CHROMOSOMAL RECOMBINATION IN *HAEMOPHILUS INFLUENZAE*

JOHAN H. STUY, JOAN F. HOFFMANN AND LEA H. DUKET

Department of Biological Science, Florida State University, Tallahassee Florida 32306

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ABSTRACT

Haemophilus influenzae cultures doubly lysogenic for defective phage HP1, with a prophage marker sequence +b+/a+c, always contained some free wild-type phage. Single ultraviolet-irradiated cells produced either no wild-type phage or large numbers of them. This suggested that the phage was not released by the original double lysogen but by internal recombinants, i.e., by double lysogens with altered prophage marker sequence such as +++/abc or +b+/++c. Thirty-one wild-type phage-producing clones have been isolated independently from cultures of this double lysogen and identified. They fell in five classes. Two classes, still possessing all three prophage markers, can be explained by CAMPBELL's (1963) prophage recombination model. The other classes had lost one or more markers. They can be explained by interchromosomal double-strand DNA breakage and rejoining. A single-DNA-strand gene conversion model is discussed in view of the fact that genetic transformation involves single-DNA-strand exchanges. A number of potentially interesting mutants has been analyzed of which only the derivatives of *rec*1 mutant DB117 (obtained from Dr. J. SETLOW) were incapable of internal recombination.

IN bacterial conjugation, transduction or transformation a donor DNA fragment is introduced into a recipient cell. Through some only vaguely understood recombinational event part of the recipient chromosome is replaced by a corresponding part of the donor DNA. This exchange involves single strands in bacterial transformation (FOX and ALLEN 1964; BODMER and GANESAN 1964; NOTANI and GOODGAL 1966). No such definite information is yet available for conjugation or transduction.

Studies of recombination between two "chromosomes" coinhabiting a bacterium have so far involved a chromosome and an episome (we do not consider here the integration of episomes into chromosomes). GALLANT and SPOTTSWOOD (1965) observed homozygous recessive R⁻F′ merodiploid cells in *Escherichia coli* cultures R^+ for alkaline phosphatase and carrying an R^- substituted F′ episome. BERG and GALLANT (1971) studied internal crossing over in partially diploid *lac*- substituted F′ strains of *E. coli*. They reported that such events generate predominantly homozygous F′ partial diploids in addition to haploid HFR and heterozygous F′ partial diploid recombinants and concluded that such recombination is essentially non-reciprocal. HERMAN (1965, 1968) studied lac^+ segregrante from cultures of *E. coli* $i^- z^- y^+/F' i^+ z^+ y^-$. He came to the conclusion that

recombination between chromosome and episome had led to recombinant (*lac* z^+ *lac* y^+) episomes. Further experiments indicated that such recombination resulted in the formation of two products each containing a region of hybrid DNA. MESELSON (1967) constructed an *E. coli* strain lysogenic for phage $\lambda_{c\ mi\ h}$ and possessing an episome carrying λ_{+++}. He examined 5735 independently descended clones after 30 generations by scoring for the types of free phage released by the cells. Analysis of his observations led him to conclude that recombination between chromosomal and episomal prophage is reciprocal. CAMPBELL (1963), on the other hand, studied the segregation of *E. coli* phage λ monolysogens. Observing an occasional double lysogen with altered prophage marker sequence, he postulated that the two tandem prophages synapse "in circular fashion". One crossover then leads to the excision of a circular phage genome and a monolysogen (after segregation) while two crossovers give rise to a double lysogen with altered marker sequence.

We have observed wild-type phage in cultures of *Haemophilus influenzae* strain Rd doubly lysogenic for defective phage HP1 (HARM and RUPERT 1963). The two prophages carried three different genetic markers in the sequence +b+/ a+c. We have speculated that such cells do not release wild-type phage. They undergo first some kind of genetic recombination giving rise to two prophages with three adjacent wild-type loci. It is these internal recombinants that spontaneously release wild-type phage (STUY and HOFFMANN 1970). We have followed up this speculation and report in this article that *H. influenzae* double lysogens indeed undergo frequent chromosomal recombination.

MATERIALS AND METHODS

Terminology: In agreement with a suggestion by TOMASZ (1969) we distinguish in this publication between transformation-negative mutant cultures which are able to bind double-stranded DNA irreversibly but fail to genetically integrate it, and mutants which cannot take up DNA. We call the former *rec*$^-$ while the latter are designated *com*$^-$. *Com*$^-$ mutants can be shown to be *rec*+ or *rec*$^-$ by analyzing their ability to perform prophage recombination (HOFFMANN and STUY, 1972). We use the term transformable to denote the entire transformation process.

