THE MECHANISMS OF TRANSFER OF R FACTORS

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A multiple-drug-resistant shigella was first isolated in Japan by Kitamoto et al. from a patient with dysentery who had just come back from Hong Kong in 1955. This strain was resistant to 4 drugs: sulfonamides, streptomycin, chloramphenicol, and tetracycline. It was not known at that time that these multiple drug resistances were transmissible from one organism to another. No such strains had been isolated in Japan before 1955 despite the extensive nationwide survey of the antibiotic sensitivities of shigella isolates. Since 1957, however, more and more strains of shigella with such unusual patterns of drug resistance have been encountered.

An organism may become resistant to an antibiotic by either spontaneous mutation and selection or by transfer of genetic information from a resistant bacterium to a sensitive one followed by selection. Transfer of genetic information can occur by processes of transformation (deoxyribonucleic acid (DNA)-mediated), transduction (bacteriophage-mediated) and sexual recombination (conjugation). Several unusual features were found in the epidemiologic studies of the multiple-drug-resistant shigella, suggesting that a different genetic mechanism was responsible: Both drug-sensitive and multiple-drug-resistant shigella strains were isolated from different patients in the same epidemic. Both these strains were usually of the same antigenic type. Both drug-sensitive and multiple-drug-resistant shigella strains were simultaneously isolated from one and the same patient. They were found again to be of the same antigenic type. The patients carrying multiple-drug-resistant shigella strains usually harbored multiple-drug-resistant Escherichia coli also. When the patients who had been excreting drug-sensitive shigella were treated with a single drug, for example, with chloramphenicol alone, they suddenly started excreting multiple-drug-resistant shigella of the same antigenic type. On the other hand, it was not
possible to obtain multiple-drug-resistant mutants from a drug-sensitive parent strain in vitro by the selection with a single antibiotic. These findings seemed difficult to explain on the basis of spontaneous mutation and selection, or by the classic methods of gene transfer.\(^6\)

In 1959, Ochiai and Akiba and their collaborators first discovered that resistance to several antimicrobial agents could be transferred among enteric bacteria in vitro.\(^7\)–\(^9\) This transmissible drug resistance, which has become known as infectious drug resistance, is not only of special interest to microbial geneticists but also of great importance in clinical medicine.

These resistance factors were further found to replicate with a pace faster than that of host chromosome in view of their rapid infective transfer among sensitive bacteria.\(^6\) Furthermore, the resistance factors can be artificially eliminated with acridines\(^10\) which are known to eliminate other cytoplasmic factors of microorganisms.\(^11\) They were found able to attach themselves to host chromosome. These results indicate that the element responsible for the multiple drug resistances belongs to episomes.\(^12\) This element is called R factor. Various types of R factors have been found to give various combinations of drug resistances to host bacteria.\(^6\)

The importance of R factors in microbiology is not only in their drug resistances but also in their transmissibility. We have, therefore, investigated the mechanisms of transmission of R factors in comparison with F factor,\(^12\) which also belongs to episomes and resembles to R factors in the mode of transmission.

MATERIALS

**Media:** Liquid cultures were prepared in Penassay broth (Difco), Lennox broth,\(^13\) minimal medium of Davis and Mingioli\(^14\) and this minimal medium without glucose, and M9 medium.\(^15\) Plating media were nutrient agar, bromothymol blue (BTB)-sugar (containing 2% sugar)-nutrient agar, minimal agar of Davis and Mingioli and M9 agar. For phage titrations, Lennox agar and Lennox soft agar (containing 0.75% agar) were employed.

**Drugs:** Selective drugs for R factor-carrying bacteria were sulfathiazole (Takeda), dihydrostreptomycin sulfate (Sankyo), chloromycetin powder (Sankyo) and tetracycline (Japan Lederle). Their abbreviations are Su, Sm, Cm and Tc, respectively. Acriflavine hydrochloride (Tokyo Kasei Kogyo) was used as an inhibitor of DNA synthesis, and 5-methyl-tryptophan (Sigma) and Cm were used as inhibitors of protein synthesis. Their abbreviations are Af and 5MT, respectively.
Strains of bacteria, R factors and other episomes: Table 1 A shows the bacterial strains employed. Table 1 B shows R factors and other episomes used. Some R factors suppress the fertility function of F factor and others do not. The type of R factors that suppress the transfer of F factor and F-mediated host chromosome is called $fi^+$ and the other type is $fi^-$, $fi$ being an abbreviation for fertility inhibition.\(^\text{16}\)

Table 1

<table>
<thead>
<tr>
<th>Strains of Bacteria, R Factors and Other Episomes Used</th>
</tr>
</thead>
</table>

A. Strains of Escherichia coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic markers</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3350</td>
<td>F$, gal$, lac$, $\lambda$, Af$^+$</td>
<td>K-12</td>
</tr>
<tr>
<td>W3350 Af$^+$</td>
<td>F$, gal$, lac$, $\lambda$, Af$^+$</td>
<td>W3350</td>
</tr>
<tr>
<td>W2252</td>
<td>Hfr, met$, $\lambda^-$ (O-lac-pro-thr--)</td>
<td>K-12</td>
</tr>
<tr>
<td>W3102/LS2 Af$^+$</td>
<td>F$, gal$, lac$, T6$, Sm$, $\lambda^+$</td>
<td>K-12: W3102</td>
</tr>
<tr>
<td>W3102/LS2 Af$^+$</td>
<td>F$, gal$, lac$, Sm$, $\lambda^+$, T6$, Af$^+$</td>
<td>K-12: W3102</td>
</tr>
<tr>
<td>PL22/Sl</td>
<td>Hfr, gal$, B1$, Sm$, $\lambda^+$</td>
<td>K-12: Hfr H: PL22</td>
</tr>
<tr>
<td>C/S</td>
<td>F$, $\lambda^-$, Sm$^+$</td>
<td>C</td>
</tr>
</tbody>
</table>

Abbreviations of genetic markers are as follows. $\text{lac}^-$: lactose-non-fermenting, $\text{gal}^-$: galactose-non-fermenting, $\lambda^-$: non-lysogenic for $\lambda$, $\text{met}^-$: methionine-requiring, $\text{O-lac-pro-thr}^-$: Transfer of chromosome in the order of lactose marker, proline marker, threonine marker, $\cdots$, T6$: phage T6 resistance, Sm$: streptomycin resistance, $\lambda^+$: lysogenic for $\lambda$, $\lambda^+$: phage $\lambda$ resistance, $\text{B1}^-$: vitamin B1-requiring.

