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Biological and Genetic Characterization of Tn*phoA* Mutants of *Salmonella typhimurium* TML in the Context of Gastroenteritis

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Tn*phoA* transposon insertion mutants of *phoN*-negative derivatives of *Salmonella typhimurium* TML (of human gastroenteritic origin) were selected by growing mutagenized recipient bacteria under a variety of growth conditions. Ninety-seven individual mutants, which expressed alkaline phosphatase, were collected and tested for their ability to invade HEp-2 cells. Seven smooth mutants had a reduced ability to invade HEp-2 cells, and three smooth mutants were consistently more invasive than their corresponding parental strains. One rough mutant was of similar invasiveness and two were of reduced invasiveness when compared with that of parental strains. The seven smooth hypoinvasive mutants, the three smooth hyperinvasive mutants, and the three rough mutant strains were tested for their abilities to invade ileal enterocytes by the rabbit ileal invasion assay described previously (3). All smooth mutants exhibited parental levels of invasiveness. The rough mutants were hypoinvasive in the rabbit ileal invasion assay. The HEp-2 system is therefore not a good predictor of behavior in gut tissue in this model. DNA sequences flanking the transposon were determined for five mutants which were hypoinvasive in the HEp-2 cell assay. The mutations were found to be insertions in two previously identified invasion genes, *invG* and *invH*, and in a gene not normally associated with invasion, *pagC*. These observations lead one to be cautious in the interpretation of the biological significance of data obtained from invasion of tissue culture monolayers when extrapolated to gut tissue.

Attempts to study *Salmonella*-associated gastroenteritis have been hindered by the lack of suitable small animal models and appropriate in vitro systems. As a result, the complex mechanisms whereby *Salmonella typhimurium* causes diarrhea are as yet incompletely understood. A correlation between the ability of *S. typhimurium* strains to invade rabbit gut epithelia and to cause gastroenteritis in humans has, however, been demonstrated (3). Here we attempt to build on this observation to study the mechanisms of invasion.

Much current work on mechanisms of bacterial invasion by enteric pathogens in general, and *S. typhimurium* in particular, involves the identification and characterization of mutants which exhibit altered invasiveness for cultured mammalian cells. Such invasion mutants have been selected by use of a variety of epithelial cell lines, including HEp-2 cells (30, 50) and polarized cell lines such as MDCK cells (17, 25). Polarized Caco-2 cells have also been used to study *Salmonella* invasion (51). The reporter transposon Tn*phoA* was developed to identify transposon insertion mutations in genes which encode surface or secreted proteins (32). This transposon was used in this work since it had been used successfully to mutate genes required for expression of the invasive phenotype.

A number of genetic loci involved in *S. typhimurium* invasion have been identified. The genes *invABC*, *invD* (12, 19), *invE* (25), *invFG* (29), and *invH* (2) are located at 59 min on the *S. typhimurium* chromosome. Immediately adjacent to the *inv* region, and overlapping with it, is the *spa* region (26), which contains nine invasion genes, the first of which, *spaL*, is *invC* (26). The predicted products of the *inv* genes, *invA*, *invE*, and *invG*, and all of the *spa* genes show various degrees of sequence similarity to proteins in *Shigella* and *Yersinia* spp. which are involved in invasion and virulence (21, 25, 26). Mutations at a separate locus, *hil*, have been shown to give rise to a hyperinvasive phenotype (30). Regions of the chromosome, of other *Salmonella* serotypes, which play a role in invasion have been shown to have homologous sequences in *S. typhimurium*; these include *sinA*, *-B*, and *-C* in *Salmonella enteritidis* (50) and *invA*, *-B*, *-C*, and *-D* in *Salmonella typhi* (13).

Electron micrograph studies have shown that the normal architecture of the brush border is disrupted during the interaction between *S. typhimurium* and polarized epithelial cell lines; this is accompanied by cytoskeletal rearrangements and an increase in intracellular free calcium (8, 16, 41). Loss of ability to cause this disruption has been associated with loss of an intact copy of either *invA* (21) or *invE* (25).

The biological significance of in vitro studies in which invasiveness of mutants is assessed in tissue culture cells must, however, be interpreted with caution. It has, for example, been shown that some Tn*phoA* mutants of *Salmonella choleraesuis* which could not transcytose MDCK cells were attenuated when administered orally to mice, whereas others were just as

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virulent as parent strains (17). If one wishes to study the mechanisms and determinants responsible for invasion and the biological sequelae of invasion in the context of gastroenteritis induced by *S. typhimurium*, then the mouse is not an appropriate model nor is the 50% lethal dose (LD_{50}) an appropriate index of gastroenteritic disease. To date, the best small animal model for this purpose is the rabbit ileal loop used originally by Giannella and colleagues (24). Derived from this model, a rabbit ileal invasion assay (RIIA) has been developed based on an asymmetric gut organ culture technique which is capable of discriminating between known virulent and avirulent strains of *S. typhimurium* (3).

In this report, we describe Tn*phoA* mutagenesis of *S. typhimurium* TML, a classical gastroenteritic diarrheagenic clinical isolate (22–24). Tn*phoA* mutants were generated and selected under different conditions. Initially, for logistical reasons, they were screened for loss of invasiveness in an HEp-2 cell assay (11). The genes which were interrupted in five hypoinvasive mutants have been sequenced and identified. Mutants which exhibited altered invasiveness for HEp-2 cells were also tested in the RIIA (3).

