Transmission of Colicinogeny between Strains of *Salmonella typhimurium* Grown Together

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SUMMARY

Ability to produce colicines I, E1, E2, K or B was transferred to *Salmonella typhimurium* strain LT2 by growth in broth with suitable colicinogenic strains of *Escherichia coli* or *Shigella sonnei*. When LT2 (*colI*), i.e. carrying the colicine I factor, or LT2 (*colB*) were grown overnight in broth with LT2 *col*⁻ (non-colicinogenic), c. 50 % of the latter became colicinogenic; LT2 (*colE2*) and LT2 (*colK*) did not transmit; LT2 (*colE1*) transmitted to only c. 0.1 % of the acceptor population. But LT2 carrying either *colI* or *colB* in addition to *colE2*, *colK* or *colE1*, transmitted both factors.

When overnight broth cultures of LT2 (*colI*) and LT2 *col*⁻ were mixed and incubated c. 40 % of the latter acquired *colI* by 20 hr. (when the viable count had doubled); but only c. 0.02 % acquired *colI* in 3 hr. The low initial transfer results from the fact that in a stock culture of LT2 (*colI*) only c. 1/5000 bacteria are 'competent donors', able to transmit *colI*. The later large increase in the proportion of colicinogenic bacteria probably results from 'epidemic spread' of the *colI* factor amongst the acceptor population, initiated by the few acceptor bacteria which originally receive it. It is supposed that most bacteria which have just acquired *colI* become competent donors. In a doubly colicinogenic strain most competent donors transmit both colicine factors.

Aeration by shaking during incubation interfered with transmission of colicinogeny, probably by abolishing the prolonged phase of slow growth of unacrated cultures. Growth in the presence of acriflavine did not 'cure' LT2 (*colI*) or LT2 (*colI*) (*colE2*) of colicinogeny, nor of ability to transmit.

LT2 (*colE1*) and LT2 (*colE2*) supported the epidemic spread of *colI* or *colB* about as well as did LT2 *col*⁻; but in LT2 (*colK*) the spread of *colI* was greatly reduced and that of *colB* somewhat reduced. The prior presence in an acceptor strain of one of the readily transmissible factors, *colI* or *colB*, did not interfere with the epidemic spread of the other. But LT2 (*colI*) did not become a competent donor on accepting *colE2* and, by inference, *colI* from LT2 (*colI*) (*colE2*).

INTRODUCTION

Colicines are antibiotics produced by some strains of Enterobacteriaceae and active on others; colicinogeny, i.e. the ability to produce a colicine, is a stable heritable property of many *Escherichia* and *Shigella* strains (for review see Fredericq, 1957). It is a character of special interest to the bacterial geneticist; for

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some colicinogenic strains of *Escherichia coli* and *Shigella* sp. when grown in broth with non-colicinogenic strains transmit their colicinogenic property to some cells of the latter (Fredericq, 1954*a*; Hamon, 1956). Colicinogeny could not be transmitted by culture filtrates. It is inferred that the character is transmitted from one bacterium to another by the transfer of a genetic determinant, during some kind of conjugation. We refer below to the postulated genetic determinant for colicinogeny as a colicine factor, indicating the colicine concerned by the symbol assigned to it by Fredericq. Thus; colicine factor I or, for short, *colI*, *colE2*, etc.

Experiments on the transmission of a colicine factor 'ER' (also termed 'K30', and falling in group E1; see Fredericq (1957)) between F^+ and F^- sublines of *Escherichia coli* strain K12 showed that only F^+ strains transmitted colicinogeny; that all recombinants from crosses of F^+ non-colicinogenic and F^- colicinogenic parents, and many from crosses of F^+ colicinogenic and F^- non-colicinogenic parents, were colicinogenic; and that in the latter cross there was no detectable linkage between colicinogeny and any other character which was segregating (Fredericq & Betz-Bareau, 1953*a,b*; Fredericq, 1954*b*). It was inferred that colicinogeny resulted from possession of a non-chromosomal factor, transmissible during conjugation mediated by the *F* agent. However, experiments on the inheritance of the same colicine factor ('K30') in crosses of *Hfr* with F^- sublines of *E. coli* K12 (Alfoldi, Jacob & Wollman, 1957; Alfoldi, Jacob, Wollman & Maze, 1958) showed that the colicinogenic or non-colicinogenic character could be mapped at a particular point on the bacterial chromosome. Thus it seems that a colicine factor may be either located on the bacterial chromosome, being then transmissible only when the relevant portion of chromosome enters another bacterium; or may occupy some non-chromosomal site, and be transmissible by conjugation even in the absence of chromosomal transfer. Jacob & Wollman (1958) defined as 'episomes' those non-essential heritable agents of bacteria which could be acquired or lost and having two alternative states; either on the chromosome, multiplying *pari passu* with it; or not on the chromosome, multiplying autonomously, sometimes more rapidly than the chromosome. The *F* factor of *E. coli*, the colicine factor 'K30' of *E. coli* and certain temperate phages (or prophages) of *E. coli* were episomes as thus defined (Jacob, Schaeffer & Wollman, 1960).

When the present work was begun, no genetic recombination by cell conjugation, mediated by the *F* factor or otherwise, had been reported in *Salmonella* or between *Salmonella* and *Escherichia* (but see Miyake & Demerec, 1959; Baron, Carey & Spilman, 1959; Zinder, 1960). We therefore investigated the transmission of colicinogeny between *Salmonella* strains grown together, a phenomenon observed by Hamon & Stocker (unpublished) and probably resulting from cell conjugation, hoping that fuller knowledge of this process would enable us to obtain recombination of the chromosomal genes of *Salmonella* by conjugation. This expectation has been realized (Ozeki & Howarth, 1961; Smith & Stocker, 1962; see also Stocker, 1960). In the present paper we describe the preparation of colicinogenic derivatives from nutritionally exacting sublines of *Salmonella typhimurium* strain LT2 and observations on the ability of such sublines to transmit their colicinogenic property to other LT2 sublines when the strains are grown together. Standard strains of *Escherichia coli* and *Shigella sonnei* producing known colicines, supplied by Professor P. Fredericq, were used as primary sources of various colicine factors; the process

of transmission from strains of these genera to *S. typhimurium* has not been investigated in detail and, in particular, we have not tested the *F* character of the donor strains.