B. Strains of R factors and other episomes

<table>
<thead>
<tr>
<th>Episome</th>
<th>Genetic markers</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-3</td>
<td>Su$, Sm$, Tc$^-$ $fi^-$</td>
<td>Isolated from Shigella strains</td>
</tr>
<tr>
<td>R-15</td>
<td>Su$, Sm$, $fi^-$</td>
<td>Segregated from 222.</td>
</tr>
<tr>
<td>N-6</td>
<td>Su$, Sm$, Tc$^+$ $fi^+$</td>
<td>Isolated by Hirotta &amp; Sneath.(^\text{17})</td>
</tr>
<tr>
<td>222</td>
<td>Su$, Sm$, Cm$, Tc$^+$ $fi^+$</td>
<td>Isolated by Jacob et al.(^\text{18})</td>
</tr>
<tr>
<td>R$^+$</td>
<td>Su$, Sm$, Cm$^+$ $fi^+$</td>
<td>F$^+$-gal$^{-}\lambda^-$</td>
</tr>
<tr>
<td>F$^+$-gal$^{-}\lambda^-$</td>
<td>gal$^+$</td>
<td>Isolated by Jacob et al.(^\text{18})</td>
</tr>
<tr>
<td>F$^+$-gal$^{-}\lambda^+$</td>
<td>gal$^+$, $\lambda^+$</td>
<td>F$^+$-gal$^{-}\lambda^+$</td>
</tr>
</tbody>
</table>

* Phage $\lambda$ induction-negative ($\lambda^{ind^-}$) was used.

Abbreviations of genetic markers are as follows.

$fi$: fertility inhibition of F factor of E. coli K-12, $\text{gal}^+$: galactose-fermenting, $\lambda^+$: non-lysogenic for $\lambda$, $\lambda^+$: prophage $\lambda$.

Abbreviations of resistance markers of R factors are as follows.

Su: sulfonamides, Sm: streptomycin, Cm: chloramphenicol, Tc: tetracycline.
F-specific phage: Phage f2 was used. This phage contains ribonucleic acid (RNA) as genetic material and absorbs only to male (F+ or Hfr) cells. It is a spherical particle with a diameter of about 200Å. Anti-f2 serum was prepared in rabbits by repeated injection of this phage.

METHODS

General principles of experiments

Transmission of R factor from donor to recipient bacteria is recognized by the conversion of the recipient bacteria to resistance to multiple drugs.

The process of the transfer of R factor may be divided, for convenience, into the following four steps: (1) Conjugation, which involves random collision of cells of R+ and R− types and the formation of effective contact between them; this contact is called specific cell pairing. The organelles of donor and recipient bacteria, which take part in this step, were also investigated. (2) Transfer of genetic material from donor to recipient cells; whether or not specific DNA synthesis is required for their transfer was studied. Host specificities of episome DNA were also taken into consideration. (3) Phenotypic expression of episomes; time and specific protein synthesis required for the phenotypic expression were examined. (4) Multiplication of episomes in infected cells (recipient); mechanisms and intracellular sites of their multiplication were studied.

Techniques

Kinetics of transfer of R factors and other episomes: Overnight cultures of donor and recipient strains were each diluted 1:10 with Penassay broth and incubated for additional 2 hr to produce logarithmic (log) phase cultures containing 2 to 5 × 10^8 cells/ml. Five tenths ml of a donor culture and 4.5 ml of a recipient were then mixed and incubated without aeration. Samples were removed at various time points and 0.1 ml of the properly diluted mating mixture was plated on selective media. For detection of the transfer of R factors (222, N-3, N-6), BTB-galactose agar plates containing 1000 μg/ml of Sm plus 25 μg/ml of Cm or Tc were used as selective media. Bacteria without the chromosomal Smr gene and with R factor carrying Smr marker cannot grow on this medium, because the level of Sm resistance conferred by R factor is rather low. The transfer of F−gal factor was detected as gal+ Smr colonies on BTB-galactose agar containing 1000 μg/ml of Sm.

The transfer of R factors at high frequencies: High-frequency resistance transfer system (HFRT) described by Watanabe was followed: Five ml broth was inoculated with about 2 × 10^4 cells of an R− Smr strain (donor) and about
10⁶ cells of an R⁻ Sm⁺ strain (intermediate donor). After overnight incubation, the mixture was diluted 1:10 with fresh broth and incubated further for 2 hr without aeration to about 10⁸ cells/ml. Most of the intermediate cells in such an HFRT donor mixture were R⁺ and since a high proportion of them had recently acquired R factor, they showed a high efficiency of transfer when mixed with the recipient R⁻ Sm⁺ strains.

Specific cell pairing: Cell-to-cell contact was observed in mixture of two strains, one of which had been vitally stained with 2,3,5-triphenyltetrazolium chloride (TTC), or a strain with a characteristic cell shape. The method of vital staining with TTC was the same as that described by Monk and Clowes.22 Usually recipient cultures were stained. To an actively growing culture at a density of about 2 × 10⁸ cells/ml, vigorously aerated in broth, a solution of TTC was added to a final concentration of 0.1% and aeration immediately stopped. After further incubation for 5 min, the culture turned bright red, and the great majority of cells were seen under the microscope to show 1 to 2 sites per cell which were intensively stained. Specific cell pairings between donor and recipient cells were thus scored as pairings between stained cells and non-stained cells or between morphologically different cells.

Treatment of cells with periodate, Af, Cm or a blender: Treatment of cells with periodate was done as follows: A log phase culture was washed by centrifugation and was resuspended in buffer (for minimal medium) in a concentration 10 times as high and a solution of periodate was added to this bacterial suspension in final concentrations of M/2000 to M/4000 and incubated for 15 min at 37°C. Periodate-treated cells were diluted 1:10 with Penassay broth at 0°C (in an ice bath). For Af or Cm treatment, 10 to 20 µg/ml of Af or 20 to 50 µg/ml of Cm was added to the culture. Blending of the culture was done with a homogenizer for 2 min at 20,000 rpm. The treated cells were used as donor or recipient cells for mating experiments.

Electronmicroscopic observation of the organelles on the cell surface: Bacteria were grown in Lennox broth to a concentration of about 5 × 10⁸ cells/ml, and phage f₂ was added to them at a multiplicity of 20. After 10 min adsorption, the infected culture was chilled in an ice bath and washed twice with buffer by centrifugation. Cells were resuspended in distilled water. A small drop of this cell suspension was placed on a collodion film overlying a microscope grid and allowed to air dry. Specimens were shadowed with chromium and observed in a Hitachi HU-11B electronmicroscope. In some cases, samples on a collodion film were sponged off with filter paper after 5 min and replaced by a drop of 2% phosphotungstic acid (PTA) solution at pH 7.2. The
PTA solution was removed immediately, and the specimens were observed.

**Test of cultures for the presence of phage-sensitive bacteria:** The method described by Meynell and Datta was followed: Bacteria were grown to 10⁹ cells/ml in Lennox broth, and phage f2 was added to this culture at a multiplicity of 20. After 10 min at 37°C for adsorption, 2 ml of the mixture was added to 2 ml of a 1/100 dilution of antiphage serum (K value 4,600). This was held at 37°C for 10 min, and 10 ml of Lennox broth was then added to it and the whole amount poured on a Millipore filter (Type HA) which was sucked to almost complete dryness before washing by pouring another 10 ml of Lennox broth. The filter was then transferred to 10 ml of Lennox broth, rinsed well to release the bacteria, and then removed. The culture was assayed for phage immediately and again after incubation for 2 hr at 37°C. Phage was assayed by the agar layer method. After appropriate dilution, 0.1 ml of the culture was mixed with 3 ml of soft agar containing about 10⁸ cells of an Hfr indicator strain and poured on Lennox agar. After overnight incubation, plaques were counted.

