Gene Transfer between *Salmonella enterica* Serovar Typhimurium inside Epithelial Cells

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Virulence and antibiotic resistance genes transfer between bacteria by bacterial conjugation. Conjugation also mediates gene transfer from bacteria to eukaryotic organisms, including yeast and human cells. Predicting when and where genes transfer by conjugation could enhance our understanding of the risks involved in the release of genetically modified organisms, including those being developed for use as vaccines. We report here that *Salmonella enterica* serovar Typhimurium conjugated inside cultured human cells. The DNA transfer from donor to recipient bacteria was proportional to the probability that the two types of bacteria occupied the same cell, which was dependent on viable and invasive bacteria and on plasmid tra genes. Based on the high frequencies of gene transfer between bacteria inside human cells, we suggest that such gene transfers occur in situ. The implications of gene transfer between bacteria inside human cells, particularly in the context of antibiotic resistance, are discussed.

Genomic databases have confirmed the enormous contribution that horizontal gene transfer (HGT) has made to the content and even the organization of bacterial genomes (for reviews see references 23, 37, 42, and 48). One theory even attributes creation of the most famous of the bacterial chromosomal structures, the operon (41), to HGT. Genes transfer between bacteria via many different kinds of vectors, ranging from conjugative plasmids and conjugative transposons to integrons and cis-acting signal sequences, which mediate transformation of organisms such as *Haemophilus* and *Neisseria*, to viruses. These vectors are collectively referred to as horizontally mobile elements (HMEs) (9, 26, 45, 52). If HGT is powerful enough to affect the organization of chromosomes, then what effect does it have on the organization and function of the elements that mediate gene transfer?

A central question in bacterial evolution, and a question that has immediate medical interest, is why antibiotic resistance and new virulence-determining genes are so often found on HMEs rather than on chromosomal genes of dominant clones of bacteria (10, 27, 29, 59). Further study of HGT in both the environment and the laboratory is necessary to answer the questions about how chromosomes and HMEs attract different kinds of genes (14, 15, 44).

We sought to address such questions concerning the evolution of antibiotic resistance by studying HGT between invasive bacteria that cause disease in humans. Many bacteria invade animal cells during pathogenesis (17). This ability to invade animal cells helps the bacteria evade both the host immune system and antibiotics (51). Eukaryotic cells protect internalized bacteria from other well-known stresses in a variety of ways. For example, some bacteria consumed by protozoans are protected from environmental and chemical stresses (4), and many antibiotics penetrate animal cells poorly or accumulate insufficiently in the intracellular compartments to kill invasive bacteria (51, 60). Thus, we asked whether the cytoplasm of animal cells could be a niche for gene transfer between bacteria protected from antibiotics that might otherwise inhibit gene transmission.

We postulated that if bacteria conjugate in the intracellular environment, such conjugation could partially explain both the evolution of virulence and the spread of antibiotic resistance in invasive pathogens. Antibiotic-susceptible bacteria that escape the effects of antibiotics by entering human cells could acquire resistance genes from pathogens residing in the same cells, from benign bacteria taken up by the cells (20), or through mixing with the normal flora upon reemergence from the cells. Conversely, normally benign bacteria could acquire virulence genes intracellularly from resident pathogens, since many new virulence traits are plasmid borne (3, 54).

These ideas are plausible because of the elevated frequencies of gene transfer between bacteria that are associated with protozoan cells (55). Transmission of the conjugative plasmid RP4 between *Escherichia coli* strains increased 3 orders of magnitude in the presence of the protozoan *Tetrahymena pyriformis*, suggesting that plasmid transfer occurred within digestive vacuoles.

Using antibiotic resistance genes as markers and the invasive pathogen *Salmonella enterica* serovar Typhimurium as a model, we sought to determine whether conjugative plasmids could be transferred between bacteria inside cultured human cells.

