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Plasmid Establishment in Competent *Haemophilus influenzae* Occurs by Illegitimate Transformation

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Establishment of plasmids in naturally competent *Haemophilus influenzae* is incompatible with transformation via the normal DNA translocation pathways. Instead, establishing plasmids appear to evade the degradation which oridinarily accompanies translocation, arriving as intact double-stranded molecules in the cytoplasm. Evidence is presented that plasmid establishment is a rare illegitimate transformation event which resembles artificial transformation. This process is compared with plasmid marker rescue transformation, and a method for greatly increasing establishment frequency is described.

Plasmid establishment in *Haemophilus influenzae* is an inefficient event. When the transforming plasmid contains no homology to DNA sequences in the cell, approximately 1 in 10^5 cells becomes a plasmid bearer (21). This is in marked contrast to the high rate of transformation of chromosomal markers, which occurs in 1 to 10% of viable competent cells. Although the phenomenon of plasmid establishment has been observed and investigated for several years, there is no agreement on the reason for its very low efficiency. This is particularly confusing in naturally competent cells, which take up large quantities of DNA. One suggestion (1) holds that plasmids are inefficiently taken up owing to a lack of specific uptake sites (9) which are present in *H. influenzae* chromosomal DNA. Decreased uptake, however, is not a sufficient explanation for the 3-log difference between plasmid establishment and chromosomal transformation frequencies. Moreover, plasmids which do contain such an uptake site exhibit the same low frequency of establishment (this work).

Plasmid establishment may occur in either naturally or artificially competent *H. influenzae*. Artificial competence (24) is induced by a high concentration of Ca^{2+} ions and a temperature shock (26). Presumably these conditions act to alter membrane permeability, allowing exogenous DNA to enter in intact, double-stranded form (4). In contrast, the development of natural competence involves the induction of a number of cellular functions for the active uptake, translocation, and integration of exogenous DNA. Donor DNA entering these cells is subject to extensive degradation (2, 25), which results in a linear single strand of less than unit length entering the cytoplasm (22). However, plasmids added to naturally competent cells may be established as intact double-stranded circular molecules within the cell at frequencies comparable to or better than those obtained in artificially competent cells (21; M. L. Pifer, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1985). These seemingly incompatible observations formed the motivation for the current work.

It should be emphasized that this work deals only with nonconjugative plasmids which bear no chromosomal homology; establishment of conjugative (26) or homologous plasmids (1) involves other mechanisms (see Discussion).

MATERIALS AND METHODS

Plasmids. The characteristics of plasmids used are given in Table 1, and a restriction map of pHVT1 is shown in Fig. 1. In the experiments described here, plasmids pHVT1 and pHVTU14 were found to be generally interchangeable, as were the corresponding ampicillin-sensitive plasmids pHVTS9 and pHVTSU3. Plasmids used for transformation were grown in *Escherichia coli* HB101 (19) and prepared by a large-scale version of the rapid alkaline method (5) followed by CsCl-ethidium bromide centrifugation (23). Plasmids prepared from *H. influenzae* were purified by a modification of the Hirt supernatant method (15) as described previously (22). Homogeneous covalently closed circular (CCC) and open circular (OC) DNAs were isolated from a mixture of the two topological forms by CsCl-ethidium bromide centrifugation.

Competence development and transformation. *H. influenzae* KW22 (K. W. Wilcox, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1974) was grown and made competent by the MIV method (14) and transformed as described previously (22). The level of competence of the cells was generally assessed at the end of the competence development procedure by the rapid uptake assay of Kahn et al. (17). Cells were either used immediately or frozen in MIV medium containing 20% glycerol at −70°C. Frozen cells were thawed on ice, pelleted, and resuspended in fresh MIV medium just before use.

Enzymes. Restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.) and used according to the suggestions of the manufacturer. Bacterial alkaline phosphatase (Worthington Diagnostics, Freehold, N.J.) was used at a concentration of 0.02 U/μg of DNA for 1 h at 65°C. End-to-end ligation of 5 μg of linear DNA was carried out with 3 Weiss units of T4 DNA ligase (New England BioLabs) in a volume of 15 μl for 1 h at 14°C. Under these conditions, formation of concatemers was favored (j:i ratio = 0.1, [19]). Production of higher-molecular-weight forms was confirmed by agarose gel electrophoresis.