**Analysis of superinfection immunity of R factors and F factor:** It is known that the frequencies of transfer of episomes by conjugation to recipients already having the same type of episomes are remarkably reduced. This phenomenon is called superinfection immunity of episomes. Strains with F⁻-gal-λ⁻, F⁺-gal-λ⁺, fi⁺ R and fi⁻ R factors, respectively, were used as donors, and F⁻ R⁻, F⁺ R⁻, F⁻ R⁺ and F⁺ R⁺ strains were used as recipients. Mating conditions and the detections of transmissions of F⁺-gal-λ⁺, fi⁺ R or fi⁻ R factors were the same as above. The transfer of F⁺-gal-λ⁺ factor results in zygotic induction of phage λ. Detection of cells in which zygotic induction occurred was done as follows: Samples of mating mixtures were taken at various time points, and plated with 3 ml of soft agar containing 1000 µg/ml of Sm and about 10⁸ cells of an indicator strain on nutrient agar containing 1000 µg/ml of Sm. After overnight incubation, plaques were counted. An induction-minus mutant of phage λ (λ-ind⁻) was used for the experiment of zygotic induction, because it gives only low background of plaques.

**Treatment with an inhibitor of DNA synthesis:** Af was used as an inhibitor of DNA synthesis. Af was added to a culture in final concentrations of 20 to 50 µg/ml before mating, during mating or during the phenotypic expression of the transferred episomes.

**Af sensitivities of used bacteria and selection of Af-resistant mutants:** Af sensitivities of the used strains were determined in M9 medium supplemented with 1% Casamino acid (Difco) and adjusted to pH 7.7 with phosphate buffer. One
loopful of bacteria was inoculated into this enriched M9 medium containing varying concentrations of Af, and incubated at 37°C overnight. Selection of Af-resistant (Af^r) mutants was made as follows: The bacteria which grew in the medium containing highest concentrations of Af were inoculated into broth containing higher concentrations of Af. This way of 5 to 6 stepwise selections gave Af^r mutants. Minimal inhibitory concentrations of Af were 10 to 20 µg/ml in Af-sensitive (Af^s), wild type bacteria and 50 to 60 µg/ml in Af^r mutants. Af resistance levels of these Af^r mutants were stable. No Af^r mutants could be obtained from the wild type bacteria by single step selection. F^-gal and R factors were transferred to wild type bacteria and Af^r mutants with the ordinary method.

Treatment with inhibitors of protein synthesis: The inhibitors of protein synthesis were added to a culture before mating, during mating or during the phenotypic expression of the transferred episomes. Bacteria were grown in M9 medium supplemented with 0.1% Casamino acid (Difco) and adjusted to pH 7.0 with phosphate buffer. 5MT and Cm were used as inhibitors. 5MT was added in final concentrations of 0.25 to 0.5 mM and the inhibitory action of 5MT was stopped by adding 0.5 to 1.0 mM L-tryptophan. Cm was added in final concentrations of 10 to 50 µg/ml and Cm was removed by filtration and washing with a Millipore filter (Type HA).

RESULTS

Kinetics of transfer of R factor at normal frequencies and high frequencies (HFRT)

An HFRT system was prepared with W3350 (222) and W3350 by the procedure mentioned above. In parallel with this, W3350 (222) alone was grown in Penassay broth at 37°C overnight and, after being diluted 1:10 with fresh broth, incubated at 37°C without aeration. One ml of donor culture was mixed with 9 ml of a recipient culture (W3102/LTS) in a 200 ml Erlenmeyer flask and incubated in a water bath at 37°C without aeration. Samples (0.1 ml) were taken at various intervals and added to 0.9 ml of a phage T6 lysate (titer: about 10^{10}/ml) and further incubated at 37°C for 20 min before plating on BTB-galactose agar containing 1000 µg/ml of Sm plus 25 µg/ml of Cm. As a control experiment, the kinetics of transfer of F^-gal factor was studied. The kinetic curves of transfer of R and F^-gal factors are shown in Fig. 1 A, and the distributions of the kinetics of the transfer were calculated as \( \frac{\Delta F}{\Delta t} \); t and F indicate time and the function of the kinetic curves of transfer, respectively (Fig.
1 B). Frequencies of transfer of R factor by HFRT system and F′-gal factor reached 1 per input donor within 30 min, whereas the frequencies of transfer of R factor by ordinary R+ strains were about 10−2 at the most. The distributions of the transfer of these episomes, however, had a peak at about the same time.

Fig. 1A  
![Fig. 1A: Kinetics of transfer of episomes.

Escherichia coli W3350 (222), W3350 (222) HFRT and W3350 (F′-gal) were used as donors, and W3102/LTS was used as a recipient. Five tenths ml of a donor culture and 4.5 ml of a recipient were mixed and incubated without aeration. At various time points, samples (0.1 ml) were taken, added to 0.9 ml of a phage T6 lysate, and further incubated at 37°C for 20 min before plating on selective media. The transfer of R factors was detected as Cmr Smr colonies on BTB-galactose agar containing 1000 µg/ml of streptomycin (Sm) plus 25 µg/ml of chloramphenicol (Cm), and the transfer of F′-gal factor was detected as gal+ Smr colonies on BTB-galactose agar containing 1000 µg/ml of Sm.

Fig. 1 B. Distributions of the transfer events in the kinetic curves.

Distributions of the transfer events were calculated as \( \frac{dF}{dt} \) in the kinetic curves; t and F indicate time and the function of the kinetic curves of transfer, respectively.

![Fig. 1B: Distributions of the transfer events in the kinetic curves.](image-url)
Specific cell pairing and frequencies of transfer of episomes

The frequencies of specific cell pairing and of the transfer of episomes were examined with the same mating mixture. W3350 (222), W3350 (N-3) and their HFRT systems were used as donors. W3350 (F′-gal), W2252 (Hfr), and these strains with R factor 222 or N-3 were used as controls. One of the recipient strains, W3120/LTS, was stained with TTC to distinguish the recipient from

Table 2
Frequencies of Specific Cell Pairing and Transfer of Episomes with Periodate-pretreated Cells of Escherichia coli

A. Specific cell pairing and transfer of R factors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Periodate pretreatment</th>
<th>Recipient</th>
<th>W3102/LTS</th>
<th>C/S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pairing*</td>
<td>Transfer**</td>
<td>Pairing*</td>
</tr>
<tr>
<td>W3350 (N-3)</td>
<td>-</td>
<td>5-0</td>
<td>4.0×10⁻⁴</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4-0</td>
<td>4.4×10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>W3350 (222)</td>
<td>-</td>
<td>5-0</td>
<td>8.0×10⁻⁴</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4-0</td>
<td>4.0×10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>W3350 (222)</td>
<td>-</td>
<td>20-0</td>
<td>2.5×10⁻⁵</td>
<td>12-0</td>
</tr>
<tr>
<td>HFRT</td>
<td>+</td>
<td>5-0</td>
<td>3.4×10⁻⁵</td>
<td>0</td>
</tr>
<tr>
<td>W3350 (F⁻ R⁻)</td>
<td>-</td>
<td>5-0</td>
<td>—</td>
<td>0</td>
</tr>
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</table>