MATERIALS AND METHODS

Culture conditions, bacteria, and plasmids. Bacteria were routinely grown at 37°C in L broth (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7.3]) and L agar (L broth with 1.5% agar). Antibiotics were used at the following concentrations: chloramphenicol, 34 μg ml^{-1}; nalidixic acid, 75 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; and kanamycin, 60 μg ml^{-1} for *S. typhimurium* and 20 μg ml^{-1} for *Escherichia coli*. 5-Bromo-4-chloro-3-indolyl phosphate (XP; Sigma, Poole, United Kingdom) was used at a concentration of 80 μg ml^{-1} as the chromogenic substrate for detecting alkaline phosphatase activity. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; NBL, Northumbria, United Kingdom), and isopropyl-β-D-thiogalactopyranoside (IPTG; NBL) were each used at 20 μg ml^{-1}. *E. coli* DH5α (Bethesda Research Labs) (see reference 28) was used for cloning. *E. coli* SM10λ*pir* (48) was used as a donor for the suicide plasmid pRT733 (52) carrying the transposon Tn*phoA* (32). *S. typhimurium* JL5070 [*phoN*::mini MudM(Amp^r)] (14, 15) was supplied by F. Heffron (Department of Microbiology and Immunology, Oregon Health Science University, Portland). *S. typhimurium* TML, a strain originally isolated from a patient with gastroenteritis (24), and noninvasive strains LT7 and SL1027 (an LT2 derivative [44]) were supplied originally by R. A. Giannella (Division of Digestive Disease, Medical Center, University of Cincinnati, Cincinnati, Ohio). Spontaneously arising nalidixic acid-resistant mutants of *S. typhimurium* TML were used for Tn*phoA* mutagenesis. First, *phoN* mutants were generated; this was necessary since the background phosphatase activity of parent colonies made selection of authentic Tn*phoA* mutants virtually impossible. The *phoN* lesion was transduced from *S. typhimurium* JL5070 into TML with bacteriophage P22 HT105/1 *int*$^-$ (46) as described previously (37). The *phoN* lesion was transduced into both a nalidixic acid-resistant derivative of TML to yield TNP-1 and wild-type TML to yield TNP-5; in the latter case, nalidixic acid resistance was selected after the introduction of the *phoN* lesion. Both TNP-1 and TNP-5 were used in Tn*phoA* mutagenesis.

Mutagenesis of *S. typhimurium* TML. Tn*phoA* was introduced into *S. typhimurium* TML-derived strains TNP-1 and TNP-5 on the suicide vector pRT733 by conjugation with the donor strain SM10 (52). Transconjugants were selected for nalidixic acid and ampicillin resistance carried by the recipient *S. typhimurium* and kanamycin resistance carried by the transposon; those which expressed alkaline phosphatase were selected for further analysis. Mutants were selected by use of the following different culture conditions: L agar incubated at 30, 37, or 42°C; Hartley digest broth (HDB; Oxoid, Basingstoke, United Kingdom) agar incubated at 37°C; L agar containing 0.3 M NaCl incubated at 37°C; MacConkey agar (BBL, Cowley, Oxford, United Kingdom) incubated at 37°C; L agar containing 2% tissue culture medium (minimal essential medium [Life Technologies, Renfrew, Scotland] containing 2 mM L-glutamine and 10% fetal calf serum [Tcm]) and incubated at 37°C; L agar coated with HEp-2 cells and incubated at 37°C; and selection of colonies growing within poured agar plates under microaerophilic conditions and incubated at 37°C. No more than two colonies were selected from an individual conjugation.

HEp-2 cell invasion assays. HEp-2 cell invasion assays were performed as described previously (11) and are briefly summarized as follows. Bacteria were grown at 37°C without shaking overnight in HDB. One milliliter of this culture was used to inoculate 90 ml of HDB, and the organisms were incubated at 37°C for a further 3 h, at which point the culture was growing exponentially. Organisms were harvested by centrifugation and resuspended to a concentration of approximately 10^8 CFU ml^{-1} in tissue culture medium. The bacterial suspensions were stored overnight on ice held at 4°C. This major change from the original procedure (11) was a logistical convenience which made easier the handling of larger numbers of samples in a working day. (Comparative analyses revealed no significant differences in the pattern of HEp-2 cell invasiveness of organisms prepared in this manner [data not shown].) Immediately before infection, the concentrated samples were adjusted to contain 2×10^5 organisms ml^{-1}. One milliliter of this suspension was added to each well in a 24-well tissue culture tray to give a 1:1 ratio of bacteria to HEp-2 cells; the organisms were centrifuged onto the monolayers at 300 × *g* for 30 min at 37°C, and the tray was incubated at 37°C for 2 h. The supernatants were removed and replaced with 1 ml of fresh medium containing 100 μg of gentamicin; the trays were then incubated for a further 1 h before invasiveness was measured. Invasiveness was expressed in the now-conventional manner as the percentage of the inoculum recovered after gentamicin treatment and release by Triton X-100 (11). This determination was based on a minimum number of four wells. For comparative purposes, invasion data were normalized to data obtained for an appropriate control on monolayers derived from the same stock of HEp-2 cells, on the same day.

For mutant bacteria, initial growth of organisms (from overnight plates) was carried out in HDB containing kanamycin at 60 μg ml^{-1}; thereafter, the culture was handled in antibiotic-free medium for two reasons. It was observed that, when nalidixic acid-resistant mutants of TML were grown in HDB containing high concentrations (75 μg ml^{-1}) of nalidixic acid, organisms were pleomorphic; the majority were long aseptate rods for which accurate viable counts were not possible. These organisms were not as invasive as wild-type TML organisms. However, the nalidixic acid resistance was stable if the organisms were precultured in the absence of nalidixic acid, and the mutant displayed only one morphotype and was as invasive as TML. Kanamycin was included in the plates used for estimating the number of organisms recovered from the HEp-2 cells.