We were for long unable to obtain transmission of colicinogeny to a substantial proportion of the cells of a non-colicinogenic LT2 strain by contact with a colicinogenic strain for short periods; the present paper therefore concerns results obtained when the colicinogenic and non-colicinogenic LT2 strains were incubated together in broth for some hours. We later found (Stocker, Smith & Ozeki, in preparation) that *Salmonella typhimurium* cultures which have just acquired the ability to produce colicine I transmit this character to a high proportion of the bacteria of a non-colicinogenic strain in a short time, because a high proportion of the cells of such newly infected cultures can conjugate; we state this conclusion now because it helps in the interpretation of the results described below.

Salmonella typhimurium strain LT2 does not produce any detectable colicine; and it is resistant to all the colicines we have used, viz. I, E1, E2, B and K.

METHODS

Media. Nutrient broth was made from a tryptic digest of beef. The minimal medium contained: K_2HPO_4 , 10.5 g.; KH_2PO_4 , 4.5 g.; $MgSO_4$, 0.05 g.; $(NH_4)_2SO_4$, 1 g.; sodium citrate, 0.47 g.; glucose, 2 g.; water, 1000 ml. The concentrations of nutritional supplements were as described by Lederberg (1950). Streptomycin was used at 1 mg./ml. These media contained 1.5% agar for plate culture, and 0.35% for soft agar. Bacterial strains were kept on Dorset egg slopes at laboratory temperature.

Bacterial strains. Table 1a shows the strains of *Escherichia coli* and *Shigella*, colicinogenic for known type(s) of colicine, from which various colicinogenic properties were transferred to strains of *Salmonella typhimurium*.

Table 1b lists the auxotrophic derivatives of *Salmonella typhimurium* strain LT2 used. A cysteine-exacting mutant, *cysD-36*, was used in many experiments, because it is very stable, reversion to prototrophy having never been observed (Clowes, 1958). Mutants resistant to streptomycin were isolated from these stocks. Some naturally colicinogenic strains of *S. typhimurium* were also used. Among strains supplied by Dr E. S. Anderson, of the Enteric Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9, 41 strains of 280 tested were found to be colicinogenic (Stocker, Ozeki & Anderson, unpublished). Strains LT7 and LT22 (Zinder & Lederberg, 1952), widely used in experiments on the genetics of *S. typhimurium*, were also observed to be colicinogenic, producing colicine I.

Table 1c records the colicine-sensitive *Escherichia coli* strains, and their derivatives, used in testing for the production of colicines, i.e. colicine-indicator strains. For the most part these were colicine-resistant mutants of sensitive strains, either obtained from Professor P. Fredericq or isolated by us from colonies appearing in colicine inhibition zones. The E group of colicines are defined by the resistance to all E colicines and to phage BF 23 of mutants obtained from sensitive strains by selection with any E colicine or with phage BF 23; within this group colicine E1 is recognized by the specific immunity to colicine E1 of strains made colicinogenic for colicine E1; and colicine E2 is similarly recognized by the specific immunity to

colicine E2 of derivatives producing colicine E2 (Fredericq, 1956). We confirmed Fredericq's observation on the resistance pattern of *E. coli* K12 lines made colicinogenic for E1 or E2, and used strain CL136, a K12 stock producing colicine E1, as an indicator sensitive to E2 but resistant to E1. We also confirmed Fredericq's observation that mutants selected for resistance to E1 are resistant to E2; but in stocks derived from *E. coli* strain ϕ of Gratia (1925) we found that mutants selected for resistance to E2 were still sensitive to E1, though less so than originally, and we used two such mutants as differential indicators.

Table 1. *Bacterial strains used*

(a) Standard colicinogenic strains, used as source of *col* factors: received from Professor P. Fredericq, Liège

Strain	Colicines produced
<i>Shigella sonnei</i> P9	I and E2
<i>Escherichia coli</i> K12-30	E1
<i>Escherichia coli</i> K77	B
<i>Escherichia coli</i> K49	K

(b) *Salmonella typhimurium* strain LT2, auxotrophic mutants

Strain no.	Character	Reference
<i>cysD</i> -36	requires cysteine	Clowes (1958)
<i>cysC</i> -7	requires cysteine	Clowes (1958)
<i>athC</i> -5	requires adenine and thiamine	Yura (1956)

Mutants of these strains resistant to streptomycin (1 mg./ml.) are indicated by *str-r* added to strain no. Colicinogenic derivatives are indicated by addition in parentheses of symbol for colicine agent concerned, e.g. *cysC*-7 (*colI*) indicates a derivative of strain *cysC*-7 producing colicine I.

(c) Colicine indicator strains

Strain no.	Sensitivity to colicine					Streptomycin sensitivity (1 mg./ml.)	Origin
	I	E1	E2	B	K		
CL 18	S	S	S	S	S	S	<i>E. coli</i> ϕ of Gratia (1925)
CL 104	S	S	S	S	S	R	<i>str-r</i> mutant of CL18
CL 147	R	S	S	r	S	S	Colicine I-res. mutant of CL18
CL 148	S	S	R	S	S	S	Colicine E2-res. mutant of CL18
CL 150	R	S	S	r	S	R	Colicine I-res. mutant of CL104
CL 151	S	S	R	S	S	R	Colicine E2-res. mutant of CL104
CL 152	R	S	R	r	S	R	Colicine I-res. mutant of CL151
— I —							
CL 56	S	S	S	S	S	S	<i>E. coli</i> K12-W677, <i>thr</i> , <i>leu</i> , <i>thi</i>
CL 135	S	S	S	R	S	S	<i>E. coli</i> K12-W1177/V*
CL 136	S	R	S	S	S	R	<i>E. coli</i> K12-30*, carries <i>colE1</i>
CL 184	R	R	S	r	S	R	Colicine I-res. mutant of CL136
CL 185	S	R	R	S	S	R	Colicine E2-res. mutant of CL136
CL 142	S	S	S	S	S	R	<i>E. coli</i> K12-Row*
CL 143	R	S	S	S	S	R	<i>E. coli</i> K12-Row/V*
CL 144	r	S	S	R	S	R	<i>E. coli</i> K12-Row/B*
CL 145	S	R	R	S	S	R	<i>E. coli</i> K12-Row/E*
CL 146	S	S	S	S	R	S	<i>E. coli</i> K12-Row/K*

S = sensitive; r = partly resistant; R = completely resistant. * = strains received from Professor P. Fredericq, of Liège, designated as shown.

