

## Insertions of Mini-Tn10 Transposon T-POP in *Salmonella enterica* sv. *typhi*

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### ABSTRACT

We have mutagenized a clinical strain of *Salmonella enterica* sv. *typhi* with mini-transposon Tn10dTet (T-POP) to obtain conditional lethal (tetracycline-dependent) mutants with T-POP insertions upstream of essential genes. Generalized transducing phage P22 was used to introduce T-POP from a *S. typhimurium* donor into a *S. typhi* recipient. Chromosomal DNA was purified from the mutagenized donor strains, fragmented, and then electroporated into *S. typhi* to backcross the original T-POP insertions. Four tetracycline-dependent mutants with two distinct terminal phenotypes were found among 1700 mutants with T-POP insertions. When grown in the absence of tetracycline, two of the four tetracycline-dependent mutants arrest at a late stage in the cell cycle, can be rescued by outgrowth in media with tetracycline, and define a reversible checkpoint late in the cell cycle. One of these insertions creates an operon fusion with a gene, *yqgF*, that is conserved among gram-negative bacteria and likely encodes an essential Holliday junction resolvase. T-POP insertions can be used not only to identify essential *S. typhi* genes but also to reveal novel phenotypes resulting from the depletion of their products.

**I**NFECTIONS caused by serovars of *Salmonella enterica* contribute to human morbidity and mortality on a global scale. Together with *S. enterica* sv. *enteritidis*, the model genetic organism *S. enterica* sv. *typhimurium* is among the leading causes of food poisoning (ALTEKRUSE *et al.* 1997). *S. typhimurium* infects a variety of warm-blooded hosts and causes a systemic lethal infection in mice. Although closely related to *S. typhimurium*, *S. typhi* infects only humans and kills >600,000 victims per year (PANG *et al.* 1998). To understand the genetic basis of the difference in host range between *S. typhi* and *S. typhimurium*, we are attempting to develop new methods to analyze the functions of *S. typhi* genes and to construct interspecific hybrids between *S. typhi* and *S. typhimurium*.

The genetic tools available for the study of *S. typhi* are limited, unlike the case for *S. typhimurium*. The most powerful genetic tool used in the study of *S. typhimurium* is generalized transduction mediated by temperate phage P22, because generalized transduction between *S. typhimurium* donors and recipients permits the construction of otherwise isogenic strains. To date, a similar generalized transducing phage for *S. typhi* has yet to be found. Although P22 can adsorb to *S. typhi* and inject its DNA into a *S. typhi* recipient, P22 cannot develop lytically in *S. typhi*. Therefore, P22 cannot be used to

mediate either intraspecific or interspecific crosses involving *S. typhi* donors. However, P22 can mediate interspecific generalized transduction between *S. typhimurium* donor strains and *S. typhi* recipients (EDWARDS and STOCKER 1988). To transfer mutations from *S. typhi* donors to *S. typhi* recipients, we substituted electroporation for generalized transduction as a method of genetic exchange. We have shown that chromosomal DNA can be isolated from an *S. typhi* donor, fragmented, and then electroporated into an *S. typhi* recipient to yield recombinants that have acquired a selectable marker from the donor (TORO *et al.* 1998).

The recent determination of the complete genome sequence of a clinical isolate of *S. typhi* (PARKHILL *et al.* 2001) has set the stage for new, systematic approaches to identify the genes involved in its virulence. We and others have found that the expression of the coliphage  $\lambda$  Red functions in *S. typhi*, as in *S. typhimurium*, permits targeted gene substitution (DATSENKO and WANNER 2000; SANTIVIAGO *et al.* 2002; our unpublished results). Thus, the construction of *S. typhi* mutants with deletions of nonessential genes and assays of their phenotypes in human cells will identify many new virulence determinants. However, because one can make deletions only of nonessential genes, this PCR-based method for gene disruption does not yield information about the roles that essential genes play in virulence.

Essential genes are most often defined by conditional lethal mutations. One subclass of these conditional loss-of-function mutations are mutations that place an essen-

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tial gene under the control of a promoter that is expressed under permissive conditions, but not under restrictive conditions. When such mutants are shifted from permissive to restrictive conditions, the product of the target essential gene is depleted by degradation and/or diluted by cell division. The terminal phenotypes of cells with subcritical concentrations of the essential gene product can be interpreted to provide clues to the function of the essential gene. This type of "depletion analysis" of essential gene functions was pioneered in *Saccharomyces cerevisiae* by fusing essential genes to the GAL1 promoter, which is active in the presence of galactose but repressed in the presence of glucose (see, for example, PATTERSON and GUTHRIE 1987). This method has been extended to *Escherichia coli* by constructing similar fusions with the arabinose-inducible *araBAD* promoter, which is active in the presence of arabinose but repressed in the presence of glucose (NISHIYAMA *et al.* 1994). Thus, we might envision a systematic genomics-based approach to explore the functions of essential *S. typhi* genes, involving the construction of arabinose-dependent transcriptional fusions with each *S. typhi* gene and the subsequent depletion analysis of each mutant. However, like other systematic genomics-based approaches to analyze gene function, this approach is labor intensive and does not preclude the more rapid success of classical genetic methods to accomplish a similar goal.

One powerful, classical genetic approach to analyze gene function on a genomic scale uses transposons with selectable genetic markers to make broad spectra of insertion mutations. DNA transposons have *cis*-acting ends and a *trans*-acting transposase, which can be separated to build more powerful tools for mutagenic analyses, including mini-transposons. Mini-transposons carry the *cis*-acting ends of a transposon flanking a selectable marker, but not the transposase gene. After an initial round of transposition in the presence of transposase, mini-transposon insertions can be separated from the source of transposase to generate stable transposon insertions that do not undergo additional rounds of transposition.