Media: SBHI agar contains 1.5% agar, 3.7% Difco Brain Heart Infusion, 10 μg of nicotine amide adenine dinucleotide (NAD) and of hemin per ml. L broth is one volume of BHI and two volumes of Eugon broth (3%; Baltimore Biological Laboratories) supplemented with NAD and hemin. YT agar is 1.5% agar containing 1% Difco Tryptone, 0.5% Difco Yeast Extract, 0.85% NaCl, NAD and hemin, 0.5% glycerol and 1 mM $CaCl_2$. Soft agar contains 0.7% agar. It had suddenly become necessary to add one or more drops of a 1% bovine serum albumin solution to YT soft agars to ensure a continuous bacterial indicator lawn. Dr. FRANK YOUNG recently suggested that present Difco Noble agar batches contain some interfering lipid material removable by shaking an aqueous slurry with methanol-chloroform (v/v). We now routinely treat our agar for YT medium this way. YT agar is used for the plating of phage HP1 at 30°C.

Mutant cultures: All experiments were carried out with derivatives of *H. influenzae* strain Rd (ALEXANDER and LEIDY 1951) and temperate phage HP1 (HARM and RUPERT 1963) both kindly supplied by Dr. C. RUPERT. Non-lysogenic mutants DB116 and DB117 were generously given to us by Dr. J. SETLOW. These mutants are sensitive to ultraviolet radiation (UV). DB117 is also transformation-negative (*rec*$^-$, SETLOW *et al.* 1968). Phage indicator cultures (STUY 1968) and defective lysogens (STUY 1969) have been described earlier (STUY and HOFFMANN 1971). Figure 1 shows a map of the prophage markers employed. For sake of brevity we call these here

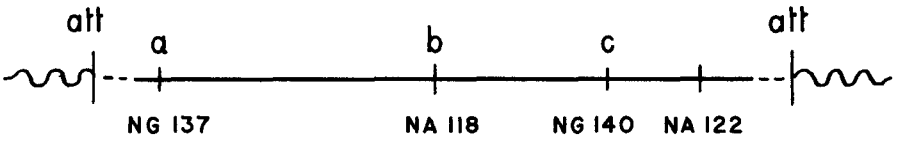

FIGURE 1.—Prophage map of *Haemophilus influenzae* phage HP1. The vegetative phage map is very similar. a, b and c notations are used in this article rather than NG137, NA118 and NG140, respectively.

a, b and c. Preparation of transformation-negative mutants using N-methyl-N′-nitroso-guanidine (NG) or by simple construction (lysogenization, transformation) has been described (HOFFMANN and STUY 1972).

Double lysogens of prophage marker sequence +b+/a+c were obtained by superinfection of +bc doubly defective monolysogens with temperature-sensitive (*ts*) phage a++ followed by selection of double lysogens capable of producing normal numbers of *ts* phage a++. DNA extracts of the purified clones were then checked for efficiency of co-transformation of the b+ and c+ prophage loci into the doubly defective +bc monolysogen (STUY and HOFFMANN 1971). Of the four possible tandem double lysogens only those with prophage marker sequence +b+/a+c are inefficient (STUY and HOFFMANN 1970; see also table 3). Where possible, identification was also carried out by analysis of monolysogenic segregants. Table 1 lists all cultures employed.

Co-transformation index: This value denotes the ratio of wild-type lysogenic transformants and antibiotic-resistant ones observed in a single transformation cross (STUY and HOFFMANN 1971). For single prophage markers this value is called transformation efficiency (STUY 1969;

TABLE 1

Haemophilus influenzae *Rd cultures used in this study; a, b and c are prophage mutations*