RESULTS

The plasmids pHVT1 and pHVTU14 (Fig. 1) (10) are *E. coli-H. influenzae* shuttle vectors which confer resistance to ampicillin and tetracycline in both cell types. These plasmids

TABLE 1. Plasmids used in this work

Plasmid	Phenotype conferred	Reference
pHVT1	Ap^r Tc^r	10
pHVTS9	Ap^r Tc^s	22
pHVTU14	Ap^r Tc^r, 11+[a]	This work
pHVTSU3	Ap^r Tc^s, 11+[a]	This work

[a] Contains 11-bp uptake sequence (9).

were used to transform naturally competent *H. influenzae* cells to tetracycline resistance. Recipient cells either had no resident plasmid (empty cells) or bore the homologous mutant plasmids pHVTS9 and pHVTSU3, which confer ampicillin resistance only (22) (see the legend to Fig. 1).

Influence of topological form on transformation. Homogeneous *Tth*I linear, *Pst*I linear, OC, or CCC pHVT1 plasmid molecules were used to transform competent cells. At saturating DNA concentrations the topological form of the donor plasmid had no significant effect on transformation frequency of cells either with or without a resident plasmid (Fig. 2). At all DNA concentrations, however, the frequency of marker rescue was higher than plasmid establishment by a factor of 10^4 to 10^5, which is in agreement with previous reports that establishment is an infrequent event. Transformation by pHVTU14 gave equivalent results, indicating that for homogeneous transforming DNAs the presence of an uptake site does not affect plasmid establishment frequencies.

Absence of degradation during plasmid establishment by linear molecules. It was surprising to note that at all DNA concentrations, linear donor plasmid established as efficiently in empty cells as did circular plasmid. Since transformation is ordinarily accompanied by degradation of the donor DNA from an end, it was of interest to determine whether there had been any loss of information from the ends of those molecules linearized at the *Pst*I site, which lies within the beta-lactamase gene for ampicillin resistance. Although the intact plasmid confers both ampicillin resistance and tetracycline resistance on the cell, tranformants had been selected solely on the basis of tetracycline resistance. Any degradation from the ends would have destroyed part of the beta-lactamase-coding sequence. Ninety-eight tetracycline-resistant colonies which resulted from establishment by *Pst*I-linearized pHVTU14 were restreaked on plates containing either 5 μg of tetracycline per ml or 25 μg of ampicillin per ml. Ninety-seven grew equally well on tetracycline and ampicillin, indicating that no information had been lost from the ends during plasmid establishment. Plasmid was prepared from six of these doubly resistant colonies and subject to restriction analysis with *Bgl*I (four sites in the plasmid) and *Rsa*I (at least 12 sites in the plasmid). The restriction patterns were indistinguishable from those of donor plasmid, indicating that no rearrangements had taken place (data not shown). Further evidence for the establishment of plasmid by linear molecules without loss of information from the ends comes from studies with pHVTU14 linearized at various sites, including the *Mlu*I, *Eco*RI, *Nco*I, and *Eco*RV sites, all of which lie within the *tetA* gene which codes for tetracycline resistance (16). The linear molecules tested were all effective at plasmid establishment as judged by transformation to tetracycline resistance (Table 2). Certainly no information can have been lost from the ends of those molecules linearized in *tetA* which succeed in estab-

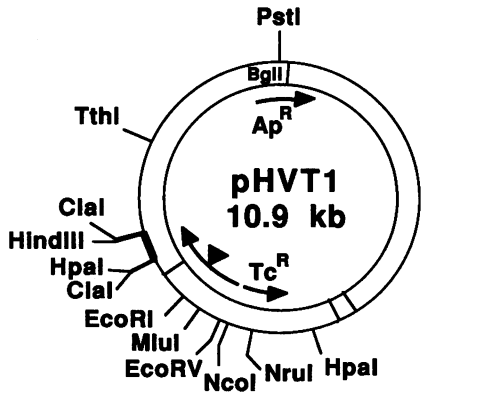

FIG. 1. Restriction map of pHVT1. Positions of genes and all relevant restriction sites are shown. Triangle indicates the location of the four-base-pair (bp) insertion in Tc^s derivatives. In the derivatives pHVTU14 and pHVTSU3, the 318-bp *Hin*dIII-*Cla*I fragment (thick line) is replaced by a 126-bp *Hin*dIII-*Hpa*II fragment which contains the 11-bp uptake site (9). These constructions preserve the *Hin*dIII site but destroy the *Cla*I site. kb, Kilobases.