How *S. enterica* serovar Typhimurium invades epithelial cells has been well described previously (17, 34, 47) and so is summarized only briefly here. Epithelial cells take up *Salmonella* when the bacteria translocate signal transduction-altering proteins to the cell. Cytoskeletal rearrangement, membrane ruffling, and bacterial uptake by macropinocytosis follow the translocation of bacterial proteins. Engulfed bacteria reside inside membrane-bound vacuoles within the cell cytoplasm. Whereas some bacteria lyse vacuoles and are released into the

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cytoplasm, *S. enterica* serovar Typhimurium remains in the vacuoles. Bacteria inside cultured mammalian cells are often distinguished from extracellular bacteria by the fact that the intracellular bacteria are protected from gentamicin in the medium (36). We exploited the fact that human cells exclude gentamicin to ensure that viable recipient and donor bacteria, introduced into a monolayer of cells at different times, could not form transconjugants in the extracellular medium. To our knowledge, this is the first demonstration of intracellular bacterial conjugation, and our results reveal both the functionality of the bacterial conjugative machinery in the intracellular environment and the potential for transfer of genes between intracellular bacteria, particularly in the presence of antibiotics.

### MATERIALS AND METHODS

**Bacteria.** ΔinvA strains were created by P22 transduction (13) of the invA::TphoA mutation from SB111 (21) to *S. enterica* serovar Typhimurium wild-type strains SL1344 (32) and 14028R-P (5). The strains and plasmids used in this study are described in Table 1.

**Cell culture.** INT-407 cells (ATCC CCL 6) were cultured in minimal essential medium with Earle's salts (Gibco) supplemented with 2 mM nonessential amino acids (Gibco), 2 mM L-glutamine (Gibco), and 10% fetal bovine serum (Gibco) at 37°C in a 10% CO₂ atmosphere.

**Conjugation experiments.** For intracellular conjugation, 24-well (1.9-cm²) trays (Nunc) were inoculated with 4 × 10⁵ tissue culture cells/ml (0.5 ml per well), and cells were grown to confluence (24 to 48 h). In some experiments, cells were grown on 10-mm membrane filter inserts with 2-μm-pore-size membranes (Anopore; Nunc). Prior to infection, monolayers were washed once with phosphate-buffered saline (PBS) (8 g of NaCl per liter, 0.2 g of KCl per liter, 1.44 g of Na₂HPO₄ per liter, 1.43 g of KH₂PO₄ per liter; pH 7.4), and they were maintained in minimal essential medium with Earle's salts supplemented with L-glutamine, nonessential amino acids, and 1% fetal bovine serum throughout the experiment.

Exponential-phase cultures of recipient (plasmid-free) bacteria were washed and diluted in PBS to a concentration of 1 × 10⁸ bacteria/ml. Wells were inoculated with ~10⁶ bacteria to give a multiplicity of infection (MOI) of approximately 10 and centrifuged at 317 × g for 5 min. Following 2 h of incubation at 37°C in the presence of 10% CO₂, monolayers were washed three times with PBS and then incubated in medium containing 100 μg of gentamicin per ml for 1 h. The procedure described above was repeated with donor (plasmid-bearing) bacteria. One hour after the second incubation with gentamicin, the gentamicin concentration was decreased to 20 μg/ml. After an additional 3 h the monolayers were washed with PBS and lysed with 0.5% deoxycholate to release the intracellular bacteria (39).

**Recombinant selection and transmission frequency calculations.** Intracellular bacteria were enumerated by serial dilution in PBS and plating on Luria-Bertani (LB) agar (Gibco) plates supplemented with antibiotics appropriate for the selection of the recipient bacteria (60 μg of nalidixic acid per ml), the donor bacteria (100 μg of rifampin per ml), and the recombinant bacteria (60 μg of nalidixic acid per ml, 100 μg of kanamycin per ml). Control crosses on agar plates were performed as described previously (26).

