Influence of the State of Fimbriation on Transmission of the Colicinogenic Factor *colI* between Strains of *Shigella flexneri*

By M. MULCZYK* AND J. P. DUGUID

Bacteriology Department, University of St Andrews, Queen's College, Dundee

(Received 19 April 1966)

SUMMARY

The colicinogenic factor *colI* derived from *Shigella sonnei* colicine type 2 (Abbott & Shannon, 1958) was transmitted between different strains of *Shigella flexneri* in serotypes 1a, 1b, 2a, 2b, 3a, 3c, 4a, 4b, 4c, 5a, X and Y when donor and acceptor strains were grown together in mixed culture in aerobic static broth for a period of 20 hr or 48 hr at 37°. The rate of transfer was influenced by the state of fimbriation of the acceptor bacteria, but not by that of the donor bacteria. The proportion of acceptor organisms acquiring *colI* was very high, e.g. 15-40 % in 20 hr and 50-95 % in 48 hr, when the acceptor was genotypically fimbriate and in the fimbriate phase (Fim⁺F); but it was much lower, e.g. 0-5 % in 20 hr and 1-15 % in 48 hr, when the acceptor was either genotypically non-fimbriate (Fim⁻) or genotypically fimbriate but in the non-fimbriate phase (Fim⁺N). Although it facilitated the transmission of *colI*, the presence of fimbriae was not essential for transmission, since transmission at a low rate was obtained in most crosses of Fim⁻ donors with Fim⁻ acceptors. Only 1 out of 17 strains of *S. flexneri* tested as acceptors failed to acquire *colI* in any test. Transmission did not take place within the first 2 hr of mixed culture; it occurred mainly during the period of slow growth after the end of the exponential phase (i.e. between 8 and 48 hr) when, with fimbriate organisms, a pellicle of bacteria had developed on the surface of the broth. The rate of transmission was very much decreased when pellicle formation was prevented by intermittent or continuous agitation of the culture. Experiments in which the donor bacteria were killed by adding streptomycin at 8 hr showed that an extensive 'epidemic' spread of *colI* took place within the acceptor population during the later stages of culture. When stationary-phase (1-10 days) broth cultures of donor and acceptor bacteria were mixed together and incubated without the addition of fresh broth, the rates of transfer at 20 and 48 hr were as high as in the mixed cultures grown from small inocula. Transmission also occurred with high frequency in a defined medium containing glucose, NH₄⁺ and nicotinic acid.

INTRODUCTION

Many strains of bacteria in different genera of Enterobacteriaceae possess the ability to produce and liberate colicines, antibiotic substances which are active against many other strains of enterobacteria (Fredericq, 1957, 1963). This ability may be transferred, during the conjugation of bacteria, from a colicinogenic to a

* Present address: Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

non-colicinogenic strain (Fredericq, 1954; Ozeki, Howarth & Clowes, 1961). The colicinogenic factor that is transferred from bacterium to bacterium has the properties of a plasmid, i.e. an autonomously reproducing cytoplasmic hereditary determinant (Lederberg, 1952), and the transfer of colicinogeny is not dependent on transfer of the bacterial chromosome as in the classical recombination phenomenon. Factors influencing the frequency of transfer of colicinogenic factor I (*colI*), which determines the production of colicine I, have been investigated by Ozeki, Stocker & Smith (1962) and Stocker, Smith & Ozeki (1963) in experiments with *Salmonella typhimurium* carrying *colI* derived from *Shigella sonnei* strain P9.

The present work was done in an attempt to discover whether the transfer of *colI* factors is influenced by the presence of fimbriae in the donor and acceptor bacteria. Fimbriae are non-flagellar filamentous appendages that are present in many strains of most genera of enterobacteria and which confer adhesive properties on the bacteria; fimbriate bacilli are known to adhere to red blood cells, causing haemagglutination, and to other kinds of animal, plant and fungal cells (Duguid, Smith, Dempster & Edmunds, 1955). It seemed possible that they might have an important role in effecting the conjugation of bacteria that is necessary for the transfer of *colI*. Cultures of *Shigella flexneri* were considered to be especially suitable for use in the investigation of the influence of fimbriation on the transfer of *colI* because both genotypically fimbriate (Fim⁺) and genotypically non-fimbriate (Fim⁻) strains occur in several serotypes of the species, and because cultures of the genotypically fimbriate strains can be grown in either a fimbriate (Fim⁺F) or a non-fimbriate (Fim⁺N) phenotype, or 'phase' (Duguid & Gillies, 1957). Comparative experiments can, therefore, be made with comparable fimbriate and non-fimbriate cultures.

METHODS

Colicinogenic factors. Most of the experiments were made with *colI* factor derived from *Shigella sonnei* strain 2, the standard colicine secretor strain no. 2 of Abbot & Shannon (1958), ref. no. 10047 of the Dysentery Reference Laboratory (DRL), Colindale, London. This factor, *colI*-CT2 is referred to as *colI* throughout the present paper.

For the purpose of comparison, a few experiments were made with the different *colI* factor, designated in this paper as *colI*-P9, which had been studied by Stocker *et al.* (1963). This factor was obtained from *Salmonella typhimurium* strain LT2 *cysD*-36 (*colI*-P9), which had been given the factor from *S. sonnei* strain P9, and was received from Dr B. A. D. Stocker. Both the *colI* donor strains, *S. sonnei* 2 and *S. typhimurium* LT2 *cysD*-36 (*colI*-P9), were streptomycin-sensitive; the former was non-flagellate and non-fimbriate (Fim⁻) and the latter was flagellate and fimbriate (Fim⁺).

Donor and acceptor strains of Shigella flexneri. These are listed, with their serotype and fimbriation genotype, in Table 1. Streptomycin-sensitive strains were used as donors of *colI*. The colicinogenic factor had been introduced into them from *Shigella sonnei* strain 2 or *Salmonella typhimurium* strain LT2 *cysD*-36 (*colI*-P9). Where subsequently, in the tables and text, a strain is described as a 'donor', it is to be understood that it carries *colI* even although it is designated by its number, e.g. 1a1, without the suffix *colI*.

Streptomycin-resistant strains were used as acceptors. All these strains were resistant to 1000 µg. streptomycin/ml. They did not produce detectable colicines and they were resistant to the colicines determined by *colI* (i.e. *colI*-CT2) and *colI*-P9. The original cultures of the acceptor strains (Table 1, second column) were colicine-resistant but streptomycin-sensitive; streptomycin-resistant mutants were derived by selection in streptomycin broth and these mutants were used as the acceptors in the experiments.

Table 1. *Donor (colicinogenic) and acceptor (non-colicinogenic) strains of Shigella flexneri used in the experiments*

Designation of strain	Parent strain*	Serotype	Fimbriation genotype
(a) Donor strains (streptomycin-sensitive)			
1a1 <i>colI</i>	NCTC 8192	1a	+
1a1 <i>colI</i> -P9	NCTC 8192	1a	+
2a1 <i>colI</i>	NCTC 8204	2a	—
2b1 <i>colI</i>	NCTC 8518	2b	+
4a1 <i>colI</i>	Edinburgh 4a1	4a	+
4c10 <i>colI</i>	DRL 127/56	4c	—
5a3 <i>colI</i>	NCTC 8523	5a	—
(b) Acceptor strains (streptomycin-resistant)			
1a1	NCTC 8192	1a	+
1b1	Wrocław 6713	1b	—
2a1	NCTC 8204	2a	—
2a19	DRL E/56/9	2a	+
2b1	NCTC 8518	2b	+
3a106	NCTC 8664	3a	+
3c2	Wrocław 6713M†	3c	—
4a1	Edinburgh 4a1	4a	+
4a2	Wrocław 4a	4a	+
4b1	DRL 181/56	4b	+
4b3	DRL E/56/6	4b	+
4c10	DRL 127/56	4c	—
5a3	NCTC 8523	5a	—
5a4	NCTC 8524	5a	+
5a12	DRL 64/56	5a	+
x105	DRL 208/63	X	+
y3	NCTC 1169	Y	+

* Parent strains were non-colicinogenic and streptomycin-sensitive. Donor strains were derived from them by infection with *colI* or *colI*-P9, and acceptors were streptomycin-resistant mutants derived from them. NCTC = National Collection of Type Cultures, Colindale, London. DRL = Dysentery Reference Laboratory, Colindale, London.