In this study, we describe the use of the mini-transposon T-POP (RAPPEYE and ROTH 1997), a Tn10-dTet element 2249 bp in length, to produce insertions in *S. typhi*. Unlike the insertions made by many other mini-transposons, T-POP insertions can be used to identify essential genes by placing their expression under the control of a tetracycline-inducible promoter. The T-POP element carries the *tetR* (repressor) and *tetA* (resistance) genes flanked by active IS10 inverted repeats required *in cis* for transposition (Figure 1). Tetracycline induces the divergent transcription of *tetR* and *tetA*, which can extend outside the ends of the transposon into adjacent genes. RAPPEYE and ROTH (1997) have shown that a subset of T-POP insertions in *S. typhimurium* are dependent on tetracycline for their growth. These insertions

separate an essential gene from its promoter and place the expression of the essential gene under the control of the *tetR* or *tetA* promoters.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions:** The clinical strain of *S. typhi*, STH2370, was obtained from the Infectious Diseases Hospital (Hospital Lucio Córdova) in Santiago, Chile. *S. typhimurium* strains MST1168 and MST4208 are derivatives of LT2 and were obtained from Stanley Maloy. MST1168 carries the ampicillin-resistant (Amp<sup>R</sup>) plasmid, pNK972, the source of Tn10 transposase. MST4208 carries the *proBA47* deletion and a complementing F'128 (*pro+* *lac+*) episome with the insertion *zzf3834::T-POP* and is the donor of the tetracycline-resistant (Tet<sup>R</sup>) T-POP mini-transposon. *E. coli* host DH5 $\alpha$  (HANAHAN 1983) was used as the host for subcloning individual insertions of T-POP elements into chloramphenicol-resistant, medium-copy-number plasmid pSU19 (MARTINEZ *et al.* 1988). Bacteria were grown routinely at 37° in Luria Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and aerated by shaking. When required, LB medium was supplemented with Amp (100  $\mu$ g/ml), Tet (10  $\mu$ g/ml), or oxytetracycline (Oxy; 10  $\mu$ g/ml). The modified E minimal medium that we used (ES medium: 0.02 g/liter MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g/liter citric acid·H<sub>2</sub>O, 13.1 g/liter Na<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 3.3 g/liter NaNH<sub>4</sub>HPO<sub>4</sub>·4H<sub>2</sub>O) was supplemented with glucose 0.2%, cysteine 50  $\mu$ g/ml, tryptophan 50  $\mu$ g/ml, and, when required, Tet (2  $\mu$ g/ml) and Amp (50  $\mu$ g/ml). Solid media contained 1.5% agar.

**Transposon mutagenesis:** A derivative of STH2370 was used as the recipient for T-POP mutagenesis. High-frequency transducing phage P22 HT105/1 *int*-201 (SCHMIEGER 1972) was grown on donor *S. typhimurium* strain MST1168 (carrying pNK972), and equal volumes (100  $\mu$ l) of a donor lysate and an overnight culture of STH2370 were mixed, incubated for 1 hr at 25°, and then seeded onto LB Amp plates. Amp<sup>R</sup> colonies were grown overnight on LB Amp plates, and single colonies were used to inoculate liquid cultures, which were grown in LB Amp medium to overnight density. Equal volumes of Amp<sup>R</sup> recipient cells and a generalized transducing lysate grown on donor strain MST4208 were mixed, incubated for 2 hr at 25°, and then spread onto either ES or LB plates with Tet. Plates were incubated 24–48 hr to select Tet<sup>R</sup> transductants, and ~100 colonies were scraped from each plate, pooled, and used to inoculate overnight cultures in LB Tet medium. Chromosomal DNA from each pool was purified and used to electroporate the *S. typhi* parental strain, STH2370, as described (TORO *et al.* 1998). Approximately 5–10  $\mu$ g of chromosomal DNA was sheared by vortexing for 5 min, mixed with washed bacterial cells in a chilled (0.2 cm) cuvette, and subjected to a pulse of 12.5 kV/cm (2.5 kV, 200  $\Omega$ , 25  $\mu$ F). After electroporation, cells were resuspended in 1 ml of LB medium and then incubated with shaking for 1 hr at 37°. Aliquots of 200  $\mu$ l of transformed bacteria were spread onto ES plates, and plates were incubated for 48 hr. Colonies were patched onto ES or LB plates with Tet and Tet + Amp to identify Amp<sup>R</sup> colonies that had retained plasmid pNK972, encoding Tn10 transposase. Backcrossed Tet<sup>R</sup> Amp<sup>S</sup> mutants were passed through subsequent phenotypic screens.

**PCR amplification and Southern hybridization:** PCR reactions were made in a volume of 50  $\mu$ l containing 1 $\times$  *Taq* PCR buffer, 1.5 mM MgCl<sub>2</sub>, dNTPs (200  $\mu$ M), 100 pmol of each primer, 0.1  $\mu$ g of DNA template, and 1.25 units of *Taq* polymerase (Invitrogen, San Diego). Standard conditions for amplification were 35 cycles at 94° for 1 min, 50° for 1 min, and 72° for 2 min, followed by a final extension step at 72° for 10 min.