Culture methods. Cultures were grown at 37°. Broth cultures were usually grown, without aeration, in loosely capped containers; in some experiments 5 ml. cultures in 20 ml. bottles (loosely capped) were grown on a shaker (throw 9 cm., 100 strokes/min.).

Test for the production of colicine. To test for the production of colicine by colonies grown either from single bacteria or from stab inocula, the bacteria were killed by chloroform vapour and the plate covered with a layer of soft agar seeded with $c. 10^8$ bacteria of an indicator strain; after overnight incubation colonies of colicinogenic bacteria were surrounded by clear zones. In experiments involving two or more colicines, the colicine or colicines produced were identified by the use of a set of test plates, each layered with a different indicator, resistant to one or more colicines.

Preparation of colicinogenic derivatives of Salmonella typhimurium LT2 by contact with standard colicinogenic Escherichia and Shigella strains

Sublines of strain LT2 producing each of the colicines I, B, E1, E2 or K, or combinations of them, were obtained by growing strain LT2 in mixed culture with appropriate 'donor' strains of *Escherichia coli* or *Shigella* sp. producing these colicines: 10 ml. broth was inoculated with about equal volumes (0.1 ml; or a loopful) of broth cultures of the colicinogenic (donor) strain and a subline of strain LT2, generally one marked by nutritional requirements, etc. After overnight incubation, colicinogenic clones of the LT2 component of the culture were isolated, the procedure used varying according to the proportion of the LT2 bacteria which had acquired colicinogeny. When this proportion was high, the overnight mixed culture was streaked on nutrient agar and *Salmonella* colonies, recognizable by their smoothness, were picked and tested for colicinogeny. When the proportion was low the mixed culture was so treated that only the *Salmonella* component would grow when the mixture was plated; either the donor bacteria were killed by exposure to a suitable colicine (a chloroform-killed broth culture of a colicinogenic strain); or the mixture was plated on a medium, e.g. streptomycin agar, on which only the *Salmonella* strain could grow. Rare colicinogenic colonies amongst a large number of non-colicinogenic ones were then detected and isolated in one of the following ways: (i) Replica plates (Lederberg & Lederberg, 1952) were tested for the presence of colicinogenic colonies; colonies corresponding to colicinogenic ones were then picked from the master plate. (ii) A first soft-agar layer inoculated with a suitable number of bacteria from the mixed culture was covered by a second uninoculated layer and after incubation a further layer containing indicator bacteria was added; after further incubation colicinogenic colonies underlying the centres of inhibition zones were picked (Fredericq, 1954*a*). (iii) When the proportion of *Salmonella* made colicinogenic was very small an inoculum of up to 10^7 *Salmonella* was plated on a medium which would support only a very limited growth of the *Salmonella*, for instance unsupplemented minimal medium when the acceptor strain (*cysD-36*) required cysteine; after incubation the plate (not chloroformed) was covered with a layer of soft minimal agar supplemented with threonine, leucine and thiamine, and containing indicator bacteria (CL56) requiring these factors. After overnight incubation the indicator bacteria (but not the cysteine-exacting *Salmonella*) had grown and tiny inhibition zones were observed in the confluent growth; a small piece of agar containing a zone was cut out and suspended in broth, and from this (by

repeating the same procedure, if necessary) the colicinogenic *Salmonella* clone could be isolated.

Singly colicinogenic derivatives of strain LT2. The standard colicine-producing strains used as donors of colicinogeny and the approximate proportion of LT2 cells which in a typical experiment became colicinogenic during overnight mixed culture are recorded in Table 2. Factors I and E2 were both obtained from *Shigella sonnei* strain P9, which produces both these colicines. After overnight culture of an LT2 subline with strain P9 about 50 % of the LT2 cells had acquired *colI*, and about 5 % *colI* and *colE2* together, but no detectable proportion had acquired factor *colE2* alone (see Ozeki & Stocker, 1958). However, a subline of LT2 with the E2 factor of strain P9, but not the I factor, was obtained, apparently by the spontaneous loss of *colI* from an LT2 stock given *colI* and *colE2* simultaneously from strain P9. It was later found that LT2 strains with factor E2 from strain P9 but without factor I could be regularly obtained either (i) by transduction, using phage PLT22 grown on an LT2 subline carrying *colI* and *colE2* (Ozeki & Stocker, 1958); or (ii) by interrupted mating of such a strain with LT2 *col*⁻; these results will be described in subsequent papers. Strain LT2 carrying *colE2*, but not *colI* derived from *Sh. sonnei* P9 behaved similarly, regardless of how it had been obtained.

Table 2. *Transfer of colicine factors from standard colicinogenic strains of Escherichia coli and Shigella sonnei to sublines of Salmonella typhimurium strain LT2 by mixed culture*

Donor of colicinogeny		Acceptor of colicinogeny		
Strain	Colicines produced	Strain	Selected by	Acceptor colonies colicinogenic
<i>Shigella sonnei</i> P9	I & E2	LT2 <i>cysD-36 str-r</i>	Streptomycin	{ c. 50 % (I only) c. 5 % (I and E2)
<i>E. coli</i> K12-30	E1	LT2 <i>cysD-36</i>	Colicine K	c. 10 %
<i>E. coli</i> K77	B	LT2 <i>athC-5 str-r</i>	Streptomycin	c. 20 %
<i>E. coli</i> K49	K	LT2 <i>cysC-7 str-r</i>	Streptomycin	c. 0.01 %

10 ml. broth was inoculated with loopfuls of broth cultures of the standard colicinogenic strain (donor) and of a subline of strain LT2 (acceptor). After overnight incubation the acceptor strain was selected and the proportion of its colonies which were colicinogenic was measured. Other LT2 sublines, and other methods of selection, have also been used, with similar results.