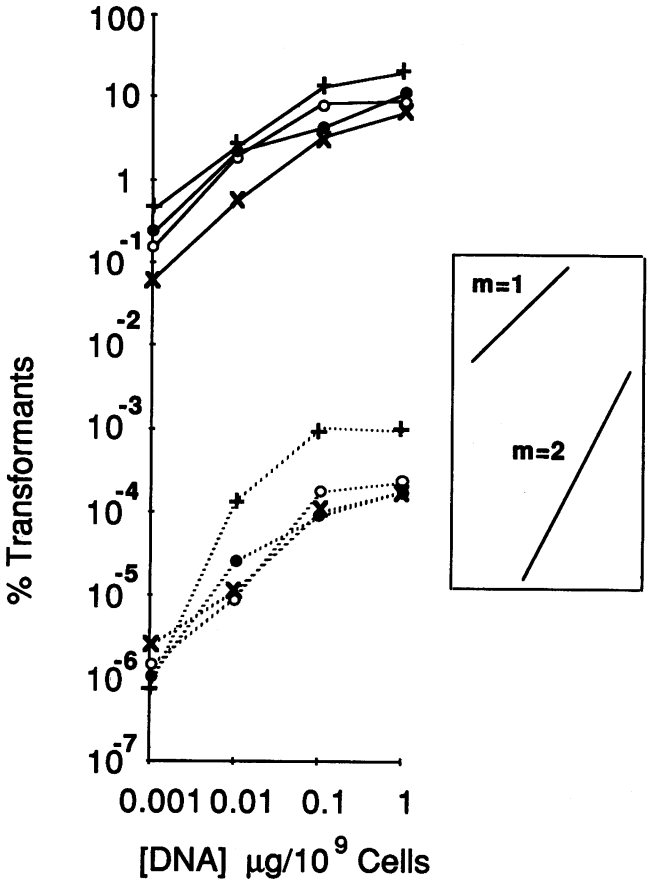

FIG. 2. Transformation of *H. influenzae* as a function of topological form and donor plasmid concentration. Graph shows percent viable cells transformed. Indicated amounts of DNA were added to ~10^9 frozen competent cells in 0.5 ml and incubated and plated as described in Materials and Methods. Recipient cells for marker rescue were KW22 bearing pHVTS9 (solid lines). Recipient cells for plasmid establishment were KW22 with no plasmid (dashed lines). Donor DNA was either closed circular pHVT1 (●), OC pHVT1 (○), *Tth*I linear pHVT1 (×), or *Pst*I linear pHVT1 (+).

lishing within the recipient cell under tetracycline selection. This indicates that plasmid establishment in naturally competent cells occurs by a very different mechanism than conventional transformation.

Preligation of linear plasmids increases transformation frequency. Additional information about the mechanism of plasmid establishment by linear molecules is given by the slope of the dose-response curve (Fig. 2). For CCC and OC molecules the *Tth*I linear molecules, the slope of the curve is approximately one, indicating that a single molecule is sufficient to tranform a cell. In contrast, for *Pst*I linear molecules, establishment frequency appears to vary as the square of the DNA concentration ($m = 2$), indicating that two donor molecules are required for a single transformation event. This suggested that in some cases, two molecules could be ligated to produce a linear dimer which then could undergo recombination between the two plasmid copies, regenerating a circle which would establish as a monomer in the cell. To test this hypothesis, I treated *Pst*I-linearized pHVT1 either with bacterial alkaline phosphatase to eliminate the possibility of end-to-end ligation or with T4 DNA ligase under conditions which favored concatemerization, before pHVT1 was used to transform empty cells. The data in Table 3 show that bacterial alkaline phosphatase treatment of *Pst*I linears effectively eliminates plasmid establishment, while ligation to multimers dramatically increases it. In addition, the slope of the dose-response curve for ligated linears is approximately one, indicating that a single multimeric molecule is required for a transformation event.

