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Sex pili and common pili in the conjugational transfer of colicin factor Ib by *Salmonella typhimurium*

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1. INTRODUCTION

Colicin factors are a class of bacterial plasmids with two principal functions. The first is to determine the synthesis of colicins, antibiotics which kill *Escherichia coli* and other enterobacteria. The second is to enable their host bacteria to act as genetic donors, not only of the colicin factor (Fredericq, 1957, 1958) but also of the bacterial chromosome itself (Ozeki, Howarth & Clowes, 1961; Smith & Stocker, 1962). Colicin factor I (*colI*) is readily transferred from a *colI*+ strain to a *col*− strain grown together overnight (Fredericq, 1956) because every bacterium which has just acquired *colI* can donate it for the succeeding four to eight generations (High Frequency Transfer; HFT). Thereafter, however, only a small proportion of *colI*+ bacteria can donate at a given time (Low Frequency Transfer: LFT: Ozeki, 1960; Stocker, Smith & Ozeki, 1963), because donor ability becomes repressed in the same way as the function of other newly introduced structural genes (Pardee, Jacob & Monod, 1959; Clark & Adelberg, 1962). The transmission of colicin factors depends on cell-to-cell contact (Fredericq, 1957, 1958) as is easily seen in HFT cultures in which clumps of conjugating bacteria occur (Ozeki, 1960; Meynell, 1961). Transfer evidently involves the synthesis of an extremely efficient organ of conjugation which can, however, be studied only in HFT cultures in which a majority of cells are able to donate. The usual method for preparing HFT cultures was found unreliable but HFT preparations of high donor ability were prepared after examining the kinetics of *colI* transfer. The HFT state proved to coincide with the appearance on the cells of a new type of pilus, that is, a filamentous appendage not concerned with motility, similar to that formed by many enterobacteria. First described by Houwink & Van Iterson (1950), these appendages were named 'fimbriae' by Duguid, Smith, Dempster & Edmunds (1955) and 'pili' by Brinton (1959). As 'pili' is already associated with the filaments determined by another sex plasmid, the F factor of *E. coli* (Crawford & Gesteland, 1964; Brinton, Gemski & Carnahan, 1964; Brinton, 1965; Caro & Schnöss, 1966), it is also used here. We distinguish 'sex pili',

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determined by sex factors like *colIb*, F, and certain Drug Resistance Factors (Datta, Lawn & Meynell, 1966; Lawn, 1966), from 'common pili' which include all other types such as Type 1 (Brinton, 1965; Duguid, Anderson & Campbell, 1966). Pili of both classes are involved in transfer of *colIb*.

2. MATERIALS AND METHODS

(i) *General*

Bacteria were grown in Oxoid Nutrient Broth No. 2, and colony counts and counts of colicin-producing colonies made by conventional methods (Fredericq, 1957) on Oxoid Blood Agar Base. The turbidity of broth cultures was measured in a nephelometer (Evans Electroselenium Ltd., Halstead, Essex).

(ii) *Bacterial strains*

(a) *Isolation of mutants.* Streptomycin-resistant (*str-r*) mutants were isolated by plating *str-s* strains on agar containing 1000 μg. streptomycin/ml. Azide-resistant (*azi-r*) mutants were isolated by exposing *azi-s* strains to ethylmethane sulphonate for 20 min. at 37°C., followed by growth in broth overnight and plating on agar containing 0·004 M sodium azide.

Table 1. *Strains of* Salmonella typhimurium

Number	Col	Other characters
M327	—	*str-s azi-s his-57*
M339	*Ib*	*str-s azi-s tryD-10*
M368	—	*str-r azi-s cysD-36*
M396	—	*str-r azi-s pil rha inl*
SL680	—	*str-r azi-s pur pro ilv-405 iM10 fla pil*
M404	—	*str-r azi-r leu-39*

Characters abbreviated according to Demerec *et al.* (1966) save for *pil* (non-piliated) in place of *fim* (non-fimbriate). Despite their genotypes, cultures of both M396 and SL680 always contained a small proportion of *fla*+ and *pil*+ cells.