B. Frequencies of specific cell pairing and transfer of F' factors or F-mediated bacterial chromosome

<table>
<thead>
<tr>
<th>Donor</th>
<th>Periodate pretreatment</th>
<th>Recipient</th>
<th>W3102/LTS</th>
<th>C/S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pairing*</td>
<td>Transfer**</td>
<td>Pairing*</td>
</tr>
<tr>
<td>W2252 (Hfr)</td>
<td>-</td>
<td>40-10</td>
<td>2.7×10⁻²</td>
<td>15-5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5-0</td>
<td>4.7×10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>W3350 (F'₉-gal)</td>
<td>-</td>
<td>40-20</td>
<td>4.5×10⁻²</td>
<td>10-5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5-0</td>
<td>1.1×10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>W3350 (F'₉-gal N-3)</td>
<td>+</td>
<td>20-5</td>
<td>2.2×10⁻³</td>
<td>5-0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5-0</td>
<td>1.1×10⁻⁵</td>
<td>0</td>
</tr>
<tr>
<td>W3350 (F'₉-gal 222)</td>
<td>-</td>
<td>7-0</td>
<td>1.1×10⁻³</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5-0</td>
<td>3.6×10⁻⁸</td>
<td>0</td>
</tr>
</tbody>
</table>

* Frequency (in %) of specific cell pairings divided by donor cells.
** Frequency of transfer per input donor cell. In the transfer of R factors, 222 and N-3, Cmr Smr and Tcr Smr colonies were counted, respectively. In the transfer of F-mediated bacterial chromosome by W2252, lac⁺ Smr colonies were scored. In the transfer of F'₉-gal factor, gal⁺ Smr colonies were counted. Method of treatment with periodate and observation of specific cell pairing, and mating conditions are described in Methods,
donor cells, and the other recipient (E. coli C/S) was easily distinguished from rod-shaped donor cells for its spherical form. The donor and recipient cultures were blended in a homogenizer or treated with periodate, Af or Cm. Equal volumes of donor and recipient cultures were mixed. After 5 min incubation, samples were taken and observed under a phase contrast microscope. Specific cell pairings were seen as pairings between donor and recipient cells and expressed as numbers of pairings divided by total donor cells observed in the visual field. After 15 min incubation, a sample was taken from the same mating mixture and plated on the selective media, and the frequency of transfer was determined. Specific cell pairings and the frequencies of transfer with donors treated with periodate are shown in Tables 2 A and B. These results indicate that specific cell pairings are in parallel with the frequencies of transmission and that periodate reduces the frequency of specific cell pairing and at the same time the frequency of transmission of R and F factors. In addition, it is apparent that $\phi^+$ R factors inhibit the formation of specific cell pairing by F factor, but $\phi^-$ R factors do not. With blended donor cultures, the formation of specific cell pairing and the frequency of transfer were reduced for a short duration of time, but recovered to normal within 5 to 10 min and 15 to 30 min, respectively. The cells treated with periodate or blended had a good recipient ability like the non-treated cells. Af and Cm did not repress the formation of specific cell pairing.

Electronmicroscopic observation of specific organelles on the cell surface

Substrains of E. coli K-12, W3350, W2252 and W3102/LTS, and E. coli C/S were used. R factor (222 or N-3) or F factor was transferred to these strains. No pili adsorbing phage f2 could be seen in F$^-$ R$^-$ cultures of E. coli K-12, W3350 and W3102/LTS, and no pili were observed in an F$^-$ R$^-$ culture of E. coli C/S. Piliated bacteria were usually present in F$^-$ K-12 strains, but all of them were common (type I) pili$^{25}$ (Plates 1,2,3, and 4). In the cultures of E. coli K-12, W3350 and W3102/LTS, and E. coli C/S carrying an R factor 222 or F factor, the infection with phage f2 clearly distinguished common type I pili from F pili, which were coated with the small spherical phage particles of f2 (Plates 2,3 and 4). Phage f2-covered pili were found on almost all cells in F$^+$ cultures and only on limited numbers of cells in the cultures of W3350 (222) and its HFR systems depending on their frequencies of transfer. These findings indicate that the pili specific for F factor are similar or identical to those formed by $\phi^+$ R factors. In the culture of the strain carrying an $\phi^-$ R factor N-3, no phage-adsorbed pili were observed. In an Hfr strain W2252 carrying an $\phi^+$ R factor 222, phage-adsorbed pili were observed in much less cells than in this strain without R factor, but this phenomenon was not found with an $\phi^-$ R factor.
N-3. Diameters of F pili and type I pili were 75 to 100 Å and 50 to 100 Å, respectively. F pili associated with these episomes vary in length from a fraction of 1 μ to over 20 μ. Type I pili seldom exceed 1.5 μ in length.

**Frequencies of phage f2-sensitive bacteria in the cultures carrying various episomes**

R factor 222 enables F− bacteria to support the multiplication of F-specific phage f2 (Table 3). The ability of phage f2 to grow in the cultures carrying

<table>
<thead>
<tr>
<th>Strain</th>
<th>Infective centers/ml in the culture</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immediately after infection (B)</td>
<td>After 2 hr incubation (A)</td>
</tr>
<tr>
<td>W2225 (Hfr)</td>
<td>8.6×10⁶</td>
<td>1.0×10⁶</td>
</tr>
<tr>
<td>W3550 (F−-gal)</td>
<td>1.2×10⁶</td>
<td>3.8×10⁶</td>
</tr>
<tr>
<td>W3550 (N-3) HFRT</td>
<td>6.0×10⁴</td>
<td>2.9×10⁴</td>
</tr>
<tr>
<td>W3550 (R-15) HFRT</td>
<td>8.0×10⁴</td>
<td>2.6×10⁴</td>
</tr>
<tr>
<td>W3550 (222) HFRT</td>
<td>2.1×10⁴</td>
<td>2.2×10⁴</td>
</tr>
<tr>
<td>W3550 (222)</td>
<td>8.0×10⁴</td>
<td>4.8×10⁴</td>
</tr>
<tr>
<td>W3550 (P− R−)</td>
<td>8.0×10⁴</td>
<td>3.0×10⁴</td>
</tr>
</tbody>
</table>

_E. coli_ strains were grown to 10⁹ cells/ml in Lennox broth, and phage f2 was added at a multiplicity of 20. After adsorption, free phage was removed by antiphage serum and filtration. The cells were resuspended, and diluted in Lennox broth. Phage was assayed on this culture immediately and again after incubation for 2 hr at 37°C. The procedures in detail are described in Methods.