DISCUSSION

The term "plasmid tranformation" has been used to refer to several different processes which utilize plasmid DNA as transforming DNA. These processes may have different mechanisms, different requirements for topological form of the DNA and physiological state of the cell, and different consequences for the cell. I propose that the following nomenclature be used to avoid confusion when discussing these phenomena. "Plasmid transformation" is most logically applied to transformation of a marker present on a resident plasmid within the recipient cell, since it is this situation which is most closely analogous to traditional (chromosomal) transformation, although "plasmid marker rescue" is more descriptive. The establishment of an independent replicon having no homology to DNA already present in the recipient cell is "plasmid establishment." For the rescue of chromosomal markers cloned on a donor plasmid but not present on a plasmid in the recipient cell, I suggest "plasmid-mediated transformation." If the donor plasmid which carries chromosomal DNA (or DNA homologous to a resident plasmid) becomes established as a replicon, "facilitated plasmid establishment" is appropriate (see reference 1). Nonhomologous DNA flanked by plasmid sequences becomes inserted into a resident plasmid via "additive plasmid transformation," by analogy with the chromosomal case (27).

TABLE 2. Plasmid establishment by various linear plasmid molecules[a]

Linearization site	Type of end	% Transformants
Expt 1		
*Pst*I	3′ 4 base	1.1×10^{-3}
*Mlu*I	5′ 4 base	0.95×10^{-3}
*Eco*RI	5′ 4 base	0.65×10^{-3}
Expt 2		
*Pst*I	3′ 4 base	8.0×10^{-4}
*Mlu*I	5′ 4 base	6.5×10^{-4}
*Eco*RV	Blunt	0.35×10^{-4}
*Nco*I	5′ 4 base	0.72×10^{-4}
*Tth*I	5′ 1 base	6.0×10^{-4}
*Hpa*I	Blunt	0.11×10^{-4}

[a] Linear pHVTU14 (1 μg) was used to transform ~10^9 frozen competent cells to tetracycline resistance.

TABLE 3. Effect of phosphatase or ligase treatment on plasmid establishment frequency[a]

Enzyme treatment	μg of DNA	% Transformants	Relative efficiency
None	1	3.9×10^{-4}	1
	0.1	6.0×10^{-5}	1
	0.01	8.7×10^{-6}	1
	0.001	1.9×10^{-7}	1
Bacterial alkaline phosphatase	1	$<10^{-7}$	
	0.1	$<10^{-7}$	
	0.01	$<10^{-7}$	
	0.001	$<10^{-7}$	
Ligase	1	9.8×10^{-2}	251
	0.1	4.4×10^{-2}	733
	0.01	3.9×10^{-3}	448
	0.001	6.2×10^{-4}	3,263

[a] *Pst*I-linearized pHVT1 was treated with the enzymes indicated as described in Materials and Methods. Indicated amounts of DNA were added to 10^9 fresh competent cells in 0.5 ml.

Plasmid establishment occurs at low frequency in *H. influenzae*; this type of transformation is several orders of magnitude less efficient than marker rescue. Establishment occurs at the highest frequency in naturally competent cells (21; M. L. Pifer, Ph.D. thesis), yet two aspects of plasmid establishment are difficult to reconcile with our current understanding of DNA processing during natural transformation.

First, it is known that extensive degradation of donor DNA ordinarily accompanies transformation. One strand of an entering molecule is completely degraded and the other stand is at least partially degraded during entry into the cytoplasm (2, 20, 22). Yet recovery of plasmids from cells transformed by linear DNA reveals that no information is lost from the ends of linear molecules during plasmid establishment.

Second, previous work from this laboratory showed that a free end is required for efficient translocation out of transformasomes; CCCs remain in the protected state (2). From the data in Table 2, however, it is obvious that CCC molecules are able to carry out both marker rescue and plasmid establishment as efficiently as topological forms which do have a free end. This paradox was partially resolved by work on the *rec-2* mutant (3) which showed the existence of an endonucleolytic activity within transformasomes. Such an activity can slowly convert CCC to OC and linear forms, exposing a free end from which translocation (and degradation) can begin. This explained the marker rescue data, but left unresolved the question of how an intact double-stranded circular plasmid can enter and establish within a cell.

The results presented here indicate that donor plasmids may at low frequency enter the cytoplasm of naturally competent *H. influenzae* cells as intact duplexes. It is probable that this is the principal route of plasmid establishment when the plasmid bears no homology to recipient