Mutant culture	Derivation	Reference*
362 str-r +++c	Wild-type lysogen; mutated with NG	a
363 str-r +bc	Superinfection of +b+ lysogen with phage +++c; segregant	a
371 nov-r +bc	Transformation of 363	a
378 str-r +++c rec⁻	NG-treatment of 362	b
430 str-r +bc rec⁻	NG-treatment of 363	b
431 str-r +bc rec⁻ com⁻	NG-treatment of 363	b
446 nov-r +b+/a+c rec+	Superinfection of 371 with *ts* phage a++	a,c
457 str-r +bc com⁻	NG-treatment of 363	b
485 UV-s rec+	DB116, from Dr. JANE SETLOW	d
486 UV-s rec1	DB117, from Dr. JANE SETLOW	d
499 str-r +bc rec⁻	Superinfection of 378 with *ts* phage +b+; segregant	b,e
514 str-r +b+/a+c com⁻	Superinfection of 457 with *ts* phage a++	b,e
515 str-r +b+/a+c com⁻	Superinfection of 457 with *ts* phage a++	b,e
523 str-r +b+/a+c rec⁻ com⁻	Superinfection of 431 with *ts* phage a++	b,e
561 str-r +b+/a+c rec1	Transformation of 446 with DNA (DB117 str-r)	e
564 str-r +b+/a+c rec⁻	Superinfection of 430 with *ts* phage a++	b,e
565 str-r +b+/a+c UV-s rec+	Transformation of DB116, lysogenization with phage +bc, superinfection with *ts* phage a++	b,e
566 str-r +b+/a+c rec1	Transformation of 446 with DNA (DB117 str-r)	e
570 str-r +b+/a+c rec⁻	Superinfection of 499 with *ts* phage a++	b,e

* a. STUY and HOFFMANN (1971); b. HOFFMANN and STUY (1972); c. STUY and HOFFMANN (1970); d. SETLOW *et al.* (1968); e. This article.

LACKS and HOTCHKISS 1960). We have normalized all values with respect to our standard novobiocin-resistance marker. The analyses reported in this article always included a wild-type monolysogenic donor as a control. Co-transformation indexes for the latter are: a+b+, 0.03–0.04; b+c+, 0.05–0.07; and a+b+c+, 0.02–0.03.

Techniques: The following references may be checked for the techniques used: preparation of transformable (competent) bacteria and their transformation, STUY (1962) and STUY and HOFFMANN (1971); preparation and assay of wild-type or mutant phage HP1, STUY (1968) and STUY and HOFFMANN (1971); transformation of defective lysogens to wild-type, STUY (1969); isolation of double lysogens, their identification and that of their monolysogenic segregants, STUY and HOFFMANN (1971); and prophage recombination, HOFFMANN and STUY (1972).

RESULTS

Single phage burst analysis: Cultures of the double lysogen 446 with prophage marker sequence +b+/a+c, grown up at 30°, always contained 1–3 × 10^5 wild-type phage per ml in addition to the expected 1–3 × 10^7 *ts* phage a++ (see also top line in Table 4). Phage a++ can arise in such cultures through a single excision event (CAMPBELL 1963) followed by lytic phage growth. However, the release of wild-type phage from cell line 446 (prophage marker sequence +b+/a+c) requires some act of recombination.

Four hundred forty-six cultures, grown up at 30° (or at 37°) from single cells in the presence of 10^8 DNase-treated *ts* phage a++ per ml contained the same numbers of wild-type phage. This indicates that the observed phenomenon is not due to reinfection. In fact, *H. influenzae* Rd cells grown at 30° in BHI media are phenotypically phage-resistant (STUY 1968). Since phage HP1 is very inactive in vegetative recombination (BOLING and SETLOW 1969; our unpublished observations involving the entire genetic map), production of wild-type phage by double cross-over (+b+ × a+c) in phage-replicating bacteria can be expected to be a rare event. We have therefore speculated (STUY and HOFFMANN 1970) that the observed wild-type phage are released by bacteria that have previously recombined their prophages leading to three wild-type loci in one complete prophage sequence, such as +++/abc or +b+/++c. Cultures with such prophage marker sequences have been found to release large numbers of wild-type phage in addition to mutant phages a++, or +b+ and ++c, respectively (STUY and HOFFMANN 1971; also see Table 3).

In agreement with this view were our observations that UV-irradiation of growing cultures of cell line 446 at 10^9 bacteria per ml always resulted in 2–5 × 10^8 phage a++, and 2–5 × 10^6 wild-type phage infectious centers per ml. After completion of phage growth, such lysates always contained 2–4 × 10^{10} free phage a++ and 2–4 × 10^8 free wild-type phage per ml. It thus appeared that the UV-induced wild-type phage-releasing bacteria produced normal-size bursts of wild phage particles.