R+ R factors is not due to the mutation of the phage, since the phage recovered from R+ cultures behaved in the same way as the parent stock in its ability to infect and multiply in such cultures. No F− strains and f− R-carrying strains gave any increase of the phage titers. The number of phage-infected bacteria in F+ or HFRT cultures could be estimated from the initial plaque counts, although this was impossible with R+ cultures, because a high background of plaques was observed in the F− R− controls. However, the fact that the phage titer increased, showed that some phage-sensitive bacteria were present in the cultures. The frequencies of the phage-sensitive bacteria calculated by this method were in good accordance with the frequencies of transfer of episomes by the cultures.

**Patterns of superinfection immunity of episomes**

The transfer of R factors from donor strains, W3550 (222) and W3550 (N-3), to recipient strains, W302/LSλ, W3102/LSλ (R₃) and W3102/LSλ...
Table 4.

Frequencies of Transfer of $R$ Factors to Various Recipient strains of Escherichia coli K-12

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>W3350 222</th>
<th>W3350 N-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3102/LSλ</td>
<td></td>
<td>Freq. Tc Smr</td>
<td>$R^+/R^-$</td>
</tr>
<tr>
<td>$R^-$</td>
<td>2.2×10^{-2}</td>
<td>---</td>
<td>2.8×10^{-3}</td>
</tr>
<tr>
<td>$R_3$</td>
<td>1.1×10^{-2}</td>
<td>5.0×10^{-2}</td>
<td>3.4×10^{-3}</td>
</tr>
<tr>
<td>R-15</td>
<td>1.6×10^{-2}</td>
<td>7.3×10^{-1}</td>
<td>8.7×10^{-5}</td>
</tr>
</tbody>
</table>

$E. \ coli$ W3350 (222) and W3350 (N-3) were used as donor strains, and W3102/LSλ, W3102/LSλ (R3) and W3102/LSλ (R-15) were used as recipients. Five tenths ml of donor culture and 4.5 ml of a recipient were mixed, and incubated for 30 min at 37°C without aeration. Transfer of $R$ factors was detected as Tc Smr colonies on BTB-galactose agar containing 1000 µg/ml of Sm plus 25 µg/ml of Tc.

Table 5

Frequencies of Zygotic Induction of $\lambda$ and gal$^+$ transmission by $F'$ factors to various recipient strains of Escherichia coli

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Zygotic induction</th>
<th>$F'$-gal transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq. Z. I.</td>
<td>$F^+/F^-$</td>
</tr>
<tr>
<td>W3102/LSλ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F^- R^-$</td>
<td>9.2×10^{-1}</td>
<td>---</td>
</tr>
<tr>
<td>$F^+ R^-$</td>
<td>1.2×10^{-2}</td>
<td>1.3×10^{-2}</td>
</tr>
<tr>
<td>$F^- R_{15}$</td>
<td>3.0×10^{-1}</td>
<td>---</td>
</tr>
<tr>
<td>$F^+ R_{15}$</td>
<td>4.5×10^{-3}</td>
<td>1.5×10^{-2}</td>
</tr>
<tr>
<td>$F^- R_3$</td>
<td>6.0×10^{-1}</td>
<td>---</td>
</tr>
<tr>
<td>$F^+ R_3$</td>
<td>7.0×10^{-2}</td>
<td>1.2×10^{-1}</td>
</tr>
<tr>
<td>PL22/Sλ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F^+$</td>
<td>4.1×10^{-1}</td>
<td>4.5×10^{-2}</td>
</tr>
</tbody>
</table>

$E. \ coli$ W3350 (F$'_3$-gal-$\lambda^-$) and W3350 (F$'_3$-gal-$\lambda^+$) were used as donor strains, and $\lambda$ induction-negative was used as phage $\lambda$. Five tenths ml of a donor culture and 4.5 ml of a recipient were mixed and incubated for 30 min at 37°C without aeration. For detection of zygotic induction, samples were poured on nutrient agar containing 1000 µg/ml of Sm with 3 ml of soft agar containing 1000 µg/ml of Sm and about $10^8$ cells of an indicator strain, $E. \ coli$ C/S. Gal$^+$ transmission was detected as gal$^+$ Smr colonies on BTB-galactose agar containing 1000 µg/ml of Sm.

(R-15), were determined and the transfer of $F^+$ factor from W3350 (F$'^+$-gal-$\lambda^-$ or F$'^+$-gal-$\lambda^+$) to W3102/LSλ carrying $F$ and $R$ factors and PL22/Sλ (Hfr) was
also studied with \( gal^+ \) transmission by \( F'^{-}gal^{-}\lambda^- \) and with zygotic induction of \( \lambda \) by \( F'^{-}gal^{-}\lambda^+ \). \( E. coli \) C/S was used as an indicator of phage \( \lambda \). The results obtained are shown in Table 4. Acceptance of \( R \) factor was remarkably reduced by the presence of a homologous \( R \) factor in the recipient, and similar suppressions were observed also for both \( gal^+ \) transmission and zygotic induction of \( \lambda \) by \( F' \) factors in \( F^+ \) recipient. \( F \) factor and \( fi^+ \) and \( fi^- \) \( R \) factors exhibit different patterns in their superinfection immunity. The transfer of \( F' \) factor is more convenient for the analysis of the mechanism of superinfection immunity, because zygotic induction which is considered to occur only after the entrance of \( F'^{-}\lambda \) factor to recipient bacteria\(^{26,27}\) and \( gal^+ \) transmission which can be detected only after multiplication of the \( gal^+ \) genes carried by \( F' \) factor,\(^{28}\) can be studied at the same time. The frequencies of zygotic induction of \( \lambda \) by \( F'^{-}\lambda \) factor were reduced by a factor of \( 10^{-1} \) to \( 10^{-2} \) equally in both \( F^+ R^- \) and \( F^+ \) \( fi^- \) \( R \) recipients (Table 5). The suppression of zygotic induction in \( F^+ \) recipients was reduced by a factor of \( 10^{-1} \) by the presence of \( fi^+ \) \( R \) factor in the recipients. The frequencies of \( gal^+ \) transmission to various recipients by \( F'^{-}gal \) were in parallel with and always lower than those of zygotic induction.

**Effects of \( Af \) on the transfer of episomes**

Fig. 2 shows the frequencies of transfer of \( R \) and \( F' \) factors from donor strain W3350 (\( R \) or \( F'^{-}gal \)) to recipient strain W3102/LS\( \lambda \) in M9 medium supplemented with 1% Casamino acid (Difco) and adjusted to pH 7.7 with phosphate buffer, when \( Af \) was added to the mating mixture in a final concentration of 20 \( \mu \)g/ml at various time points. These results suggest that the transfer mechanism was blocked in some stage by \( Af \). Effects of \( Af \) on the episomes immediately after their transfer were also examined: Mating mixtures were interrupted by blending at various time points. Blended samples were diluted in broth containing 1000 \( \mu \)g/ml of Sm plus 20 \( \mu \)g/ml of \( Af \) and incubated for 20 min at 37°C. Detecions of transfer of episomes were done in the same ways as above. Kinetic curves of transfer of episomes treated with \( Af \) after interruption of mating are shown in Fig. 3. These results indicate that \( Af \) inhibits the phenotypic expression of \( R \) factor. This effect of \( Af \) cannot be observed with \( F' \) factors, because phenotypic expression is allowed on the selective media in the transfer of \( F' \) factor. Donor and recipient bacteria were also separately treated with 20 \( \mu \)g/ml \( Af \) for 20 min at 37°C, chilled in an ice bath and washed twice with broth at 0°C. The frequencies of transfer of \( R \) and \( F' \) factors in the matings with donor and recipient pretreated with \( Af \) were about equal to those in the matings with non-treated cells.
Fig. 2. Effect of Af on the transfer of episomes.

