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A new DNA-cloning vector for *Haemophilus influenzae* Rd.

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Abstract. A new, high-efficiency, DNA-cloning vector pJ1-8 was derived in two steps from the chimeric plasmid pD7 consisting of RSF 0885 (*amp*^{r}) and *Haemophilus influenzae* chromosomal DNA. pJl-8 has only one *Eco*RI site and a molecular weight of only 2.5×10^6. No detectable *amp*^{r} transformation was obtained with pJl-8 DNA. However, *amp*^{r} transformation increases markedly if *Haemophilus influenzae* chromosomal DNA segments are spliced into it, providing a very facile assay for detecting inserts.

Keywords. Cloning vector; *Eco*RI site; efficient insert detector.

Introduction

A small, multicopy plasmid RSF0885 has been utilized as a vector for cloning DNA in *Haemophilus influenzae* (Setlow *et al*.,1981; Notani, 1981; Notani *et al*., 1981). RSF0885 by itself transforms *H. influenzae* with a very low efficiency for the *amp*^{r} marker that it contains. Plasmid RSF0885 has a single *Pvu*II site; cleavage of which with the enzyme generates blunt ends. However, ligation of *Pvu*II-cut *H. influenzae* chromosomal DNA to RSF0885 increases the *amp*^{r} transformation 100 to 1000-fold presumably because RSF0885 does not contain the required 11 base-pair uptake sequence (Sisco and Smith, 1979) which is provided by the spliced chromosomal DNA. A chimeric plasmid pD7 ($M_r \sim 12 \times 10^6$) containing a large chromosomal DNA insert in RSF0885 was isolated and characterized (Notani, 1981). pD7 is cut at 4 sites with *Eco*RI. All the sites are presumably in the chromosomal DNA. A new cloning vector pJl-8 was derived from pD7 in two steps by reducing the number of *Eco*RI sites to one. This cloning vector was even smaller than RSF0885 and by itself did not transform *H. influenzae*. However, ligation of chromosomal DNA segments increased the *amp*^{r} transformation significantly. Thus, practically each transformant colony contained a chromosome insert. We report the isolation and characterization of a new vector pJ1-8 and other derivatives from pD7.

Materials and methods

Bacterial strains

Haemophilus influenzae Rd (pD7) contains plasmid pD7, which is a spliced chimeric plasmid containing RSF0885 and chromosomal DNAs in the size ratio of approximately 1:3, respectively. Other derived strains are described in this paper.

Abbreviations used: Mr, Molecular weight; BHI,brain heart infusion;BSA, bovine serum albumin; DTT, dithiothereitol; DEAE, diethyl aminoethyl.

Media, growth and storage conditions

H. influenzae was grown in 3.7% Difco brain heart infusion (BHI) broth at 37° C with supplements of nicotinamide adenine dinucleotide (NAD) and hemin. Cells were made competent by aerobic-anaerobic-aerobic method (Goodgal and Herriott, 1961). Cultures grown from single colony isolates were stored in 15% glycerol at –3°C.

DNA isolation and purification

Chromosomal DNA was extracted according to the procedure of Marmur (1961). Plasmid DNA was isolated by the method of Hirt (1967) with minor modifications (Notani, 1981). To remove chromosomal DNA, purification was done by ethidium bromide-CsCl equilibrium density-gradient centrifugation (Radloff *et al*., 1967) or by sedimenting through sucrose gradients (Notani, 1981). For [^{3}H]-labelling of DNA, 8 ml of cells were grown in BHI with 30 µCi [^{3}H]-thymidine (Sp. act. 20 Ci/mM, source Bhabha Atomic Research Centre, Bombay) added per ml.

Restrictions nucleases, DNA cutting and ligation

Restriction nucleases were purchased from Bethesda Research Laboratories, Gaithersburg, Maryland, USA. T4 ligase was a gift from Dr. S. Mitra.