Analyses of single phage bursts supported this view. A number of growing cultures were UV-induced and then diluted to about one wild-type phage-producing infectious center per five aliquots of 0.5 ml of L broth. After sufficiently long incubation, each aliquot was plated for wild-type phage. Table 2 shows the distribution of phage in bursts obtained with our standard wild-type monolysogenic

TABLE 2

Distribution of wild-type phage in single bursts produced by several UV-induced HP1-lysogenic H. influenzae *cell lines*

Culture	None	Number of aliquots with indicated numbers of +++ phage* 1–24	25–49	50–99	100–149	150–199	200–299	300–399	400–500
221 (HP1)	33	0	0	1	9	2	2	1	1
221 (HP1)	41	2	1	5	1	2	2	1	1
452 (HP1, HP1)	47	0	0	1	3	1	1	1	0
446 +b+/a+c, 37°	41	0	2	2	3	0	3	1	2
446 +b+/a+c, 30°	38	5	3	3	3	1	2	0	0

* The average burst size produced by UV-induced or phage-infected cultures is 90–100 particles per cell.

culture 221, a wild-type double lysogen and the triply defective double lysogen 446 (prophage marker sequence +b+/a+c). The numbers of wild-type phage in bursts produced by 446 cells at 30° or at 37° are clearly very much higher than those predicted from rare vegetative phage recombination. Since the average phage burst produced under our conditions equals 90–100 particles, induced 446 cells appeared to release predominantly half, whole, or multiple size bursts of wild-type phage (multiple size bursts always seemed to occur at frequencies greater than expected from the Poisson distribution). This, of course, is very unlikely. Instead we propose that some 446 cells first undergo internal recombination. Some of these recombination events lead to altered prophage marker sequences which now allow the bacteria to produce (normal numbers of) wild-type phage. However, it can still be argued that UV- or spontaneous induction might first lead to recombination between the two prophages and thereafter to prophage excision. Indeed, CURTISS (1968) has published a detailed article on the stimulation of UV-irradiation on internal recombination (between chromosome and exogenote) in *E. coli*.

Isolation and identification of internal recombinants: To test our hypothesis that 446 cultures always contained bacteria with altered prophage marker sequence (internal recombinants) we developed two methods for the isolation of such recombinants. Firstly, subclones of cell line 446 were diluted to 0.1–0.2 cells per broth aliquot and incubated at 30°. Since the bacteria are phenotypically phage-resistant under these conditions, we avoided the complications arising from re-infection by spontaneously released phage. The turbid aliquots (designated 446 subsubclones since they grew up from single cells) were titered for free wild-type phage. Subsubclones containing abnormal numbers of this phage were streaked on agar and single colonies again analyzed for their ability to produce wild-type phage. Those subsubclones releasing abnormal numbers of phage were then frozen at −90° in the presence of 15% glycerol (v/v) and eventually further analyzed. This is a time-consuming method but it has the advantage that one can observe subsubclones releasing abnormal numbers of mutant phages, such as ++c, or no phages at all. In the second method, a large number of 446 subclones were first analyzed with respect to b^+c^+ co-transformation (see below) and the

presence of wild and *ts* phage a+++ (ratio should be about 1%). Confirmed 446 subclones were stored at −90°. Each subclone was diluted and plated in soft SBHI agar on hard SBHI agar to give about 300 small colonies after 12–14 hr of incubation at 30°. The plates were then overlayered with antibiotic-sensitive indicator cells (novobiocin in this case) in soft SBHI agar and incubated at 37° for 6–8 hr. About half of the colonies had produced a small clear halo in the top soft agar layer (indicating production of some wild-type phage) while the other half of the colonies showed no such halos. Most plates also contained one or more colonies surrounded by a large halo of clear agar which signified the release of many wild-type phage. These colonies were stabbed and suspended in SSC (0.15 M NaCl, 0.015 M Na citrate; to kill free phage). After 5 min at room temperature they were streaked on antibiotic-containing agar. Three or four colonies of each streak were inoculated in L broth, grown up at 30° and titered for free wild-type phage. In nearly all cases all colonies gave the same results and one was then stored frozen. We did not isolate more than four subsubclones from each 446 subclone and discarded those that could have been sisters. All but four subsubclones (i.e., 456, 489, 490, and 560) were isolated by the second method.