Escherichia coli W3350 (222) and W3350 (F'8-gal) were used as donor strains, and W3102/LSa was used as a recipient. Five tenths ml of a donor culture and 4.0 ml of a recipient were mixed. Mating mixtures were incubated without aeration. At various time points, 0.5 ml of 200 µg/ml of Af was added to each mating mixture. After 30 min, all the mating mixtures were interrupted by blending, and the transfer of R and F' factors were scored as Cmr Smr and gal+ Smr colonies, respectively. The procedures in detail are described in Methods.

Fig. 3. Effect of Af on episomes immediately after their transfer to recipient cells.

Escherichia coli W3350 (222) and W3350 (F'8-gal) were used as donor strains and W3102/LSa was used as a recipient. One ml of a donor culture and 9 ml of a recipient were mixed and incubated without aeration. At various time points, samples (1 ml) were taken and blended. Blended samples were diluted in broth containing 1000 µg/ml of Sm plus 20 µg/ml of Af and incubated for 20 min at 37°C. The transfer of R and F' factors was detected as Cmr Smr and gal+ Smr colonies, respectively.

Solid lines indicate the kinetics of R factor, and dotted lines indicate that of F's-gal factor.
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Effects of Af on the transfer of episomes in the mating of Af\(^s\), wild type, and Af\(^r\) mutant strains

Af\(^r\) mutants were isolated from W3350 and W3102/\(\lambda\). R (222) and F\(^{\prime}\)-gal factors were transferred to W3550 wild type (Af\(^s\)) strain and Afr mutants. The transfer of these episomes was examined in the matings of Af\(^s\) and Afr donor and recipient strains. The frequencies of transfer in matings for 30 min and the effects of Af are shown in Table 6. These results indicate that

<table>
<thead>
<tr>
<th>Af sensitivity of</th>
<th>Af in mating mixture</th>
<th>R (222)</th>
<th>F(^{\prime})-gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Recipient</td>
<td>Cm(^r) Sm(^r) /donor</td>
<td>Af +/-</td>
</tr>
<tr>
<td>Af(^s)</td>
<td>Af(^s)</td>
<td>1.4x10(^{-2})</td>
<td>1.1x10(^{0})</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.6x10(^{-2})</td>
<td>1.9x10(^{-3})</td>
</tr>
<tr>
<td>Af(^s)</td>
<td>Af(^r)</td>
<td>9.3x10(^{-3})</td>
<td>1.2x10(^{0})</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.5x10(^{-3})</td>
<td>8.1x10(^{-3})</td>
</tr>
<tr>
<td>Af(^r)</td>
<td>Af(^s)</td>
<td>7.7x10(^{-3})</td>
<td>2.5x10(^{-1})</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.9x10(^{-3})</td>
<td>5.4x10(^{-1})</td>
</tr>
<tr>
<td>Af(^r)</td>
<td>Af(^r)</td>
<td>6.2x10(^{-3})</td>
<td>1.4x10(^{0})</td>
</tr>
</tbody>
</table>

Wild type, Af-sensitive (Af\(^s\)) and Af-resistant (Af\(^r\)) strains of Escherichia coli W3350 (222) and W3350 (F\(^{\prime}\)-gal) were used as donors and Af\(^s\) and Afr strains of E. coli W3102/\(\lambda\) as recipients. Five tenths ml of a donor culture, 4.0 ml of a recipient and 0.5 ml of Af solution (200 \(\mu\)g/ml) were mixed in M9 medium supplemented with 1% Casamino acid and adjusted to pH 7.7 with phosphate buffer. In control experiments, 0.5 ml of Af solution was replaced by 0.5 ml of medium. These mixtures were incubated at 37\(^{\circ}\)C for 30 min and plated on selective media. The effect to Af is indicated by the ratio of the frequencies of transfer in Af-containing to those in Af-free media.

The inhibition of transfer of these episomes by Af was only slight when donor bacteria were resistant to this acridine dye regardless of whether the recipient bacteria were Af\(^s\) or Af\(^r\).

Effects of 5MT and Cm on the transfer of episomes and their phenotypic expression

The transfer of R (222 or N-6) and F\(^{\prime}\)-gal factors from donor strains W3350 (222 or N-6) and W3350 (F\(^{\prime}\)-gal) to recipient strain W3102/LTS was examined. The kinetics of the transfer of these elements in the presence and absence of 5MT or Cm during mating and during phenotypic expression are shown in Figs. 4 A and B. In the transfer of R factor, these kinetics can be easily explained by the assumption that these inhibitors inhibit only the phenotypic expression.
Fig. 4. Effect of inhibitors of protein synthesis in the mating culture or in the culture for phenotypic expression after interruption of mating.

A: 0.5 mM 5MT as an inhibitor, B: 10 μg/ml Cm as an inhibitor.

Escherichia coli W3350 (222 or N-6) and W3350 (F's-gal) were used as donor strains and W3102/LTS was used as a recipient. Donor and recipient bacteria were grown in M9 medium (pH 7.0) supplemented with 0.1% Casamino acid. Five tenths ml of a donor culture and 4.5 ml of a recipient were mixed with or without an inhibitor of protein synthesis, 5MT or Cm (final concentrations of 0.5 mM and 10 μg/ml, respectively), and incubated at 37°C for 30 min. In the transfer of R (222) and F's-gal factors with 5MT, mating mixtures were blended, and 0.5 ml of each blended mixture was added to 4.5 ml of fresh broth containing 1000 μg/ml of Sm plus 1 mM L-tryptophan or 1000 μg/ml of Sm plus 0.5 mM 5MT. These mixtures were incubated for further 20 min at 37°C for phenotypic expression. In the transfer of R (N-6) and F's-gal factors with Cm, mating mixtures were filtered with Millipore filters (Type HA), washed and resuspended in fresh medium. Five tenths ml of each suspension was added to 4.5 ml of broth containing 1000 μg/ml of Sm or 1000 μg/ml of Sm plus 10 μg/ml of Cm, and incubated for further 20 min at 37°C for phenotypic expression.

The transfer of R factors, □—□; without inhibitor, ○—○; with inhibitor only in the culture for phenotypic expression, ■—■; with inhibitor in the mating mixture but without inhibitor in the culture for phenotypic expression, and ●—●; with inhibitor both in the mating mixture and in the culture for phenotypic expression.