DNA. In light of the current model of transformation, which holds that donor DNA is initially sequestered in the transformasome, a membranous structure on the cell surface (17), I propose that exit from the transformasome can in rare cases occur without the usual directional translocation and degradation. In these instances, although most donor molecules are partially degraded and thus prevented from establishing in the cell, an occasional DNA molecule enters the cytoplasm in intact double-stranded form. This may come about because a rare transformasome lacks the normal translocation proteins and allows passive diffusion of the DNA into the cell. This process would bear a resemblance to the entry of DNA into artificially competent cells, except that the DNA uptake mechanisms present in the naturally competent cell are utilized in place of changes in membrane permeability to allow the DNA to enter. Others have noted that plasmid establishment is affected differently by changes in temperature (21) and divalent cation concentration (13) than is chromosomal transformation. This underscores the similarity to artificial competence, because both a heat shock and a high divalent cation concentration are used in the artificial competence procedure. The fact that plasmid establishment nonetheless occurs at the highest frequency in naturally competent cells is easily accounted for by their greater affinity for DNA and the very efficient uptake into transformasomes, leaving only the cytoplasmic membrane to be penetrated.

Since this method of DNA entry appears to circumvent the normal mode of translocation in naturally competent cells, I have termed it illegitimate transformation. This is a rare occurrence, as only one in 10^5 to 10^7 competent cells becomes a plasmid bearer. During plasmid marker rescue transformation, this low rate of plasmid establishment is obscured by the far more frequent transformation by homologous recombination (Fig. 2). Similarly, the occasional escape of an intact plasmid into the cell would be invisible to radiochemical investigation (such as that used in references 2 and 17), as the percentage of input molecules which are involved would be too low to detect.

If illegitimate transformation is in fact the principal route of plasmid establishment, one would predict that plasmid establishment frequencies in *rec-1* and *rec-2* cells, in which marker rescue frequencies are greatly diminished, would be the same as in wild-type cells, since establishment would rely neither on homologous recombination (defective in *rec-1* [18]) nor on the translocation mechanism (defective in *rec-2* [3]). This has been shown to hold true for the plasmid pRSFO885 (21). My preliminary results with pHVTU14 were consistent with independence from *rec-1* and *rec-2* function, but this has been difficult to reproduce. There may be additional defects in our *rec-1* and *rec-2* strains which adversely affect plasmid establishment.

The insensitivity of illegitimate transformation to the topological form of the donor plasmid correlates well with the findings of Gromkova and Goodgal for plasmid establishment in *Haemophilus parainfluenzae* (13). They showed that circular and linear pRSFO885 could establish at equal frequencies. This is in marked contrast to the situation in *Bacillus subtilis*, in which a single nick is sufficient to inactivate a closed circular donor plasmid for establishment (8). Notani et al. (21) have reported that linear pRSFO885 is unable to transform empty *H. influenzae* cells; I found this not to be the case for the pRSFO885-based plasmids pHVT1 and pHVTU14, regardless of the overhang left by the linearizing enzyme (Table 2). The transforming activity of the linear and OC preparations was not due to residual CCC DNA, since dose-response data indicate that transformation by all topological forms reached a maximum at roughly the same donor DNA concentration. This would not have been true were the transforming activity due primarily to a minor contaminant in the preparation. A ligation or repair mechanism within the cell would likely be required to seal nicks in OC DNA and circularize linear DNA such that these forms can establish as efficiently as closed circular plasmid. Such a ligation activity which acts on donor DNA has previously been reported in competent *H. influenzae* cells (11). This activity apparently resides within the transformasome, as in vivo-ligated DNA recovered after uptake could be redigested to yield the input form, indicating that it had not yet been subject to degradation.

Concatemeric ligation of linear donor plasmid has the striking effect of increasing the frequency of establishment by 100- to 1,000-fold. One may hypothesize that a concatemeric linear plasmid is able to recombine with itself to produce a unit-length circular plasmid. This could occur even after a portion of the linear concatemer had been degraded. Such a mechanism would resemble that observed in *B. subtilis*, in which monomer plasmids cannot establish but dimers can (7). This is also analogous to work in *B. subtilis* (12) and *H. influenzae* (6, 28) which showed that multimeric phage DNA was significantly more active in transfection than was monomer phage. This aspect of phage transfection could be similar to plasmid establishment by linear molecules. Preligation of linear plasmids should prove useful as a tool to increase plasmid establishment frequencies.

Whether simple entry of a circular duplex plasmid molecule into the cytoplasm of a cell is sufficient to constitute establishment is unknown for these plasmids. An additional process of association with cellular components may also be required, such as binding of various host proteins to the replication origin or attachment of the plasmid to the inner membrane of the cell. For linear transforming molecules, circularization is certainly required, but it is not certain where and when this takes place. These questions will need to be investigated before the process of plasmid establishment in *H. influenzae* is thoroughly understood.

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