† Strain Wrocław 6713M is a serological mutant derived from the serotype 1b strain Wrocław 6713 (Mulezyk, 1960).

Indicator of colI. The indicator strain, sensitive to colicine I and used to detect it, was *Escherichia coli* strain K12-Row (Fredericq; DRL 10044), received from Dr J. D. Abbott.

Culture media. The nutrient broth used was Difco heart infusion broth. Difco heart infusion agar plates were used with or without streptomycin (1 mg./ml.). The defined medium was the glucose ammonium salts minimal medium of Ozeki *et al.* (1962) supplemented with nicotinic acid 1 µg./ml. Trypsin was not used in media.

Fimbriate and non-fimbriate cultures. Fimbriate-phase (Fim⁺F) cultures were obtained by serial culture of Fim⁺ strains at 37° in static tubes of 10 ml. broth. Non-fimbriate-phase (Fim⁺N) cultures were obtained by serial culture of Fim⁺ strains on plates of nutrient agar (Duguid & Gillies, 1957). Before use, the cultures were tested for haemagglutinating activity with a 3% suspension of guinea-pig red cells (method of Duguid & Gillies, 1957) to confirm that they were richly fimbriate or entirely non-fimbriate. The notation used to designate the fimbriation genotype and phenotype (phase) of donor and acceptor cultures is shown in Table 2.

Table 2. *Designations used to indicate the state of fimbriation of donor and acceptor cultures*

Designation	Fimbriation status of culture
Fim ⁻	Genotype non-fimbriate
Fim ⁺	Genotype fimbriate
Fim ⁺ N	Genotype fimbriate but phenotype (or phase) non-fimbriate
Fim ⁺ F	Genotype fimbriate and phenotype (or phase) fimbriate

Preparation of inocula for mixed cultures. The mixed broth cultures in which transmission of *colI* was observed were inoculated with about 10⁷ bacteria of the donor strain and about 10⁷ bacteria of the acceptor strain. These bacteria were taken from 24 hr broth cultures of predetermined fimbriation genotype and phenotype. Phenotypically fimbriate (Fim⁺F) cultures were obtained by carrying a Fim⁺ strain through 4 or 5 serial 48 hr cultures in broth. Genotypically fimbriate but phenotypically non-fimbriate (Fim⁺N) cultures were obtained by making 4 or 5 serial 48 hr cultures on agar plates. The final 48 hr culture of the Fim⁺F or Fim⁺N organisms was subcultured in broth for 24 hr to prepare the inoculum for the experimental mixed culture. Fim⁺N organisms remained in the non-fimbriate phase during this single 24 hr period of growth in broth. Genotypically non-fimbriate (Fim⁻) organisms were inoculated into the mixed culture from a 24 hr broth culture that had been inoculated from a 48 hr broth culture.

Tests for transmission of colI in mixed cultures. Crosses were made by growing the donor and acceptor strains in mixed culture in aerobic static broth for 20 and 48 hr periods at 37°; 10 ml. broth in a test-tube stoppered with cottonwool was incubated under ordinary aerobic conditions without disturbance by movement, mixing or artificial aeration. Because sampling of the culture for examination involved mixing and disruption of any surface pellicle, separate tubes of mixed cultures were grown from similar inocula for each time of sampling (usually 20 and 48 hr) so that the culture and pellicle remained undisturbed until the sample was taken.

After the culture had been inoculated with about 10⁷ donor bacteria and about 10⁷ acceptor bacteria and had been incubated for the test period, it was mixed thoroughly by drawing up and down in a pipette so that any surface pellicle and any deposit were completely and uniformly dispersed. Tenfold dilutions of the culture were then prepared in sterile saline and 0.1 ml. volumes spread on plates of streptomycin nutrient agar. After incubation for 24 hr, the plates were treated with chloroform vapour for 30 min. and then overlaid with a thin layer of nutrient agar (1% agar). They were dried and inoculated by flooding with a broth culture of the indicator strain, *Escherichia coli* K12-Row. After overnight incubation the

proportion (%) of colonies that were colicinogenic, i.e. surrounded by zones of inhibition of growth of the indicator strain, was determined. Generally this result was based on the observation of 200–700 acceptor-strain colonies.

RESULTS

Transfer of colI between genotypically fimbriate (Fim⁺) strains of Shigella flexneri

The first experiments were made with the streptomycin-sensitive Fim⁺ strain 1a1 of *Shigella flexneri* as donor of *colI*. This strain had been rendered colicinogenic by infection with *colI* from *S. sonnei* strain 2. Like other Fim⁺ strains of *S. flexneri*, it could be obtained in a fimbriate phase (Fim⁺F) by serial culture in broth and in a non-fimbriate phase (Fim⁺N) by serial culture on agar. The acceptor strain was a streptomycin-resistant mutant derived from the original streptomycin-sensitive non-colicinogenic stock of strain 1a1; it, too, could be obtained in a fimbriate and a non-fimbriate phase. Crosses of the donor and acceptor strains were tested by growing the two strains together in mixed cultures in tubes of 10 ml. nutrient broth

Table 3. *Frequency of transfer of colI between fimbriate-phase (F) and non-fimbriate-phase (N) organisms of the Fim⁺ strains 1a1 and 2a19 of Shigella flexneri during growth in mixed culture in aerobic static broth for 20 or 48 hr at 37°*

Tubes of 10 ml. broth were inoculated with, per tube, 10⁷ bacteria of the donor (streptomycin-sensitive) culture and 10⁷ bacteria of the acceptor (streptomycin-resistant) culture, and were then incubated without disturbance for either 20 or 48 hr; separate tubes were set up for each time of incubation. After growth, the culture was thoroughly mixed and dilutions of it plated on streptomycin agar so as to yield colonies derived only from the acceptor-strain bacteria. These colonies were tested for colicinogeny.

Cross		Viable count of streptomycin-resistant (acceptor strain) bacteria ($\times 10^{-6}$ /ml.) in mixed culture at		% of acceptor-strain colonies found to be colicinogenic (and no. colicinogenic/no. tested*) at	
Donor bacteria	Acceptor bacteria	20 hr	48 hr	20 hr	48 hr
1a1 F	1a1 F	140	95	37 (302/816)	93 (516/554)
1a1 F	1a1 N	71	42	< 0.3 (0/417)	22 (52/246)
1a1 N	1a1 F	130	89	40 (301/752)	95 (492/518)
1a1 N	1a1 N	62	46	< 0.3 (0/364)	16 (42/262)
1a1 F	2a19 F	130	66	27 (103/380)	81 (150/185)
1a1 F	2a19 N	37	33	1.4 (3/216)	4.3 (4/93)
1a1 N	2a19 F	160	150	25 (111/446)	86 (372/432)
1a1 N	2a19 N	93	78	0.8 (2/258)	1.4 (3/219)

* Numbers in parentheses are: no. of acceptor-strain colonies found to be colicinogenic/no. of acceptor-strain colonies tested.

incubated at 37° under aerobic conditions without agitation, disturbance or artificial aeration. Four crosses were made with the organisms in different combinations of phases: (1) donor Fim⁺F $\times$ acceptor Fim⁺F, (2) donor Fim⁺F $\times$ acceptor Fim⁺N, (3) donor Fim⁺N $\times$ acceptor Fim⁺F, (4) donor Fim⁺N $\times$ acceptor Fim⁺N. The results are shown in Table 3.

