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Addition, Deletion, and Substitution of Long Nonhomologous Deoxyribonucleic Acid Segments by Genetic Transformation of *Haemophilus influenzae*

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A complete *Eco*RI digest of *Haemophilus influenzae* phage HP1 deoxyribonucleic acid (DNA) was mixed with incomplete digests of various *H. influenzae* R plasmids, sealed with T4 ligase, and transformed into an HP1 lysogen. Most of the chloramphenicol- and tetracycline-resistant transformants did not produce phage although they possessed all the phage genes examined. They also did not transfer antibiotic resistance by conjugation. DNA lysates from them transformed other lysogens to resistance and to loss of phage production at different but quite high frequencies (addition of long DNA segments). They themselves could be transformed efficiently to strains with a wild prophage (deletion of long DNA segments). It was concluded that lysogenic cultures had been constructed with various DNA inserts in their prophages carrying antibiotic resistance genes from the R plasmids. The site of insertion was determined by genetic crosses. DNAs with inserts that transferred with lower efficiency were more sensitive to ultraviolet radiation. This supports the view that insert transfer efficiencies reflect the sizes of the insert.

Additive transformation, or the addition to the genome of recipient cells of very long (nonhomologous) DNA segments by genetic transformation, has been reported for *Bacillus subtilis* (1, 5), *Streptococcus pneumoniae* (15), and *Haemophilus influenzae* (3, 21, 23). This transfer is characterized by its low efficiency. Interspecific (heterologous) transformation is similarly characterized (4, 9, 15, 27), and it has been proposed that both additive and heterologous transformation occur through synapsis between regions in the donor DNA flanking the added segment (4, 9, 15, 21, 23). Evidence was presented recently (23) that in *H. influenzae* additive transformation involving chromosomally integrated R plasmids, as well as transfection with DNA from single lysogens (24), occurs by integration of the added segment (R plasmid or phage genome) as a single strand. The efficiency of additive transformation as observed for transfer of integrated plasmids is quite low (23) as compared to that of a high-efficiency point mutation. It is attractive to assume that the low efficiency is a consequence of the large size of the added DNA segment (30 to 40 megadaltons [Md] for the integrated plasmids). We have in this study examined this possible size effect by using donor DNA with rather well-characterized DNA inserts. We have also studied the deletion of these inserts and the replacement of one insert with another one.

MATERIALS AND METHODS

Strains. All *H. influenzae* strains used were derived from the BC200 strain isolated by Barnhart and Cox (2). The *uvr-1* mutant strain was obtained from Jane Setlow (her DB112; see reference 14). This strain does not excise UV-induced thymine dimers. We used for all test crosses the lysogenic strains Rd1021 *strA1* ($HP1^+$) and Rd1023 *vioA2* ($HP1^+$) and several Rd *novA1* strains with a defective HP1 prophage. Phage HP1 was given to us by Claude Rupert (8). The conjugative R plasmids pRI234 Cm^r Tc^r, pJS1057-2 Ap^r, and pJS1782 Cm^r Tc^r and the small plasmid RSF0885 Ap^r have been described (6, 11, 20, 21).

Our reference antibiotic resistance markers (16, 20, 21) were the high-efficiency mutation *novA1* and the low-efficiency mutations *strA1*, *vioA2*, and *novAB*. All transformation efficiencies mentioned in the text and tables are the ratios of transformants studied to those for the reference marker, and they have all been normalized with respect to the efficiency of the *novA1* marker.

Phage map. Figure 1 shows the map positions of the various phage mutations used. Some 60 mutations have been mapped. Vegetative phage DNA cosediments with *Escherichia coli* phage T7 DNA in sucrose density gradients; its size is thus about 25 Md. Cotransformation of three markers spanning about 80% of the map is roughly 30% (18).