The transfer of F's-gal factor, △—△; without inhibitor and ▲—▲; with inhibitor in the mating mixture.
of drug resistances. No effect of these inhibitors was shown in the transfer of F-gal factor, because its phenotypic expression was allowed on the selective media. These results indicate that 5MT and Cm do not inhibit the transfer itself but do inhibit the phenotypic expression of these elements.

DISCUSSION

It is known that R factors are extrachromosomal genetic elements which render their bacterial hosts resistant to antibacterial agents. They are also known to enable their host to conjugate with other strains which thereby acquire the R factor and in turn become drug-resistant. That portion of R factor which determines conjugation is known as the resistance transfer factor (RTF), and an R factor may thus be thought of as a variable number of genes determining drug resistances linked to an RTF. During conjugation brought about by an R factor, part of the chromosome of the R+ donor cell may also occasionally be transmitted; thus, RTF resembles the sex factor, F of E. coli K-12, which belongs to episomes. In the present investigation, the analysis of the mechanisms of transmission of R factors was carried out on the physiological as well as morphological aspects of their transmission.

The capacity to conjugate may be regarded as a specific differentiation of the donor cell which makes possible infective transfer of the conjugation factors. From this point of view, the process of conjugation may be comparable with the infective transmission of bacteriophage particles. Transfer of phages takes place by means of free extracellular particles each possessing its own apparatus for attaching and injecting its nucleic acid into a new host cell, whereas in conjugation, it occurs by the formation of a temporary intercellular connection. The formation of cellular union may be taken to include the sequence of events which culminate in the formation of an intercellular connection between mating cells. These events include random collision between donor and recipient cells, pairing by means of the surface structures specific for their transfer, and the formation of some stable connecting structure. Measurement of the amount of transfer which has taken place at successive time by stopping further transfer should give not only the minimum time required for transfer of R factors, but also a precise indication of the kinetics of cellular union between donor and recipient cells. Allowance must, of course, be made for the time taken for actual transfer. It takes about 3 min for the transfer of R and F factors, and single-peaked distribution of transfer events and the homologies of their distribution patterns of established (normal state) R factor and its HFRT systems and F-
factor may indicate that the transfer processes are the same in all cases, and that the differences of transmission frequencies in these states are due to the differences in the number of transmissible competent cells in the donor populations. Thus, all the F+ cells are always competent for the transfer of F factor, and competent cells are $10^{-2}$ to $10^{-4}$ in the population of the cells which have established R factors and are almost all ($10^9$) in the population of the cells of HFRT systems. This phenomenon is also observed in the transfer of colicino- genic factor I (Col I), and it is known that the cells which have recently acquired these types of conjugation factors conjugate with very high efficiencies, but lose the ability to conjugate within a few generations. In the case of col I-mediated conjugation, it has already been shown that loss of ability to conjugate is due to inability to form contacts with recipient cells. Then, we examined the cell contacts between donor and recipient cells in the transfer of R factors.

In the transfer of R factors, it was also shown that the frequencies of transmission are in parallel with the frequencies of specific cell pairings, and that inhibition of the transfer of F factor by $fi^+$ R factors is caused by the inhibition of formation of specific cell pairings. It was shown that blending or periodate treatment of donor bacteria which is known to reduce the frequencies of transfer of F factor and R factors, also reduces the ability of the donor bacteria to form effective contacts, indicating that R factor-mediated conjugation also involves a periodate-sensitive surface component, and that none of the inhibitors of the synthesis of DNA, RNA or protein inhibit the formation of effective cell contacts. Therefore, the organelles which exist on the surface of donor bacteria and which are specific for mating, must have the following characters.

1. They exist only on the cell surface of donor bacteria.
2. They are easily removed by blending and regenerate rapidly.
3. They are inactivated by treatment with periodate.
4. They are always fully yielded and are, therefore, not affected by inhibitors of the synthesis of DNA, RNA or protein.

In F+ bacteria, several specific surface properties have already been reported. The first demonstration of a physiological difference between donor and recipient cells of E. coli K-12 was provided by Maccacaro, who showed that F+ cells have a greater affinity to acidic dyes than female (F-) cells, and precipitate out of suspension in less acid media. It was also observed that F+ cells have a greater tendency to autoagglutinate. F+ cells have also been found to be less motile than F- cells, and evidence was presented that male and female cells can be separated by countercurrent distribution. A characteristic surface antigen, termed $f^+$, has been detected in strains with F factor. Anti-
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body to the $f^+$ antigen cross-reacts weakly to the surface of cells harboring the $F^\circ$-lac factor of Salmonella typhosa (or typhi), indicating that this factor controls the production of an analogous and related antigen. In addition, the presence of surface antigens which are serologically unrelated to $f^+$ have been demonstrated in cells of wild strains of E. coli harboring conjugation factors different from F. Phages which absorb to F$^+$ or Hfr cells of E. coli but not to F$^-$ cells have been isolated by several workers. The genetic material of some of these male-specific phages is RNA and is single-stranded DNA in others.

These observations are satisfied by the organelles which were found on the surface of F$^+$ bacteria by electronmicroscope. This organelle was named as "F pilus" and is rod-shaped, 85 Å in external diameter and 1 to 40 µ in length, and resembles the tail core of T-even bacteriophages. It was found that F pilis are the receptor of male-specific phages, and are the same substance as $f^+$ antigen. All the physiological properties specific for donor bacteria seem to be easily explained by the presence of F pilis. Axial hole of this F pilus is large enough to pass through bacterial and phage nucleic acids, and it was in fact shown that the nucleic acids of male-specific phages pass through the F pilis. Therefore, this structure has been regarded as organelle to form the conjugation bridge for the transfer of F factor.

In the transfer of R factors, Meynell and Datta found that R$^+$ bacteria have pili specific for R factors using several strains of R factors which were collected in Europe, and that these pili are similar or probably identical to F pilis. It was also demonstrated that R factors which form F pilis all belong to $f^+$ type and that $f^-$ R factors do not produce F pilis. In R$^+$ bacteria, the frequencies of cells with F pilis showed a good correlation to the frequencies of transfer of R factors. These points were investigated with our R factors by electronmicroscopy and assays of male-specific phage-sensitive bacteria. Our results with our R factors completely confirmed the reports by Meynell and Datta: (1) $f^+$ R factors produce F pilis in proportion to their transfer frequencies. (2) The inhibition of transfer of F factor by $f^+$ R factors is due to the inhibition of F pilis formation of F factor by $f^+$ R factors. (3) $f^-$ R factors do not produce F pilis. They may produce other type pili. This idea may not be unreasonable in view of the finding that specific surface antigens which are serologically unrelated to $f^+$ exist in cells of wild strains of E. coli harboring conjugation factors different from F factor.

Thus, also in the transfer of R factors, it may be considered that specific pili act as the organelles for transferring episome DNA from donor to recipient...
bacteria by conjugation. The presence of specific receptor on the surface of recipient bacteria to which specific pili are attached has been assumed but not demonstrated.