RIIA. The invasiveness of mutants in the RIIA was carried out as described previously (3) with stripped rabbit ileal mucosa mounted in a purpose-built asymmetric organ culture apparatus which allowed selective challenge of the mucosal side of gut tissue; a brief summary of the technique is given here. By using this apparatus, it was possible to bathe the mucosal and serosal surfaces in different solutions. The mucosal surface was bathed in a solution containing 60 mM choline chloride, 30 mM choline HCO_3, 20 mM KCl, 111 mM glucose, and 10% Tcm; this was designated mucosal medium. The serosal surface was bathed in a solution containing 60 mM NaCl, 30 mM $NaHCO_3$, 20 mM KCl, and 111 mM glucose (the standard W.H.O. rehydration solution); this was designated serosal medium. These conditions allowed the tissue to be maintained intact and functional for up to 4 h (3). Bacteria were prepared as described for the HEp-2 cell assay except that they were finally resuspended in mucosal medium and stored overnight on ice held at 4°C. As stated above, this was a logistical convenience which made easier the handling of large numbers of samples by one individual in a working day. The mucosal surface of the rabbit ileal tissue (50 mm^2 in each chamber) was challenged with 3 ml of stored suspensions, each containing approximately 10^8 bacteria, and incubated for 2 h at 37°C. The supernatants were then removed from each chamber, and residual extracellular bacteria were killed by the addition of fresh medium containing 100 μg of gentamicin ml^{-1} for 1 h at 37°C. The effectiveness of gentamicin under these conditions in killing noninternalized organisms had been demonstrated previously (3). The tissue was then removed from the apparatus and washed in isotonic saline, and a disk of tissue (8-mm diameter) was cut out from each chamber with a cork borer. The tissue was homogenized on ice in 3 ml of Triton X-100 (1% in phosphate-buffered saline) in an Omnimixer (Sorvall Inc., Newtown, Conn.) at maximum speed for 30 s to release intracellular bacteria. Invasiveness was expressed as the percentage of the inoculum recovered from the tissue.

Because of (i) the growing awareness of the effect of temperature on the expression of invasive phenotypes of enteric bacteria (34) and (ii) the fact that much of the comparative work had been done with stored organisms, a separate set of experiments was carried out to compare the levels of invasiveness of bacteria (wild type, mutants, and their immediate parents) prepared as described above, but without storage overnight on ice at 4°C in mucosal medium.

Characterization of Tn*phoA* mutants. Mutants were examined for motility by the hanging-drop method using light microscopy and the semisolid agar culture test (9). Lipopolysaccharide (LPS) composition was examined by use of O-specific agglutination sera and polyacrylamide gel electrophoresis (54). The invasiveness of mutants was measured both in HEp-2 cells and by the RIIA.

Colony and Southern blot analysis of Tn*phoA* mutants. Colony blots onto nylon filters were screened with the radioactively labelled oligonucleotides 5′-GTAATATCGCCCTGAGCAGC-3′ complementary to part of the *phoA* sequence (47) and 5′-TCACATGGAAGTCAGATCCT-3′ complementary to IS*50* sequences (4) present on Tn*phoA*. Chromosomal DNA was digested to completion with restriction enzymes which were known not to have a site in Tn*phoA*, namely, *Asp*718I and *Eco*RV (4, 6, 32, 35, 47). Fragments were separated on a 0.5% agarose gel and capillary blotted onto a nylon membrane (Boehringer Mannheim). The membranes were probed with a *Nco*I fragment of Tn*phoA* carrying the kanamycin resistance marker, which had been labelled with α-^{32}P by random multiprime primer labelling (Amersham). Bands were visualized by exposure to X-ray film (Amersham) overnight.

Cloning chromosomal segments flanking Tn*phoA* insertions. Molecular biological techniques were carried out by standard procedures (43). Fragments of chromosomal DNA from the mutated *S. typhimurium* strains were generated by

complete digestion with restriction endonuclease *Asp*718I. The fragments were cloned into the medium-copy-number vector pSU19 (which is a chloramphenicol-resistant derivative of pSU2713 [33]) and transformed into *E. coli* DH5α. Transformants bearing Tn*phoA* and flanking chromosomal sequences were selected by virtue of the kanamycin resistance encoded by the transposon. A single transformant was selected, and plasmid DNA was isolated.

Inverse PCR amplification of DNA segments flanking Tn*phoA* insertions. In the case of mutant 64, inverse PCR was used to obtain DNA upstream of the Tn*phoA* insertion. The *phoA* primer mentioned above was used together with a second primer, 5′-ATATTACTGCACCCGGCGGT-3′, which also recognizes sequences in the first 100 bp of *phoA*, but on the opposite strand. Inverse PCR was modified from published procedures (40, 53). Chromosomal DNA was digested to completion with *Sau*3A, and the resulting fragments were circularized by ligation at a DNA concentration of 0.5 μg ml^{-1} in the presence of 0.5 U of DNA ligase μl^{-1} (Life Technologies). Amplification was performed in a Techne PHC2 dri block with 30 cycles of denaturation at 93°C for 30 s, annealing at 60°C for 30 s, and extension by *Taq* polymerase (Boehringer Mannheim) at 72°C for 60 s.

Sequence analysis of flanking segments. DNA sequencing of cloned DNA was performed on double-stranded template by the dideoxy chain termination method (45), using Sequenase (U.S. Biochemical) as described in the manufacturer's instructions. The *phoA* and IS*50* primers described previously were used to generate sequence data outwards into the *Salmonella* DNA. Further synthetic oligonucleotide primers were designed to allow the sequence of *invG* to be fully confirmed on both strands. Sequence analysis of PCR fragments was performed by a modification of the Sequenase protocol in which 10% dimethyl sulfoxide was added to the termination mixes supplied in the kit. After annealing of the primer, the template mixture was cooled rapidly in liquid nitrogen. ^{35}S-dATP (Amersham), dithiothreitol, and Sequenase were added as the mixture thawed, and aliquots were dispensed into the termination mixes. Termination reactions were allowed to proceed for 5 min at room temperature and then for a further 5 min at 37°C before addition of 2 μl of a chase mix containing 0.25 mM each deoxynucleoside triphosphate, 50 mm NaCl, and 10% dimethyl sulfoxide. The reaction mixtures were incubated for a further 10 min at 37°C before addition of the formamide stop buffer. The two primers used for inverse PCR were used to obtain the sequence upstream of the Tn*phoA* insertion on both strands.