After 20 hr incubation of the mixed culture, *colI* had been transmitted to a large

percentage (37 and 40 %) of the acceptor bacteria in the crosses in which the acceptor culture was in the fimbriate phase; but it had not been transmitted even to a barely detectable proportion (about 0.3 %) of the acceptor bacteria in the crosses in which the acceptor culture was in the non-fimbriate phase. Similar results were obtained regardless of whether the donor culture was in the fimbriate or the non-fimbriate phase.

After 48 hr incubation of the mixed culture the proportion of Fim⁺F acceptor bacteria that were colicinogenic had become still larger (93 and 95 %). By this time some of the Fim⁺N acceptors also had become colicinogenic, but the proportion (22 and 16 %) was much less than that in the case of the Fim⁺F acceptors. The late acquisition of *colI* by the originally non-fimbriate (Fim⁺N) acceptor bacteria could possibly have been the result of the conversion of some of these bacteria to the fimbriate phase in the later stages of culture in broth. After 48 hr a few of the cultures that originally were in the non-fimbriate phase showed a small degree of haemagglutinating activity and, therefore, must have contained a small proportion of fimbriate bacteria.

These results, indicating that the state of fimbriation of the acceptor bacteria, but not that of the donor bacteria is important in determining the frequency of transfer of *colI*, were confirmed in further experiments in which strain 1a1 as donor was crossed with Fim⁺F and Fim⁺N cultures of eleven strains of other serotypes as acceptors. The results of 10 of the 11 heterotypic crosses, 1a1 × 2a19, 2b1, 3a106, 4a1, 4a2, 4b1, 5a4, 5a12, X105, Y3, were very similar to those of the homotypic crosses (1a1 × 1a1); the results of the cross 1a1 × 2a19 are shown in Table 3. In crosses with the eleventh strain, 4b3, as acceptor the results were completely negative. Neither the fimbriate nor the non-fimbriate form of strain 4b3 acquired *colI*; this strain seemed to be absolutely incompetent as an acceptor.

Two other Fim⁺ strains, 2b1 and 4a1, were investigated as donors. The acceptors were streptomycin-resistant mutants of the same strains and similar mutants of strains 1a1, 2a19, 3a106, 4a2, 5a12. The crosses gave similar results to those in which strain 1a1 was donor.

*Transfer of colI from genotypically non-fimbriate (Fim⁻)
to genotypically fimbriate (Fim⁺) strains*

Crosses were tested between the Fim⁻ strain 5a3 of *Shigella flexneri* as donor and fimbriate-phase (Fim⁺F) and non-fimbriate-phase (Fim⁺N) bacteria of twelve different Fim⁺ strains as acceptors. The results are given in Table 4. Just as in the crosses with Fim⁺ donors, the rate of transfer of *colI* from the Fim⁻ donor was high when the acceptor bacteria were in the fimbriate phase and low when they were in the non-fimbriate phase. This finding was obtained with 11 of the 12 acceptor strains. The twelfth strain, 4b3, proved to be absolutely incompetent as an acceptor, regardless of whether it was tested in the fimbriate or non-fimbriate phase.

Crosses were also made with the Fim⁻ strains 2a1 and 4c10 of *Shigella flexneri* and the Fim⁻ strain 2 of *S. sonnei* as donors. They gave similar results to those obtained with strain 5a3 as donor.

Table 4. *Transfer of colI from Shigella flexneri* bacteria of *Fim*⁻ strain 5a3 as donors to fimbriate-phase (*Fim*⁺*F*) and non-fimbriate-phase (*Fim*⁺*N*) bacteria of 12 different *Fim*⁺ strains as acceptors during growth in mixed culture for 20 or 48 hr

Crosses were made in 10 ml. broth inoculated with 10⁷ donor and 10⁷ acceptor bacteria and tested as described in Table 3. In each cross and at each period of incubation, between 200 and 700 acceptor-strain colonies were tested for colicinogeny (only 50 colonies in culture with acceptor 1a1 *Fim*⁺*N* at 20 hr.).

Acceptor strain	Viable count ($\times 10^{-6}$ /ml.) of the acceptor-strain bacteria in the culture in which they were				% of acceptor-strain bacteria found to be colicinogenic in the culture in which they were			
	<i>Fim</i> ⁺ <i>F</i>		<i>Fim</i> ⁺ <i>N</i>		<i>Fim</i> ⁺ <i>F</i>		<i>Fim</i> ⁺ <i>N</i>	
	20 hr	48 hr	20 hr	48 hr	20 hr	48 hr	20 hr	48 hr
1a1	98	70	29	20	10	97	< 2.0	2.8
2a19	110	80	64	51	14	57	< 0.3	1.6
2b1	100	79	56	40	20	75	< 0.3	0.5
3a106	130	93	69	42	20	54	2.7	5.6
4a1	130	91	67	52	36	92	4.3	7.0
4a2	120	86	73	43	16	74	3.9	11.4
4b1	140	94	69	54	22	75	3.0	4.2
4b3	115	82	61	48	< 0.2	< 0.3	< 0.3	< 0.4
5a4	110	76	62	46	17	62	1.3	3.1
5a12	96	76	44	39	14	58	< 0.5	0.5
x105	130	92	68	47	19	54	2.1	9.1
y3	125	88	71	49	16	44	1.2	5.0

Table 5. *Transfer of colI between different genotypically non-fimbriate strains of Shigella flexneri* during growth in mixed culture for 20 or 48 hr

Crosses were made in 10 ml. broth inoculated with 10⁷ donor and 10⁷ acceptor bacteria and tested as described in Table 3. The viable counts ($\times 10^{-6}$ /ml.) of the acceptor-strain bacteria ranged between 25 and 120 in the different 20 hr cultures, and between 22 and 76 in the 48 hr cultures.

Cross		% of acceptor-strain bacteria found to be colicinogenic (and no. colicinogenic/no. tested) in mixed culture tested at	
Donor (<i>Fim</i> ⁻) strain	Acceptor (<i>Fim</i> ⁻) strain	20 hr	48 hr
2a1	1b1	< 0.9 (0/122)	< 1.1 (0/86)
	2a1	5.2 (26/502)	6.2 (20/314)
	3c2	< 0.5 (0/192)	< 0.7 (0/143)
	4c10	4.6 (6/128)	13.3 (23/172)
	5a3	< 0.3 (0/346)	< 0.3 (0/317)
4c10	1b1	< 1.0 (0/104)	< 1.2 (0/82)
	2a1	1.4 (5/354)	2.4 (6/248)
	3c2	< 0.6 (0/176)	< 1.1 (0/87)
	4c10	< 0.8 (0/123)	< 1.1 (0/92)
	5a3	< 0.3 (0/326)	< 0.5 (0/216)
5a3	1b1	< 0.5 (0/207)	1.6 (2/124)
	2a1	10.7 (42/392)	21.0 (62/295)
	3c2	< 0.4 (0/244)	2.9 (4/136)
	4c10	10.1 (19/188)	19.1 (25/131)
	5a3	6.2 (17/274)	13.2 (21/159)