Superinfection immunities of R and F factors were studied to investigate the receptor sites for F pili and the intracellular multiplication of these episomes. The results obtained are as follows: Superinfection immunities of R and F factors are essentially different from that of lysogenic bacteriophage λ, and the greater part of the inhibition of acceptance of superinfecting non-viral episomes is due to the inhibition of acceptances on the surface of the recipient cells which already have homologous episomes. In the transfer of F factor, a half of this acceptance inhibition on the surface is caused by the presence of F pili, and another half may be caused by the function other than by specific pili. It is known that superinfection immunity between homologous R factors is not observed in the transduction with phage P1. This was considered to be because the transfer of episome DNA by transduction takes place through different sites on the cell surface from those by conjugation. Jacob et al. proposed in their replicon hypothesis that F factor replicates at specific sites on the inner side of the cell membrane and that this attachment site may exist immediately underneath the transfer organelle on the cell wall. On the basis of this hypothesis and the results of transduction experiment, the following assumptions may be allowed. The receptor for specific pili on the surface of cells may exist on the external side of the intracellular sites of attachment for a specific episome, and if these sites are already occupied by a homologous episome, the acceptance of newly infecting episome may be inhibited by the inability of contact formation due to altered receptors and specific pili which are made on the surface of recipient cells by the homologous episome. This hypothesis seems to be supported by Echols' report that superinfection immunity between F factors may take place on the surface of recipient cells. He studied on F superinfection of F' strains of E. coli by F'-lac-P and F'-gal, and the transfer of superinfecting F' was judged by alkaline phophatase (P) synthesis directed by F-transferred P' gene. The mechanism of the superinfection immunity of R factors may be analogous to that of F factors. The acceptance inhibition of superinfecting R factor, however, may be due only to the alteration of receptor on the surface of recipient cells which is caused by the presence of a homologous R factor, because most of the R' recipient cells have no specific pili. Mutual exclusion between homologous types of R factors in the same cells was also shown in the experiment of superinfection immunity. This may be explained by the hypothesis of competition between homologous
types of R factors in occupying the few specific sites for replication.  

Conjugation step, thus, begins first with random collision between donor and recipient cells and is completed by the effective connection between a specific pilus of donor cell and a receptor of recipient cell. Then, the completion of this connection may give signal to donor cell and the transfer of episome DNA from donor cell to recipient cell through pilus would start.  

It is known that in some stages the transfer of episomes is reduced by some of the inhibitors of DNA synthesis. Af was used as an inhibitor of DNA synthesis. Af inhibits the transfer of episomes depending on the time of its addition to the mating mixture. To see what stage of the transfer is inhibited by this inhibitor, the transfer of episomes was studied in the matings between Af-sensitive and -resistant cells with and without Af. It was found that Af inhibits the transfer of episomes only when it is added at the time of DNA transmission in donor bacteria. This may be the inhibition of DNA synthesis specific for episome transfer proposed in the replicon hypothesis, because Af does not inhibit specific cell pairing between donor and recipient cells. In other words, this indicates that episome transfer requires specific replication. This result also confirms the report by Gross and Caro who showed the transfer of radioactive genetic material from donor labeled only during mating.

Following the replication for transfer, episome DNA is transferred through specific pili from donor to recipient cells. It is known that immediately after entrance of foreign DNA into new host cells, the transferred DNA is checked for its host specificity by the restriction mechanisms of recipient cells. In this way, transferred DNA which is not restricted or escapes this restriction reaches its replication site and will be allowed to replicate.

Af also inhibits the phenotypic expression of R factor. This may be explained by the inhibition of transcription by this dye. It is known that in E. coli K-12, drug resistances of R factors are expressed within 10 min after transmission. It was shown that the expression of drug resistances is suppressed by inhibitors of protein synthesis. This and the fact that it takes about 10 min to express the drug resistances after receiving R factor indicate that the expression of drug resistances requires specific protein synthesis.

SUMMARY

Kinetic studies of transfer of R and F factors showed that transfer events distribute with a single-peaked homologous pattern in established (normal state)
R factor as well as its HFRT systems and F' factor. These results indicate that the differences of transmission frequencies in these states are due to the differences in the number of transmissible competent cells in the donor populations. The transfer of these episomes requires cell-to-cell contact between donor and recipient bacteria. The frequencies of formation of specific cell pairing counted under a phase contrast microscope were in good accordance with the frequencies of transfer of these episomes, and inhibiting agents of this cellular union disclosed some physiological properties of the organelle which takes part in cell-to-cell contact.

Electronmicroscopic observation of the donor cells demonstrated the presence of specific pili on their surface. The frequencies of cells having specific pili counted by electronmicroscopy and the frequencies of phage f2-sensitive bacteria in donor cultures carrying various episomes were in good accordance with the frequencies of their transfer. Furthermore, the physiological properties specific for donor bacteria seem to be easily explained by the presence of specific pili on their surface. All of these findings suggest that these specific pili may be organelles for forming conjugation bridges for the transfer of episomes.

Analysis of superinfection immunities of R and F factors indicated that the greater part of the inhibition of acceptance of superinfecting non-viral episomes is due to the inhibition of acceptance on the surface of recipient cells which already have homologous episomes and that mutual exclusion between homologous episomes in the same cell is also involved. These results suggest the presence of specific receptors on the cell surface and of specific sites for their replications inside the cell. The sites for replication may be different among F, f° R and f+ R factors, and may be present in small numbers assuming from the data of superinfection immunities. Specific pilus for each episome is probably formed immediately outside of this specific site in view of the rapid transfer of episomes.

Acriflavine, an inhibitor of DNA synthesis, was shown to inhibit the transfer of episomes but not to inhibit specific cell pairing and evidence was obtained that this inhibition occurs in donor bacteria. These results suggest that the transfer of episomes may require specific DNA replication for their transfer.

The phenotypic expression of drug resistances was found to require specific protein synthesis, because the expression of drug resistances is suppressed by inhibitors of protein synthesis.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

Plate 1. Type I pili.
Type I pili of Escherichia coli W3350 (F−) are indicated by arrow. Flagella are also observed. This specimen was shadowed with chromium.
PF: Type I pili, FL: Flagella.

Plate 2. F pili by fi+ R factor.
F pili of Escherichia coli W3350 (222) coated with small spherical phage particles are indicated by arrow. Type I pili and flagella are also observed. This specimen was shadowed with chromium.
PFR: F pili by R factor, PI: Type I pili, FL: Flagella, f: Small spherical phage f2.

Plate 3. F pili by F factor.
F pili of Escherichia coli W2252 (Hfr) are coated with small spherical phage particles. This specimen was shadowed with chromium.
PF: F pili by F factor, PI: Type I pili, FL: Flagella, f: Small spherical phage f2.

Plate 4. F pili by F factor.
Escherichia coli W3102/LTS (F+) was negatively stained with PTA. F pili coated with small spherical phage particles, type I pili and flagella are also observed.
PF: F pili, PI: Type I pili, FL: Flagella, f: Small spherical phage f2.