Analysis of sequence data. Nucleotide and derived amino acid sequence data were analyzed with programs from the University of Wisconsin Genetics Computer Group (10). The OWL database (7) was searched for sequence similarities by using FASTA (42). Facilities provided by SEQNET, SERC Daresbury Laboratory, Warrington, United Kingdom, in addition to those of the Academic Computing Service at the University of Birmingham were used.

RESULTS

Isolation and preliminary characterization of TML Tn*phoA* insertion mutants. Ninety-seven independent Tn*phoA* insertion mutants of TNP-1 or TNP-5, which expressed alkaline phosphatase, were isolated as described above. Radioactively labelled oligonucleotides complementary to IS*50* (4) and to *phoA* (47) sequences present on Tn*phoA* were used separately to screen all of the mutants by DNA-DNA hybridization. Only kanamycin-resistant mutants that hybridized to both probes were used in the study.

Three mutants (numbers 41, 44, and 85) expressed modified LPS and were assumed to be rough mutants. All mutants were found to be prototrophic and motile. One mutant (number 40) had a reduced growth rate as compared with that of its parent.

HEp-2 cell invasiveness of Tn*phoA* mutants of *S. typhimurium* TML. Independent Tn*phoA* mutants were tested for their ability to invade HEp-2 cells. From a preliminary screen (one test of four wells per mutant), mutants were grouped into three categories: (i) the majority which showed invasiveness ranging from 51 to 130% of parental invasiveness, (ii) those which showed less than 51% of parental invasiveness, and (iii) those which showed greater than 130% of parental invasiveness (Fig. 1a). Groups ii and iii were selected for retesting since they fell at the extremes of the distribution (Fig. 1) and would be expected to contain mutants which were significantly different from their respective parents. Seven smooth mutants, numbers 25, 40, 45, 56, 64, 81, and 83, were reproducibly hypoinvasive (Fig. 1b and 2).

Three smooth mutants, numbers 32, 76, and 97, were hyperinvasive (Fig. 1b and 2). The avirulent strain SL1027 of *S. typhimurium* had been found previously to be relatively more invasive than virulent strains when centrifuged onto monolayers but not when the centrifugation step was omitted (11). The hyperinvasive phenotype observed in these mutants was independent of the inclusion of a centrifugation step (data not shown). Two of the three rough mutants, numbers 41 and 85, were approximately 50% less invasive than their respective parents, whereas the third, number 44, was of similar invasiveness to its parent in HEp-2 cells.

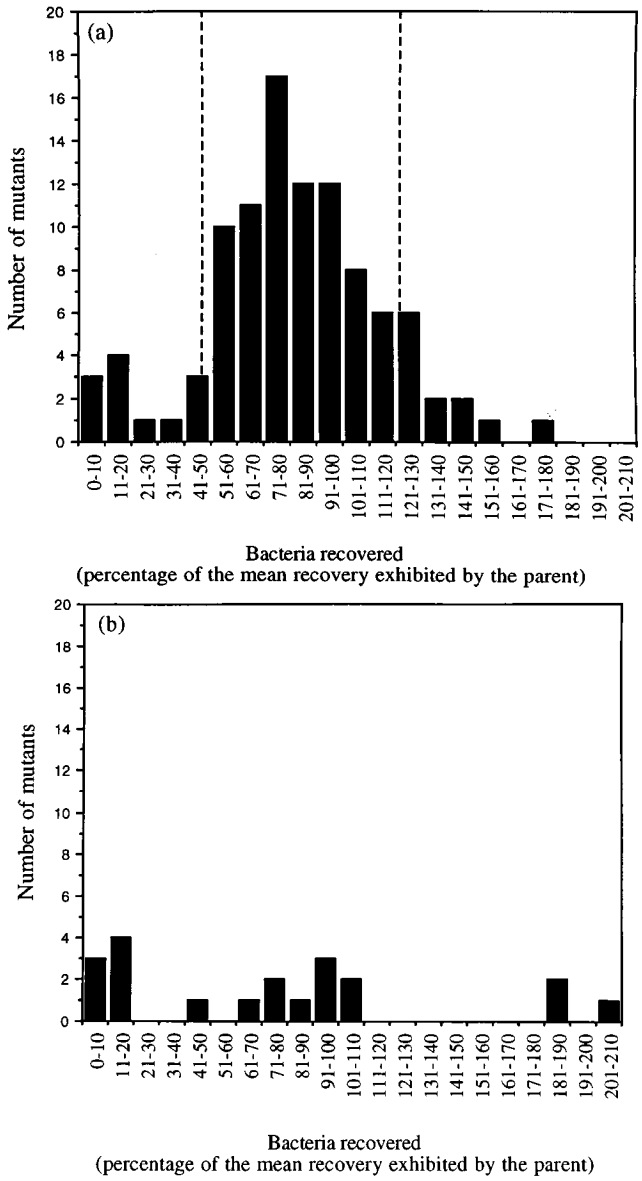

FIG. 1. Distribution of recoveries of Tn*phoA* mutants of *S. typhimurium* TML from HEp-2 cell invasion assays based on one test of four wells for each mutant (a) and reanalysis of distribution of recoveries of those Tn*phoA* mutants which scored outside of the dashed vertical lines in panel a (b).

Invasiveness of Tn*phoA* mutants of *S. typhimurium* TML in the RIIA. (i) Inocula stored at 4°C before use. Strains TNP-1 and TNP-5 were as invasive as strain TML in the RIIA when challenge inocula were held at 4°C (Fig. 3).