We have previously described two methods which independently allowed us to identify the order of the a, b, and c markers in double lysogens. One method is the analysis of monolysogenic segregants as first described by CAMPBELL (1963). The other method is the determination of co-transformation values for the a^+ and b^+, b^+ and c^+, or a^+, b^+ and c^+ loci using the double lysogen as a donor and a^-b^-, b^-c^-, or $a^-b^-c^-$ monolysogens as recipients. These values are normalized with respect to the internal reference novobiocin or streptomycin resistance marker. The normalized values (co-transformation indexes) turn out to be much higher for marker pairs residing in the same prophage. For instance, an $a^+b^+c^+/a^-b^-c^-$ donor double lysogen gives a much higher a^+b^+ co-transformation index than an $a^+b^-c^-/a^-b^+c^+$ double lysogen.

We have used both methods for the identification of 32 subsubclones. All 32 turned out to have altered prophage marker sequences and we thus classify them as internal recombinants. They appeared to fall in five classes which are listed in Table 3. Recombinants in the first two classes still possess the original three prophage markers but those in the next two classes appeared to have lost either the a^- or the b^- marker. We think it significant that we did not observe recombinants which had lost only the c^- marker, because such cells cannot release wild-type phage according to our hypothesis. The fifth class of recombinants consists of subsubclones which had lost more than one deficiency locus. We isolated one recombinant which had lost the wild c^+ locus (547 N-1). Its isolation was possible because one can score the plaques (and halos) produced by the mutant phage ++c.

Internal recombination in mutant cultures: We have studied internal recombination in a number of potentially interesting mutants. This was done by converting these mutants into cell lines possessing the prophage marker sequence +b+/a+c (see Table 1). This sequence was confimred by analysis of monolysogenic segregants and by determining the b^+c^+ co-transformation index (Table 4).

TABLE 3

Identification of wild-type phage-producing H. influenzae *clones isolated from single-cell cultures of cell line 446 with prophage marker sequence +b+/a+c*

Subsubclone	Free phage	Co-transformation index a^+b^+	b^+c^+	$a^+b^+c^+$	Monolysogenic segregants	Suspected prophage marker sequence
CLASS 1						
527 C-1	9.6×10^6	.074	.052	.028	8 abc; 3 +bc; 2 ++c; 1* ab+; 1* +b+	+++/abc
456 Z-1	1.2×10^7	.071	.028	.021	12 abc; 2 ++c; 1* a+c	+++/abc
CLASS 2						
530 D-1	2.4×10^7	.031	.0053	.011	16 ++c; 9 a+c; 5 abc; 4 ab+; 3* a++; 1* +b+	ab+/++c
532 E-1	2.8×10^7	.067	.0047	.0070	8 a+c; 5 abc; 2 ++c; 1 ab+; 2* a++	ab+/++c
534 F-1	1.3×10^7	.054	.0029	.0067	11 a+c; 5 ab+; 4 ++c; 3* a++; 1* +bc	ab+/++c
540 J-1	4.8×10^7	.078	.022	.029	12 a+c; 9 abc; 6 ++c; 3 ab+; 5* +b+	ab+/++c
541 K-1	†	.043	.0051	.0048	4 a+c; 3 ++c; 2 abc; 2 ab+	ab+/++c
543 L-2	2.2×10^7	.054	.016	.011	15 abc; 11 ab+; 13 a+c; 4 ++c; 4* +b+; 1* a++	ab+/++c
545 M-1	1.5×10^7	.040	.0019	.0039	5 ab+; 5 ++c; 4 a+c; 1 abc; 1* a++; 1* +bc	ab+/++c
548 N-2	9.0×10^6	.045	.0045	.0033	11 a+c; 3 abc; 2 ab+; 1 ++c; 1* a++	ab+/++c
549 O-1	1.7×10^7	.076	.014	.0093	22 ab+; 6 a+c; 5 abc; 2* a++; 1* +b+	ab+/++c
CLASS 3						
489 Y-1	3.4×10^6	.11	.074	.080	12 ++c; 3 a+c; 1* a++	+++/a+c
531 D-2	3.5×10^6	.048	.038	.064	20 ++c; 2 a+c; 2* a++	+++/a+c
533 E-2	†	.086	.027	.062	17 ++c; 2 a+c; 2* a++	+++/a+c
536 F-3	4.0×10^6	.058	.036	.051	12 ++c; 3 a+c; 1* a++; 1* abc	+++/a+c
538 H-1	8.1×10^6	.043	.065	.038	11 ++c; 4 a+c; 1* a++	+++/a+c