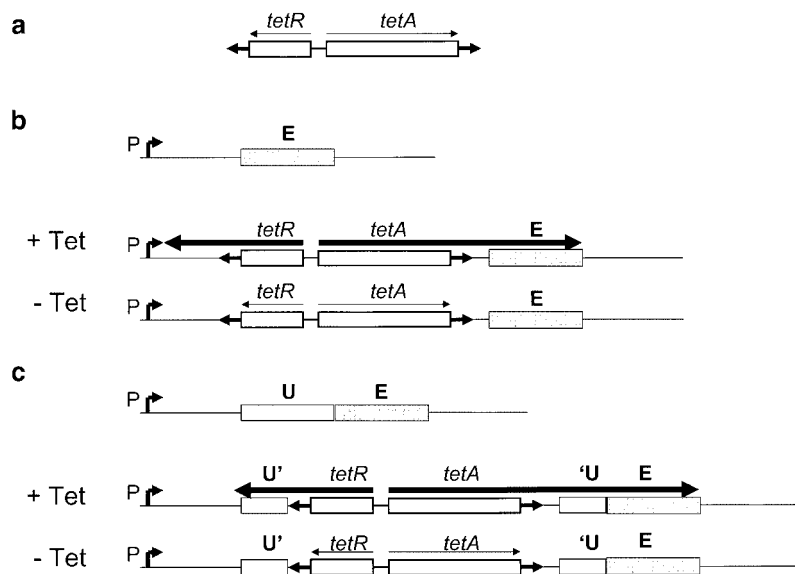


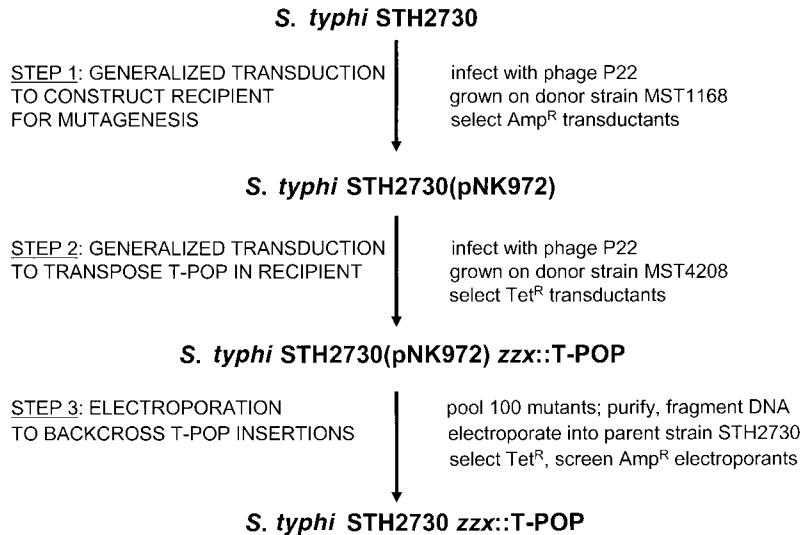
FIGURE 1.—Insertions of transposon T-POP can generate Tet-conditional mutants. (a) The T-POP transposon (RAPPEYE and ROTH 1997) has the divergently transcribed *tetR* and *tetA* genes (rectangles) flanked by IS10 inverted repeats (arrowheads). To generate Tet-conditional mutants, insertions of the T-POP element (b) can separate an essential gene, *E* (shaded rectangle), from its promoter *P* (bent arrow) or (c) can inactivate a nonessential gene (*U*, open rectangle) upstream of an essential gene, *E*, in the same transcription unit. Mutants are Tet dependent because induced expression of the outward-pointing *tetRA* promoters in the presence of tetracycline (+Tet) can allow the transcription of adjacent, essential genes (thick arrows). In the absence of tetracycline (-Tet), transcription initiated from the TetR-repressed outward-pointing *tetRA* promoters (thin arrows) is not sufficient to express adjacent, essential genes at levels required for growth.

Primers *tetR* (CGGGATCCATCACGGAAAAAGGTT) and *tetA* (CGGGATCCTGGGTTATCAAGGGTCATTAT) were used to amplify an internal 1996-bp fragment of T-POP with the *tetRA* genes. This fragment was purified and labeled using the Detector random primer DNA biotinylation kit (KPL). Salmonella chromosomal DNA was prepared as described (SANTIVIAGO *et al.* 2002) and cleaved with *EcoRI* (Invitrogen). Fragments were separated on a 0.8% agarose gel, transferred to a nylon membrane, and crosslinked by UV irradiation. Hybridization using the *tetRA* fragment as probe was performed in solutions without formamide at 65°, and membranes were washed twice for 15 min at 65° in 0.5 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2), 2% SDS, and 1 mM EDTA. Hybridization was detected using the nonradioactive DNA Detector HRP Southern blotting kit (KPL) and XAR-5 Kodak film.

**Screens for mutant phenotypes:** Each of 1700 backcrossed mutant strains was screened for three different phenotypes: Tet-dependent growth, sensitivity to low pH (5.0), and the ability to invade human HEp-2 epithelial cells. Mutants unable to grow in the absence of Tet were screened by patching single colonies onto LB and LB Tet plates, which were incubated overnight. Efficiencies of plating (EOPs) of each mutant were calculated as the titer of colony-forming units (cfu) of overnight cultures grown in LB medium on LB plates with Tet divided by that on LB plates without Tet. To screen for mutants defective in growth at low pH, aliquots (5  $\mu$ l) of overnight cultures of each mutant grown in LB medium were spotted onto LB plates (pH 7.0) and LB plates adjusted to pH 5.0 by the addition of citric acid. Acid-sensitive mutants were screened as those unable to grow at pH 5.0 after overnight incubation and were found to have EOPs <10-fold of that of the wild-type strain on LB plates at pH 5.0. Mutants defective in the invasion of human epithelial cells were screened by using a modification of the *in vitro* assay described by LISSNER *et al.* (1983). HEp-2 cells (ATCC CCL23) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (DMEMFS) at 37° in a 10% CO<sub>2</sub>/90% air atmosphere. Cells for bacterial assays were prepared by seeding 5  $\times$  10<sup>4</sup> cells into each well of a 96-multiwell tissue culture plate. Prior to assays, plates were incubated overnight at 37° in a 10% CO<sub>2</sub>/90% air atmosphere. Each mutant bacterial strain was grown overnight in LB medium (without antibiotics) and aliquots (5  $\mu$ l) were added to each well containing HEp-2 monolayers. After a 1-hr incubation in a 10% CO<sub>2</sub>/90% air atmosphere to allow for bacterial entry into the cells, mono-

layers were washed twice with phosphate-buffered saline (PBS: NaCl, 0.8%, KCl, 0.02%, Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 0.13%, KH<sub>2</sub>PO<sub>4</sub>, 0.02%). Then 100  $\mu$ l DMEMFS containing 200  $\mu$ g/ml gentamicin was added to each well, and plates were incubated for 2 hr to kill extracellular bacteria. Medium was removed, and cells were washed twice with PBS. Monolayers from each well were lysed with 0.5% sodium deoxycholate in PBS and diluted by the addition of 100  $\mu$ l of sterile PBS. Aliquots (5  $\mu$ l) from each well were spotted onto LB plates and incubated overnight at 37°. Mutants defective in invasion were detected as those that formed few colonies, if any, in the seeded spot. To quantify the ability of *S. typhi* to invade epithelial cells, titers of intracellular bacteria (cfu at *t* = 2 hr) were determined by plating dilutions of the lysed epithelial cells on LB plates (CONTRERAS *et al.* 1997). Invasion indices were calculated as the percentage of cfu at *t* = 2 hr/initial cfu of the mutant strain divided by the percentage of cfu at *t* = 2 hr/initial cfu of the wild-type strain.