Transforming DNA. We always prepared transforming DNA by mixing 0.2 ml of a young stationary-phase culture with 0.02 ml of a solution of 1 mg of pronase per ml and 1% sodium dodecyl sulfate in 0.15 M NaCl-0.015 M sodium citrate and incubating this mixture at 42°C for 2 h. We then added 5 ml of 0.1 M

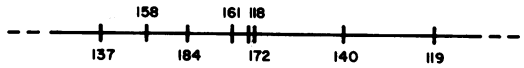

FIG. 1. *Genetic map of prophage HP1. Distance between the NG137 and NA118 loci is 105 map units; between NA118 and NG140 it is 72 units (17). Distances between terminal markers and prophage ends are not known. The vegetative phage map is identical (18).*

NaCl–0.1 mM EDTA and swirled the solution gently. One volume of this was added to 10 volumes of competent cells.

Plasmid DNA. Plasmid DNA was isolated as described earlier (7, 20). We used 1% agarose gels for the demonstration of free plasmid DNA (13, 20).

Phage HP1 DNA. HP1 DNA was isolated as reported in an earlier study (19). The phenol-extracted water phase was mixed with 2 volumes of 95% ethanol, and the precipitated DNA (after overnight storage at −20°C) was dissolved in electrophoresis buffer (20).

Transformation. Transformation techniques were those described before (16, 17). We used the agar overlay method. Final antibiotic concentrations were 5 μg/ml for ampicillin with 0.1 μg of tetracycline per ml (22) and 3 μg/ml for chloramphenicol and tetracycline.

Conjugation. Transfer of antibiotic resistance by conjugation was done using the standard filtration method (20).

Preparation of ligase-sealed DNA. *Eco*RI enzyme and T4 ligase were purchased from Miles Laboratories. We used their recommended reaction mixtures. About 2 μg of phage HP1 DNA in 50 μl of reaction mixture was digested with 20 U of *Eco*RI for 8 h at 37°C. We always saw three bands on agarose gels (15 h at 50 V and 15 mA) at about 45 mm from the well and separated by 2 mm. About 1 μg of plasmid DNA in 50 μl was digested for only 30 min. We were at the time experiencing difficulties with the *Eco*RI enzyme for which we still have no explanation; we had to use more enzyme than recommended. *Eco*RI was inactivated by heating the samples at 70°C for 5 min. To 25 μl of digested phage DNA we added 25 μl of digested plasmid DNA. ATP at 0.1 mM (final concentration) and T4 ligase (0.05 U) were also added, and the mixtures were held at 4°C for 24 h, then kept at −20°C. An *Eco*RI-digested phage DNA sample run as a control was found to be virtually completely sealed.

RESULTS

Isolation of antibiotic-resistant transformants. About 1 μl of *Eco*RI-digested phage HP1 DNA and R plasmid DNA mixtures, sealed with T4 ligase, was added to 2 ml of a competent culture of the HP1 lysogen Rd1021. Transformants were scored by plating 0.4 ml of each suspension by the agar overlay method. Single colonies were transferred to fresh broth, grown, and then frozen at −80°C before further study.

The ligated DNA prepared from HP1 and the small plasmid RSF0885 yielded no Ap^r transformants. The DNA from the conjugative plasmid pJS1057-2 gave one Ap^r transformant. The DNA from the conjugative plasmid pRI234 gave 2 Cm^r and 5 Tc^r transformants, whereas that from the plasmid pJS1782 gave 7 Cm^r and 20 Tc^r clones. Only seven of the latter Tc^r clones were studied. These original transformants were named Rd c1 through Rd c9 for Cm^r and Rd t1 through Rd t12 for Tc^r.

Some general properties of the transformants. The one Ap^r, four of the Cm^r, and one of the Tc^r transformants behaved in conjugation crosses as if they possessed a conjugative R plasmid. Agarose gel electrophoresis analysis of their DNA showed that they carried a large plasmid. The *Eco*RI digestion patterns of these plasmids were very similar to those of the plasmids used in the ligation with HP1 DNA fragments. We conclude therefore that these strains had received the original undigested plasmid.

The two Cm^r and five Tc^r transformants from pRI234 and the six Tc^r transformants from pJS1782 did not produce phage. They were insensitive (immune?) to this phage. They had acquired only one resistance which they did not transfer by conjugation. Their resistances were readily transferred by transformation but only to lysogenic recipients. We tentatively concluded that these strains carried a plasmid-derived DNA segment spliced into their prophages. We call these segments here "inserts" and name them c1, c6, t1, t2, etc., and the strains carrying them are here called Rd c1, Rd t1, etc.

