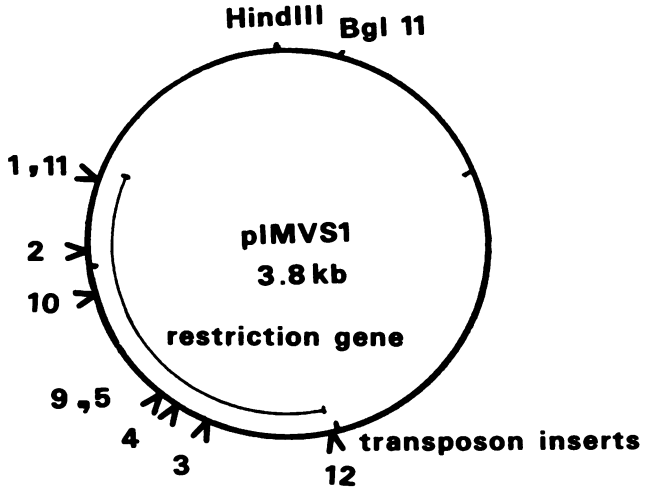

FIG. 3. Insertion map of pIMVS1 indicating the relative positions of Tn*3* insertions with respect to known restriction sites.

To examine this phenomenon further, single plaques were picked from a lawn of *E. coli* K-12 harboring either pIMVS1 or a Tn*3* derivative and propagated in *E. coli* K-12. When the efficiencies of plaquing of these phages were examined, no difference was detected in *E. coli* K-12 containing pIMVS1 or a Tn*3* derivative. Thus, the propagation of bacteriophage lambda in the presence of some pIMVS1 gene product, other than that inactivated by Tn*3* insertion, protected it from the effects of an intact pIMVS1. Such an effect is typical of a DNA restriction and modification system.

DISCUSSION

The transmission of *Salmonella* strains is often difficult to document by using traditional epidemiological tools, such as serotyping and biotyping. Many salmonellae, such as *S. typhimurium*, are so commonly isolated that epidemiologists need other means of subdividing strains (10). Phage typing and plasmid profile analysis together provide a means for subgrouping and characterizing isolates from common sources and so are valuable tools for epidemiological surveys (6, 9, 14–17). In this report, plasmid analysis has been used to differentiate an epidemic of salmonellosis caused by *S. typhimurium* phage type 135 isolates from nonepidemic background strains by using a procedure that was rapid and easy to perform and that gave reproducible results (3). A broad size range (0.5 to 150 kb) of plasmid DNA was detected, and it was in pure enough form for further characterization with restriction endonucleases.

Plasmid analysis revealed the presence of a small 3.8-kb plasmid, pIMVS1, in salmonellae isolated during this outbreak. Southern hybridization analysis demonstrated that this plasmid was unique to isolates from humans and did not hybridize to similarly sized plasmids identified at the same time in strains from chickens. Thus, the strains analyzed from chickens were unlikely to be responsible for, or to be involved in, the origin of this outbreak of salmonellosis.

Salmonellae are known to occur naturally as either high- or low-virulence subclones. The subsequent gain or loss of a serotype-specific plasmid carrying virulence determinants may produce a change in the organism and its virulence for certain hosts (13, 18). pIMVS1 may have contributed to the increased virulence of the strains in which it was found. Initial investigations failed to associate pIMVS1 with potential virulence determinants such as toxin production, antibiotic resistances, and adhesion (results not shown). Further characterization of pIMVS1 was undertaken, and attempts were made to clone pIMVS1. Cloning was not successful.

Transposon mutagenesis using Tn*3* was successful in tagging pIMVS1. Restriction analysis revealed that all inserts were within a 1.5-kb region adjacent to the single *Hin*dIII site. Thus, if all the Tn*3* insertions were inactivating a single function, such as a restriction enzyme, we can put a lower limit of 55,000 daltons on the size of this product. We would predict that all of these insertions would be within the gene for the restriction enzyme encoded by pIMVS1. The *Hin*dIII and *Bgl*II sites, on the other hand, would be predicted to be within the region coding for the modification enzyme.

These results would explain why cloning of pIMVS1 via either its *Hin*dIII or its *Bgl*II site proved unsuccessful. Inactivation by either of these enzymes of the gene coding

for the modification function in the presence of an active restriction enzyme site would have proved lethal to the host cell. What is the function of a restriction and modification system with respect to the virulence or host specificity of *S. typhimurium* phage type 135? This question cannot be answered at present, but it provides an interesting basis for speculation.

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