**Cloning and sequencing of T-POP insertions:** Chromosomal DNA from backcrossed mutants with T-POP insertions was digested with *PstI*, which does not cleave within the T-POP element, and ligated to the *PstI* site of plasmid pSU19. Ligation mixtures were electroporated into *E. coli* DH5 $\alpha$ , and transformants were selected on LB Oxy plates. Unlike Tet, Oxy does not induce the *tetRA* genes and can be used to select for medium- or high-copy-number plasmid carrying subcloned T-POP inserts. Plasmid DNA was purified using the QIAGEN (Chatsworth, CA) miniprep kit and used as template in PCR reactions with the *tetA* and *tetR* primers; in each case, mutant templates yielded a product of the predicted size (~2 kb). The complete sequences of the inserts in two plasmids, including the entire T-POP transposon and flanking *S. typhi* DNA, were determined. The sequence of the entire T-POP element has been deposited in GenBank as accession no. AY150213. Sequences of the *S. typhi*/T-POP insertion join points in other mutants were determined using a primer (T-POP-EX1; CCTT TTTCCGTGATGGTA) internal to the T-POP element and extending outward from *tetR*. Mutant alleles are designated by numbers corresponding to the coordinate in the genome sequence of *S. typhi* strain CT18 (PARKHILL *et al.* 2001) of the first base pair in the 9-bp direct repeat generated by T-POP (Tn10) insertion; these are *tyrS*::T-POP-1603310 (upstream of *tyrS*), *yggE*::T-POP-3114068, *yabB*::T-POP-141278 (upstream of *yabB*), *yabB*::T-POP-141737, *phoQ*::T-POP-1226903, *yehF*::T-POP-1804115, and *fliD*::T-POP-2012950 (see Figure 4).



14 different electroporants resulting from the same backcross starting with an initial pool of 100 mutagenized progeny each have different insertion mutations. We find that the same method can be extended to *S. paratyphi* A, *S. paratyphi* B, and *S. dublin*; these serovars, like *S. typhi*, do not permit P22 lytic development (our unpublished results).

## RESULTS

**Insertion mutagenesis of *S. typhi* with T-POP:** We made stable insertions of the T-POP mini-transposon in *S. enterica* sv. *typhi* in three steps: two rounds of interspecific generalized transduction, using *S. typhimurium* as the donor and *S. typhi* as recipient, and a subsequent electroporation step with *S. typhi* as both donor and recipient (Figure 2). In the first step, we used phage P22 grown on *S. typhimurium* strain MST1168 to move plasmid pNK972 (which expresses Tn10 transposase from the *tac* promoter) into *S. typhi* clinical isolate STH2370. In the second step, we used P22 grown on *S. typhimurium* strain MST4208 to introduce fragments of an F' episome with a T-POP insertion into recipient *S. typhi* STH2370 (pNK972). Because the recipient strain in this transducing cross does not have homology with the donor DNA flanking the T-POP insertion, Tet<sup>R</sup> recombinants derived from the recipient must arise by transposition of the T-POP element. Confirming this point, after the second generalized transducing cross, we obtained Tet<sup>R</sup> colonies with a frequency of  $2 \times 10^{-5}$ /recipient cell. In a control experiment in which the same transducing lysate was used with recipient strain STH2370 (without pNK972), we did not obtain Tet<sup>R</sup> colonies ( $<10^{-9}$ /recipient cell), showing that transduction of the T-POP element depends on the plasmid source of transposase.

At this point, the T-POP insertion mutations present in the mutagenized recipient are not stable (can still transpose), because they are present in a genetic background that makes Tn10 transposase. To backcross the T-POP insertions, DNA was extracted from cells derived from pools of 100 colonies, sheared by vortexing for 5 min, and then used to electroporate the parental *S.*

FIGURE 2.—Flow chart of mutagenesis of *S. enterica* sv. *typhi* with transposon T-POP. In step 1, we used generalized transduction with phage P22 to construct a recipient strain that expresses Tn10 transposase from plasmid pNK972 (WAY *et al.* 1984). To obtain positive, reproducible results with this method, we found it necessary to reconstruct the recipient STH2370(pNK972) strain immediately (24 hr) before each mutagenesis (step 2). Serial passage of the recombinant STH2370 (pNK972) strain enriches for the loss of transposase activity made by pNK972, most likely because pNK972 is somewhat toxic to *S. typhi* and accumulates mutations that lower the specific activity of transposase (our unpublished results). Although the mutants obtained after T-POP mutagenesis in step 2 have independent mutations, those obtained after step 3 do not. This is because they are derived from backcrosses starting with pools of 100 mutants obtained from step 2. Nonetheless, the results presented below (Figure 3) show that