Detailed analyses of four separate experiments with mutant 45 are given in Fig. 4; these are typical of the data obtained

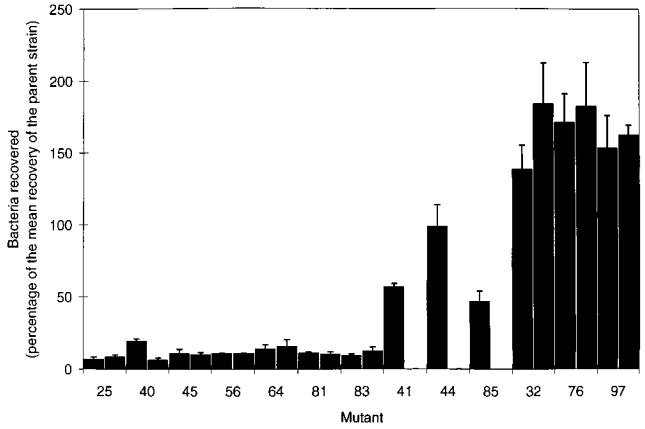

FIG. 2. Recoveries of seven hypoinvasive mutants (numbers 25, 40, 45, 56, 64, 81, and 83), three hyperinvasive mutants (numbers 32, 76, and 97), and three rough mutants (numbers 41, 44, and 85) of *S. typhimurium* TML from HEp-2 cell invasion assays. Individual experiments are shown as separate columns; error bars represent standard deviations of recoveries from four wells. The rough strains were assayed only once in these experiments.

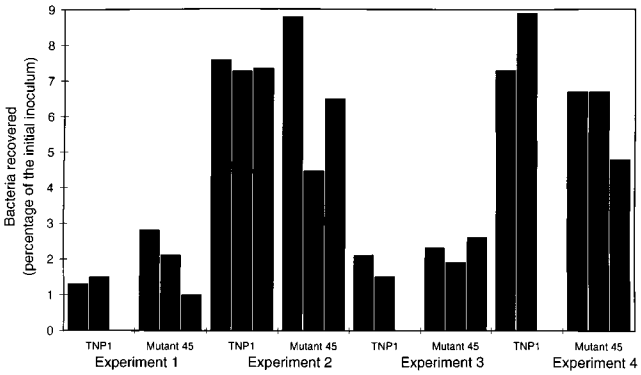

FIG. 4. Recoveries from RIIAs of Tn*phoA* mutant number 45 and parent strain TNP-1. Organisms were prepared the day before the assay and stored overnight at 4°C. The data are from four separate experiments carried out on different days with tissue from different rabbits. Each vertical column represents one experimental chamber in the organ culture assay. Note the following: (i) there is reproducibility between replicate chambers; (ii) there is no significant difference between the parent and mutant 45; and (iii) there is large interanimal variation. The last point emphasizes the need for strict internal controls to be used each time for meaningful interexperimental comparisons to be made.

with all other mutants in this group (numbers 25, 56, 64, 81, and 83). The data show variation between experiments but consistency within each experiment. Such variability between experiments has been observed previously, is operator independent, and is almost certainly due to unpredictable variation in the susceptibilities of gut from different animals. However, the data are consistent within experiments. It is therefore possible, by including relevant controls and normalizing the data, to distinguish between invasive and noninvasive strains (3): there was no statistically significant difference between the parent strain and mutant 45 (Fig. 4).

Mutants 25, 45, 56, 64, 81, and 83, which were hypoinvasive for HEp-2 cells, were not significantly different from their parents (Fig. 5). Mutants 32, 76, and 97, which were hyperinvasive for HEp-2 cells, exhibited only parental levels of invasiveness (Fig. 5). The three rough mutants (numbers 41, 44, and 85) were about 10-fold less invasive than their parents (Fig. 5). The viabilities of the rough mutants were similar to those of their parents in mucosal medium. However, since these organisms were found to have the potential to autoagglutinate, extra care was taken to disperse them with vigorous mixing at all possible stages during the assay.

Mutant 40 was initially thought to be less invasive than its parent. However, in addition to having a reduced growth rate in HDB, it did not (in contrast to the other mutants) survive well in the medium used to bathe the mucosal side of the tissue in the RIIA. When increased inocula were used to compensate for the loss of viability, mutant 40 was found to be as invasive as the parental strain.

A control noninvasive strain (SL1027) was used in these experiments (see Fig. 5) and, as described previously (3), was consistently less invasive than strain TML.

(ii) Inocula prepared at 37°C and used without storage at 4°C. Because of the growing awareness of the effect of temperature on the expression of invasive phenotypes of enteric

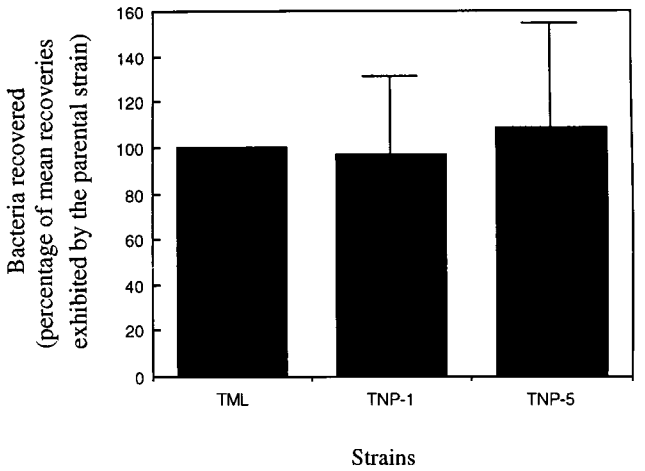

FIG. 3. Recoveries of strains TNP-1, TNP-5, and TML from RIIAs. Organisms were prepared the day before the assay and stored overnight at 4°C. The data are from four separate experiments carried out with tissue from different rabbits; error bars represent the standard errors of the means.