DNA (1–1.5 µg) digestion was done in *Eco*RI reaction mixture (100 µl) containing 50 mM NaCl, 100 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 50 µg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT) and 2 units of *Eco*Rl. Plasmid DNA digestion was carried out at 37° C for 1 to 1.5 h and that of chromosomal DNA at the same temperature for 20 min. Plasmid and chromosomal DNAs were mixed in the ratio of 1:3 respectively. Ligation mixture contained ligase salts (6 mM KCl, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT) and ligase additions (1 mM ATP pH 7.0, 10 µg/ml BSA, 10 mM DTT 1 mM spermidine). A maximum of 2 units of T4 DNA ligase was added to the ligase mixture containing 2µg of DNA and kept at 14°C overnight.

Agarose gel electrophoresis

One per cent agarose gels were used in all the experiments. Gel electrophoresis was carried out in Tris acetate buffer pH 7.9 to 8.2 for about 3 h (4 V/Cm). Both the gel and buffer contained ethidium bromide (0.1 µg/ml). Gels were visualized under an ultra-violet (UV) (350 nm) lamp. Photographs were taken with a 35 mm camera using UV filter.

Electro-elution of DNA fragment

Electro-elution of DNA fragment from agarose gel and purification of eluted DNA was carried out as described in Schleif and Wensink, 1981 (pages 124 and 94) except that Tris acetate buffer instead of Tris borate buffer was used for DNA elution from the gel.

Measurement of [^{3}H]-labelled DNA binding to competent cells

Transformation mixture for this purpose consisted of the following: 0.1 ml [^{3}H]-labelled DNA (big or small fragment) + 0.1 ml competent cells + 0.8 ml BHI

broth. The mixture was incubated at 37° C for 10 min. 0.5 ml of each mixture was washed 2X in cold and resuspended in 0·1 ml standard saline citrate (SSC). The entire content was spotted on Whatman 3 MM paper discs, treated with trichloroacetic acid, dried and counted in a Beckman scintillation counter.

Estimation of plasmid DNA concentration

This was done as described in Schleif and Wensink (1981) by making a set of standards and visually comparing them with the samples.

Results

*Eco*RI-digested *H. influenzae* chromosomal DNA was ligated with *Eco*RI-cut pD7 DNA. Ligated mixture was used to transform *H. influenzae* ampicillin-sensitive cells. Ampicillin-resistant colonies were picked and grown individually. Lysates were prepared and screened for the presence of altered plasmids. Only one out of ten isolates showed plasmid smaller than pD7. One smaller plasmid, designated pJ1, was characterized further. Figure 1 shows DNA profiles of pJl and pD7 sedimented through 5–20% sucrose gradients. pD7 sediments faster than pJ1. pJl

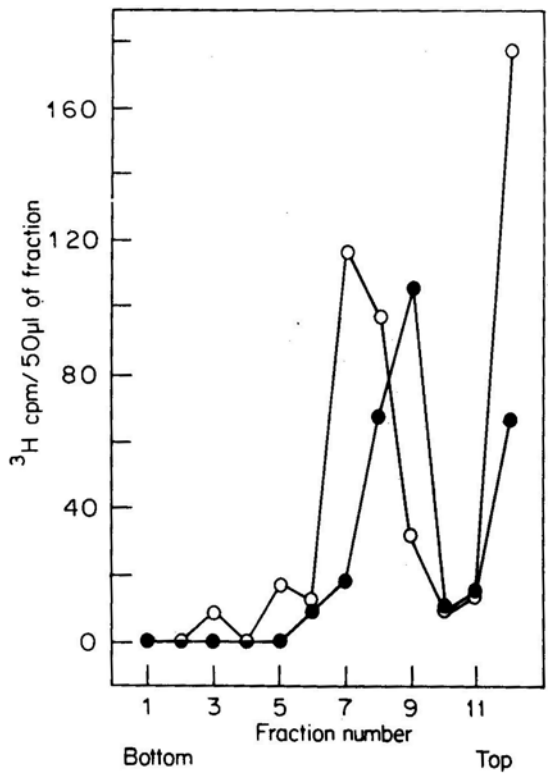


Figure 1. pD7 (O), and pJ1 (●), DNA sedimentation profiles through 5 to 20% sucrose gradients. Centrifugation done at 108500 *g* for 180 min at 20° C.

transforms with a somewhat lower efficiency than pD7 (table 1). When pJ1 DNA was cut with *Eco*RI it gave only two bands indicating that pJ l has ony two *Eco*RI sites Molecular weight (M_r) of these two *Eco*RI bands was determined by running *Hpa*-cut T7 DNA standards (McDonell *et al*., 1977) in parallel in the agarose gels (figure 2). The M_r of two fragments was estimated to be 3.9×10^6 and 2.5×10^6 respectively.