(*b*) *Salmonella typhimurium.* These strains are described in Table 1 and were all derived from strain LT2, except for M396 which was derived from strain 7491, isolated by Professor J. P. Duguid. Strain 7491 is attacked by a temperate phage carried by strain LT2; strain M396 is 7491 lysogenized by this phage. *ColIb* is one of the two main types of *colI* now recognized (Stocker, 1966).

(*c*) *Escherichia coli.* The indicator strain used to detect *colIb* was M388 (*colE1 str-r azi-r*) derived from strain K12.

(iii) *Electron microscopy*

Specimens were prepared on formvar-carbon grids and negatively stained with uranyl acetate (for details, see Datta, Lawn & Meynell, 1966).

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3. RESULTS

(i) *The spread of* colIb *in* col– *cultures*

HFT cultures are usually obtained by inoculating 10 ml. broth with 5×10^5 *colI*+ cells and 10^7 *col*– cells, incubating at 37°C. overnight, and next day diluting 1/20 in fresh broth and incubating for a further 2 hours at 37°C. (Stocker, Smith & Ozeki, 1963). During incubation, the occasional *colI*+ cell capable of donation will transfer *colI* to a *col*– cell which thus becomes an HFT donor for a few generations and so in turn transmits *colI* to other *col*– cells. In this way, *colI* spreads through the previously *col*– culture so that, by the end of the standard procedure, at least 40% of the cells should be *colI* and able to donate. Although this method produces cultures with sufficient donors for many purposes, it has two serious disadvantages. The first is that the original *col*+ strain, which is LFT, may outgrow the second strain so that their ratio may change from the initial value of 1/20 to perhaps 3/1. Thus, a majority of cells in the mixed culture may be LFT although most of the initially *col*– cells have become HFT. Secondly, even with strains that are often satisfactory, the proportion of HFT donors varies unpredictably (e.g. Silver & Ozeki, 1962, Table 1, column c), and, with certain species like *Salmonella typhi*, the method may fail completely (Meynell, 1961).

These disadvantages of the standard method evidently arise because *colI* transfer is largely left to occur in the poorly defined conditions of a 'stationary' phase bacterial culture. The alternative method used here aimed at improving the production of HFT cultures by defining the conditions of bacterial growth more precisely. Transfer of *colIb* to the test *col*– strain was allowed to occur for only a limited time, all further spread being restricted to cells of the test strain. Furthermore, the initial proportion of *colI*+ in the test strain was fixed approximately by mixing it with an appropriate amount of a standard overnight HFT culture. In a typical experiment, 10 ml. of an unshaken overnight culture of the test strain (*col*– *str-r azi-s*) was mixed with 0·1 ml. of an unshaken overnight culture of a standard HFT preparation of *str-s azi-s* cells. After 20 min. at 37°C., the mixture was diluted 1/100 into broth containing 200 μg. streptomycin/ml. to prevent further transfer from the HFT preparation and to kill the *str-s* HFT cells. The culture was then allowed to grow exponentially without shaking to a concentration of 2×10^8/ml., as judged by turbidity, and at appropriate intervals was diluted 1/4 into warm streptomycin-broth to maintain exponential growth by keeping all nutrients in excess and to avoid entering the stationary phase in which the bacteria are, in effect, starved. Cultures could be transferred to a refrigerator at 4°C. for 1–2 days without interrupting the spread of the colicin factor on returning to 37°C. The percentage of *colIb* cells in the test culture was determined by plating on streptomycin-agar (200 μg./ml.) and the percentage capable of *colIb* transfer measured by mixing it in the ratio of 1/20 with a *col*– *str-r azi-r* recipient for 20 min. at 37°C., followed by plating on agar containing 200 μg. streptomycin/ml. and 0·004 M azide.

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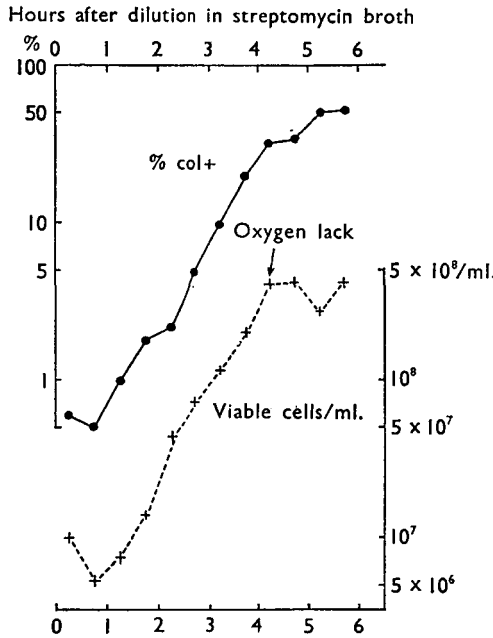
Experiments of this kind showed that (Text-fig. 1):