Since the HP1 prophage is weakly linked to the *vioA2* mutation, antibiotic resistance genes in the inserts should cotransform with the *vioA2* mutation. We transformed the lysogen RD1021 with DNA from *vioA2* lysogens having either the c1 or the t1 insert in their DNA. Cotransformation of Cm^r with *vioA2* was 5.7% relative to Cm^r alone, whereas Tc^r cotransformed at 3.5% relative to Tc^r alone. Cotransformation of the $NG137^-$ prophage mutation and *vioA2* was about 4%. Our observations are thus consistent with the conclusion that we had constructed strains with DNA inserts in their prophages. To obtain further evidence, we transformed the defective lysogen Rd *novA1* (HP1 $NG137^-$) with DNA from strains Rd c1 and Rd t1. Cotransformation of Cm^r and Tc^r with the prophage $NG137^+$ marker was 9 of 17 Cm^r and 8 of 16 Tc^r transformants. This close linkage establishes that the inserts are indeed in the HP1 prophage.

We have frequently transformed inserts into other recipients. Examination of these secondary transformants has never given us an indication that their properties had been altered by the transfer.

Insert strain Rd t1 was very poorly transformable. This was consistent with an earlier obser-

vation (20) that plasmid pJS1782 possesses a gene or genes which render its host nontransformable.

Mapping of the inserts. HP1 DNA is cut by *Eco*RI into fragments which run very close together as three bands in a 1% agarose gel and only slightly faster then undigested DNA (unpublished data). We believe that there are only three fragments which differ relatively little in size, but very small fragments may have escaped detection. We think that there are only two *Eco*RI sites, i.e., between the prophage mutations NG137$^-$ and NA118$^-$ and between NA118$^-$ and NG140$^-$. To distinguish between these two sites we made use of earlier observations (25, 26) that cotransformation of two prophage loci is much reduced if the donor DNA cannot perfectly synapse with the recipient prophage.

Competent cultures of the singly defective lysogens Rd NG137$^-$, Rd NA118$^-$, and Rd NA119$^-$ and of the doubly defective lysogens Rd NG137$^-$ NA118$^-$, Rd NA118$^-$ NG140$^-$, and Rd NG137$^-$ NG140$^-$ were mixed with DNA from the wild-type lysogen Rd1021 and from all original transformants believed to have a DNA insert in their prophages. In these crosses we scored transformants having a wild prophage (17) and demonstrating resistance to streptomycin (*strA1* reference marker). The observed transformation efficiencies are listed in Table 1. The values for the single prophage markers NG137$^+$, NA118$^+$, and NA119$^+$ varied somewhat, but they were close to the control value obtained with DNA from the wild-type lysogen 1021. The cotransformation efficiencies of the NG137$^+$ and NA118$^+$ markers were very much lower than the control value for all insert-carrying donor DNAs. Efficiencies for the markers NA118$^+$ and NG140$^+$ were only slightly reduced, whereas the efficiencies for the third marker pair were again much lower than the control value. This establishes the insertion sites for all inserts: they are between the NG137 and NA118 prophage loci, probably at the left *Eco*RI site. The slightly lower efficiencies seen from the single markers NG137$^+$ and NA118$^+$ can be explained by the cotransformation of the insert with those markers (see above; this would keep the recipient a defective lysogen).

Additive transformation efficiencies of the prophage inserts. In the experiments listed in Table 1 we also measured the ratios of Cm^r or Tc^r transformants (addition of a c or t insert) to *strA1* transformants. Table 2 shows the efficiencies (normalized with respect to the *novA1* marker) with which the various inserts were transferred. There appeared to be four main groups with respect to this efficiency: inserts c1 and c6 (both from pRI234): inserts t8. t10, t11, and t12 (all from pRI234); inserts t2, t4, t5, t6, and t7 (all from pJS1782); and inserts t1 (from pJS1782) and t9 (from pRI234).

