A Plasmid Cloning Vehicle for *Haemophilus influenzae* and *Escherichia coli*

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A new plasmid cloning vehicle (pDM2) was used to introduce a library of *Haemophilus influenzae* chromosomal fragments into *H. influenzae*. Transformants of the highly recombination-defective *rec-1* mutant were more likely to contain exclusively recombinant plasmids after exposure to ligated DNA mixtures than was the wild type. pDM2 could replicate in *Escherichia coli* K-12.

The construction and use of a new plasmid cloning vehicle, pDM2, is reported in this note. *Haemophilus influenzae* BC200 (Rec⁺) and *H. influenzae rec-1* mutants have been previously described (3, 4, 9). *Escherichia coli* C600 *hsdR* was obtained from F. W. Studier. *H. influenzae* cells were grown in supplemented brain heart infusion broth (10) and made competent in M-IV medium (12). *E. coli* cells were transformed by the CaCl$_2$ technique (8). pDM2 is a hybrid plasmid constructed by joining plasmid RSF0885 (6) to a portion of p2265. The latter plasmid is a large (34-kilobase) *H. influenzae* conjugative plasmid which encodes resistance to tetracycline and chloramphenicol (1, 2).

The restriction endonuclease map of RSF0885 (Fig. 1A) shows the positions of various sites in kilobase pairs from the unique *Pvu*II site. The *Bst*EII site shown is the only position cut by this enzyme at 37°C. Enzymes that do not cut RSF0885 include *Bcl*I, *Bgl*II, *Cla*I, *Eco*RI, *Hpa*II, *Sal*I, *Sma*I, *Sst*II, *Tth*III, and *Xho*I. Our RSF0885 lacks a *Pst*I site that was located in the non-Tn*A* portion of the RSF0885 studied by Brunton et al. (5). Assuming that the difference in the molecular weights of RSF0885 reported by Brunton et al. (5) and by us (11) reflects a real difference in the sizes of the plasmids studied by the two groups, it seems possible that either our plasmid has undergone a deletion or the other form of RSF0885 may bear an insertion.

A restriction endonuclease map of pDM2 is shown in Fig. 1B. The chloramphenicol gene resides in the largest *Bam*HI fragment. pDM2 represents an improvement over RSF0885 as a cloning vehicle because it carries two antibiotic resistance genes. The insertion of a DNA fragment into a restriction endonuclease site within one of the antibiotic resistance genes should eliminate expression of that gene and allow discrimination between transformants carrying insert-bearing plasmids and transformants carrying recircularized cloning vehicles. The absence of *Pst*I and *Pvu*I sites in the chloramphenicol gene-bearing fragment should allow the recovery of ampicillin-sensitive recombinant plasmids with *Pst*I and *Pvu*I fragments inserted into the ampicillin gene.

Chromosomal DNA from a strain carrying eight antibiotic resistance markers was digested with *Pst*I and ligated to *Pst*-cut pDM2. Cells transformed by the ligation mixture were plated after DNA uptake and selected for chloramphenicol resistance to eliminate untransformed cells. All of the chloramphenicol-resistant colonies were screened to determine their sensitivity to ampicillin.

Transformation by recombinant plasmids is likely to be many orders of magnitude higher than that by recircularized pDM2, since pDM2 contained no specific DNA uptake sites (data not shown), whereas recombinant plasmids almost always would. In addition, the establishment of an intracellular plasmid containing a chromosomal insert rather than one without an insert is more heavily favored in recombination-proficient hosts by a factor of about 1,000 (11; unpublished data).

Table 1 represents all of our attempts to find ligation and transformation conditions that would maximize the frequency of ampicillin-sensitive transformants. Since these data were not collected in a systematic study of the variables, and because some of the statistics are inadequate, a direct comparison of results obtained with the different conditions cannot always be made. Nevertheless, certain generalizations are possible.

The great bias favoring uptake and establishment of recombinant plasmids by *H. influenzae*
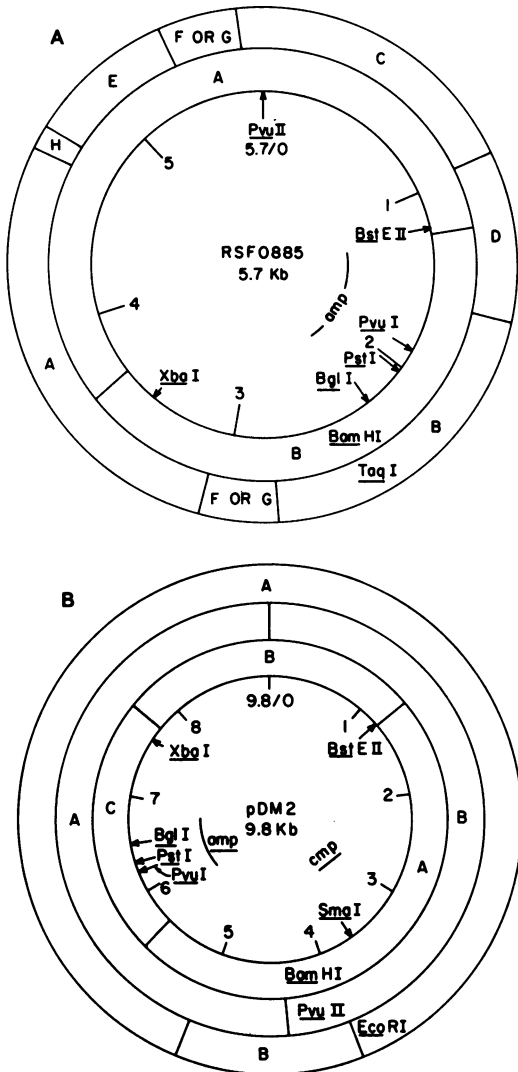
FIG. 1. Restriction endonuclease maps of plasmids RSF0885 (A) and pDM2 (B).