Transfer of colI to genotypically non-fimbriate (Fim⁻) strains as acceptors

The results of crosses between strains that are Fim⁻ in genotype, i.e. which never form fimbriae, are given in Table 5. Three streptomycin-sensitive Fim⁻ strains carrying *colI* were tested as donors and 5 streptomycin-resistant non-colicinogenic Fim⁻ strains as acceptors. After 48 hr incubation of the mixed culture the proportion of acceptor bacteria that had acquired *colI* was, in most crosses, either small or undetectable. Nevertheless, it is noteworthy that some transfer of *colI* from Fim⁻ donor to Fim⁻ acceptor was detected in at least one cross with each of the 3 donors, and in at least one cross with each of the 5 acceptors. In 3 crosses with strain 5a3 as donor and in one with strain 2a1 as donor the percentage of colicinogenic acceptor bacteria was somewhat higher (21, 19, 13 and 13 %) than in the other Fim⁻ × Fim⁻ crosses, but it was still not nearly as high as in comparable crosses with fimbriate acceptor bacteria (e.g. 44–97 %, Table 4).

Other crosses were made between Fim⁺F bacteria of strain 1a1 as donors and the Fim⁻ strains 1b1, 2a1, 3c2, 4c10 and 5a3 as acceptors. The percentage of colicinogenic acceptor bacteria at 48 hr was never greater than 21 and in most crosses it was much less. These results confirm the relative incompetence of Fim⁻ bacteria, as compared with Fim⁺F bacteria, as acceptors of *colI*, and also show that this incompetence applies when the donor bacteria are fimbriate as well as when they are non-fimbriate.

Table 6. *Transfer of colI between donor and acceptor bacteria of Shigella flexneri Fim⁺ strain 1a1 in mixed cultures inoculated with the donor and acceptor bacteria in different proportions*

Tubes of 10 ml. broth were inoculated with the stated numbers (per tube) of donor bacteria in the fimbriate (F) phase and acceptor bacteria in the fimbriate (F) or non-fimbriate (N) phase, and were incubated for 20 or 48 hr; separate tubes were set up for each period of incubation. Between 532 and 628 acceptor-strain bacteria from each culture (tube) were tested for colicinogeny.

Cross Fimbrial phase and no. of bacteria inoculated		Viable count (× 10 ⁻⁶ /ml.) of donor + acceptor bacteria (and of acceptors only) at		% of acceptor-strain bacteria found to be colicinogenic at	
Donor bacteria	Acceptor bacteria	20 hr	48 hr	20 hr	48 hr
F, 10 ⁷	F, 10 ⁴	420 (0.37)	380 (0.29)	22	69
F, 10 ⁷	N, 10 ⁴	410 (0.16)	370 (0.13)	< 0.2	2.9
F, 10 ⁴	F, 10 ⁷	390	330	0.7	34
F, 10 ⁴	N, 10 ⁷	380	320	< 0.2	< 0.2

Influence of the relative numbers of the donor and acceptor bacteria in the mixed culture

In the experiments (e.g. see Tables 3, 4) in which Fim⁺F and Fim⁺N bacteria of the same strain were compared as acceptors of *colI* in crosses with the same donor culture, the viable count of the acceptor-strain bacteria in the mixed culture after 20 or 48 hr was 2–3 times greater when the acceptors were Fim⁺F than when they were Fim⁺N. This finding agrees with the observations of Duguid & Gillies (1957) and Duguid & Wilkinson (1961) that fimbriate bacteria grow to higher concentra-

tions in aerobic static broth than do non-fimbriate bacteria; they do this apparently as a result of their ability to grow as a pellicle on the surface of the broth where they have free access to atmospheric oxygen. The question therefore arises as to whether the infrequency of transfer of *colI* to non-fimbriate acceptor bacteria was due to the growth and metabolism of these acceptor bacteria being depressed in the post-logarithmic phase of culture through their being overgrown by the fimbriate donor bacteria. This explanation is not supported by the results of mixed-culture experiments in which donor and acceptor bacteria of strain 1a1 were inoculated into broth in different proportions (Table 6). The relative numbers of the donor and acceptor bacteria did not have a very great influence on the frequency of transfer of *colI*. Indeed, in a cross in which non-fimbriate (Fim^+N) acceptor bacteria in the inoculum outnumbered fimbriate (Fim^+F) donor bacteria by 1000 to 1 ($\text{F}, 10^4$; $\text{N}, 10^7$), no transfer of *colI* was detected at 48 hr.

Transfer of colI-P9 from Salmonella typhimurium to Shigella flexneri

Ozeki *et al.* (1962) showed that when *Salmonella typhimurium* strain LT2 *cysD*-36 carrying *colI*-P9 was grown overnight in mixed culture in broth with a non-colicinogenic line of the same strain, about 50 % of the latter bacteria acquired *colI*-P9. We made tests with strain LT2 *cysD*-36 *colI*-P9, which is Fim^+ , to determine whether it was as efficient in donating *colI*-P9 to *Shigella flexneri* as it was to *S. typhimurium*, and whether the frequency of transfer of *colI*-P9 was influenced by the state of fimbriation of the acceptor bacilli in the same way as was the transfer of *colI* from *S. flexneri* donors. *S. typhimurium* LT2 *cysD*-36 *colI*-P9 was crossed with 5 Fim^- and 12 Fim^+ strains of *S. flexneri* as acceptors; the Fim^+ acceptors were tested both in the fimbriate and the non-fimbriate phase. Mixed cultures were grown in 10 ml. broth from inocula of 10^7 donor and 10^7 acceptor bacteria. The proportions of acceptor-strain bacteria found to be colicinogenic after 20 and 48 hr were invariably low in the crosses with Fim^- acceptors (under 2.5 %) and Fim^+N acceptors (under 7 %), but were high (e.g. 40 %) in those with Fim^+F acceptors. These results of crosses with the *S. typhimurium* donor were very similar to the results of the crosses with *S. flexneri* donors. A minor difference was that in the crosses with *S. typhimurium* the proportion of colicinogenic acceptor bacteria did not continue to increase when incubation of the mixed culture was prolonged from 20 to 48 hr, but instead became somewhat smaller again. The absence of continued transfer between 20 and 48 hr may have been due to the fact that the *S. flexneri* acceptor bacteria became very much overgrown by the *S. typhimurium* donor bacteria. At 48 hr the viable counts ($\times 10^6$)/ml. in the mixed culture were: *S. typhimurium* 570 and *S. flexneri* 5.7 when *S. flexneri* was Fim^+F , and *S. typhimurium* 570 and *S. flexneri* 2.6 when *S. flexneri* was Fim^+N .

Kinetics of transfer of colI

The frequency of transfer of *colI* was observed after different periods of incubation from 2 to 72 hr in crosses between Fim^+F bacilli of *Shigella flexneri* strain 1a1 as donors and Fim^+F bacteria of *S. flexneri* strains 1a1 and 2a19 as acceptors. A separate tube of mixed broth culture was set up for examination after each period of incubation and about 500 acceptor-strain colonies in platings from each culture were

examined for colicinogeny. The results of the experiment with strain 1a1 as acceptor are given in Table 7; almost identical results were obtained in a similar experiment with strain 2a19.