Insert size. The differences in insert transfer efficiencies suggested to us that low-efficiency inserts are larger than high-efficiency ones. Low-efficiency insert-carrying DNA can then be expected to be more sensitive to UV. We examined this by exposing standard DNA lysates to UV and assaying the surviving activities on a *uvr*$^+$ and a *uvr-1* recipient lysogen. We somewhat unexpectedly observed that activity decreased exponentially with dose (R. B. Walter and J. H. Stuy, Photochem. Photobiol., in press). We give in Table 3 only the inactivation by 12 J/m^2. These results show that the UV sensitivity of insert-carrying DNA increased with decreasing transfer efficiency. If UV lesions in the insert-flanking DNA regions reduce the probability of

TABLE 1. *Efficiencies of transformation of singly and doubly defective H. influenzae lysogens to wild-type lysogens with DNA from insert-carrying donor DNA*

Insert	Origin	Defective HP1 prophage mutation					
		NG137$^-$	NA118$^-$	NA119$^-$	NG137$^-$ NA118$^-$	NA118$^-$ NG140$^-$	NG137$^-$ NG140$^-$
None[a]		0.16	0.21	0.15	0.045	0.072	0.043
c1	pRI234	0.077	0.055	0.18	0.00018	0.022	0.0010
c6	pRI234	0.044	0.044	0.18	0.00019	0.032	0.0013
t1	pJS1782	0.053	0.050	0.17	0.00055	0.015	0.00072
t2	pJS1782	0.099	0.061	0.18	0.00046	0.015	0.0022
t4	pJS1782	0.026	0.034	0.12	0.00019	0.0088	0.0011
t5	pJS1782	0.072	0.032	0.17	0.00024	0.053	0.0014
t6	pJS1782	0.050	0.037	0.13	0.00046	0.015	0.0034
t7	pJS1782	0.033	0.037	0.088	0.00044	0.014	0.0012
t8	pRI234	0.11	0.083	0.17	0.00044	0.061	0.0034
t9	pRI234	0.080	0.035	0.15	0.00066	0.061	0.0016
t11	pRI234	0.088	0.055	0.13	0.00072	0.012	0.0015
t12	pRI234	0.051	0.048	0.13	0.00055	0.012	0.0030

[a] Wild-type lysogen.

TABLE 2. *Transformation efficiencies of singly and doubly defective H. influenzae lysogens to insert-specified antibiotic resistance with DNA from insert-carrying donor strains*

Donor	Defectively lysogenic recipients						
	Wild type	NG137	NA118	NA119	NG137 NA118	NA118 NG140	NG137 NG140
Rd c1	0.18	0.029	0.048	0.051	0.031	0.029	0.013
Rd c6	0.15	0.0053	0.037	0.040			
Rd t1	0.021	0.00050	0.0018	0.0066			
Rd t2	0.046	0.0019	0.014	0.020			
Rd t4	0.043	0.0016	0.013	0.023			
Rd t5	0.035	0.0020	0.016	0.014			
Rd t6	0.046	0.0019	0.018	0.019			
Rd t7	0.044	0.0024	0.013	0.017			
Rd t8	0.072	0.0088	0.030	0.052			
Rd t9	0.026	0.0028	0.0066	0.0072	0.0018	0.0053	0.0055
Rd t10	0.094	0.022					
Rd t11	0.11	0.0083	0.031	0.045			
Rd t12	0.099	0.017	0.032	0.047			

TABLE 3. *UV sensitivity of insert-adding H. influenzae DNA*

Donor	Recipient		Dose reduction	Transfer efficiency[a]	37% survival dose (J/m^2)	Size[b] (Md)
	uvr$^+$	*uvr-1*				
Rd c1	0.083	0.018	1.6	0.18	5.3	5.9
Rd t11	0.042	0.013	1.4	0.11	4.1	7.7
Rd t2	0.011	0.0063	1.3	0.046	3.4	9.3
Rd t9	0.010	0.0096	1.05	0.026	2.8	11
Rd t1	0.0065	0.0018	1.1	0.021	2.2	14
pJS1261				0.005	0.9[c]	35[c]

[a] From Table 2.
[b] Target size.
[c] From reference 23.

insert transfer (which is quite likely), the observed UV sensitivities indicate a target that is larger than the insert alone. Using the 37% survival doses calculated for the *uvr*$^+$ recipient, and a size of 35 Md for the integrated plasmid pJS1261 (17), we arrive at insert sizes (or rather target sizes) as shown in the last column of Table 3.