TABLE 3—Continued

Subsubclone	Free phage	Co-transformation index a^+b^+	b^+c^+	$a^+b^+c^+$	Monolysogenic segregants	Suspected prophage marker sequence
CLASS 4						
490 Y-2	1.7×10^7	.091	.028	.0021	11 ++c; 6 +b+; 1 +bc	+b+/++c
529 C-3	1.0×10^7	.030	.0047	.0025	8 ++c; 8 +b+; 1 +bc; 1* abc	+b+/++c
537 G-1	1.9×10^7	.051	.0087	.0068	7 ++c; 7 +b+; 3 +bc	+b+/++c
539 I-1	3.8×10^7	.041	.0092	.0028	5 ++c; 5 +b+; 3 +bc; 2* a+c	+b+/++c
544 L-3	1.5×10^7	.044	.011	.0095	21 ++c; 19 +b+; 13 +bc; 1* ab+	+b+/++c
550 P-1	8.5×10^6	.092	.012	.0081	27 +b+; 5 ++c; 3 +bc; 1* ab+	+b+/++c
552 R-1	2.7×10^7	.082	.015	.0059	10 +b+; 3 ++c; 3 +bc	+b+/++c
554 T-1	2.5×10^7	.055	.014	.0061	14 +b+; 2 +bc; 1 ++c	+b+/++c
CLASS 5						
528 C-2	7.0×10^7	.10	.12	.11	none	+++/+++
535 F-2	4.0×10^7	.13	.076	.041	15 +b+; 5 ++c; 2 +bc; 1* ab+	+b+/+++/++c
542 L-1	2.0×10^7	.090	.11	.041	22 +b+	+b+/+++
546 M-2	1.2×10^7	.053	.042	.015	8 ++c; 3 a++; 1 a+c	a++/++c
547 N-1	none‡	.034	.000	.0000	13 ++c; 3 +bc	+bc/++c
551 Q-1	1.4×10^7	.10	.11	.051	none	+++/+++
553 S-1	1.6×10^7	.085	.075	.058	30 +bc; 3 ++c; 2* +b+	+++/+bc
560 ZZ	1.1×10^7	.11	.060	.030	12 +b+; 5 ab+	+++/ab+

* Unexpected segregants.
† Lost.
‡ About 1×10^7 free phage ++c.

TABLE 4

Properties of mutant cultures of H. influenzae *double lysogens with prophage marker sequence +b+/a+c*

Culture	Free phage		UV-induction		Co-transformation index			Monolysogenic segregants
	Wild	a++	Wild	a++	a^+b^+	b^+c^+	$a^+b^+c^+$	
446 rec+	1.8×10^5	1.5×10^7	4.5×10^6	3.8×10^8	.0078	.0036	.0014	8 ++c; 7 a+c; 2 +b+
514 com−	7.2×10^4	3.0×10^6	6.0×10^6	5.8×10^8		.004		10 ++c; 3 a+c; 1 +b+
515 com−	9.3×10^4	3.3×10^6	1.0×10^7	8.4×10^8		.003		8 ++c; 7 a+c; 2 +bc; 1 +b+
523 rec− com−	7.7×10^4	3.6×10^6	8.5×10^6	6.0×10^8	.002	.003	.0001	10 +b+; 9 +bc; 7 a+c; 4* a++ 3 ++c; 2* abc
561 rec1†	none	2.2×10^4	none	none		.0013		none
564 rec−	1.2×10^5	7.3×10^6	7.8×10^6	5.4×10^8	.003	.002	.002	11 +b+; 8 a+c; 5 +bc; 2* a++ 1* abc
565 UV-s‡	8.0×10^5	5.5×10^6	5.0×10^6	1.1×10^8	.004	.0065	.008	6 +bc; 5 ++c; 3 a+c; 1 +b+; 3* abc
566 rec1†	none	1.2×10^4	none	none	.001	.0015	.0003	none
570 rec−	4.2×10^4	2.6×10^6	2.0×10^6	1.5×10^8	.002	.003	.0005	9 ++c; 6 a+c; 3 +bc

* Unexpected segregants.
† Derived from Dr. J. SETLOW's DB117 (SETLOW *et al.*, 1968).
‡ Derived from Dr. J. SETLOW's DB116 (SETLOW *et al.*, 1968).