(1) the percentage of *colIb*+ cells in the test population increased exponentially during exponential bacterial growth, provided the bacterial concentration exceeded 5×10^6/ml. so that random contacts occurred with reasonable frequency. The shortest time taken for the percentage of *colIb*+ cells to double was 30 min., using a flagellated (*fla*+) and common piliated (*pil*+) strain with the same bacterial division time. The two times were not correlated, however, because the *pil*– test strains, M396 and SL680, divided in 40 min. and 1 hour respectively whereas the times taken for % *colIb*+ to double were 1 hour and 3 hours;

(2) the rate of spread of *colIb* fell after the bacteria ceased to grow at their maximum rate;



Text-fig. 1. The spread of *colIb* through a culture of strain M368 which was not diluted repeatedly to maintain exponential growth. The viable count ceases to rise after reaching 5×10^8/ml. and, at the same time, the rate of spread of *colIb* falls.

(3) when measuring the number of donors, it was important to use stationary phase, not exponentially-growing recipient bacteria and to mix for the shortest time that allowed maximal transfer of *colIb* (20 min. appears sufficient: Stocker *et al.*, 1963, Table 4). Otherwise, *colIb* may start to spread in the recipient culture, so over-estimating the true number of donors in the test population;

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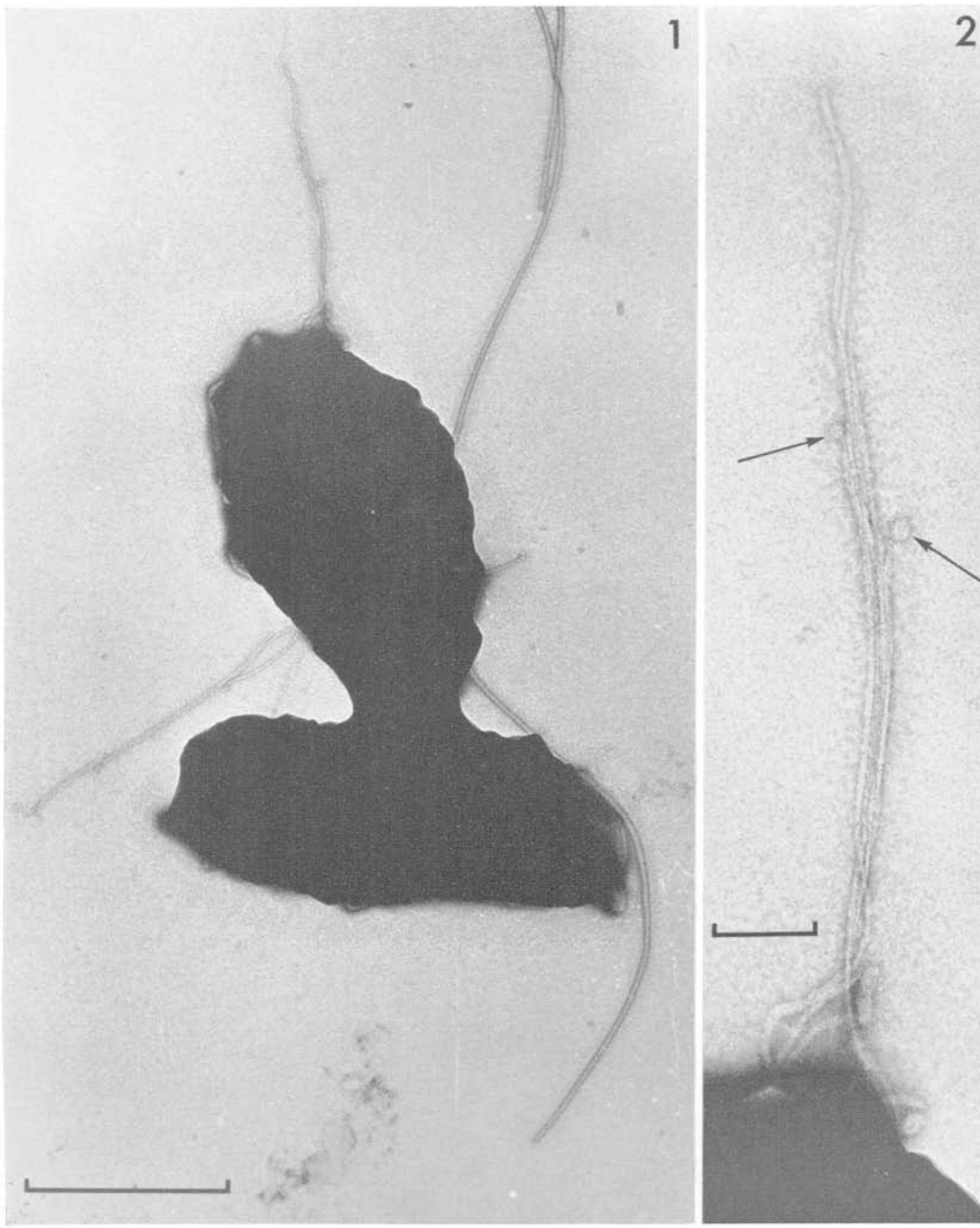