Deletion of inserts by transformation. The insert-carrying strains do not produce phage probably because the larger genome cannot be packaged in the phage head. This enabled us to measure quantitatively the exact deletion of an insert by exposing competent insert-carrying cultures to DNA from a wild-type lysogen. Insert-deleted prophage transformants can produce phage, and they were scored as described earlier (17). We studied only one strain of each group. Table 4 shows that deletion of inserts by transformation is very efficient, roughly equal to a high-efficiency point mutation. One might consider that the genetic marker in this reaction is the pair of nucleotides between which the insert is located in the recipient prophage. Small markers can be expected to transfer efficiently.

TABLE 4. *Efficiencies of insert addition, insert deletion, and insert substitution in H. influenzae genetic transformation*

Reaction	Insert studied			
	c1	t11	t2	t9
Addition	0.18	0.11	0.046	0.016
Deletion	0.69	0.63	0.56	0.52
Substitution of c1		0.16	0.053	0.025
Substitution of t11	0.14			
Substitution of t2	0.16			
Substitution of t9	0.27			

In agreement with this assumption is our finding that deletion transformation is very UV resistant (Walter and Stuy, in press).

Substitution of one insert with another one. Our insert-carrying transformants are resistant to either chloramphenicol or tetracycline. This allowed us to study the replacement of one insert with another one. We transformed strain Rd c1 with DNA from strains Rd t2, Rd t9, and Rd t11, and we transformed strains Rd t2, Rd t9, and Rd t11 with DNA from strain Rd c1. We scored for transformants resistant to the anti-

biotic specified by the insert. Table 4 shows that the substitution efficiencies were roughly equal to those found for adding alone. This indicates that in the replacement reaction the size of the replacing insert is most important.

DISCUSSION

The purpose of this study was to examine factors which influence the transfer by genetic transformation of long nonhomologous DNA segments and to obtain evidence for the model proposed to explain this phenomenon (21). For this we needed donor cells with well-characterized DNA segments (inserts) in their chromosomes. We constructed such cells by fusing *Eco*RI-digested phage DNA with digested plasmid DNA and using this DNA for the transformation of certain recipients to resistance to antibiotics specified by the plasmid-derived inserts. We selected the HP1 prophage as the insertion region because we had available an extensively developed genetic map of this phage (17, 18). We could thus perform the crosses necessary for the identification of the insertion sites. This system would also allow us to study quantitatively the exact deletion of the inserts by genetic transformation. We chose plasmid-carried antibiotic resistance genes for the inserts because these can be easily followed in transfer reactions. Yoneda and co-workers (28) by this technique inserted α-amylase genes of *Bacillus amyloliquefaciens* into the φ3T prophage of *B. subtilis*. However, they did not study the transformation transfer of these inserts.

We believe that we have established that our insert-carrying strains have long nonhomologous plasmid-derived DNA segments in their prophages. The questions to be answered first are the following. (i) Where exactly in the prophage do the inserts map? (ii) What are their sizes? (iii) How well are they characterized?

To answer the first question we performed substitution crosses (those in Table 4) and scored for doubly resistant transformants. We reasoned that if donor and recipient inserts map at exactly the same (*Eco*RI) site, substitution transformants should always have lost their own insert (Fig. 2C). These crosses, however, yielded a small number of doubly resistant transformants. We explain these by assuming that the c1, t9, and t11 inserts possess regions of homology (see Fig. 2E and F). This is not unreasonable since an incomplete digest of the pRI234 plasmid was used in the strain construction. We now have evidence that this is the case (manuscript in preparation). The above three inserts map at the same site, but the t2 insert maps some distance away (Fig. 2D).