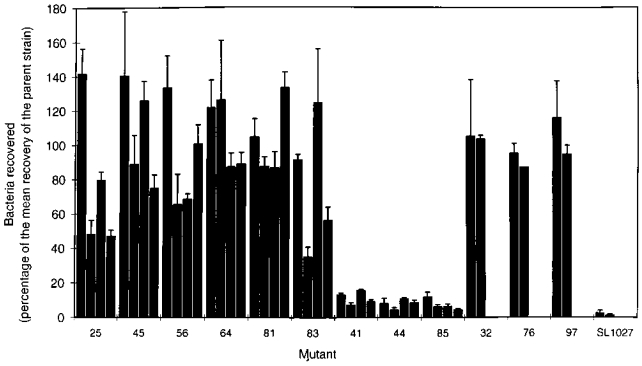

FIG. 5. Recoveries from RIIAs of six mutants which were hypoinvasive for HEp-2 cells (numbers 25, 45, 56, 64, 81, and 83), three mutants which were hyperinvasive for HEp-2 cells (numbers 32, 76, and 97), and three rough mutants (numbers 41, 44, and 85) of *S. typhimurium* TML normalized to the levels of invasiveness of their respective parent strains. SL1027 is included as a control noninvasive strain. Organisms were prepared the day before the assay and stored overnight at 4°C. Each column represents a separate experiment carried out with tissue from a different rabbit and represents the mean of the recovery from three experimental chambers in the organ culture assay; error bars represent standard deviations.

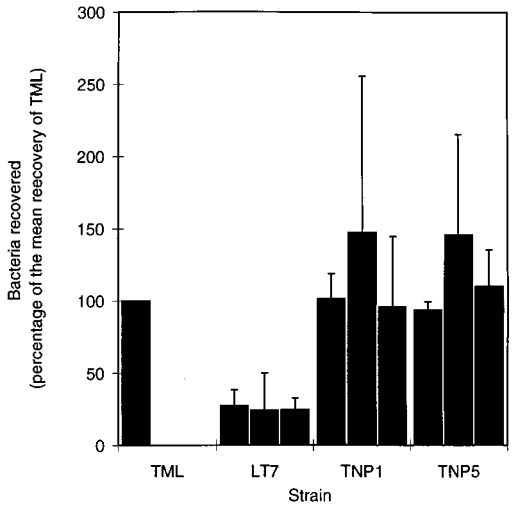

FIG. 6. Recoveries from RIIAs of strains TNP-1 and TNP-5, their parent strain TML, and strain LT7. Organisms were prepared on the day of the assay without storage at 4°C. The data are from three separate experiments carried out with tissue from different rabbits; error bars represent the standard deviations.

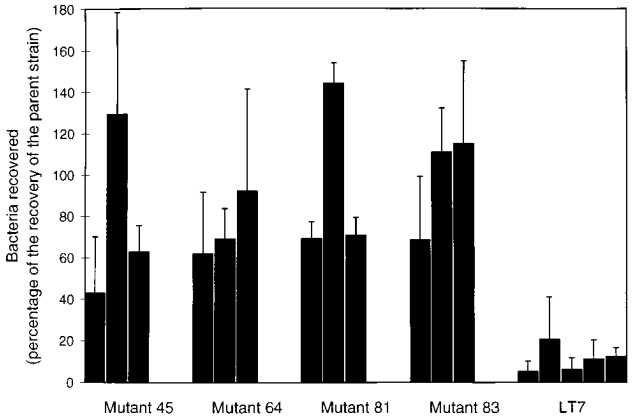

FIG. 7. Recoveries from RIIAs of mutants 45 (*invH*), 81 (*invH*), 64 (*pagC*), 83 (*invG*), and their respective parent strains. Organisms were prepared without cooling on the day of the assay. Each column represents a separate experiment carried out with tissue from a different rabbit and represents the mean of the recovery from three experimental chambers in the organ culture assay; the error bars represent standard deviations. LT7 is included as a control noninvasive strain.

bacteria (34), and the potential significance of the genetic data described below, the levels of invasiveness of the mutants were reexamined relative to those of their respective parents with challenge inocula prepared freshly on the day of the experiment without any storage at 4°C. For logistical and cost reasons only, the comparisons were kept to those described. Mutants 25 and 56 were not included since 25 was shown to have a double Tn*phoA* insertion and 56 was one of three *invH* mutants (see below). LT7 was used instead of SL1027 for two reasons. First, it belonged to the same group of noninvasive strains as did SL1027 (3). Second, we wished to characterize this strain for future work since it was an isolate which, unlike SL1027, had not been genetically manipulated in the laboratory (44).

TNP-1 and TNP-5 were not significantly different from parental strain TML (Fig. 6). Four mutants (numbers 45, 64, 81, and 83; all hypoinvasive for HEp-2 cells) were each tested on three separate occasions with tissue from different rabbits and in different combinations; there was no significant difference between the mutants and their respective parents (Fig. 7).

In another two experiments carried out on separate days with tissue from different rabbits, a direct comparison was made between fresh and stored challenge inocula derived from TNP-1 and mutant 45. Single colonies from fresh MacConkey plates of each strain were used to prepare organisms stored at 4°C for use in the RIIA as described in Materials and Methods. On the next day, fresh cultures were prepared from the same plates and used without storage at 4°C; both types of inocula were tested on gut derived from the same rabbit. The relative patterns of invasiveness did not change, but the absolute levels did: stored organisms were ca. 10-fold less invasive.

Identification of the lesions in the hypoinvasive mutants. A probe derived from the kanamycin resistance marker of Tn*phoA* was used for Southern blot analysis of restriction digests of chromosomal DNA from the six hypoinvasive mutants. The analysis revealed that mutant 25 contained a double Tn*phoA* insertion but that the others were single insertions. Since the double insertion would make interpretation of invasion data difficult, mutant 25 was not further characterized.