Confirmed cultures were then studied with respect to spontaneous production of phage at 30° and with respect to UV-induced phage producers (Table 4). In some cases we studied more than one double lysogen with the given mutation. We have not identified internal recombinants as we have done for the wild-type (*rec*+) culture 446. Nevertheless, we believe that the data presented in Table 4 are sufficient proof that all mutants but one were capable of normal internal recombination. These mutants include two *rec*- mutants (523 and 564), two *com*- mutants (523 and 514/515), one mutant with reduced transforming ability only at 37° but with seemingly normal prophage recombination (HOFFMANN and STUY 1972; mutant 570 which was here studied exclusively at 37°) and a *rec*+ UV-sensitive mutant (565, derived from Dr. J. SETLOW's DB116 which is incapable of thymine dimer excision; see SETLOW *et al.* 1968). The only mutant incapable of internal recombination (and of segregating monolysogens) was 561/566 which was derived from Dr. J. SETLOW's DB117. This culture produces greatly reduced levels of *ts* phage a++ and is non-inducible by UV (Table 4; this has been reported by BOLING and SETLOW 1969, for the wild-type lysogen). When plated at 30° in soft YT agar, all colonies produced a clear zone in the top soft agar layer containing indicator bacteria. This demonstrated the absence of defective monolysogenic segregants in about 10,000 cells plated. It also showed that enough *ts* phage a++ had been released by the colonies to give the clear zones in the top soft agar layer. When some 20,000 cells were plated in soft SBHI agar at 37°, no colonies were found with such clear zones. This indicated the absence of internal recombinants capable of producing wild-type phage.

DISCUSSION

We have demonstrated the presence of double lysogens with altered prophage marker sequence in cultures of the double lysogen 446 which possesses the prophage marker sequence +b+/a+c, even when the latter were grown up from single cells. Our identification of these altered cell lines was based on two independent analyses: identification of monolysogenic segregants and determination of co-transformation indexes of wild prophage loci. Examination of Table 3 will reveal that neither method gave absolutely satisfactory results. Thus some co-transformation indexes turned out (reproducibly) high, perhaps indicating that the donor bacteria were triply rather than doubly lysogenic. And we occasionally observed unexpected segregants which may have been derived from secondary internal recombinants. Most of the observed abc monolysogenic segregants were checked for, and found to possess the NG122+ locus. This demonstrates that they were not contaminants. Although at present we have no explanation for the observed discrepancies, we think that both methods together give a reasonable identification of the prophage marker sequence in double lysogens.

We have ruled out the possibility that re-infection of 446 cells by spontaneously released phage resulted in an altered prophage marker sequence because our experimental conditions prevented such a complication. However, it can be argued that the doubly lysogenic bacteria frequently excise one prophage. The high incidence of defective monolysogens (1–3%) in nearly all our doubly lysogenic

bacterial cultures suggests this strongly. Such excised prophages might either insert again at a different site giving rise to class 1 and 2 internal recombinants, or they might exchange markers with the tandem prophage sequence in the other chromosome to give class 3, 4 and 5 internal recombinants. Our observations that mutants 430 and 431 (Table 1) are deficient in prophage recombination (recombination between superinfected phage genome and resident prophage; see HOFFMANN and STUY 1972) while they show normal internal recombination do not support this possibility. We think it therefore much more likely that the observed double lysogens with altered prophage marker sequence arose through recombination events between two chromosomes or within one chromosome.

Two basically different recombination mechanisms can then be considered. One involves exchanges of double-stranded DNA segments while the other considers single-DNA-strand gene conversion. Double-stranded marker exchanges between two prophages in different chromosomes as shown in Figure 2A explain the occurrence of class 3 and 4 internal recombinants while exchanges of more than one marker give rise to class 5 recombinants. Such exchanges have been reported to occur between chromosome and episome in *E. coli* (see Introduction). Recently, RUSSELL *et al.* (1970) observed that *E. coli* strains possessing two adjacent identical genes for the minor tyrosine tRNA, segregated one-gene (10^{-7} to 10^{-5}) and three-gene derivatives (10^{-5} to 10^{-4}), presumably by unequal recombination between the two chromosomes. The low frequencies may be due to the small size of the repeated gene sequence. The drawback of the model of Figure 2A is that it must involve double crossover events and that it fails to consider the much more frequent (?) single crossovers.