The transmission frequency was calculated by determining the number of recombinants per limiting intracellular parent (28, 31). Because the number of bacteria that internalize in tissue culture cells can and did vary from experiment to experiment, frequencies had to be calculated for a common limiting parent for meaningful comparisons between experiments. Generally, fewer donors than recipients were recovered from tissue culture cells, but on occasion the recipient was the limiting parent.

In any conjugation assay, some recombinants can form after the donor and recipient conjugants are transferred to the selection plates (31). The proportion of recombinants that formed after plating was determined by mixing lysates of donor-infected cells with lysates of recipient-infected cells on LB agar plates supplemented with nalidixic acid and kanamycin. Extracellular bacteria in the culture medium were concentrated by centrifugation (15,000 × g for 3 min), rinsed once in PBS, and then enumerated by plating on LB agar. The resulting colonies were transferred by replica plating to LB agar supplemented with nalidixic acid and kanamycin to test for recombinant markers (43).

All recombinant colonies were transferred by replica plating to LB agar plates supplemented with rifampin to test whether they were nalidixic acid-resistant donors that arose spontaneously.

### RESULTS

**Plasmids were transmitted between invasive *S. enterica* serovar Typhimurium cells during infection of cultured human cells.** Two conjugative plasmids were tested for transmission between bacteria internalized in human cells. The RP4 (IncP) and F (IncF1) plasmids used express conjugation genes constitutively (25, 35). Moreover, there are interesting differences in their frequencies of transmission in liquid and solid environments (8). Whereas F plasmids are transmitted at high frequencies in both environments, IncP plasmids are transmitted between bacteria at much higher frequencies on solid surfaces.

The transmission frequencies of RP4 and F were measured by mating experiments performed on LB agar plates as previ-
The numbers in parentheses are the numbers of recombinant colonies observed.

The recipient bacteria were SL1344 N and BA770 in RP4 and F matings, respectively.

The values are maxima. Five of nine replicates (pRK21558) and three of nine replicates (Jp143) produced no detectable transconjugants. The values are averages of values obtained with replicates when transconjugants were detected.
*Salmonella*-containing vacuoles reportedly coalesce 12 h after infection, and bacteria have been observed inside one large perinuclear vacuole (16, 18). If vacuole fusion were required for intracellular conjugation, intracellular recombinants might not be detected before 12 h after donor internalization. However, we observed intracellular recombinants by 3 h after donor invasion (Fig. 1).

The number of intracellular recombinants per limiting parent increased 2 orders of magnitude over 6 h, from our limit of detection of $10^{-6}$ recombinant per limiting parent to an average of $7 \times 10^{-4}$ recombinant per limiting parent (Fig. 1). This time-dependent increase in recombinant frequency was not due to faster growth of newly formed recombinants than of the parent bacteria. In reconstruction experiments, cells infected by newly recombinant bacteria did not accumulate intracellular recombinant bacteria any faster than they accumulate parental bacteria (data not shown). Thus, the increase in recombinant frequency was likely due to accumulation of new recombinants.

**Gene exchange did not occur extracellularly.** Some proportion of extracellular recipient bacteria may have escaped killing by gentamicin or may have emerged from cells and mated with extracellular donors during the time that donor bacteria were infecting cells. If this were the source of the recombinants, then the recombinants should have been created even if the donor bacteria could not invade. Therefore, the intracellular gene transfer experiment with invasive donors (see above) was repeated using the noninvasive donors $\Delta invA$ SL1344$^{\text{R}}$ and $\Delta invA$ 14028$^{\text{R}}$-P, which are compromised for invasion by TyphoA insertions in the invA gene (21). InvA is an inner membrane protein that may form part of the type III secretion system (22). Although invA mutants can bind to the cell surface, they invade at least 100 times less frequently than the wild type (21).