Fig. 1. Two cells from an HFT preparation of strain SL680, one without any appendages and the other bearing two flagella and two groups of Ib pili.

Fig. 2. One of the groups of Ib pili shown in Fig. 1 at higher magnification, showing two terminal knobs (marked by arrows).

The calibration bar corresponds to 1μ in Fig. 1 and 0·1μ in Fig. 2.

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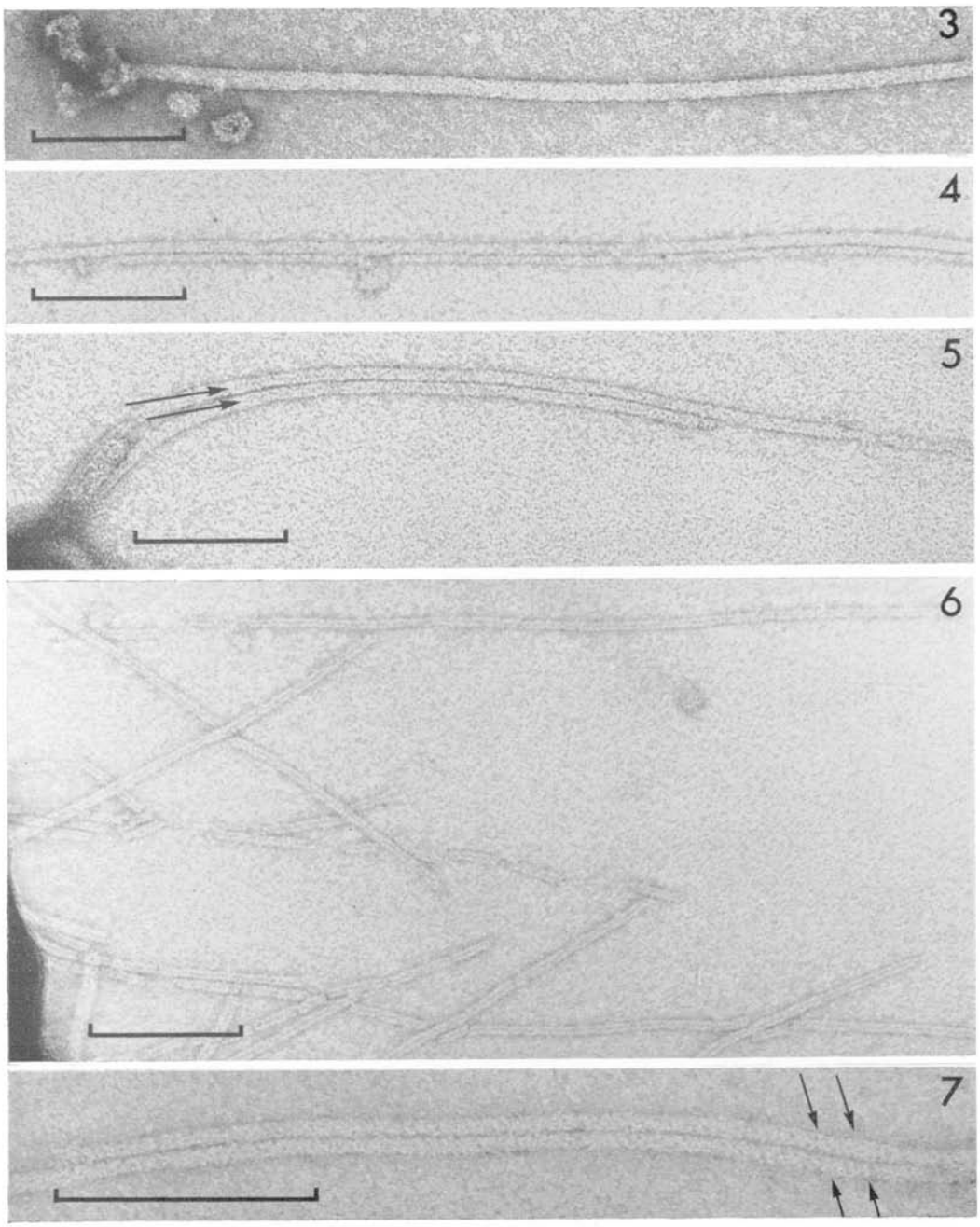