Doubly colicinogenic derivatives of strain LT2. To obtain doubly colicinogenic derivatives we used as acceptor strain an LT2 subline carrying one colicine factor, and incubated it in broth with a donor strain (*Escherichia coli*, *Shigella sonnei* or LT2) carrying some other factor. With one exception (Table 3c) singly colicinogenic LT2 sublines accept a second, different, colicine factor in the same way that non-colicinogenic LT2 does. LT2 (*colI*) (*colE2*), a doubly colicinogenic LT2 derivative used in many experiments, was prepared by several methods: (i) in a single step by growth in mixed culture with a donor strain carrying both factors; (ii) by transduction of *colE2* by phage PLT22 from LT2 (*colI*) (*colE2*) into a stock already carrying *colI*; (iii) by transmitting *colI* by mixed culture to stocks carrying *colE2* only, obtained either by transduction of *colE2* into a non-colicinogenic stock, or by the spontaneous loss of *colI* from a stock carrying *colI* and *colE2*.

Test for transmission of colicine factors between LT2 sublines

To test for the transmission of colicinogeny between LT2 sublines we used the technique described above, of overnight incubation of broth inoculated with equal numbers of bacteria of the donor and acceptor strains, in this case genetically labelled LT2 sublines. In most experiments we used a streptomycin-sensitive donor and a resistant acceptor, plated the mixed culture on streptomycin agar and tested for colicinogenic acceptor bacteria by the methods described above. In the case of colicine K no streptomycin-sensitive subline carrying *colK* was available for use as donor; but as the donor strain (*cysC-7 str-r (colK)*) was nutritionally exacting, LT2 wild-type was used as acceptor, and was selected by two serial passages of the mixed culture in liquid minimal medium.

RESULTS

The colicinogenic derivatives of *Salmonella typhimurium* LT2 obtained by contact with standard colicinogenic donor strains (see Methods) were indistinguishable from the strains from which they had acquired colicinogeny, in respect both of their range of activity and of the appearance of the inhibition zones they produced. That is, the colicine produced by the derivative seems to be identical with that produced by the original strain.

A method for detecting the production of colicine by individual bacteria of these colicinogenic LT2 sublines has been described elsewhere (Ozeki, Stocker & de Margerie, 1959), together with observations on the proportion of the bacteria of such strains which liberated colicine, spontaneously or after ultraviolet irradiation. The colicinogenic LT2 derivatives varied in their stability. Sublines producing colicine I were stable, even on prolonged storage at room temperature on Dorset egg slopes; on a single occasion, however, a variant producing only colicine E2 was found in a stock initially producing both I and E2. Derivatives carrying *colE1* or *colE2* were also in general stable. Colicinogeny for colicines B and K was less stable; for when Dorset egg stock slopes of LT2 lines carrying *colB* or *colK*, kept at room temperature for a year or more, were streaked out, only 10–50 % and c. 0.2 %, respectively, of the colonies were colicinogenic.

When a culture of an F⁺ strain of *Escherichia coli* is incubated in a sub-lethal concentration of an acridine dye or a cobalt salt, many of the bacteria lose the F factor and become F⁻ (Hirota, 1956; Hirota & Iijima, 1957). We therefore tested acriflavine for effect on the *colI* and *colE2* agents in *Salmonella typhimurium* LT2. Strains *cysD-36 (colI)* and *cysD-36 (colI) (colE2)* were grown in broth with acriflavine, 80 µg/ml.; after 24 hr. at 37° the count of viable bacteria had increased from c. 10⁶/ml. to c. 7 × 10⁷/ml.; all of several hundred colonies tested retained their colicinogenic character unchanged. All of 20 clones isolated from the acriflavine-treated *cysD-36 (colI)* culture transmitted colicinogeny in the same way as the untreated parent strain.

Singly colicinogenic LT2 sublines were tested for their ability to transmit colicinogeny to non-colicinogenic sublines during overnight mixed cultured in broth. Table 3a records the results of a typical experiment; similar results were obtained when the donor or acceptor lines carried other genetic markers, and when other methods of selection of the acceptor strain were used. It appeared that in strain LT2

colicine factors I and B are readily transmissible by mixed culture; that factors E2 and K are not transmitted to a detectable extent; and that factor E1 is transmitted at a very low rate. All of 20 'wild' colicinogenic strains of *Salmonella typhimurium* found to produce colicine I readily transmitted colicinogeny to LT2 *cysD-36 str-r*; whereas none of 12 'wild' strains found to produce colicine E2 transmitted colicinogeny to a detectable extent.

Table 3. *Transfer of colicine factors between LT2 strains during overnight mixed growth*

- (a) Singly colicinogenic donor and non-colicinogenic acceptor strains.
 (b) Doubly colicinogenic donor and non-colicinogenic acceptor strains.
 (c) Colicinogenic donor and colicinogenic acceptor strains.

Donor strain	Acceptor strain	State of acceptor bacteria after mixed growth			
		Producing indicated colicine			Non-colicinogenic
(a) <i>cysD-36 (colI)</i>	<i>cysD-36 str-r</i>	I, 50 %			50 %
<i>cysD-36 (colB)</i>	<i>cysD-36 str-r</i>	B, 20 %			80 %
<i>cysD-36 (col E1)</i>	<i>cysD-36 str-r</i>	E1, 0.1 %			99.9 %
<i>cysD-36 (col E2)</i>	<i>cysD-36 str-r</i>	E2, < 0.001 %			100 %
<i>cysC-7 str-r (colK)</i>	LT2 (wild-type)	*K, < 0.001 %			100 %
(b) <i>cysD-36 (colI) (colB)</i>	<i>cysD-36 str-r</i>	I, 3.5 %	B, 5.5 %	I & B, 55 %	36 %
<i>cysD-36 (colI) (colE1)</i>	<i>cysD-36 str-r</i>	I, 43 %	E1, 0 %	I & E1, 5 %	52 %
<i>cysD-36 (colI) (colE2)</i>	<i>cysD-36 str-r</i>	I, 55 %	E2, 0 %	I & E2, 22 %	23 %
LT2w.t.†(<i>colI</i>)(<i>colK</i>)	<i>cysD-36 str-r</i>	I, 55 %	K, 0 %	I & K, 1 %	44 %
(c) <i>athC-5 (colI)</i>	<i>cysD-36 str-r (colB)</i>	I & B, 33 %	B, 67 %		
<i>athC-5 (colI)</i>	<i>athC-5 str-r (colE1)</i>	I & E1, 50 %	E1, 50 %		
<i>athC-5 (colI)</i>	<i>cysC-7 str-r (colE2)</i>	I & E2, 40 %	E2, 60 %		
<i>athC-5 (colI)</i>	<i>cysC-7 str-r (colK)</i>	I & K, 0.4 %	K, 99.6 %		
<i>athC-5 (colB)</i>	<i>cysC-7 str-r (colI)</i>	B & I, 35 %	I, 65 %		
<i>athC-5 (colB)</i>	<i>athC-5 str-r (colE1)</i>	B & E1, 29 %	E1, 71 %		
<i>athC-5 (colB)</i>	<i>cysC-7 str-r (colE2)</i>	B & E2, 58 %	E2, 42 %		
<i>athC-5 (colB)</i>	<i>cysC-7 str-r (colK)</i>	B & K, 8 %	K, 92 %		