Recombinants were rarely and inconsistently detected in the intracellular mating assay when donors were noninvasive (Table 4), even though invA and wild-type donors transmit RP4 and F at frequencies comparable to those of recipient bacteria mixed on LB agar plates (Table 2).

No recombinants containing RP4 were recovered when invA mutant donors were used, whereas a small number of recombinants containing F were recovered. The difference reflects the fact that the invA mutation has different effects on the invasion efficiencies of SL1344 and 14028-P. On average, the number of intracellular $\Delta invA$ 14028$^{\text{R}}$-P(F42::mini-Tn10Kn) bacteria recovered was 3% of the number of inv$^+$ bacteria recovered, whereas the number of intracellular $\Delta invA$ SL1344$^{\text{R}}$(RP4) bacteria recovered was 0.1% of the number of inv$^+$ bacteria recovered. It appears that a threshold of 10$^5$ intracellular donors is required for detection of the formation of recombinants by this assay.

**Mechanism of plasmid transmission was conjugation.** It was still possible that plasmids that were released from dead inter-

![Graph showing accumulation of intracellular transconjugants over time.](https://example.com/graph.png)

**FIG. 1.** Accumulation of intracellular transconjugants over time. Intracellular recipients (SL1344$^{\text{R}}$ (●), donors [SL1344$^{\text{R}}$(RP4)] (■), and transconjugants (○) were enumerated over time beginning 6 h after recipient invasion began (or 2.75 h after donor invasion began). The number of transconjugants formed after plating (○) was not significantly different at the first time point. Each value is an average based on three independent experiments performed in triplicate. Error bars indicate standard errors.

**TABLE 4. Intracellular plasmid transmission requires donors to be invasive**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Replicate</th>
<th>Invasive donors (RP4)</th>
<th>Noninvasive donors (RP4)</th>
<th>Invasive donors (F42::mini-Tn10Kn)</th>
<th>Noninvasive donors (F42::mini-Tn10Kn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>$8 \times 10^{-5}$ (142)</td>
<td>$\leq 3 \times 10^{-7}$ (0)</td>
<td>$2 \times 10^{-5}$ (60)</td>
<td>$\leq 4 \times 10^{-7}$ (0)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>$7 \times 10^{-5}$ (126)</td>
<td>$\leq 3 \times 10^{-7}$ (0)</td>
<td>$3 \times 10^{-5}$ (63)</td>
<td>$8 \times 10^{-7}$ (2)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>$7 \times 10^{-5}$ (128)</td>
<td>$\leq 3 \times 10^{-7}$ (0)</td>
<td>$2 \times 10^{-5}$ (45)</td>
<td>$8 \times 10^{-7}$ (2)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>$2 \times 10^{-5}$ (43)</td>
<td>$\leq 8 \times 10^{-8}$ (0)</td>
<td>$2 \times 10^{-5}$ (123)</td>
<td>$4 \times 10^{-7}$ (4)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>$4 \times 10^{-5}$ (84)</td>
<td>$\leq 8 \times 10^{-8}$ (0)</td>
<td>$8 \times 10^{-5}$ (428)</td>
<td>$1 \times 10^{-7}$ (1)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>$2 \times 10^{-5}$ (48)</td>
<td>$\leq 8 \times 10^{-8}$ (0)</td>
<td>$8 \times 10^{-5}$ (335)</td>
<td>$2 \times 10^{-7}$ (2)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>$3 \times 10^{-5}$ (382)</td>
<td>$\leq 5 \times 10^{-8}$ (0)</td>
<td>$2 \times 10^{-5}$ (272)</td>
<td>$\leq 2 \times 10^{-7}$ (0)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>$3 \times 10^{-5}$ (417)</td>
<td>$\leq 5 \times 10^{-8}$ (0)</td>
<td>$3 \times 10^{-5}$ (266)</td>
<td>$\leq 2 \times 10^{-7}$ (0)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>$3 \times 10^{-5}$ (523)</td>
<td>$\leq 5 \times 10^{-8}$ (0)</td>
<td>$2 \times 10^{-5}$ (112)</td>
<td>$\leq 2 \times 10^{-7}$ (0)</td>
</tr>
</tbody>
</table>