No transfer of *colI* was detected after the first 2 hr of incubation, but after 4 hr a small proportion (0.3 %) of acceptor bacteria possessed *colI* and the proportion of acceptors with *colI* increased slowly up to 12 hr (4 %) and then more rapidly up to 24 hr (30 %). By 48 hr, almost all (97 %) of the acceptor bacteria were colicinogenic. The transfer of *colI* thus took place mainly in the period of slow post-logarithmic growth, which began after the first 6 hr of incubation. Most transfers took place after the 12th hr, by which time a pellicle had developed on the surface of the culture.

Table 7. *Kinetics of transfer of colI between fimbriate-phase donor and acceptor bacteria of Shigella flexneri strain 1a1 during growth in mixed culture at 37°*

Nine tubes of 10 ml. broth were each inoculated with 10^7 Fim⁺F donor bacteria and 10^7 Fim⁺F acceptor bacteria and incubated under aerobic static conditions at 37° for different periods up to 72 hr. After incubation, the culture was homogenized and tested for the viable count of acceptor-strain bacteria and the proportion of acceptor-strain bacteria which were colicinogenic. A separate tube of mixed culture was used for each period of incubation so that it and any pellicle it formed were undisturbed until the time of testing.

Period of incubation of mixed culture (hr)	Viable count of acceptor-strain bacteria ($\times 10^{-6}$ /ml.)	% of acceptor-strain bacteria found to be colicinogenic (and no. colicinogenic/no. tested)
0	1.0	.
2	5.6	< 0.2 (0/528)
4	68	0.3 (2/688)
6	130	0.4 (2/548)
8	180	2.0 (13/647)
10	190	3.2 (18/571)
12	190	4.1 (23/562)
24	180	30 (187/624)
48	120	97 (585/603)
72	89	100 (442/442)

There seem to be two possible explanations for the very low frequency of transfer of *colI* in the early hours of incubation of the mixed cultures. (1) The initial concentration of the inoculated bacteria (donors and acceptors each about 10^6 /ml.) may have been so low that collisions and contacts between bacteria, and thus opportunities for transfer of *colI*, were rare until the numbers of bacteria had increased as a result of several hours' growth. Against this explanation, however, should be noted the fact that transfer was still relatively infrequent throughout the period between the 4th and 12th hours of incubation, i.e. in a period when the number of viable bacteria was nearly maximal. A possible reason for the finding that the majority of transfers took place after 12 hr is that the best opportunities for contact and transfer of *colI* between bacteria existed in the pellicle of bacteria which developed on the surface of the broth after about the 10th hour of incubation and in which most of the slow post-logarithmic growth of bacteria presumably took place.

(2) Only a small proportion of the bacteria of the donor cultures may have been competent to transmit the *colI* factor. Ozeki *et al.* (1962) found that the low fre-

quency of transfer of *colI*-P9 during the first 6 hr of incubation of mixed cultures of donor and acceptor stocks of *Salmonella typhimurium* LT2 *cysD*-36 was due to the fact that only about 0.02 % of the bacteria in the donor cultures were competent to transmit the *colI*-P9 factor. They explained the high rate of transfer after prolonged (20 hr) incubation as being the result of an 'epidemic' spread of *colI*-P9 which took place through the acceptor population during the period of slow post-logarithmic growth. Apparently a large proportion of the freshly infected acceptor bacteria become competent donors, able to conjugate with other acceptor bacteria and pass *colI*-P9 into them. Stocker *et al.* (1963) showed that 50 % of the *S. typhimurium* bacteria which had freshly acquired *colI*-P9 became competent donors and were able to pass the factor into acceptor bacteria with high frequency. Preparations with a high proportion of competent donor bacteria were named HFC preparations (high-frequency colicinogeny-transferring); when mixed with acceptor bacteria at concentrations of more than 10^8 bacteria/ml., they transmitted *colI*-P9 to about 50 % of the acceptor bacteria in 1 hr.

Attempt to prepare an HFC culture of Shigella flexneri

The technique of Stocker *et al.* (1963) was used. About 5×10^5 Fim⁺F bacteria of streptomycin-sensitive *Shigella flexneri* strain 1a1 carrying *colI* or *colI*-P9 as original donors and about 10^7 Fim⁺F non-colicinogenic streptomycin-sensitive 1a1 bacteria as intermediate donors were inoculated into 10 ml. broth and the mixed culture incubated for 18 hr. The culture was then diluted tenfold with fresh broth and incubated for a further 2 hr. After the first 18 hr of incubation the proportion of colicinogenic bacteria was, in different experiments, between 17 and 33 %, and after dilution and secondary incubation for 2 hr it was only about half as much (9-21 %). These values are much lower than those obtained by Stocker *et al.* with *Salmonella typhimurium*, namely 30-70 % after the first 18 hr and 50-90 % after secondary 2 hr incubation. Thus *S. flexneri* did not behave in the same manner as *S. typhimurium* and our attempt to prepare an HFC culture was not pursued. A possible reason for the different behaviour of *S. flexneri* may lie in its non-motility, since Stocker *et al.* found that motility was important in the development of HFC preparations of *S. typhimurium*. They found that when the intermediate (non-colicinogenic) strain was non-motile, the proportion of the bacteria that acquired colicinogeny after primary incubation for 18 hr and secondary incubation for 2 hr was less than 1 %, whereas when the intermediate strain was motile the proportion was about 70 %.

'Epidemic' spread of colI within the acceptor population

Although we failed to demonstrate by the production of an HFC culture that *Shigella flexneri* organisms which have been freshly infected with *colI* are more effective in transmitting *colI* than the bacteria of a long-established colicinogenic culture, it still seemed possible that such bacteria, like comparable *Salmonella typhimurium* organisms, may be exceptionally competent as donors. If they are specially competent, then it is likely that the majority of the acceptor bacteria that become colicinogenic during 48 hr in a mixed culture do so as a result of the *colI* factor spreading 'epidemically' from one acceptor bacterium to another, and not by acquiring the factor directly from the bacteria of the donor strain. An experiment

was made to determine whether such an 'epidemic' spread of *colI* took place within the acceptor population. The method of the experiment involved adding streptomycin (1 mg./ml.), to a mixed culture to kill the streptomycin-sensitive donor bacteria after the culture had been incubated for only 8 hr and when only a few bacteria of the streptomycin-resistant acceptor strain had acquired *colI*; the culture was then re-incubated for the remainder of a period of 20 or 48 hr. It was found that the proportion of acceptor bacteria possessing *colI* increased very greatly during the secondary incubation and, since all the donor strain bacteria were then dead, the increase must have been the result of an 'epidemic' spread of *colI* taking place through the population of acceptor-strain bacteria.

Table 8. *Transfer of colI between donor and acceptor Shigella flexneri organisms grown in mixed cultures with and without the addition of streptomycin after the first 8 hr*

For each cross, 4 tubes of 10 ml. broth were inoculated with 10^6 streptomycin-sensitive donor bacteria and 10^7 streptomycin-resistant acceptor bacteria per tube. After incubation at 37° for 8 hr, each culture was mixed until homogeneous and samples were taken from it for estimation of the viable count of acceptor-strain bacteria and the proportion that were colicinogenic. Streptomycin (1 mg./ml.) was added to two of the cultures and all four were then reincubated. A streptomycin-treated and an untreated culture were examined after a total of 20 hr incubation and the other two cultures at 48 hr.