Loopfuls of overnight broth cultures of acceptor and donor strains were inoculated together into 10 ml. broth. After 18 hr. incubation the acceptor bacteria were selected on streptomycin agar and scored for colicinogeny with appropriate indicator strains.

* As the donor strain, *cysC-7 str-r (colK)* was streptomycin-resistant, but auxotrophic, the prototrophic acceptor bacteria were selected on minimal medium.

† Wild-type.

The *F* agent can be transmitted by contact from *F*⁺ strains of *Escherichia coli* to *Salmonella typhimurium* (Zinder, 1960); and one of our standard colicinogenic strains, *E. coli* K12-30, used as source of the *colE1* factor, was known to be *F*⁺ (we have not investigated the *F* character of the other standard colicinogenic strains; see Table 2). We therefore considered the possibility that our colicinogenic derivatives of LT2 had become *F*⁺ as well as colicinogenic. However, they did not show the kind of fertility found by Zinder (1960) in *F*⁺ derivatives of LT2; and all were sensitive to phage SP6 which does not attack LT2 lines carrying the *F* agent (Zinder, 1961). We conclude that none of our colicinogenic LT2 derivatives carries the *F* agent.

Though factors E1, E2 and K are scarcely, if at all, transmitted by singly colicinogenic donor strains they are readily transmitted by donor strains which carry,

in addition, one of the readily transmissible factors, I or B (see Table 3*b*). Most of the experiments described in this paper concern factors I and E2, as representatives of the classes of factors which are, respectively, transmissible and non-transmissible by singly colicinogenic donor strains.

Transfer of the colI factor

When *cysD-36* (*colI*) (donor) and *cysD-36 str-rcol-* (acceptor) were grown together in broth (inoculum *c.* 10^5 bacteria/ml. of each strain), more than 50 % of the acceptor population became colicinogenic during overnight incubation, by which time the total population was *c.* 10^9 bacteria/ml. Undiluted broth cultures of the same donor and acceptor strains, grown overnight without aeration, were mixed in equal parts and re-incubated, without addition of fresh broth; after 3 hr. only *c.* 0.02 % of the acceptor bacteria were colicinogenic, and after 6 hr. *c.* 0.1 %; but after 20 hr. incubation the proportion of colicinogenic acceptor bacteria had increased to *c.* 50 %. No such transfer of colicinogeny occurred when the viable donor culture was replaced by either (1) a bacterium-free culture filtrate, or (2) the pasteurized (60°, 45 min.) supernatant of a centrifuged culture, or (3), a culture killed by shaking with chloroform. We conclude that transmission of colicinogeny between *Salmonella typhimurium* sublines grown together requires contact of 'live' cells, presumably because it occurs by conjugation, as has been inferred in other genera.

The transfer of colicinogeny during 20 hr. incubation of an undiluted mixture of unaerated overnight broth cultures of donor and acceptor strains might be thought to indicate that transmission of the *colI* factor was occurring under conditions where there was no bacterial growth. However, in our broth, incubated at 37° without aeration by shaking, the growth of strain LT2 slows down when the count reaches $1-2 \times 10^8$ /ml., and after overnight incubation there are about 10^9 viable bacteria/ml.; slow growth continues on longer incubation, the number of viable bacteria increasing to *c.* 2×10^9 /ml. in a further 24 hr. Thus the transmission of colicinogeny to *c.* 50 % of the acceptor bacteria took place in conditions permitting slow bacterial growth.

If a broth culture of strain LT2 is aerated by shaking during incubation the number of viable bacteria increases rapidly until it reaches $4-6 \times 10^9$ /ml. and then growth ceases. When aerated 18 hr. cultures of donor and acceptor strains were mixed, without dilution, very few of the acceptor bacteria became colicinogenic during 20 hr. further incubation, with or without shaking of the mixture. The almost complete absence of transmission of the *colI* factor under these conditions presumably results from the environmental conditions which are preventing bacterial growth (probably lack of any available energy source).

Shaking of a mixture of overnight unaerated cultures of donor and acceptor strains greatly reduced the proportion of acceptor bacteria which became colicinogenic during 20 hr. incubation; e.g. to 0.8 %, compared with 45 % in an unshaken control. As stated above nearly all the acceptor bacteria which acquire *colI* in an unshaken mixture do so between the sixth and twentieth hour of incubation, during which time there is slow bacterial growth. In the shaken mixture by contrast growth would have ceased by the sixth hour of incubation and presumably no transmission of colicinogeny could occur thereafter.

We therefore conclude that all the observed inhibitory effects of shaking on the transfer of *colI* can be accounted for by the effects of aeration by shaking on bacterial growth, without postulating any direct effect, e.g. on pair formation.