*typhi* STH2370 strain (TORO *et al.* 1998). Each backcross yielded  $\sim 100$  colonies on minimal ES Tet plates (at a frequency of electroporation of  $\sim 5 \times 10^{-8}$ /recipient cell). About 10% of the backcrossed mutants were also Amp<sup>R</sup>. These were not analyzed further, because they presumably acquired both a T-POP insertion and the plasmid source of transposase. To demonstrate that transposition had occurred by a genetic test, we screened for auxotrophs among 106 backcrossed Tet<sup>R</sup> mutants resulting from one pool of 100 independent T-POP insertion mutants by first plating the backcross onto LB Tet plates and then screening for growth on minimal ES Tet plates. We found that 17/106 of the mutants (16%) were auxotrophs, suggesting that the initial pool of mutants we chose had a fortuitously high fraction of auxotrophs, but confirming that the T-POP mutagenesis of *S. typhi* generates auxotrophs. To demonstrate that transposition had occurred by a physical test, we purified genomic DNA from a subset of mutants and used both PCR and Southern hybridization analysis to detect the presence of transposon insertions. When chromosomal DNA isolated from backcrossed Tet<sup>R</sup> mutants was used as template in PCR amplifications with *tetA* and *tetR* primers, we repeatedly obtained a product of  $\sim 2$  kb in length, as expected (data not shown). In addition, we used the *tetRA* PCR product as probe for the Southern analysis of DNA prepared from several mutants. DNA was cleaved with *EcoRI*, which cuts at a single site within the T-POP element, cleaved DNA was resolved by agarose gel electrophoresis, and the products of cleavage were hybridized with a *tetRA* probe. In each case, the probe was found to hybridize with two chromosomal fragments, consistent with the idea that each backcrossed mutant contains a single T-POP insertion (Figure 3).

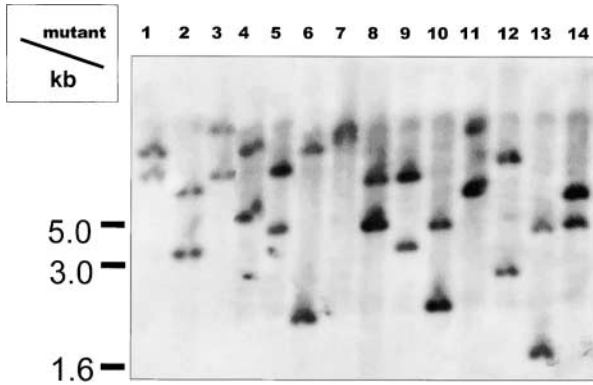


FIGURE 3.—DNA hybridization analysis of T-POP insertions in *S. typhi*. A total of 14 different Tet<sup>R</sup> electroporants resulting from the backcross of 100 independent *S. typhi* mutants with T-POP insertions were grown in overnight cultures with LB Tet medium. Chromosomal DNA prepared from these cultures was cleaved with *EcoRI* (which cleaves once within T-POP), resolved by gel electrophoresis, and then probed with a fragment internal to the *tetRA* genes. In each case, we found that the probe illuminates two fragments of different sizes, showing that each mutagenized and backcrossed recombinant has one and only one T-POP insertion. The finding that each of the 14 strains has a T-POP insertion at a different chromosomal locus shows that the backcross step in step 3 of our mutagenesis scheme (Figure 2) does not enrich significantly for particular insertion mutations.

**T-POP insertions in *S. typhi* give rise to mutants with Tet-dependent phenotypes:** RAPPEYE and ROTH (1997) have shown that among the mutants generated by T-POP insertions in *S. typhimurium* are mutants that can grow in the presence but not the absence of tetracycline. These Tet-dependent, or “Tet-conditional,” mutants presumably carry T-POP insertions that separate an essential gene from its promoter or insertions in a non-essential gene upstream of essential genes in the same operon (Figure 1). Among 1700 backcrossed mutants with T-POP insertions that we screened, we found four mutants dependent on Tet for their growth. The locations of the T-POP insertions in these mutants and the operons they define are depicted in Figure 4.

The first insertion lies between the *pdxH* and *tyrS* genes, which encode pyridoxine (pyridoxamine) 5'-phosphate (PNP/PMP) oxidase and tyrosyl-tRNA synthetase, respectively. The chromosomal organization of these and flanking genes is conserved between *S. typhi* and *E. coli* K12. In *E. coli*, *tyrS* is transcribed from both the *pdxH* promoter and a second, relatively strong promoter in the intergenic region between *pdxH* and *tyrS* (LAM and WINKLER 1992). As shown in Figure 5, the *pdxH-tyrS* intergenic region in *S. typhi* is 126 bp, nearly identical in sequence to that of *E. coli*. The T-POP insertion in this first mutant strain places *tyrS* under the control of the *tetA* promoter and lies between the  $-35$  and  $-10$  sequences corresponding to the *E. coli tyrS* promoter.

The second insertion that confers a Tet-dependent phenotype is within the potential open reading frame (ORF) designated *yggE*, which is in the same transcrip-

tion unit as the translationally coupled ORF *yggF*. This result shows that *yggF* is an essential gene, whereas *yggE* is not. Again, the chromosomal organization of these and flanking genes is conserved between *S. typhi* and *E. coli* K12. The function of the essential *yggF* gene has yet to be determined.

The third insertion is in *yabB*, in the same transcription unit as, and upstream of, the essential *ftsL*, *ftsI*, *murE*, *murF*, and other genes known to be required for membrane biosynthesis and cell division in *E. coli* K12 (HARA *et al.* 1997). This result suggests that the *yabB* gene is not essential, as has been shown to be the case in *E. coli* K12 (DASSAIN *et al.* 1999; MERLIN *et al.* 2002). Again, the chromosomal organization of these genes is conserved between *S. typhi* and *E. coli* K12. Presumably, the T-POP insertion in the fourth mutant strain, immediately upstream of *yabB*, separates the *yabB* operon from a critical element of its promoter and thereby confers a Tet-dependent phenotype.

Because *S. typhi* and *S. typhimurium* are exposed to a low-pH environment when they infect their mammalian hosts, and their virulence depends on the ability to survive passage through this acid environment, we also screened among the 1700 backcrossed mutants for those defective in growth on media with pH 5.0. We found two mutants that plate with efficiencies >10-fold lower on media with pH 5.0 than on media with pH 7.0, with insertions in the nonessential *phoQ* and *ychF* genes (Figure 3). Our finding that a *phoQ*:T-POP insertion confers acid sensitivity in *S. typhi* is consistent with previous results that show that acid tolerance in *S. typhimurium* is dependent on the PhoPQ two-component regulatory system (BEARSON *et al.* 1998).