Avg  $4 \times 10^{-5} \pm 1 \times 10^{-5}$ (1,893) (0) $4 \times 10^{-5} \pm 1 \times 10^{-5}$ (1704) (11)

*a* In RP4 experiments, the donor was either SL1344$^{\text{R}}$ or $\Delta invA$ SL1344$^{\text{R}}$ and the recipient was SL1344$^{\text{R}}$. In F experiments, the donor was either 14028$^{\text{R}}$-P or $\Delta invA$ 14028$^{\text{R}}$-P and the recipient was BAY70. Plasmid transmission frequencies are expressed as the number of transconjugant colonies per input donor.

*b* The numbers in parentheses are the numbers of recombinant colonies observed.
nalized donors transformed competent internalized recipients. Transformation was an unlikely mechanism of transmission because Salmonella is not naturally competent for DNA uptake. Nevertheless, vacuoles might induce competence in unknown ways.

If the mechanism of transmission is conjugation, it should be sensitive to mutations in the tra (transfer) genes of the plasmids. To distinguish decisively between conjugation and transformation as the mechanism of RP4 transmission, a ΔtraJ derivative of RP4 (57) and a traB derivative of F (31) were introduced into the donor strains. TraJ forms part of the relaxosome complex required for nicking and unwinding of single-stranded plasmid DNA (24). traB is necessary for conjugal pilus formation (35). The traJ deletion significantly reduced plasmid transmission on agar plates (Table 2). Likewise, traJ was necessary for the formation of intracellular recombinants (Table 3). Similarly, F plasmids with mutations in traB produced no intracellular recombinants (Table 3).

In addition, if transformation contributes to the formation of recombinants, the effect should be enhanced with smaller plasmids. However, no recombinants were detected when donor bacteria carried either the 4.4-kb plasmid pBR322 or the 10.5-kb plasmid pSUP104 (49) (data not shown). Conjugation, not transformation, was therefore probably the mechanism of intracellular plasmid transmission measured in these experiments.

Transconjugants did not form from conjugation on the well surfaces beneath the monolayers. Since Salmonella has been shown to traverse cell monolayers (18, 19), it was possible that conjugation occurred on the well surfaces beneath the cells in pockets not penetrated by gentamicin-containing medium. To control for this possibility, we performed two experiments. In the first experiment, tissue culture cells were seeded onto permeable membrane supports (see Materials and Methods) inserted inside wells. This allowed both sides of the monolayer to be bathed in gentamicin, thus exposing any potential bacterial escapees. Transconjugants were recovered at a frequency indistinguishable from that observed when cells were grown on a plastic surface (Table 5).

Many fewer transconjugants were recovered in experiments in which membranes were used. Since the surface area of an insert membrane was approximately one-half that of a well, the bacteria released from cells grown on two inserts were pooled to make one replicate. Despite this, the recovery of intracellular bacteria was poor, a result of both poor adhesion of cells to the membrane and reduced bacterial invasion efficiency. In a second experiment, cells were released from the monolayer by treatment with trypsin (0.25% trypsin, 1 mM EDTA; Gibco) after the second gentamicin-bathing period and reseeded into wells as a cell suspension in gentamicin-containing medium. Cells did not reaggregate during this experiment. Again, transconjugants were detected at a frequency indistinguishable from that observed with undisturbed monolayers (Table 5).

As a further control for extracellular mating, cells were infected with either recipient or donor bacteria and then were released from the monolayer with trypsin after the second gentamicin-bathing period. Transconjugants were detected only at the frequency at which they formed after plating of the lysate (Table 5). Together, these experiments confirmed that transconjugants were not formed by extracellular conjugation between emergent bacteria and suggested that donors and recipients must concurrently infect the same cell in order for transconjugants to form.