DNA sequence data were obtained from the remaining five hypoinvasive mutants (numbers 45, 56, 64, 81, and 83) by using two primers which allowed sequences to be read both upstream and downstream of the transposon insertion. Analysis showed that three of the mutations (numbers 45, 56, and 81) were insertions in the same gene and that the other two were insertions in two separate loci. Since the mutants were selected on the basis of their ability to express alkaline phosphatase from a fusion between the leader sequence of the mutated gene and the transposon-borne alkaline phosphatase, the reading frame of the mutated gene could be deduced from that of the *phoA* gene. The deduced amino acid sequences of the disrupted genes were used to search the OWL database (7) for sequences showing similarity.

Sequence data from mutants 45, 56, and 81 showed 100% identity with the *S. typhimurium invH* gene (2) over a stretch of 93 amino acids. We conclude that mutants 45, 56, and 81 are disruptions in the *invH* gene of *S. typhimurium* TML. The mutations were in the same reading frame: mutant 81 was located downstream from the predicted signal sequence and mutants 45 and 56 were located at the same position within the hydrophobic region containing the signal sequence.

A 53-amino-acid sequence, predicted from the sequence adjacent to the Tn*phoA* insertion in mutant 83, showed similarity with the *Shigella flexneri* MxiD protein and with *Yersinia enterocolitica* and *Yersinia pestis* YscC (1, 27, 36). Both of these proteins are involved in the secretion or surface positioning of proteins involved in the pathogenicity of these organisms. Since the DNA sequence data showed no significant similarity to the sequence of any of the *S. typhimurium* genes published at the time, the complete sequence of the disrupted gene was determined. The position of the insertion giving rise to mutant 83 is inside the mature protein sequence, 163 bp downstream of the putative ATG start codon. The nucleic acid sequence at the 3′ end of the disrupted gene overlapped, with 100% identity, the first 260 bp of the sequence of the *invE* gene (accession number M90714 in the GenEMBL database). Thus, mutant 83 is located upstream of *invE*, and both genes are transcribed in the same direction. The nucleotide sequence of this gene is 99% identical to the recently published sequence of *S. typhimurium invG* (29). The ATG start codon for *invE* overlaps the stop codon of *invG* in the sequence ATGA. We have entered the sequence of the *S. typhimurium invG* gene in the EMBL

database, accession number X75302. Insertion of Tn*phoA* into *invG* may have a polar effect on transcription of *invE* and other *inv* and *spa* genes which are downstream of *invG*.

The derived amino acid sequence of the polypeptide encoded by *invG* is 51% identical to that of *S. flexneri* MxiD (1) and 28% identical to that of YscC from *Y. enterocolitica* (36). This suggests a similar role for *invG* in the excretion or surface positioning of proteins involved in invasion.

The insertion in mutant 64 falls in *pagC*, a gene which has been implicated in survival within macrophages (38); the insertion is in the last residue of the signal sequence.

DISCUSSION

S. typhimurium TML used in these experiments was originally isolated from a case of human gastroenteritis; the study of invasion mutants of this strain may therefore be expected to be relevant to gasteroenteritic disease. The mutants we isolated and report here were screened initially by use of HEp-2 cells and, subsequently, by the RIIA, which is unsuitable for primary screening of large numbers of mutants.

Since several virulence genes have been shown to be highly regulated in pathogenic bacteria (20, 34), Tn*phoA* mutants were isolated initially by using different growth conditions for selection of blue, alkaline phosphatase-positive colonies. This approach was taken to obtain as wide a range of positive fusions as possible. Mutants that exhibited both hyper- and hypoinvasiveness were identified, and these were distributed within 4 of the 10 groups selected under different sets of growth conditions. However, expression of the gene fusions in these mutants was not found to be dependent on the conditions under which they were selected.

The transposon mutants were selected in spontaneously arising nalidixic acid-resistant mutants of TML. This was necessary to allow for selection of recipient *S. typhimurium* after conjugation. Nalidixic acid resistance arises from alterations in DNA gyrase and could have an effect on supercoiling of DNA, which in turn could have an effect on the invasion phenotype. There are three reasons why we think that this is not the case in this work. First, neither of the nalidixic acid-resistant derivatives of TML (TNP-1 and TNP-5) showed any change in invasiveness relative to that of their parents in the RIIA (Fig. 3 and 6). Second, the hypoinvasive mutants showed a reduction in invasiveness for HEp-2 cells to about 10% of that seen for wild-type organisms, which is in agreement with the observations of other workers (26, 50). Third, these mutants also showed wild-type invasiveness for rabbit ileal epithelium. These observations suggest that the gyrase mutations giving rise to the nalidixic acid resistance were not having an effect on the invasion phenotype of the mutants.

Two of the rough variants of TML (numbers 41 and 44) were 50% less invasive for HEp-2 cells, and the other (number 85) appeared to be as invasive as its wild-type parent. All three mutants were, however, relatively noninvasive in the RIIA. Since these mutants have not been fully characterized, it is not possible to distinguish between two possible explanations for their reduced invasiveness. First, it could be that LPS is involved in assisting invasion. Second, the rough phenotype may not be a result of the insertion of the transposon into a gene involved in the synthesis of LPS but may result from the selection of spontaneously arising rough mutants which may serve as better recipients for conjugation; in this case, the noninvasive phenotype could have resulted from a separate mutation. However, these data have been included here because they emphasize the ability, already demonstrated previously (3), of the RIIA to distinguish between strains with differing levels of invasiveness. Had any of the smooth mutants been consistently hypoinvasive, we are confident that the system would have detected them.

Mutants of *S. typhimurium* which are hyperinvasive for tissue culture cells have been described previously (30). The mutants described in this study which were hyperinvasive for HEp-2 cells were not hyperinvasive in the RIIA, indicating that other factors may be involved in the invasion of complex differentiated gut epithelium.