Finally, we also screened among the 1700 backcrossed mutants with T-POP insertions for mutants defective in the ability to invade human epithelial cells and found one mutant with a pronounced hypoinvasive phenotype in HEp-2 cells. This mutant carries an insertion in the *fliD* gene and has an invasion index of 20% relative to that of the wild type (see MATERIALS AND METHODS). This mutant also has the same nonmotile phenotype as a *S. typhimurium* mutant with a *fliD*:Tn10 insertion (KUTSUKAKE *et al.* 1990; data not shown). A mutation in the *fliC* (flagellin) gene of *S. enteritidis* shows a similar defect in the invasion of human Caco-2 (epithelial) cells in tissue culture (VAN ASTEN *et al.* 2000), and transposon insertions in *S. typhi* that impair motility, in general, are defective in invasion (LIU *et al.* 1988).

**Terminal phenotypes of *S. typhi* mutants with Tet-conditional T-POP insertions:** The depletion of a gene product, by degradation and/or by dilution, can result in a variety of terminal phenotypes. Most severe, the depletion of an essential enzyme involved in cell division, such as FtsI or FtsW, can result in the immediate cessation of cell growth and subsequent cell lysis (BOYLE *et al.* 1997; HARA *et al.* 1997). On the other hand, the depletion of a stable essential gene product in functional excess, such as Ffh, can have a more subtle pheno-

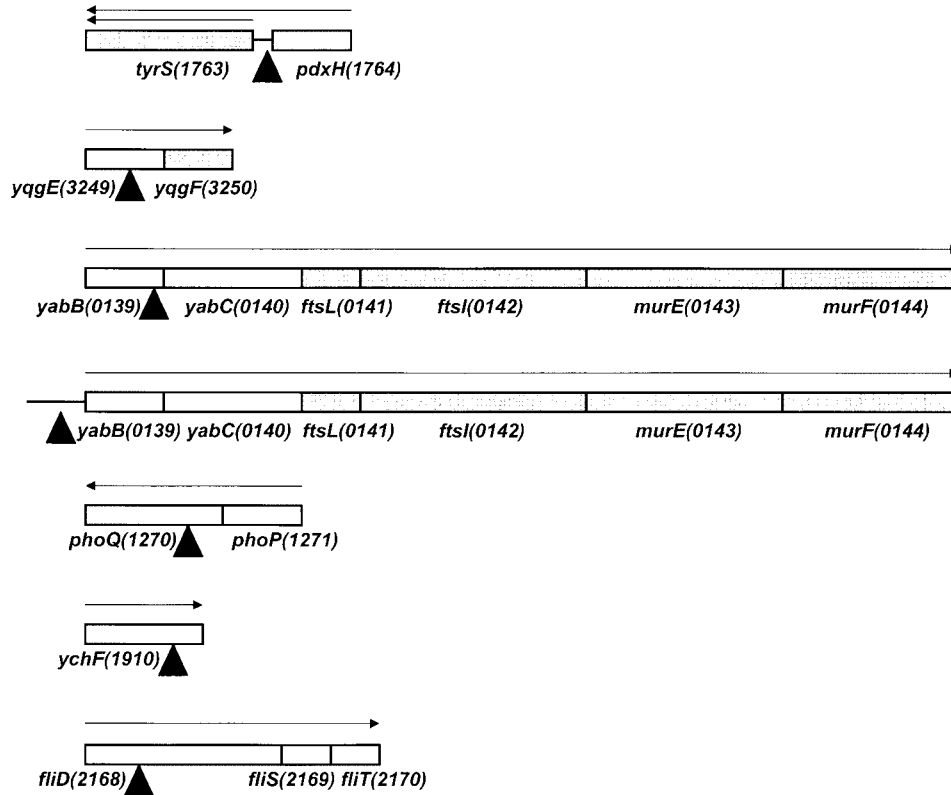


FIGURE 4.—Insertions of transposon T-POP with Tet-conditional, pH-sensitive, and hypoinvasive phenotypes. Shown are the locations of T-POP insertions (solid triangles) within transcription units of the *S. typhi* genome for the mutants described in this article. Genes (rectangles) are indicated by their common functional names determined in *S. typhimurium* (e.g., *tyrS*) or by the names of their closest homologs in *E. coli* K12 (e.g., *yqgE*) and are depicted at approximate physical size; essential genes are indicated as shaded rectangles. The directions of transcription of each operon with respect to the *S. typhi* genome are indicated by arrows above the genes. Only the first six genes in the *yabB* operon are shown (HARA *et al.* 1997). Note that the T-POP insertions in the four Tet-conditional mutants (top) are upstream of known essential genes or upstream of a gene of as-yet-undetermined function.

type (HERSKOVITS and BIBI 2000). Cells undergoing depletion can grow and divide for a number of generations prior to the cessation of growth. To investigate the terminal phenotypes of *S. typhi* mutants with Tet-dependent insertions, we grew cultures of these mutants to exponential phase in rich medium with Tet, washed the cells to eliminate Tet in the growth medium, and then passaged the cells in rich medium without Tet. At various times after the initiation of nonpermissive passage, we titered these cultures for viable cells. We also examined the cells microscopically to determine whether the depletion of an essential gene product results in morphological changes as part of the terminal phenotype, because, for example, the depletion of the essential FtsW protein results in cell filamentation and lysis (BOYLE *et al.* 1997).

As shown in Figure 6, the four mutants display differ-

ent phenotypes when grown without Tet. The two Tet-conditional mutants with T-POP insertions affecting the *yabB* operon cease growth within 1 hr after Tet is removed, filament and lyse, and cannot be recovered by plating onto media with Tet. Less than 0.1% of these cells form colonies after 1 hr of growth without Tet on plates with or without Tet. This result is consistent with the result when the *E. coli yabB* operon is placed under the control of the conditional *lac* promoter and passage of these lactose-dependent mutants in media without an inducer of the *lac* operon also results in filamentation and cell lysis (HARA *et al.* 1997).