Proportion of competent donors in overnight broth cultures of LT2 (colI)

The very small proportion of acceptor bacteria which acquired the *colI* factor during the first few hours of incubation of a mixture of undiluted overnight broth cultures of donor and acceptor strains suggested that perhaps only a small fraction of the bacteria in a broth culture of an LT2 line carrying *colI* are 'competent donors', able to transmit *colI* (even though all bacteria in such a culture carry the factor). This hypothesis was tested, and the proportion of competent donors estimated, as follows: (i) graded numbers (e.g. 300, 3000 or 30,000) of bacteria of strain *cysD-36 (colI)* in 0.2 ml. of saline were added to 0.2 ml. volumes of an overnight broth culture of a streptomycin-resistant acceptor strain; (ii) after either 15 min. or 20 hr. incubation 10 ml. of streptomycin broth were added to each tube, to prevent further growth of the streptomycin-sensitive donor bacteria; (iii) after overnight incubation each tube was tested for the presence of colicinogenic bacteria of the streptomycin-resistant acceptor strain. In such an experiment (Table 4) the final test for

Table 4. *Estimation of proportion of competent donors in a broth culture of strain cysD-36 (colI) by the dilution method*

No. of bacteria of donor strain added	No. of tubes giving positive or negative test for presence of streptomycin- resistant colicinogenic bacteria after incubation in streptomycin broth added at			
	15 min.		20 hr.	
	+	-	+	-
3×10^4	12	0	12	0
3×10^3	4	8	5	7
3×10^2	0	12	1	11

0.2 ml. volumes of saline dilutions of an overnight unshaken broth culture of the donor strain *cysD-36 (colI)* were mixed with 0.2 ml. volumes of a similar culture of strain *cysD-36 str-r*, 24 tubes being set up for each size of inoculum. After 15 min. incubation 10 ml. streptomycin broth was added to half the tubes of each set; the remainder were similarly treated after 20 hr. After 24 hr. at 37° all tubes were streaked on streptomycin-agar plates, which after incubation were layered with a streptomycin-resistant indicator; reconstruction experiments showed that this final test would detect as few as 0.01 % of streptomycin-resistant colicinogenic bacteria.

colicine production was either completely negative or strongly positive, the latter indicating that a large fraction of the acceptor bacteria had become colicinogenic; and the distribution of positive tubes was the same whether 15 min. or 20 hr. had been allowed, without streptomycin, for transmission from the donor to the acceptor strain. We infer that positive tubes received, in the inoculum of donor bacteria, at least one competent donor bacterium, able to transmit colicinogeny, and that negative tubes received none. The mean number of effective donor bacteria inoculated per tube, calculated by the Poisson series from the proportion of negative tubes (15/24), is *c.* 0.48; as each tube received *c.* 3000 bacteria the proportion of

effective donors in the broth culture of strain *cysD-36 (colI)* was *c.* 1/6000. The proportion of competent donors was also estimated by a different method. Samples of an overnight broth culture of a streptomycin-resistant acceptor strain were inoculated with graded numbers of bacteria of a streptomycin-sensitive donor strain. After 15 min. at 37°, samples (*c.* 10⁶ acceptor bacteria/plate) were plated on streptomycin agar, and after incubation over-layered with a streptomycin-resistant indicator. The number of colicine inhibition zones produced indicated the number of streptomycin-resistant acceptor bacteria made colicinogenic, and hence the number of effective donor bacteria. The calculated proportion of effective donor bacteria was 1/5000 to 1/7800 (Table 5).

Table 5. *Estimation of proportion of competent donors in a broth culture of strain cysD-36 (colI) by plating method*

No. of bacteria inoculated/plate		No. of colicine zones/plate*	No. of donor bacteria/zone
Donor strain <i>cysD-36 (colI)</i>	Recipient strain <i>athC-5 str-r</i>		
1.5 × 10 ⁶	<i>c.</i> 10 ⁶	192	7.8 × 10 ³
3.2 × 10 ⁵	<i>c.</i> 10 ⁶	51	6.3 × 10 ³
1.3 × 10 ⁵	<i>c.</i> 10 ⁶	24	5.4 × 10 ³
2.9 × 10 ⁴	<i>c.</i> 10 ⁶	4	7.3 × 10 ³
1.5 × 10 ⁴	<i>c.</i> 10 ⁶	3	5.0 × 10 ³

1 ml. volumes of an overnight unshaken broth culture of the streptomycin-resistant recipient strain were seeded with graded numbers of bacteria from a similar culture of the streptomycin-sensitive donor strain. After 15 min. at 37° 0.1 ml. volumes of 1/100 dilutions were spread on streptomycin-agar plates; after 15 hr. incubation these were tested with a streptomycin-resistant indicator (CL104).

* Average of three plates.

The large number of acceptor bacteria which on long incubation become colicinogenic, even in tubes receiving only one competent donor, do so, we believe, as a result of the serial transmission of the *colI* factor amongst the acceptor bacteria. To estimate the number of acceptor bacteria which become colicinogenic as a result of the inoculation of a single effective donor bacterium, graded numbers of bacteria of a donor strain were added to 1 ml. volumes of an overnight culture of a streptomycin-resistant acceptor strain and the tubes were re-incubated, without the addition of fresh broth; after 20 hr. (by which time there was only a *c.* twofold increase in the number of bacteria) the number of colicinogenic acceptor bacteria in each original tube was estimated by subculturing serial decimal dilutions of each tube in streptomycin broth and testing each subculture after incubation for the presence of streptomycin-resistant colicinogenic bacteria, as above. This experiment showed that original tubes which had received *c.* 3000 bacteria of the donor strain contained, 20 hr. later either (i) <10 and presumably no colicinogenic acceptor bacteria or (ii) >10⁵. The positive tubes had, by inference, received one (or a very few) effective donors. It is unlikely that the one effective donor in the inoculum had mated, successively, with 10⁵ acceptor bacteria during the 20 hr. incubation; the epidemic spread of the *colI* factor amongst the acceptor population seems the only plausible explanation of these results.

Transfer of the colE2, colK, colE1 and colB factors

Singly colicinogenic LT2 sublines carrying either *colE2* or *colK* did not transmit their colicinogenic properties to any detectable extent to non-colicinogenic LT2 during overnight growth in broth (Table 3a); transfer of colicinogeny to $1/10^5$ of the acceptor population would have been detected. LT2 sublines carrying only *colE1* transmitted colicinogeny to *c.* 0.1 % of an acceptor population during overnight mixed culture. LT2 sublines carrying only *colB* transferred colicinogeny to *c.* 20 % of an acceptor population during overnight mixed culture, we assume as a result of the epidemic spread of the *colB* factor amongst the acceptor population.