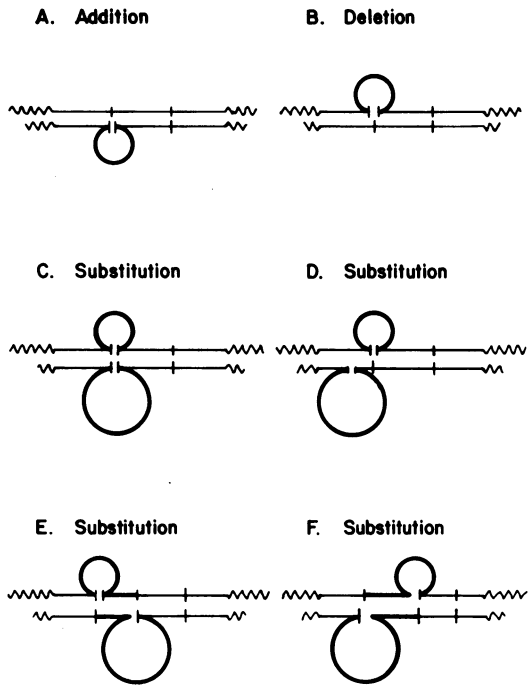

FIG. 2. *Models for insert addition (A), deletion (B), and various possible substitutions (C, D, E, and F). Top line, Recipient single strand of the synapsed structure; bottom line, donor single strand. Thin straight line, prophage DNA; zigzag, chromosomal DNA; thick line, insert DNA; crossbars, EcoRI sites.*

With respect to the second question above, there can be no doubt that the inserts are long because they carry active plasmid genes. Laufs and co-workers (10, 12) have shown for plasmid pRI234 that the Tc determinant of about 4.5 Md is flanked by an inverted repeat of about 1 Md. This might put the size of a t insert at ca. 6.5 Md. The Cm region of about 1.5 Md is flanked by possibly the same, or similar, inverted repeats, and the size of the c1 insert could be between 1.5 and 3.5 Md. The actual sizes can only be established by isolation of the inserts in pure form and then applying them to gel electrophoresis. We have not yet been able to do this. However, we think that the above considerations and our UV studies strongly indicate that insert sizes range from 2 to perhaps 10 Md.

It is perhaps not surprising that the transfer of such long nonhomologous DNA segments to the recipient genome by transformation is inefficient and size dependent. But how can one explain that? Adams (1) studied the addition of long DNA segments (about 6 Md) in *B. subtilis* and found that this process is very inefficient. Adams speculated that the donor DNA is cut after uptake into such short segments that at

best only short insert-flanking regions are left. Most fragments are believed to have been cut within the insert. Insert addition is much more efficient in *H. influenzae*. We think that the main reason is that in this bacterium very long donor DNA (single) strands are integrated into the recipient DNA. Since three prophage markers some 10 Md apart (single stranded) cotransform 30% (19), the integrated single donor DNA strand is on the average 14 Md. The insert size is a relatively small part of that, and it is thus not frequently cut during the integration process (or before that). We consider two explanations for the inverse correlation between insert size and additive transformation efficiency. In the first one, a double strand-specific exonuclease digests away a portion of the synapsed (but not yet sealed in) donor strand, starting from either end. The single donor insert, looping out of that complex as a single strand, may distort the complex to some extent and thus cause the exonuclease to digest away long portions. If the insert is short, flanking regions are long, and the exonuclease will not often reach the insert (Fig. 2A). Thus flanking regions protect the insert. This model also explains why insert deletion is such an efficient process and why there appears to be no size dependence. In the second explanation, the single-stranded insert, looping out of the synapsed structure between donor and recipient DNA, is vulnerable to cutting by endonuclease(s). Longer inserts are more likely to be cut and thus eliminated. This model also explains the high efficiency of insert deletion if we assume that the looped-out recipient insert is protected by single-strand-specific protein. We prefer at present the second explanation because we observed that insert addition, deletion, and substitution all show exponential decrease with increasing UV dose (Walter and Stuy, in press).

Table 2 shows that the additive transformation efficiencies of all inserts dropped dramatically when the recipient lysogen possessed a linked prophage point mutation. A second mutation had no further effect. We also established that insert deletion and insert substitution were similarly affected. The effect was much smaller if the point mutation was in the donor DNA. A report about these observations will shortly be submitted.

ADDENDUM IN PROOF

We have recently inserted the linear form of *E. coli* plasmid pBR322 (2.7 Md) in the left *Eco*RI-sensitive site of prophage HP1. Transfer of this insert was more efficient and more UV resistant than the transfer of the c1 insert.

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