In contrast, the Tet-conditional mutants with T-POP insertions affecting the downstream *tyrS* and *yqgF* genes have more subtle terminal phenotypes. These mutants lose the ability to form colonies within 4 hr after growth in the absence of Tet and, before this time, can be

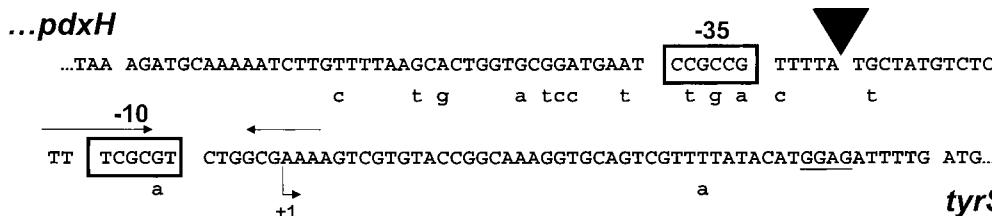


FIGURE 5.—A T-POP insertion upstream of the *S. typhi tyrS* gene has a Tet-dependent phenotype. Uppercase letters indicate the intergenic region between the stop codon of *pdxH* and the start codon of *tyrS*. Differences between this inter-

genic region and its *E. coli* K12 counterpart (lowercase letters) are indicated below the *S. typhi* sequence. The  $-35$  and  $-10$  elements of the *E. coli tyrS* promoter (boxed), as well as its start site ( $+1$ ) are shown (LAM and WINKLER 1992). The homology of the region upstream of *tyrS* with the 3' end of 16s rRNA (Shine and Dalgarno sequence) is underlined. The T-POP insertion (triangle) interrupts the *S. typhi tyrS* promoter, which is predicted to initiate transcription at a region of dyad symmetry (arrows).

rescued by plating onto rich media with Tet (data not shown). After growth without Tet, >90% of the cells are found as doublets with well-formed septa. Presumably, these mutants are blocked in a step of cell division required for the separation of daughter cell pairs.

As one of the controls for this experiment, we also examined the phenotypes of mutants with T-POP insertions in the genes *phoQ*, *ychF*, and *fliD*, after growth in media with and without Tet, and found an unexpected result. Mutants with insertions in the *phoQ* and *ychF* genes have the same (wild-type) morphology when grown in media with or without Tet, as expected (data not shown). In contrast, the mutant with the *fliD*:T-POP insertion has a wild-type phenotype when grown in media without Tet, but filaments when grown in media with Tet (Figure 6). Presumably, in this mutant, outward transcription from the *tetA* promoter in the presence of Tet results in the  $\sigma^{28}$ -independent expression of the upstream, divergently transcribed *fliC* (flagellin) gene, and expression of flagellin, normally repressed by the *fliD* gene product in the absence of a flagellar basal body, is toxic but not lethal.

#### DISCUSSION

We have shown that a combination of interspecific generalized transduction and electroporation can be used to make otherwise isogenic derivatives of a clinical isolate of *S. typhi* with insertions of the mini-transposon T-POP and that these T-POP insertions can be used to identify essential genes by placing their expression under the control of a tetracycline-inducible promoter. Our work extends the initial demonstration by RAPPLEYE and ROTH (1997) that T-POP insertions can be used to identify essential genes in *S. typhimurium*. Recently, JUDSON and MEKALANOS (2000) have described a similar *mariner*-based mini-transposon, TnAraOut, which allows the identification of essential genes by generating transcriptional fusions of these genes with the arabinose-inducible *araBAD* promoter. Together, our results show that, by combining the power of untargeted transposon mutagenesis with the ability to make conditional transcriptional fusions, *S. typhimurium*, *S. typhi*, and *Vibrio cholerae* genes of unknown function can be shown to be essential.

Each of these mini-transposons has its advantages and disadvantages. The *mariner*-based TnAraOut mini-transposon has a much broader target specificity than does Tn10-based T-POP. On the other hand, T-POP offers the advantages that it includes two divergent promoters (*tetR* and *tetA*) with different basal and tetracycline-induced levels of outward transcription, whereas TnAraOut has only the single, outward-pointing *araBAD* promoter that initiates transcription at a very low basal level or at a very high induced level.

Despite these differences, we find that the spectrum of essential genes identified by Tet-dependent T-POP