Although singly colicinogenic LT2 (*colE2*) strains did not transmit *colE2*, overnight incubation of a doubly colicinogenic LT2 (*colI*) (*colE2*) strain with a non-colicinogenic acceptor resulted in the acquisition of both factors by some (e.g. 22 %) acceptor bacteria and of *colI* alone by a larger fraction (e.g. 55 %; see Table 3b); acceptor bacteria which had acquired *colE2* only were not detected (cf. transmission of *colI*, and of *colI* and *colE2* together, by *Shigella sonnei* P9 to LT2, Table 2). LT2 (*colI*) (*colE2*) stocks behaved alike in transmission tests, regardless of whether they had acquired the two factors simultaneously, or successively, in either order (see Strains, in 'Methods'). All the 12 'wild' *Salmonella typhimurium* strains producing colicine E2 transmitted *colE2*, with *colI*, after the latter factor had been introduced into them; a clone of *Sh. sonnei* P9 which had lost the transmitting ability of the doubly colicinogenic parent strain was found to have lost the ability to produce colicine I; ability to transmit was regained when factor *colI* was re-introduced.

Thus LT2 and several other strains of *Salmonella typhimurium* and *Shigella sonnei* strain P9 when carrying only *colE2* do not transmit this factor, but when carrying both *colE2* and *colI* transmit both factors.

The carriage of *colI* by a donor strain likewise resulted in some transmission of *colK*, not transmitted by singly colicinogenic strains, and facilitated the transmission of *colE1* (Table 3b). In the case of the latter *c.* 5 % of an acceptor population acquired *colE1* (with *colI*) during overnight growth with a doubly colicinogenic donor, as against *c.* 0.1 % when the donor carried *colE1* only.

From analogy with the transmission of *colI* we infer that most of the acceptor bacteria which acquire *colE2* (or *colK* or *colE1*), together with *colI*, during overnight growth with a doubly colicinogenic donor strain do so through the epidemic spread amongst the acceptor population of the two factors in association. In an experiment similar to that recorded in Table 4 the proportion of competent donor cells in a broth culture of *cysD-36* (*colI*) (*colE2*) was measured; as in *cysD-36* (*colI*) this proportion was only one in several thousand. In this experiment the tubes inferred to have received only one (or a very few) competent donor cells of *cysD-36* (*colI*) (*colE2*) gave rise to streptomycin-resistant (acceptor) cultures producing colicine E2 as well as colicine I; this suggests that in the doubly colicinogenic strain all or nearly all competent donor cells can transmit both factors. If many acceptor bacteria which have just acquired both *colI* and *colE2* (or *colK* or *colE1*) from such competent donors themselves become competent donors of both factors, epidemic spread of the two factors in association would be expected. The larger number of acceptor bacteria which acquire *colI* alone, instead of *colI* and *colE2*, would be accounted for if during conjugation *colI* is sometimes transmitted without *colE2*.

A few experiments indicated that the presence of *colB* in a donor strain facilitated the transfer of *colE2*, *colK* and *colE1* in the same way that the presence of *colI* did, though not to quite the same extent.

As singly colicinogenic donor strains carrying either *colI* or *colB* transmitted colicinogeny to a high proportion of acceptor bacteria during overnight mixed culture (Table 3*a*), we could not use a comparison of the transmitting ability of singly and doubly colicinogenic donor strains as a test for the ability of one of these factors to assist the transfer of the other. However, when a donor strain carrying both *colI* and *colB* was incubated with a non-colicinogenic acceptor more than half the acceptor bacteria acquired both factors, while 36% acquired neither, a distribution incompatible with the hypothesis of independent transmission of the two factors (Table 3*b*). This suggests that competent donor bacteria carrying two transmissible factors resemble competent donors carrying one transmissible and one 'non-transmissible' factor, in that they usually transmit both factors.

Transfer of colI and colB factors to colicinogenic acceptor strains

When an acceptor strain already producing colicine E2 or E1 was incubated overnight with a donor strain producing colicine I (or B), a high proportion of the acceptor bacteria acquired the ability to produce colicine I (or B), just as they did when the acceptor strain was non-colicinogenic (Table 3*c*); and the acquisition of *colI* or *colB* did not result in loss of the colicine factor already carried by the acceptor strain. When a 'donor' strain carrying *colI* was incubated with an 'acceptor' carrying *colB*, or vice versa, many bacteria of the 'acceptor' strain acquired the colicine factor carried by the 'donor', becoming doubly colicinogenic (Table 3*c*). Thus for most pairs of colicine factors tested the prior presence in an acceptor strain of one factor failed to prevent the epidemic spread, in this acceptor, of some other, transmissible, factor, introduced by a donor strain.

However, when an acceptor strain carrying *colK* was incubated overnight with LT2 (*colI*) or LT2 (*colB*) only 0.4 and 8%, respectively, of the acceptor bacteria acquired *colI* or *colB*, as against *c.* 50% and *c.* 20%, respectively, when the acceptor was *col*⁻. This limited spread of *colI* in LT2 (*colK*) has been observed in several experiments.

We could not directly test whether the prior presence of one *colI* factor in an acceptor strain would prevent the epidemic spread in this strain of some other *colI* factor, introduced by a donor strain, because all the *colI* agents we examined were indistinguishable. But as LT2 (*colI*) (*colE2*) transmits both factors to many bacteria of a non-colicinogenic acceptor strain (Table 3*b*), presumably through the epidemic spread of the two factors in association, we could test whether *colE2* was transmitted in this way when the acceptor strain already carried *colI*. Strain *cysD-36* (*colI*) (*colE2*) was used as donor, and either *cysD-36 str-r col*⁻ or *cysD-36 str-r* (*colI*) as acceptor; after overnight incubation of broth inoculated with *c.* 10⁷ bacteria/ml. of donor and of acceptor strains, *c.* 15% of the non-colicinogenic acceptor population, but no detectable proportion (and thus certainly less than 0.01%) of the acceptor population carrying *colI* had acquired *colE2*. This inhibition by the prior presence of *colI* of the acquisition of *colE2* from a donor carrying *colI* and *colE2* suggests that there is no epidemic spread of *colI* and *colE2* together in a population already carrying *colI*, and so presumably no spread of *colI* in such a population.