Figs. 3, 4 and 5. Ib pili all printed at the same magnification to show their variable width and sinuous profile compared to common pili. Fig. 5 shows indistinct axial 'holes', seen in only a few micrographs (marked by arrows).

Fig. 6. Common pili, showing the prominent axial 'hole' revealed by negative stains.

Fig. 7. Two Ib pili, one of which shows a helical substructure with a period measuring about 50 Å (marked by arrows). The calibration bars in Figs. 3–7 correspond to 0·1μ.

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(4) during the period of rapid spread, about 50% of *colIb*+ cells of the test strain, whether or not they formed common pili, could donate *colIb* in 20 min. The remaining 50% of *colIb*+ cells had presumably acquired the factor sufficiently long beforehand for donor ability to have become repressed.

Knowing the rate at which *colIb* spread from a trial experiment like that of Text-fig. 1, it was possible to estimate the period of exponential bacterial growth required for % *colIb*+ to increase from its predetermined initial value to 100%. This turned out to be quite considerable with *pil*– strains, which were used here because, in the absence of common pili, any new surface structures could be detected easily. With strain SL680, some 15 hours was needed for the *colIb*+ cells to increase from 0·6% to 14% because each doubling in % *col*+ took 3 hours. The *pil*– *col*+ cells evidently donated efficiently, as 50% of an HFT culture could donate to a *pil*+ *col*– recipient within 20 min. Also, *pil*– *col*– cells accepted the factor as readily as *pil*+ *col*– cells from a *pil*+ HFT culture. Presumably, the reason for the slow rate of spread, which explains why the usual method completely failed to give HFT cultures with *pil*– strains, lies in the known adhesive properties of most common pili (Duguid *et al.*, 1955, 1966). It appears that, unless at least one member of a prospective mating pair is *pil*+ so that the two cells adhere non-specifically, they are separated by Brownian motion before transfer has time to take place.

(ii) *Morphological findings*

The appearance of conjugating ability in HFT cultures, which was lost as *colIb* became repressed, suggested that the HFT state might be associated with the transient appearance of new structures on the cells. When HFT cultures of the *pil*– strains, M396 and SL690, were examined, a new type of pilus was found (the 'Ib pilus'), whose appearance differed from that of common pili and from that of the

Table 2. *Morphological characteristics of Ib, F and Type 1 pili*

	Maximum length (μ)	Width (Å)	Axial hole	Adsorption of F phages	Terminal knobs present
Ib	1·5	60–120	Indistinct	No	Yes
F	20·0	75–135	More distinct	Yes	Yes
Type 1	1·5	45–100	Prominent	No	No