Double-stranded marker exchanges between two prophages in the same chromosome have been discussed by CAMPBELL (1963; see Figure 2B). Although

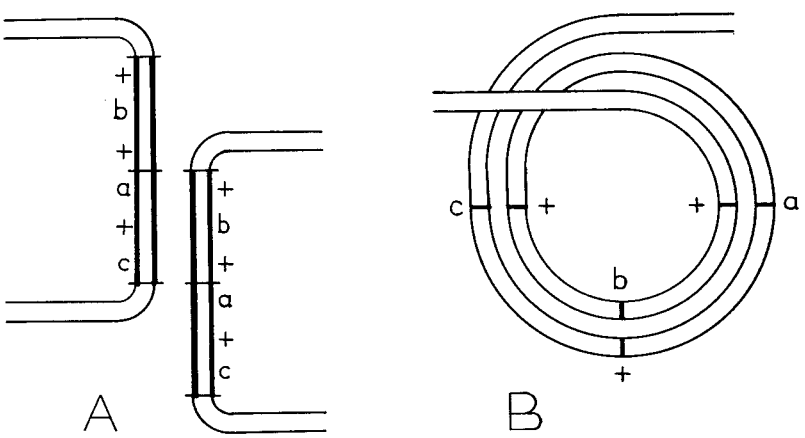

FIGURE 2.—A. Interchromosomal recombination by double-stranded breakage and rejoining; double crossovers flanking the a, b or c locus lead to marker exchanges between prophages in different chromosomes. B. CAMPBELL's (1963) model for "circular" synapsis of two tandem prophages; double crossovers flanking the a, b or c locus lead to intrachromosomal marker exchanges between the two prophages.

such exchanges also involve double crossovers, single crossover events are readily observed. They lead to either monolysogenic segregants or to phage production initiated by the excised circular phage genome. CAMPBELL's model thus readily explains the occurrence of class 1 and 2 internal recombinants.

In view of the fact that genetic transformation occurs through exchange (or insertion) of single DNA strands, we believe that one should also seriously consider single-strand gene conversion as a mechanism for the observed internal recombination. We have already speculated (STUY and HOFFMANN 1970) that transformation is just an aspect of chromosomal recombination. HOTCHKISS (1970) has published an elegant unified model for DNA–DNA recombination which can be interpreted as single-strand insertion and gene conversion. BOON and ZINDER (1971) presented evidence of recombination between f1 bacteriophage mutant genomes favoring a gene conversion model. Their results cannot be reconciled with the reciprocal breakage-rejoining model for recombination and they give a number of alternative models. SPATZ and TRAUTNER (1970) published an elegant demonstration of gene conversion occurring in *Bacillus subtilis* transfection with heteroduplex bacteriophage DNA. They showed that conversion for a given phage marker was asymmetric with regard to the strand which was preferentially corrected but that this asymmetry was not a simple matter of purine or pyrimidine conversion. Basically then, we envision that gene conversion can result in altered prophage marker sequences in *H. influenzae* double lysogens. First, single strands of different prophages in the same or in different chromosomes synapse, and then one strand induces the "correction" of the other strand. However, the fact that we cannot score both internal recombination products prevents us from setting up more detailed models.

Finally, we like to comment on the teleological function of internal recombination. The most attractive assumption is that internal recombination is one way by which bacteria can repair chromosomal damage (RUPP and HOWARD-FLANDERS 1968; HOWARD-FLANDERS *et al.* 1968; RADMAN *et al.* 1970). Indeed, UV-irradiation (CURTISS 1968) and thymidine starvation (GALLANT and SPOTTSWOOD 1965) of *E. coli* increased considerably subsequent recombination between chromosome and episome. Very recently, RUPP *et al.* (1971) demonstrated the physical exchange of single-strand DNA segments between sister chromatids in UV-irradiated thymine dimer excision-deficient *E. coli* mutants. The events observed by us in "normally grown" *H. influenzae* cultures may then reflect efforts to repair DNA duplication mistakes. Earlier observations (STUY and HOFFMANN 1971; 1970) that rapidly growing *H. influenzae* bacteria do not seem to have their recombination system "mobilized" suggested to us that such cells may not be undergoing internal recombination. Preliminary experiments have supported this view. When cell growth is slowed down (as occurs during oxygen or nutrient starvation) the recombinational system appears to become active (STUY and HOFFMANN 1971; 1970). The competent state of transformable bacteria may then have evolved to ensure the bacteria of another possibility of obtaining a "DNA partner" for internal recombination, i.e., the cells take up homologous DNA from their surrounding with the purpose of using it in chromosomal repair.

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