Cross		Treatment at 8 hr	Viable count ($\times 10^{-6}$ /ml.) of acceptor-strain bacteria at			% of acceptor- strain bacteria found to be colicinogenic at		
Donor bacteria	Acceptor bacteria		8 hr*	20 hr	48 hr	8 hr†	20 hr	48 hr
1a1 Fim ⁺ F	1a1 Fim ⁺ F	Untreated	320	360	340	0.44	30	76
		Streptomycin	320	355	345	0.45	0.37	62
1a1 Fim ⁺ F	2a1 Fim ⁻	Untreated	290	305	215	0.28	12	24
		Streptomycin	290	310	220	0.29	0.24	0.47
5a3 Fim ⁻	1a1 Fim ⁺ F	Untreated	335	345	335	0.69	37	87
		Streptomycin	335	340	260	0.34	1.8	69

* Results are averages for the four tubes of the cross. † Results are averages for the two tubes that were later examined at 20 and 48 hr.

Table 8 shows the results of experiments made in this way on three crosses of *Shigella flexneri* strains: (1) donor Fim⁺F $\times$ acceptor Fim⁺F, (2) donor Fim⁺F $\times$ acceptor Fim⁻; (3) donor Fim⁻ $\times$ acceptor Fim⁺F. In each of the crosses a small proportion (0.28–0.69%) of the acceptor bacteria had acquired *colI* by the end of the first 8 hr of incubation. In the cultures with Fim⁺F acceptors that were treated with streptomycin at 8 hr and then reincubated until 48 hr, the proportion of acceptor bacteria possessing *colI* rose to values (62 and 69%) nearly as high as those (76 and 87%) in the control cultures without streptomycin. On the other hand, the proportion of colicinogenic acceptor bacteria remained very low (under 0.5%) in the streptomycin-treated cultures in which the acceptor was Fim⁻. Thus it appeared that *colI* spread epidemically through the population of acceptor bacteria when these were fimbriate (Fim⁺F), but did not do so when they were non-fimbriate (Fim⁻). The continuing acquisition of *colI* by Fim⁺F acceptor bacteria after the donor-strain bacteria had been killed with streptomycin must have been due to an epidemic

spread of *colI* that originated from the few acceptor bacteria that had acquired *colI* from the donor-strain bacteria before the addition of streptomycin. It may be assumed that the acceptor bacteria did not acquire *colI* from the killed donor bacteria, since it was found that when streptomycin was added at 2 hr instead of 8 hr, i.e. before any acceptor bacteria had become colicinogenic, none of the acceptor bacteria became colicinogenic during secondary incubation for 48 hr.

An interesting difference in the results between the streptomycin-treated and the untreated crosses with Fim⁺F acceptors (Table 8) is seen in the finding that in the streptomycin-treated cultures the increase in the proportion of colicinogenic acceptor bacteria was delayed until after the 20th hour of incubation, whereas in the untreated cultures it was already very great at 20 hr (compare 0.37 with 30 %). This finding seems to indicate that in the untreated cultures the large increase in colicinogenic acceptors that took place between 8 and 20 hr was mainly due to transfer of *colI* from the donor-strain bacteria to the acceptor-strain bacteria, and not to epidemic spread among the acceptors. It may perhaps also be concluded that the proportion of the donor-strain bacteria in the culture at 8 hr that were 'competent donors' of *colI* was at least much greater than about 0.5 %, which was the proportion of the tenfold larger population of acceptor bacteria that by 8 hr had become colicinogenic. In contrast, Ozeki *et al.* (1962) found that only about 0.02 % of the bacteria in an established colicinogenic stock of *Salmonella typhimurium* strain LT2 were 'competent donors'. However, these various conclusion are valid only on the assumption that the streptomycin added to our cultures had no effect other than that of killing the donor-strain bacteria.

Control tests indicated that the increase of colicinogenic bacteria in the streptomycin-treated cultures was not due to the emergence, survival and multiplication of streptomycin-resistant mutants of the colicinogenic donor strain. The rates of mutation of strains 1a1 and 5a3 to resistance to 1 mg. streptomycin/ml. are very low, less than 1 per 10¹¹ bacteria, and the number of viable donor-strain bacteria in the mixed cultures at 8 hr cannot have exceeded about 10⁹/10 ml. tube. Fifty pure cultures of the donor strain 1a1 and 50 of the donor strain 5a3 were grown for 8 hr in tubes of 10 ml. broth inoculated with about 10⁶ bacteria; streptomycin was then added and incubation continued until 48 hr. None of these control cultures yielded a growth of streptomycin-resistant mutants. Serological agglutination tests were made on 400 streptomycin-resistant colicinogenic colonies obtained in platings grown from the streptomycin-treated mixed cultures of 5a3 donor and 1a1 acceptor bacteria after they had been incubated for 20 and 48 hr (Table 8) and on another 400 such colonies from the corresponding cultures grown without the addition of streptomycin; all 800 colonies were found to be of the acceptor (1a) serotype. This finding confirms that the streptomycin-resistant colicinogenic organisms were not donor strain (5a) bacteria which had acquired resistance to streptomycin either by spontaneous mutation or by transfer of a resistance factor from the acceptor bacteria.

Pellicle formation and the transmission of colI

In cultures of *Shigella flexneri* in aerobic static broth the development of fimbriae is associated with the formation on the surface of the broth of a thin pellicle composed of aggregated bacteria. In cultures inoculated with fimbriate-phase organisms, e.g. Fim⁺F 1a1 bacteria, the pellicle appears after about 10 hr of incubation

(Duguid & Wilkinson, 1961). Since, in our crosses with Fim⁺F acceptors, the main increase in the proportion of colicinogenic acceptor bacteria took place only in the period of incubation after the first 10–12 hr (e.g. see Table 7), it seemed possible that the conjugation of bacteria and the transfer of *colI* were effected principally in the pellicle. To investigate this possibility the following experiment was done.

A tube of 10 ml. broth was inoculated with 10⁷ Fim⁺F donor bacteria and 10⁷ Fim⁺F acceptor bacteria and incubated under aerobic static conditions for 8 hr. The culture was then homogenized and examined for colicinogenic acceptor bacteria; the proportion of these was found to be about 1 %. The culture then was re-incubated for 12 hr, but during this second period of incubation it was gently agitated at intervals of 1 hr. The intermittent agitation was sufficient to prevent the formation of a surface pellicle, but it could not have effectively aerated the culture or have interfered mechanically with the conjugation of bacteria, since the culture was static for the greater part of the 12 hr period. After the total of 20 hr of incubation, the proportion of colicinogenic acceptor bacteria was found to have increased only to 7 %. By contrast, the proportion in an unagitated control culture increased from 1 % at 8 hr to 34 % at 20 hr. Both the agitated and control cultures showed strong haemagglutinating activity, indicating that they were richly fimbriate. When the agitated culture was left undisturbed for a further 28 hr at 37°, a surface pellicle appeared and the proportion of colicinogenic acceptor bacteria increased to 95 %. These findings seem to indicate that the formation of a surface pellicle facilitates the transmission of *colI*.

Influence of aeration of the mixed culture on the transfer of colI

When broth cultures (2 ml. broth in a 20 ml. bottle) inoculated with Fim⁺F donor and Fim⁺F acceptor bacteria of strain 1a1 were agitated continuously in a rotating machine while being incubated for 20 hr, the proportion of colicinogenic acceptor bacteria did not increase above 2 %. The cultures did not form a pellicle, although they were moderately richly fimbriate. The poor transfer of *colI* may, as in the previous experiment, have been due to the effect of agitation in preventing pellicle formation. However, an additional factor may have played a part in this experiment in which agitation was continuous and aeration therefore efficient. In the continuously aerated culture, the growth proceeds rapidly and gives a maximum yield of bacteria within about 6 hr. After this time it ceases abruptly, probably as a result of exhaustion of the oxidizable energy-yielding nutrients in the medium. Ozeki *et al.* (1962), who found that transfer of *colI*-P9 in mixed cultures of *Salmonella typhimurium* grown in aerobic static broth took place mainly during the prolonged phase of slow post-logarithmic growth, and that aeration by continuous shaking prevented transfer of the *colI* factor, considered that the inhibition produced by aeration might be due to the effect of aeration in eliminating the phase of post-logarithmic growth.