Frequency of transconjugants decreased with decreasing likelihood of coinfection. For intracellular conjugation to occur, donor and recipient bacteria must make contact inside cells. Consistent with this fact, the frequency of transconjugants was found to decrease as the ratio of invading bacteria to cells (the MOI) decreased, which decreased the probability of a donor and a recipient occupying the same cell at the same time (Fig. 2A).

Plasmid transmission was measured in experiments conducted at MOIs ranging from 0.1 to 100. In each experiment, the same number of donors as recipients was introduced into a cell culture. Thus, the ratio of the total number of bacteria to tissue culture cells was always twice the MOI reported. The number of recipients internalized after the gentamicin-bathing period. The initial number of intracellular recipients increased as the MOI increased. MOIs greater than 10 had no additional effect on the number of internalized bacteria (Fig. 2B).

The plateau observed in the number of internalized bacteria is consistent with previously reported observations that Salmonella invasion kinetics reach saturation at MOIs greater than 40. Saturation is attributed to the presence of a limited number of binding sites on the cell surface (33, 40). The initial number of donors that invaded cells that had been preinfected with

### Table 5. Plasmid transmission frequencies in adherent and nonadherent cells

<table>
<thead>
<tr>
<th>Monolayer on permeable filter support, coinfected</th>
<th>Total no. of recombinants recovered</th>
<th>Transmission frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT-407 cells</td>
<td>338 (1,260)</td>
<td>$2 \times 10^{-4}$ ($3 \times 10^{-4}$)</td>
</tr>
<tr>
<td>Nonadherent, coinfected</td>
<td>4,475 (5,278)</td>
<td>$2 \times 10^{-4}$ ($3 \times 10^{-4}$)</td>
</tr>
<tr>
<td>Nonadherent, mixed after independent infection</td>
<td>22 (1,406)</td>
<td>$2 \times 10^{-6}$ ($3 \times 10^{-6}$)</td>
</tr>
<tr>
<td>Recombinants formed after plating</td>
<td>22 (1,406)</td>
<td>$9 \times 10^{-6}$ ($3 \times 10^{-6}$)</td>
</tr>
</tbody>
</table>

- The donor and recipient bacteria were SL1344<sup>R</sup>(RP4) and SL1344<sup>N</sup>, respectively.
- The numbers in parentheses are the numbers of recombinant colonies recovered in the corresponding positive control experiments. [INT-407 monolayers infected with series with SL1344<sup>R</sup> and SL1344<sup>N</sup>(RP4)].
- In most cases plasmid transmission frequencies are expressed as the total number of transconjugants per limiting intracellular parent as determined in three representative experiments performed in triplicate; two experiments were performed in triplicate with the coinfected monolayers on permeable filter supports. The transmission frequencies in parentheses are the frequencies from the corresponding positive control experiments.
- Coinfected cells were infected with both recipient and donor bacteria.
- Nonadherent monolayers were treated with trypsin after the second gentamicin-bathing period.
- Independently infected cells were separately infected with either donors or recipients.

### Notes
- The numbers in parentheses are the numbers of recombinant colonies recovered in the corresponding positive control experiments. [INT-407 monolayers infected with series with SL1344<sup>R</sup> and SL1344<sup>N</sup>(RP4)].
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- Independently infected cells were separately infected with either donors or recipients.
transconjugants was lower at MOIs greater than 10 for un-
determined reason, but this may have been because cells infected
by large numbers of bacteria became more permeable to gen-


e. 2A. Transconjugant frequency measured as a function of MOI (B) Invasion of tissue culture cells by recipients (●) and donors (○) measured as a function of MOI. Each value is an

average based on three independent experiments performed in tripli-
cate.