DISCUSSION

The determinants for the production of five distinct colicines were thus transferred from *Escherichia coli* or *Shigella sonnei* into *Salmonella typhimurium* strain LT2, wherein each caused the production of a colicine indistinguishable by its spectrum of activity from that produced by the donor strains. Colicines E1 and E2, produced either by the original donor strains or by the LT2 derivatives, were distinguishable from each other not only by their lack of activity on indicator strains themselves producing the homologous colicine (Fredericq, 1956) but also by mutants resistant to colicine E2 but still sensitive to E1.

In the case of factors *colI*, *colE1* and *colE2* the newly introduced factors were never or almost never lost by the LT2 derivatives; but on storage at room temperature for many months non-colicinogenic bacteria become predominant in initially pure colicinogenic LT2 cultures carrying *colK* or *colB*. The reason for this difference in stability is unknown. Growth in the presence of acriflavine did not cause loss of *colI* or *colE2*. Perhaps the stability of these two factors in *Salmonella typhimurium* LT2 during storage and when exposed to acriflavine during growth results from their integration into the bacterial chromosome; for acriflavine treatment, though it 'cures' F⁺ *Escherichia coli* strains of their autonomous F agent, does not affect the integrated F agent of Hfr strains (Hirota, 1960).

The factors differed in their transmissibility by singly colicinogenic *Salmonella typhimurium* strains; *colE2* and *colK* were not transmitted at all, *colI* and *colB* on prolonged incubation were acquired by a high proportion of the cells of a non-colicinogenic acceptor, and *colE1* was of intermediate transmissibility. The ability of the source strain of *Escherichia coli* carrying the *colK* agent to transmit it to *S. typhimurium* may reflect an inherent difference in the behaviour of this factor in the two host species, or may result from the presence of some assisting factor, for instance the F agent, in the colicinogenic *E. coli* strain.

Only a small fraction (10^{-3} to 10^{-4}) of the bacteria in a broth culture of an LT2 strain carrying *colI* actually transmits the factor; and it appears that during long incubation of a mixed culture, *colI* spreads 'epidemically' in the acceptor population. The phenomenon of epidemic spread of *colI* (or *colB*) amongst non-colicinogenic populations can be explained by postulating that many or all bacteria newly infected by either (or both) these factors become 'effective donors', able to transmit. Experimental proof of the correctness of this postulate was later obtained (Stocker, Smith & Ozeki, in preparation). Transmission to a high proportion of an acceptor population, and so, by inference, epidemic spread, took place only when conditions were such that there was slow bacterial growth, at high bacterial concentrations, for some hours. Aeration of mixed cultures interfered with transmission apparently because of the effect of aeration on the 'growth cycle' of a broth culture, rather than any direct effect on the ability of colicinogenic bacteria to transmit their agent. This effect of aeration is therefore probably unrelated to its effect in producing an F⁻ (i.e. non-fertile) phenotype in F⁺ strains of *Escherichia coli* K12 (Cavalli, Lederberg & Lederberg, 1953).

The factors not transmitted at all by singly colicinogenic LT2 strains, viz. *colE2* and *colK*, and the poorly transmissible *colE1*, were transmitted, with the transmissible factor concerned, to many acceptor bacteria by doubly colicinogenic donor

strains carrying *colI* or *colB* as well as *colE2*, *colK* or *colE1*. These observations, and the data on the transmission of both their colicine factors by individual effective donors in doubly colicinogenic strains, suggest that when a bacterium becomes an effective donor, either spontaneously in an established colicinogenic strain carrying *colI* or *colB* or as a result of newly acquiring one of these factors, it transmits all the colicine factors it possesses.

During prolonged incubation the transmissible factor *colI* (or *colB*) was acquired by a high proportion of the bacteria of acceptor strains already carrying some colicine factor other than that of the donor strain; except in the case where the donor carried *colI* and the acceptor *colK* (Table 3c). One may infer that, with this one exception, bacteria already carrying some other factor are susceptible to the epidemic spread of *colI* or *colB*; and therefore that a bacterium carrying some other factor can accept *colI* or *colB* and then becomes an effective donor. On the other hand, *colE2* was not transmitted from LT2 (*colI*) (*colE2*) to a high proportion of an acceptor carrying *colI*. One may probably infer that a bacterium already carrying *colI* does not become an effective donor when it accepts *colE2*, and presumably *colI* also. Thus a bacterium already carrying *colI* apparently does not become an effective donor when it acquires an additional *colI* factor.

The epidemic spread of *colI* initiated by a single effective donor may involve $> 10^5$ acceptor bacteria, during a period in which the number of bacteria is only doubled. Under these conditions *colI* is clearly multiplying faster than the bacteria carrying it, and so may be said to be multiplying autonomously, by definition a property of an episome in its non-integrated state. By the same argument applied to epidemic spread initiated by doubly colicinogenic donors, factors *colE2*, *colK* and *colE1* are likewise capable of autonomous multiplication, at a rate faster than their hosts. The present data do not show whether colicinogenic bacteria which are not effective donors (all bacteria in strains carrying only *colE2* or *colK*; and nearly all in strains carrying *colI* or *colB*) are those in which the colicine factor(s) are integrated into the host chromosome; for their failure to transmit their factor(s) may result merely from failure to pair with acceptor bacteria.

The readily transmissible factors *colI* and *colB* in some ways resemble the *F* factor of *Escherichia coli*. Thus the presence of *colI* or *colB* in LT2 enables it to transmit the otherwise non-transmissible *colE2* and *colK*; and F^+ but not F^- sublines of *E. coli* K12 transmit colicine factors ER (= *colE1*) and S_2 (= *colE2*) (Fredericq, 1954b). Furthermore, under some conditions, the presence of the *colI* agent in a line of *S. typhimurium* results in conjugation and chromosomal recombination (Ozeki & Howarth, 1961; Smith & Stocker, 1962; see also Clowes, 1961). These similarities in behaviour of the *colI* agent in *Salmonella typhimurium* and the *F* agent in *E. coli* probably arise from the ability of each of these episomes to confer on its host the capacity to conjugate. It is likely that the large majority of the population carrying *colI* or *colB* are not 'competent donors' because the agent, though still present, does not confer ability to conjugate. It may be that the non-transmissibility (in the absence of assistance) of the *colE2* and *colK* factors in *S. typhimurium* is due to their inability to confer this ability to conjugate on even a small fraction of bacteria carrying them.

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