Transfer of colI in mixtures of separately grown cultures of donor and acceptor strains

In all the experiments described above the mixed cultures were grown from relatively small inocula, e.g. about 10⁷ donor and 10⁷ acceptor bacteria, and this procedure allowed the bacteria to pass through a substantial period of rapid logarithmic

growth. Other experiments were made under different conditions. The donor and acceptor strains were first grown separately for 24 hr in tubes of aerobic static broth. These post-logarithmic cultures, which contained $1-4 \times 10^8$ viable bacteria/ml., were mixed together in a tube, 5 ml. with 5 ml., and the mixture was incubated for a period of 8, 20 or 48 hr without the addition of any fresh broth. This procedure caused the donor and acceptor bacteria to be mixed in high concentrations from the outset and it permitted only a slow and limited growth in the mixture; the total count (turbidity) increased about twofold in 48 hr.

Table 9. *Transfer of colI between 24 hr fimbriate-phase (F) and non-fimbriate-phase (N) broth cultures of Shigella flexneri strain 1a1 after mixture and incubation without the addition of fresh broth*

For each cross, 3 tubes were set up containing a homogeneous mixture of 5 ml. of a 24 hr broth culture of the donor bacteria and 5 ml. of a 24 hr broth culture of the acceptor bacteria. The mixtures were incubated under aerobic static conditions at 37° for 8, 20 or 48 hr before harvesting and testing.

Cross		Viable count ($\times 10^{-6}$ /ml.) of acceptor-strain bacteria at			% of acceptor-strain bacteria found to be colicinogenic at		
Donor bacteria in phase	Acceptor bacteria in phase	8 hr	20 hr	48 hr	8 hr	20 hr	48 hr
F	F	120	100	75	1.2	66	91
F	N	51	42	31	< 0.4	< 0.4	22
N	F	140	120	100	0.9	50	92
N	N	54	38	32	< 0.3	< 0.5	4.8

Table 9 shows the results of crosses between Fim⁺F and Fim⁺N cultures of *Shigella flexneri* strain 1a1. Separate tubes of each mixture were put up for examination after 8, 20 and 48 hr. There was little transfer of *colI* during the first 8 hr after mixing, although throughout this period the donor and acceptor bacteria were exposed to each other in high concentrations and under conditions favourable to slow post-logarithmic growth. A few colicinogenic acceptor bacteria were found at 8 hr in the mixtures in which the acceptors were in the fimbriate phase, but the proportion of these bacteria (about 1 %) was no higher than it was in comparable cultures grown for 8 hr from small inocula. The delay in transfer of *colI* may have been due to the time taken for re-establishment of a surface pellicle after the mixing of the cultures.

In the crosses in which the acceptor was Fim⁺F the proportion of colicinogenic acceptor bacteria increased sharply after the first 8 hr and reached fairly high values (50 and 66 %) at 20 hr, and very high values (91 and 92 %) at 48 hr. The crosses in which the acceptor was Fim⁺N did not yield colicinogenic acceptor bacilli until 48 hr and then did so only in a proportion much lower (4.8 and 22 %) than that in the crosses with Fim⁺F acceptors. These results are very like the results obtained for mixed cultures grown from small inocula.

When the donor and acceptor cultures were grown for several (up to 7) days before they were mixed, the proportions of colicinogenic acceptors after 20 and 48 hr of incubation of the mixture were almost as high as those in the mixtures of 24 hr cultures. When, however, the parent cultures were still older the rate of transfer of

colI was decreased. Thus, in a cross of donor 5a3 Fim⁻ with acceptor 1a1 Fim⁺F, the percentages of colicinogenic acceptor bacteria at 20 and 48 hr were 36 and 97 when the age of the parent cultures before mixture was 24 hr, 75 and 98 when the age was 48 hr, 36 and 95 when it was 5 days, 20 and 76 when it was 10 days, 1 and 27 when it was 15 days, and < 0.2 and 8 when it was 20 days. The diminishing rate of transfer in the mixtures of the older cultures may have been due to the diminishing viability of the bacteria in them.

In the experiments just described, the parent cultures and the mixture of them were incubated under aerobic static conditions, which permitted them to form a pellicle and undergo a prolonged phase of slow post-logarithmic growth. When, instead, the separate parent cultures (donor 1a1 Fim⁺F and acceptor 1a1 Fim⁺F) were grown for 24 hr with continuous agitation and aeration, no detectable transfer of *colI* took place when the mixture was incubated under aerobic static conditions for 48 hr. Ozeki *et al.* (1962) obtained a similar result with aerated cultures of *Salmonella typhimurium* and they attributed the absence of transmission to the energy sources in the medium being exhausted before the cultures were mixed.

Transfer of colI in defined medium

A high rate of transmission of *colI* was demonstrated in mixed cultures of donor 5a3 Fim⁻ and acceptor 1a1 Fim⁺F bacteria grown in liquid glucose ammonium salts medium supplemented with nicotinic acid. Strain 1a1 formed a delicate pellicle on the surface of this medium and the culture gave a positive haemagglutination reaction, indicating that it was fimbriate. Separate cultures of the donor and acceptor bacteria were grown in the defined medium to provide the inocula for the mixed cultures. The mixed cultures, which were inoculated with 10⁷ donor and 10⁷ acceptor bacteria, were grown in 10 ml. defined medium under aerobic static conditions for 48 hr. At the end of this period, in different experiments, between 37 and 58 % of the acceptor bacteria were colicinogenic. When the bacteria from donor and acceptor cultures which had been grown for 48 hr in defined medium were washed twice in saline, resuspended in equal proportion and to their original concentration in fresh defined medium, and then incubated for another 48 hr, the proportion of the acceptor bacteria, that became colicinogenic ranged from 46 to 74 %. When such mixtures of washed donor and acceptor bacteria (> 10⁸ viable bacteria/ml.) were incubated in defined medium that lacked either glucose or nicotinic acid, both of which were essential for growth, there was little or no transfer of *colI* (< 1 %).

DISCUSSION

The colicinogenic factor *colI* was found to be transmitted between the bacteria of different strains and serotypes of *Shigella flexneri* when these bacteria were grown together in mixed culture in aerobic static broth for 20 or 48 hr at 37°. Transmission, though at a low rate, was observed in crosses in which both the donor and acceptor organisms were genotypically non-fimbriate (Fim⁻) and it is therefore clear that the presence of fimbriae is not *essential* for transmission. By 'fimbriae', in this context, we mean the ordinarily numerous kind of non-flagellar filaments that are easily demonstrable by electron microscopy and haemagglutination tests. Scanty 'F fimbriae' ('F pili') of the kind demonstrated by Crawford & Gesteland (1964)

and Brinton, Gemski & Carnahan (1964) in *Escherichia coli* and thought by the latter authors to serve as an organ of conjugation in enterobacteria, would not have been detected by us if they had been present in the strains of *S. flexneri* that we have designated Fim⁻.