FIG. 2. Intracellular conjugation frequency increases with the probability of coinfected. (A) Transconjugant frequency measured as a function of MOI. (B) Invasion of tissue culture cells by recipients (●) and donors (○) measured as a function of MOI. Each value is an

average based on three independent experiments performed in triplicate.

recipients at each MOI was therefore also determined. As expected, fewer donors infected cells that had been preinfected
with a high number of recipients (Fig. 2B). Again, this was
likely due to internalization of binding sites during the first
infection (33).

The frequency of transconjugants (Fig. 2A) was proportional
to the frequency of coinfection (Fig. 2B). The transconjugant
frequency increased from $3 \times 10^{-5} \pm 2 \times 10^{-5}$ transconjugant
per limiting parent (where detectable) at an MOI of 0.1 to $6 \times
10^{-4} \pm 1 \times 10^{-4}$ transconjugant per limiting parent at an MOI
of 10. The peak frequency at an MOI of 10 corresponded to
the highest number of both donors and recipients internalized.
Therefore, the intracellular conjugation frequency was depen-
dent on the probability of coinfection. The frequency of transconjugants was lower at MOIs greater than 10 for un-
known reasons, but this may have been because cells infected
by large numbers of bacteria became more permeable to gen-
ticin or were otherwise lost due to the cytotoxicity associ-
ated with a heavy infection.

**DISCUSSION**

*Salmonella* transferred plasmids within cultured INT-407
human cells. Mating was detected at frequencies greater than
$10^{-4}$ per limiting parent for RP4 and F. This frequency is only
3 orders of magnitude less than that detected on agar plates
containing much higher concentrations of bacteria. The INT-
407 cell line that we used was not a special case because
transconjugants also formed in dog kidney (MDCK) and hu-
man colon cancer (CaCo-2) cells (data not shown). These
observations support suggestions that antibiotic resistance
genes can and do transfer within humans and animals (50, 58).

Gene transmission between bacteria occurs inside animal
cells by conjugation. In this study, the requirement that donors
be invasive, the requirement that both parental types occupy
the same cell at the same time, and the requirement that
transconjugants remain inside the cell at all times prior to
harvest showed that gene transmission was intracellular.

The dependence on *tra* genes for the formation of recombi-
nants demonstrated that genes were transmitted by conjuga-
tion and not by transformation. The detection limits of these
experiments ranged from $\sim 10^{-5}$ to $\sim 10^{-7}$ recombinant per
limiting parent (Table 3). This range can be attributed to the
variation in internalization of bacteria from experiment to ex-
periment. In some replicates, fewer than the average number
performed in triplicate, no recombinant colonies were recov-
ered when donors carried *traJ* or *traB* plasmids, compared with
the 1,893 and 1,704 recombinant colonies obtained with *Tra*+
RP4 and F, respectively.

What is the frequency of intracellular gene transmission
between invasive pathogenic bacteria? The overall frequency
of transmission, $\sim 10^{-4}$ transconjugant per limiting parent, is
the result of (i) how often donors and recipients infect the
same cell, (ii) how often the bacteria could form contacts
within the cells, and (iii) the frequency of plasmid transmission
between bacteria in contact with each other. Perhaps dual
infeciton of single human cells by donor and recipient bacteria
is common and the frequency is a true reflection of gene
transmission efficiency. Alternatively, conjugation within hu-
man cells may be as effieient as conjugation on agar plates, but
donors and recipients are less often in direct contact because
coinfection of human cells is infrequent or contact between
bacteria inside human cells is rare. Experiments are under way
to distinguish between these two explanations. Preliminary mi-

roscopy results suggest that the proportion of cells that are
infected after the first round of invasion is high (approximately
80% on average) and that the cells tend to be infected equally
with a few bacteria ($\sim 1$ to 20 bacteria) as opposed to a small
subpopulation of cells that are infected with many bacteria. We
are in the process of determining the frequency of coinfection.