'F type pili' now known to be synthesized by bacteria possessing any one of a series of related plasmids—such as F, the fertility factor of *E. coli* (Crawford & Gesteland, 1964; Brinton *et al.*, 1965), colicin factors V or B, and *fi*+ Drug Resistance Factors (Datta, Meynell & Lawn, 1966). Ib and common pili are shown in Plates I and II and their morphological characteristics in Table 2. The Ib pili often occurred in clusters, several of which might be seen on one cell (Plate I). An axial 'hole' was only occasionally detected in Ib pili (Plate II, Fig. 5) by contrast with the obvious hole seen in common pili (Plate II, Fig. 6), and Ib pili also had a more sinuous profile and their width was greater and more variable (Plate II, Figs. 3–6). A helical sub-

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structure, with a period of about 50 Å, was just visible (Plate II, Fig. 7). Terminal 'knobs' occur in Ib pili, as they do on F but not on common pili (Lawn, 1966), although in the *Salmonella* strains examined, they were uniformly small, unlike those on F pili present on *E. coli*. Indirect evidence suggests that the knobs are not concerned in conjugation but are probably pieces of cell wall carried away from the bacterial body when a new pilus first emerges (Lawn, 1966, and unpublished observations). The Ib pili appear to be of two widths, even on the same cell (Plate II, Figs. 3–5). They did not appear to adsorb either of the F specific phages, MS2, which attaches along the length of F pili, or M13, which attaches to their tips.

Table 3. *Correlation of incidence of cells with Ib pili and donor ability*

Duration of exponential growth (hours)	Cells with Ib pili/total counted	% cells with Ib pili*	% cells transferring *colIb* in 20 min.
	Strain M396		
3·5	0/200	0	1·4
4·0	0/100	0	2·0
4·8	0/200	0	10·0
6·3	8/200	4	26·0
8·3	21/200	10·5	26·0
24·0	0/100	0	1·4
	Strain SL680†		
3·1	0/25	0	0·36
3·8	0/25	0	0·56
5·8	0/25	0	2·2
9·6	9/200	4·5	4·3
11·0	3/100	3·0	6·2
12·0	5/100	5	4·0
13·7	3/50	6	23·0
16·0	1/50	2	14·5
17·8	19/200	9·5	28·0

* These estimates carry large sampling errors. Thus, if 10/200 cells are found to have Ib pili, the observed estimate of 5% has 95% confidence limits of 1·8–10·3%.

† The culture was kept at 4°C for 2 days between the third and fourth and again for 2 days between the fifth and sixth counts.

Ib pili were never seen in *col*– stocks of M396 or SL680 nor in LFT preparations of their *colIb*+ derivatives. Nor were they seen when the *colIb*+ component of the HFT preparation used as source of *colIb* was replaced by its *col*– ancestor. This control excluded the possibility that the Ib pilus was determined by an unsuspected plasmid, such as may form the satellite bands found unexpectedly during CsCl density gradient centrifugation of bacterial DNA (Rownd, Nakaya & Nakamura, 1966). The conclusion that Ib pili are determined by *colIb* and not another plasmid, was reinforced by finding that the number of cells bearing Ib pili was related to the number able to donate *colIb* in 20 min., regardless of the rate of spread of the factor in the *col*– strain (Table 3). The correlation was not perfect as the percentages carry

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sizeable sampling errors (see Table 3) and as some pili will have been obscured by the bacterial bodies.

Finally, it was shown that treatment in a high speed blendor under conditions in which common pili and flagella were not only detached but their regeneration prevented, also lowered the proportion of cells able to donate *colIb* (Table 4). Blending in itself was not sufficient to prevent transfer as blending of an overnight LFT culture did not affect transfer in the succeeding 20 min., and it was supposed that the Ib pili regenerated as common pili were seen to regenerate within this time. Regeneration of common pili occurred even when chloramphenicol was present in a concentration that inhibited bacterial growth (30 μg./ml.), and was only prevented if the cells were blended three times at 15 min. intervals, being incubated meanwhile in chloramphenicol-broth at 37°C. Pili therefore appear to be synthesized from a pool of precursor which has to be exhausted, by a combination of repeated blending and periods for regeneration, before donor ability is permanently diminished.

Table 4. *Effect of blending on donation of colicin factor Ib*

	Viable counts of donors* (/ml.) $\times 10^{-8}$			% donor cells transferring *colIb*†			% decrease in transfer due to blending‡
Chloramphenicol	−	+	+	−	+	+	
Blending§	−	−	+	−	−	+	
Exp. 1	2·7	3·4	3·2	2·0	1·3	0·27	79%
2	1·5	2·2	1·9	7·7	1·5	0·54	63%
3	3·2	5·5	4·9	3·9	4·6	0·9	79%

* Conventional HFT preparation of M339 + M327.