A high rate of transmission of *colI* was obtained only in those crosses in which the acceptor *Shigella flexneri* organisms were both genotypically and phenotypically fimbriate (Fim⁺F). Under these conditions, and regardless of whether the donor organisms were fimbriate or non-fimbriate, usually between 50 and 95 % of the acceptor bacteria became colicinogenic during growth for 48 hr in mixed culture. When the acceptor bacteria were either genotypically non-fimbriate (Fim⁻) or genotypically fimbriate but in the non-fimbriate phase (Fim⁺N), the proportion of them which became colicinogenic was usually only between 0.2 and 20 %. The expression of fimbriation in the acceptor bacteria thus had the effect of promoting a high rate of transmission, whereas the state of fimbriation in the donor bacteria was without effect.

In many respects the conditions and course of transmission of *colI* observed in our experiments with fimbriate *Shigella flexneri* organisms resembled those observed by Ozeki *et al.* (1962) and Stocker *et al.* (1963) in similar experiments with the fimbriate strain LT2 *cysD*-36 of *Salmonella typhimurium*. Thus, in both species the transmission of *colI* factor in mixed cultures in aerobic static broth took place mainly in the period of slow post-logarithmic growth, and in the case of both species there was evidence that this transmission involved an extensive 'epidemic' spread of the *colI* factor within the acceptor population, i.e. from freshly infected acceptor bacteria, to other acceptor bacteria. In both species, too, transmission was almost completely absent when the mixed cultures were aerated by continuous agitation. A difference in behaviour between the species was noted in our failure to obtain an HFC preparation of *S. flexneri* that contained a high proportion of colicinogenic organisms after 2 hr of secondary incubation of the mixture of the original donor and the intermediate strain. Since, however, Stocker *et al.* also failed to obtain a high proportion of colicinogenic organisms in such cultures when they used a non-motile mutant of *S. typhimurium* as the intermediate strain, our poor result with non-motile *S. flexneri* is not surprising. Ozeki *et al.* and Stocker *et al.* did not use any Fim⁻ strain or any proven non-fimbriate-phase culture of *S. typhimurium*, so that it is not known whether the presence of fimbriae in the acceptor bacteria promotes transmission of *colI* in *S. typhimurium* in the same way as it does in *S. flexneri*.

The reason why transmission of *colI* is promoted by the presence of fimbriae in *Shigella flexneri* acceptors has not been determined. The finding that fimbriae (of the ordinarily demonstrable kind) are not essential for transmission makes it seem unlikely that they act as specific organs for transfer and intromission of the *colI* factor. It may be, however, that they facilitate the adhesion and conjugation of organisms as a necessary preliminary to transfer. There is good evidence to show that fimbriae have the effect of causing bacteria to adhere to various kinds of animal, plant and fungal cells, but none to show that they cause bacteria which are submerged in a liquid medium to adhere to one another. On the other hand, it has been found that the presence of fimbriae in *S. flexneri* organisms causes these, when cultured in aerobic static broth, to grow aggregated together in large numbers in a

pellicle that appears on the surface of the broth after incubation for about 10 hr (Duguid & Gillies, 1957); some Fim⁻ strains also may form a pellicle, but they do this at a later stage in culture, e.g. after 2-3 days. It may be supposed that the aggregation of fimbriate bacteria in a pellicle increases the opportunity for conjugation and transfer of *colI* between them. That pellicle formation is indeed responsible for the high rate of transfer obtained in crosses with fimbriate acceptors is suggested by our finding that relatively few acceptor bacteria became colicinogenic in mixed cultures in which pellicle formation was prevented by gentle shaking at intervals of 1 hr.

Another way in which fimbriation may help to promote transfer of *colI* is by its increasing, through the effect of pellicle formation, the amount of growth during the post-logarithmic phase in the mixed culture. Duguid & Wilkinson (1961, see fig. 3) found that in fimbriate-phase cultures of *Shigella flexneri* strain 1a1 in aerobic static broth the total count of bacteria increased sevenfold between the 6th hr of incubation, when logarithmic growth ended, and the 48 hr. In non-fimbriate-phase cultures the corresponding increase was only twofold. Since the transmission of *colI* in mixed cultures takes place mainly in the post-logarithmic period, it is understandable that the better growth given in this period by fimbriate as compared with non-fimbriate acceptor bacteria, may favour a higher rate of acquisition of *colI*.

If, as has been suggested, fimbriae promote transfer of *colI* through their effects in causing the aggregation and conjugation of bacteria in a pellicle and in increasing the amount of post-logarithmic growth, it may be asked why it is only the acceptor's fimbriation status that is important and why high rates of transfer are obtained with non-fimbriate donors. A possible explanation of the relative unimportance of the fimbriation status of the donor is afforded if we accept the conclusion, suggested by the experiments in which donors were killed by addition of streptomycin after 8 hr, that a high rate of transfer is the result of an extensive epidemic spread of *colI* among the acceptor bacteria. In a cross of non-fimbriate donor bacteria with fimbriate acceptor bacteria a few acceptor bacteria must first acquire *colI* from the non-fimbriate donors. Subsequently, *colI* may be transmitted freely from one fimbriate acceptor bacterium to another during the prolonged period of their post-logarithmic growth in the surface pellicle. This spread of *colI* would be little affected by the failure of the original non-fimbriate donor bacteria to participate equally in the post-logarithmic and pelliculate growth.

We wish to thank Dr B. A. D. Stocker for providing us with strains and for much helpful advice and criticism. M. M. was the holder of a British Council Scholarship.

REFERENCES

- ABBOTT, J. D. & SHANNON, R. (1958). A method for typing *Shigella sonnei* using colicine production as a marker. *J. clin. Path.* **11**, 71.
BRINTON, C. C., GEMSKI, P. & CARNAHAN, J. (1964). A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K12 and its role in chromosome transfer. *Proc. natn. Acad. Sci. U.S.A.* **52**, 776.
CRAWFORD, E. M. & GESTELAND, R. F. (1964). The adsorption of bacteriophage R-17. *Virology* **22**, 165.
DUGUID, J. P. & GILLIES, R. R. (1957). Fimbriae and adhesive properties in dysentery bacilli. *J. Path. Bact.* **74**, 397.

- DUGUID, J. P. & WILKINSON, J. F. (1961). Environmentally induced changes in bacterial morphology. *Symp. Soc. gen. Microbiol.* **11**, 69.
- DUGUID, J. P., SMITH, I. W., DEMPSTER, G. & EDMUNDS, P. N. (1955). Non-flagellar filamentous appendages ('fimbriae') and haemagglutinating activity in *Bacterium coli*. *J. Path. Bact.* **70**, 335.
- FREDERICQ, P. (1954). Transduction génétique des propriétés colicinogènes chez *Escherichia coli* et *Shigella sonnei*. *Séanc. C. r. Soc. Biol.* **148**, 399.
- FREDERICQ, P. (1957). Colicins. *A. Rev. Microbiol.* **11**, 7.
- FREDERICQ, P. (1963). On the nature of colicinogenic factors: a review. *J. theoret. Biol.* **4**, 159.
- LEDERBERG, J. (1952). Cell genetics and hereditary symbiosis. *Physiol. Rev.* **32**, 403.
- MULCZYK, M. (1960). Studies on antigenic variability of *Shigella flexneri* 1b. *Archiv. Immun. Terap. doświad.* **8**, 1.
- OZEKI, H., HOWARTH, S. L. & CLOWES, R. C. (1961). Colicine factors as fertility factors in bacteria. *Nature, Lond.* **190**, 986.
- OZEKI, H., STOCKER, B. A. D. & SMITH, S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *J. gen. Microbiol.* **28**, 671.
- STOCKER, B. A. D., SMITH, S. M. & OZEKI, H. (1963). High infectivity of *Salmonella typhimurium* newly infected by the *colI* factor. *J. gen. Microbiol.* **30**, 201.