should result in high yields of recombinant clones, even when cells are exposed to ligation mixtures having different compositions of recombinant and recircularized plasmids. However, experiments 1 and 2 (Table 1) indicated that the relative yield of recombinant clones decreased with an increase in the concentration of ligated DNA during transformation. The relative yields of recombinant clones from the most extreme ligation conditions (experiments 1, 2, and 3 versus experiment 7) implied that ligation conditions reducing the proportion of recombinant plasmids (7) tended to favor the formation of clones bearing exclusively recombinant plasmids.

We observed a surprisingly high fraction of

ampicillin-resistant transformants even under conditions where the input plasmid DNA concentration was very low (Table 1, experiments 1 and 10), indicating that the overwhelming majority of the BC200 transformants and a substantial minority of *rec-1* transformants did not contain exclusively recombinant plasmids. We propose that the insert-bearing plasmids enter the competent cells and frequently undergo recombination that regenerates the original cloning vehicle. We would predict from this hypothesis that ampicillin-resistant transformants would arise infrequently when a competent *rec-1* strain is exposed to the ligated DNA. In experiments 8, 9, and 10 with the recombination-deficient host, there were considerably more transformants containing exclusively recombinant plasmids than with the recombination-proficient host. With the same ligation conditions and approximately the same DNA concentration for transformation, there was a difference of more than a factor of three between hosts (experiments 7 and 8). In the *rec-1* mutant the overall level of transformation to chloramphenicol resistance by the library was not greatly elevated by the presence of the insert-bearing plasmids, even though most of these plasmids contained uptake sites (there were 3×10^4 BC200 chloramphenicol-resistant transformants per ml in experiment 1 but 85 *rec-1* chloramphenicol-resistant transformants per ml in experiment 10). Given the depressed level of transformation to chloramphenicol resistance in the *rec-1* mutant, it is likely that recircularized pDM2 made a relatively large contribution to the total yield of *rec-1* transformants. The inability of the *rec-1* strain to generate ampicillin-resistant transformants from plasmids bearing chromosomal inserts implied that most of the ampicillin resistance in the BC200 transformants arose by a *rec-1*-dependent pathway.

The hypothesis that pDM2 is regenerated by recombination implies that we might find some transformants that contain a mixture of pDM2 and an insert plasmid, assuming that the recombination event occurred after the original plasmid had replicated at least once. Such a situation was observed in some of the BC200 ampicillin-sensitive clones recovered from experiment 1 (Table 1). Of 144 ampicillin-sensitive transformants spotted onto chloramphenicol-ampicillin plates, 18 contained a few ampicillin-resistant colonies, indicating that these clones contained a mixture consisting primarily of ampicillin-sensitive cells with a few resistant cells. On the other hand, in experiment 9, with almost the same amount of plasmid DNA input as in experiment 1, only 1 in 105 *rec-1* ampicillin-sensitive transformants showed any evidence of a few colonies of ampicillin-resistant cells. These data

TABLE 1. Conditions affecting the relative yield of ampicillin-sensitive plasmid transformants

Expt no.	Concn of DNA in ligation mixture (μg/ml)		Concn of plasmid DNA used for transformation by ligated mixture (μg/ml)	Host cell	No. of chloramphenicol-resistant colonies tested	% of chloramphenicol-resistant colonies that were ampicillin sensitive
	Plasmid	Chromosomal				
1	20	30	0.0008	BC200	1,348	11
2	20	30	0.08	BC200	658	6
3	40	20	0.16	BC200	222	0.5
4	10	5	0.10	BC200	45	<2
5	10	10	0.10	BC200	44	11
6	1	5	0.01	BC200	45	9
7	1	10	0.01	BC200	45	20
8	1	10	0.004	rec-1	201	67
9	1	10	0.0012	rec-1	185	58
10	0.1	10	0.00028	rec-1	110	62

suggested that the few ampicillin-resistant cells in the otherwise ampicillin-sensitive BC200 clones arose by recombination.

E. coli was transformed by pDM2 with an efficiency of about 5×10^3 transformants per μg, approximately the same as observed in *H. influenzae*.

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