Analysis of the genes interrupted in the smooth hypoinvasive mutants revealed that this group consisted of mutations in three separate loci. Three of the mutations were in *invH*, a gene whose sequence shows no significant homology with any other sequences in the GenEMBL database. It is transcribed in the opposite direction from the other *inv* genes (2), and a Tn*phoA* insertion in this gene is unlikely to exert a polar effect on other characterized invasion genes. The demonstration that mutants of *S. typhimurium* defective in *invH* are noninvasive for HEp-2 cells is in agreement with the findings of two other groups. Altmeyer et al. (2) cloned and sequenced *invH* from *S. choleraesuis* and *S. typhimurium* and demonstrated that it was necessary for adherence to and penetration of Henle-407 and MDCK cells. This gene is widely distributed among *Salmonella* serotypes (2). Introduction of a mutation into *invH* in several *Salmonella* serotypes rendered these serotypes defective in their ability to invade cultured cells; this was most marked in host-adapted *Salmonella gallinarum*, *S. choleraesuis*, and *S. typhi* (2). Stone et al. (50) have also shown that three mutations in *S. enteritidis invH* significantly reduced invasiveness for HEp-2 cells (1% of that of the wild type) and, to a lesser extent, for CHO and MDCK cells (about 25% of that of the wild type). Our *invH* mutants are approximately 10% as invasive as their immediate parents in the HEp-2 cell assay, which is within the range observed by other authors.

InvG shows significant similarity at the amino acid level to virulence proteins in other enteric pathogens, in particular to the MxiD protein of *S. flexneri* and the YscC protein of *Y. enterocolitica* and *Y. pestis*, which are encoded on the virulence plasmids of these organisms (1, 27, 36). These plasmids encode a large number of proteins which play a variety of roles in the virulence of these organisms. For example, the virulence plasmid of *S. flexneri* encodes the Ipa proteins (invasion plasmid antigens), some of which are involved in the process of entry of *Shigella* into cultured epithelial cells (5, 55). These plasmid-encoded virulence factors are all excreted or exposed on the surface of the bacteria but do not have conventional signal sequences (1, 27, 36). Excretion of these proteins is facilitated by a group of genes, including *mxiD* and *yscC*, which are also located on these plasmids. The sequence similarity with MxiD and YscC suggests that InvG may be required for the excretion or surface positioning of proteins which play a role in invasion of cultured epithelial cells by *S. typhimurium*.

In addition to *invG*, a number of other genes in the *S. typhimurium inv* locus have been reported to show significant sequence similarity with genes of the virulence plasmids of *Yersinia* and *Shigella* spp. (21, 25, 26) which are also part of the virulence factor excretory mechanism. It has been suggested that *S. typhimurium* may have acquired these genes from the *Shigella* or *Yersinia* spp. at some time in its evolutionary history (21, 25) or that the genes may have a common evolutionary origin (26).

The mutation in *pagC*, a gene which has been implicated in survival within macrophages (38), gives rise to a hybrid *pagC*-alkaline phosphatase protein which is identical to that described by Miller et al. (39). They propose that while *pagC* is in fact involved in survival within macrophages, this particular

pagC-alkaline phosphatase hybrid is aberrantly localized in the cytoplasmic membrane, which prevents the proper positioning of proteins necessary for invasion, giving rise to a hypoinvasive phenotype. This reinforces the need for caution when interpreting the biological significance of phenotypes arising from the expression of fusions to alkaline phosphatase.

There is now a rapidly emerging perception that invasiveness in *Salmonella* spp. is a multifactorial phenotype. A large number of genes have been identified in *Salmonella* spp. as being important in the invasion of cultured mammalian cells (2, 13, 19, 21, 25, 26, 50). However, it is clear from the work reported here that one cannot arbitrarily extrapolate invasion data derived from cultured cells to differentiated gut epithelia. This is further substantiated by the work of Finlay et al. (17), who showed that some Tn*phoA* mutants of *S. choleraesuis* which could not transcytose MDCK cells were just as virulent as parent strains when administered orally to mice.

For logistical reasons, invasion-defective mutants are nearly always selected initially by screening on tissue culture cells. Further characterization of such mutants may be carried out in whole-animal studies (e.g., by comparing oral and intraperitoneal LD_{50} ratios [19]) or by measuring survival within macrophages (18). However, in whole-animal experiments, it is not always easy to distinguish events concerned with the primary invasion of enterocytes from subsequent events in the pathogenesis of disease, such as negotiating the hypertonic environment of the lamina propria (49) or survival in macrophages. In contrast, the system used here allows statements to be made about the early events in invasion restricted to the negotiation of gut enterocytes (3). Use of this assay leads us to suggest caution in the interpretation of data, derived solely from tissue culture assays, as reflecting what happens in intact explanted gut epithelial tissue: mutations in two genes (*invG* and *invH*) in the well-characterized *inv* region of *S. typhimurium* as well as a mutation which has a nonspecific effect on invasion had no detectable effect on the invasion in the RIIA. The products of the *invG* and *invH* genes in *S. typhimurium*—important for the invasion of HEp-2 cells but not for that of explanted rabbit ileal epithelia—may well be important in other aspects of the pathogenesis of *S. typhimurium*-induced gastroenteritis. One could also argue that, in the complex tissue of the intestinal mucosa, alternative routes of invasion which are not present in relatively simple and nonrepresentative tissue culture cells are available.

Finally, we comment on the effect of temperature on the levels of invasiveness of the organisms tested. Storage at 4°C significantly affected the degree but not the pattern of relative invasiveness. We take the fact that the same relative patterns could be observed when invasiveness was reduced by 1 order of magnitude as indicative of the power of the system to discriminate between strains of different levels of invasiveness when compared under the same conditions. The fact that inocula stored at 4°C were 10-fold less invasive than comparable inocula prepared and used fresh may be interpreted in the light of the fact that prolonged inhibition of protein synthesis abolishes the ability of *S. typhimurium* to enter HEp-2 cells (31).

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