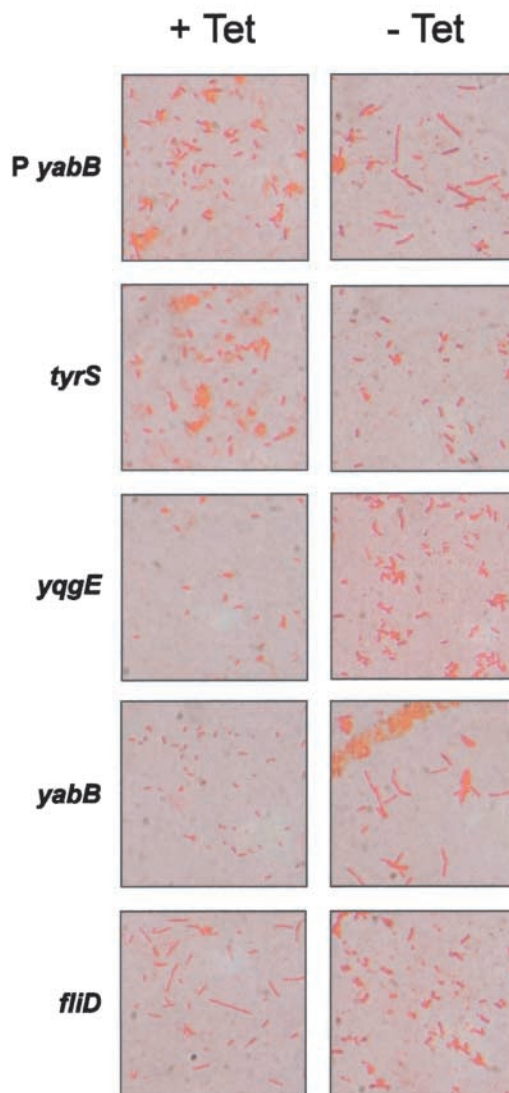


FIGURE 6.—Terminal phenotypes of *S. typhi* mutants with T-POP insertions that confer Tet-conditional phenotypes. Mutants with T-POP insertions (in sites indicated at the left) were grown in rich (LB) medium with Tet to exponential density, washed three times with medium without Tet, and then incubated in medium without Tet for 4 hr. Photographs were taken using a Nikon model Labophot-2 microscope with visible light at a magnification of  $10 \times 100$  and show cells before (+Tet) and 4 hr after (–Tet) incubation without Tet. An enlarged photograph showing that most (>90%) arrested cells of the mutant with an insertion of T-POP in *yqgE* are present as unseparated doublets with well-defined septa may be viewed at <http://www.genetics.org/supplement/>.

insertions in *S. typhi* is similar to that found by JUDSON and MEKALANOS (2000) in *V. cholerae*, using transposon TnAraOut. Among 16 arabinose-dependent mutants generated by TnAraOut in *V. cholerae*, 3 were found to have insertions upstream of tRNA synthetase genes, 8 were found to be upstream of homologs of other known, essential genes, and 5 were found to be upstream of genes of as-yet-undetermined function. Among four tetracycline-dependent mutants generated by T-POP in *S.*

*typhi*, one was found to have an insertion upstream of a tRNA synthetase gene, two were found to be upstream of homologs of other known, essential genes, and one was found to be upstream of a gene, *yggF*, of as-yet-undetermined function. Because the *yggF* gene is predicted to encode a resolvase in the RuvC family (ARAVIND *et al.* 2000), we speculate that this gene may encode an essential Holliday junction resolvase.

We have also shown that T-POP insertions may be used to identify genes that are conditionally essential for the response of *S. typhi* to an environmental stress, in our case, the response to low pH. Again, this has enabled us to identify both a gene known to be involved in the response to low pH, *phoQ*, and a gene of, as-yet-undetermined function, *ychF*. The *ychF* gene is predicted to encode a small GTP-binding protein and has homologs present in every sequenced genome to date (MITTENHUBER 2001); it may play a role in a fundamental stress response overlapping that to low pH. YchF is in the same family of small GTP-binding proteins as the product of one of the essential *V. cholerae* genes identified by JUDSON and MEKALANOS (2000).

We have extended the use of T-POP insertions to characterize essential genes in an important way, by simply observing the phenotypes of tetracycline-dependent mutants when passaged under nonpermissive conditions in the absence of tetracycline. Passage under nonpermissive conditions presumably results in the depletion of an essential gene product. Consistent with this idea, we find that *S. typhi* mutants with T-POP insertions in the *yabB* operon filament and lyse when placed under nonpermissive conditions and display the same phenotype as that of derivatives of *E. coli* depleted of the same set of gene products (HARA *et al.* 1997). However, mutants of *S. typhi* with T-POP insertions upstream of the essential *tyrS* and *yggF* genes display a very different phenotype when passaged under nonpermissive conditions. These mutants arrest cell division and accumulate unseparated cell doublets with well-defined septa; this terminal phenotype may be reversed by the addition of Tet after passage under nonpermissive conditions. We interpret these results to mean that depletion of these gene products results in a block at a specific, reversible checkpoint late in the *S. enterica* cell cycle. Like the late M-phase checkpoint in the eukaryotic cell cycle, this checkpoint may depend on both the elongation step of translation (blocked in the *tyrS* mutant) and the separation of paired chromosomes (presumably blocked in the *yggF* mutant). Also, because this block in the cell cycle may be reversed by the addition of Tet after passage under nonpermissive conditions, this series of treatments may provide a new way to synchronize the cell cycle in a population of *S. enterica* cells grown in liquid culture.

The results of several other studies have argued that, as is the case for eukaryotic cells, enteric gram-negative bacteria have multiple checkpoints to ensure that a rep-

lication cycle has been completed prior to mitotic division in the cell cycle. Walker and colleagues have shown that the *E. coli* UmuCD proteins participate in a DNA damage checkpoint (OPPERMAN *et al.* 1999; SUTTON and WALKER 2001). In addition, the depletion of several *E. coli* gene products involved in DNA replication suggests that there are checkpoints that coordinate division with cell size (BOTELLO and NORDSTROM 1998) and that make nucleoid segregation a prerequisite for mitotic division (BRITTON *et al.* 1998; DASSAIN *et al.* 1999).

Although our screen for mutants of *S. typhi* deficient in the ability to invade human epithelial cells resulted in only 1 mutant among the 1700 that we screened, a mutant with an insertion of T-POP in the *fliD* gene, this mutant has a surprising phenotype that is important for two reasons. When grown in the presence of Tet, this mutant overproduces flagellin (data not shown), and the majority of cells grown under these conditions form long, conjoined filamentous arrays. This result suggests that the Tet-dependent overproduction of flagellin in this mutant may result in a phenotype similar to that of the SOS response, but not so severe as to be lethal, a phenotype that we are currently exploring in greater detail. More important, this result suggests that there may be a class of T-POP insertions that have lethal phenotypes in the presence, but not in the absence, of Tet. Such mutants can be screened among mutants with T-POP insertions after selection for the transposon in the presence of oxytetracycline (which is not an inducer of the *tetRA* genes), and initial screens have yielded such mutants (our unpublished results). Therefore, in the future, we plan to use T-POP insertions to identify the subset of both essential and nonessential genes whose products must be maintained in low intracellular concentrations to ensure efficient growth.

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