Our experiments required donor and recipient bacteria that
were initially in separate vacuoles to somehow meet despite
that fact that *Salmonella* is known to remain inside vacuoles for
extended periods. The fact that the mechanism of gene trans-
fer was found to be conjugation rather than transformation
suggests that the bacteria did meet, because conjugation requires bacterium-bacterium contact. We propose two models to explain how the bacteria find one another. In the first model, we imagine that a small proportion of intracellular bacteria of each parental type escape the vacuole. Transconjugants are subsequently formed by conjugation in the cytoplasm. In the second model, we imagine that transconjugants are formed after fusion of donor- and recipient-containing vacuoles, which is known to occur within 12 to 24 h (16, 18).

To our knowledge, there is no evidence that 

Salmonella

escapes from vacuoles. If conjugation occurs outside vacuoles, then it has to involve a proportion of intracellular bacteria smaller than the proportion which could be detected by the experiments of other workers designed to determine the intracellular location of invasive 

Salmonella

. The vacuole fusion model is supported only if vacuole coalescence occurs, at least at some level, before 12 h. To our knowledge, the time course of vacuole coalescence has not been investigated. We are currently monitoring the intracellular location of transconjugants using donor and recipient bacteria marked with fluorescent proteins of different colors.

Of what relevance are these observations to plasmid and bacterial evolution? Bacterial conjugation is probably the primary mechanism of antibiotic resistance gene transmission (27, 46). The ubiquity of plasmid-borne resistance may be explained in part by the stamina of conjugation itself (27). Conjugation can occur even in environments that otherwise kill a bacterium, allowing plasmids to replicate by horizontal gene transfer in the presence of antibiotics and other environmental toxins that prevent bacterial reproduction (11, 27, 28).

HMEs carry a range of genes in addition to resistance determinants, including genes that accentuate virulence and symbiosis potential in many different microbes (29), from soil microbes (2) to members of the human flora (3, 9, 34, 38). What is the mechanism that creates linkage among various catabolic pathways, virulence, and resistance genes (29)? Here we considered the possibility that in at least one environment, gene transfer concentrates virulence and resistance determinants, providing an opportunity for the evolution of genetic linkage. When the invasive form of 

Salmonella

is inside human cells, it can exchange plasmids with other strains. Since virulence is partially dependent upon invasion, the most virulent bacteria are probably inside cells more often than less virulent bacteria are. The most virulent pathogens are also more likely to attract the attention of antibiotic-dispensing clinicians who effectively remove other microbes from the extracellular niche and concentrate resistant microbes in the patient (61). Mammalian cells infected by 

Salmonella

can subsequently internalize other species of bacteria (20), which must be antibiotic resistant in patients treated with antibiotics. These other species may then transfer antibiotic resistance genes to 

Salmonella

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Furthermore, it is known that antibiotic treatment causes overgrowth of resistant microbes, increasing translocation of these organisms from the gut to the mesenteric lymph nodes (7). Translocation most likely occurs by intracellular passage of bacteria through the intestinal epithelium (6). Thus, antibiotic usage may increase the potential for intracellular gene exchange, giving the pathogen and less virulent strains the opportunity to acquire the genes that confer antibiotic resistance and virulence characteristics. The phenomenon of in situ mat-

ing provides an evolutionary mechanism for the coevolution of resistance and novel virulence traits.

Tissue culture experiments have established the plausibility of gene transmission in humans and animals, particularly inside their cells, but have not established that the process is relevant to evolution. The frequency of coinfection, intracellular contact between donors and recipients, and expression of conjugative functions may be different in the gut environment. The results of tissue culture experiments do, however, directly support the possibility that gene transmission occurs during antibiotic therapy.

HGT is a surprisingly robust and common phenomenon, defying the most concerted efforts to control microbial diseases (27, 29, 30). Understanding the evolution of gene transfer should be as relevant to developing a lasting strategy for controlling infectious diseases as mining the genome for new drug targets or restricting the application of antibiotics.

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