† In 20 min. at 37°C. to strain M404 at the ratio of 1 donor : 20 recipients.

‡ Calculated from the rates of transfer in chloramphenicol broth, with and without blending.

§ Blended three times in chloramphenicol broth for 5 min. on each occasion, with two intervals of 15 min. at 37°C. to allow regeneration of pili.

4. DISCUSSION

Since donor ability is soon repressed in a *colIb* cell, there are two general ways for obtaining cultures with higher proportions of donor cells: by exposing repressed cells to an inducing agent like ultra-violet irradiation which is, at best, only moderately effective (Monk & Clowes, 1964); or by introducing the factor into *col*− cells, in which it then remains unrepressed for a few generations. The second method depends on the epidemic spread of *colIb* in a *col*− population, and its success therefore depends to a large extent on whether the cultural conditions permit replication (Text-fig. 1). This being so, to obtain reproducible results, it is best to keep the cultural conditions as constant as possible either, as we have done, by repeatedly diluting in fresh broth so that all nutrients are in excess (which seems to give the fastest spread of *colIb*), or by using some form of continuous culture apparatus, in which one or more nutrients is necessarily deficient which would probably restrict

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the rate of epidemic spread. The same considerations apply to the study of any transmissible agent, like the F factor, whether or not it is repressible.

The role of common pili in the spread of *colIb* has also been studied by Mulczyk & Duguid (1966), who showed that *pil*– *col*– cultures of *Shigella flexneri* remained largely *col*– after many hours' incubation with *colI*+ strains. Their observations are not inconsistent with our finding that *pil*– and *pil*+ strains accept *colIb* equally well from a *pil*+ *col*+ donor, because the experiments took different forms. The final % *col*+ *pil*– cells observed by Mulczyk and Duguid resulted from epidemic spread through the *pil*– strain in which *pil*– cells acquiring *colI* would have to act as donors to *pil*– *col*– cells, whereas our experiments involved only a single transfer during 20 min. mixing of a *pil*+ *col*+ donor with the *pil*– *col*– recipient. Such observations show how comparisons of the frequency of gene transfer between pairs of unrelated strains may be biased by unsuspected physiological factors and do not necessarily depend on purely genetic events.

The pilus formed while the donor function of *colIb* is expressed is distinct morphologically from both common pili and from F pili. Nor does it adsorb either of two unrelated F-specific phages, MS2 and M13, which confirms the observation that HFT cultures of *colIb*+ *E. coli* are not attacked by phage MS2 (Datta *et al.*, 1966). Its exact function is, of course, as open to question as that of the F pilus, for in neither case is it known whether the pilus acts as a canal down which donor genes pass to the recipient or merely provides the initial link between the mating cells which is followed by fusion of the cell walls and gene transfer. That the Ib pilus has a role in conjugation seems certain from the blendor experiments (Table 4) and because the incidence of pili paralled donor ability (Table 3).

All types of pili may be synthesized from a precursor pool of appreciable size, since F pili (Brinton, 1966) and common pili both regenerate in the presence of chloramphenicol. The transfer of *colIb* is unaffected by a single blending in chloramphenicol-broth which removed all common pili, although it is diminished by repeated treatments (Table 4). Transfer was not totally abolished, however, possibly because regeneration and consequent exhaustion of the pool did not occur synchronously throughout the bacterial population so that a fraction of cells remained able to regenerate their pili, even after three treatments in the blendor.

SUMMARY

The kinetics of spread of colicin factor Ib (*colIb*) in *col*– cultures of *S. typhimurium* was studied. The rate of spread was greater with *pil*+ strains (those forming common pili) than with *pil*– strains. The difference reflects inefficient pairing between *pil*– cells, which donate or receive *colIb* efficiently only when mated with *pil*+ cells. The donor function of *colIb* is known to be repressed a few generations after it is acquired by a *col*– cell. While donor ability is manifest, a new type of pilus (the 'Ib pilus') is formed which is morphologically distinct from common pili and other sex pili such as that determined by the F factor. The Ib pilus is presumably involved in the transfer of *colIb* by conjugation.

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