Table 1. Transformation (*amp*^{r}) activity of pD7 and pJ1 DNAs.

DNA source	DNA (ng/ml)	*Amp*^{r} transformants 10^{-4} ml
pD7	100	504
	10	107
	1	8
pJ1	100	153
	10	38
	1	4

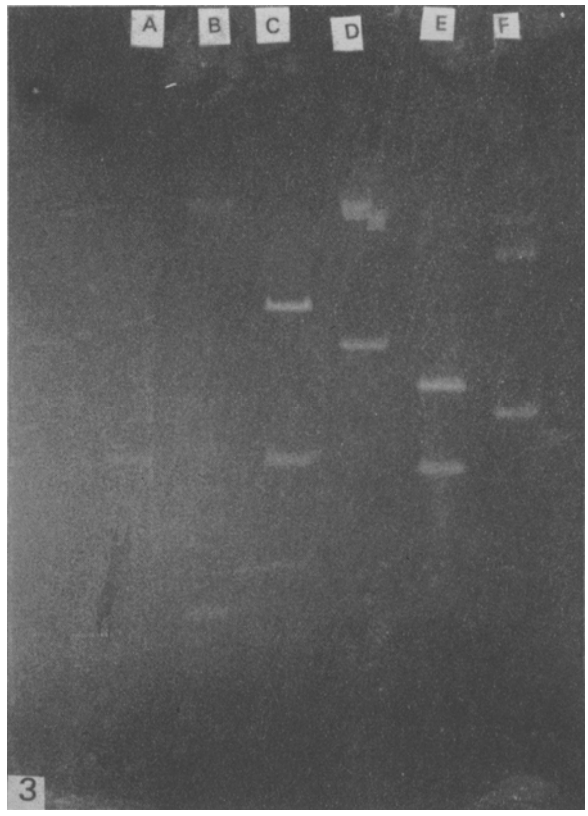
pD7 or pJ l DNA was isolated from 200 ml culture grown under identical conditions. DNA was purified by passing through sepharose 4B column. DNA concentration was estimated by matching intensity of the band with DNA bands of known concentrations (Schleif and Wensink, 1981).

Since *Haemophilus* transformation requires the presence of an 11-bp uptake sequence in the input DNA, it was of interest to determine which of the two fragments contained the required sequence. [^{3}H]-Labelled plasmid DNA in the two bands was separated by gel electrophoresis and electroeluted separately. Both the bands were then purified by passing through diethylaminoethyl (DEAE)-sephacyl ion exchange column. The two fragments were separately mixed with competent cells. After washing, binding of acid-insoluble [^{3}H]-label to the cells was determined (table 2). There was little binding of smaller fragment whereas the bigger fragment was bound substantially.

Construction of EcoRI -chromosomal library

Chromosomal DNA from wild-type *H. influenzae* cell culture was digested with *Eco*RI and ligated to *Eco*RI-cut pJ1 DNA. Competent cells were exposed to (i) uncut pJ1 DNA, or (ii) *Eco*RI-cut pJ1 or (iii) *Eco*-RI cut but self-ligated pJ1 or (iv) *Eco*RI cut pJ1 and *Eco*RI cut chromosomal DNA ligated mixture. Transformation mixture was plated and *amp*^{r} colonies were scored. Uncut pJ1 gave the highest transformation but *Eco*RI-cut plasmid yielded no transformants. Self-ligated pJ1 DNA restored the transformation to about 7% of the uncut plasmid and pJ1 ligated to chromosomal DNA to less than 2%. pJ1 DNA concentration was the same (250 mg) in all the cases.

Ampicillin-resistant colonies obtained from the transformation with ligated chromosomal DNA were picked and clear lysates were made to determine the size of the plasmid. Altogether 28 clones were analysed by agarose gel electrophoresis. Out of 28 clones, 19 were found to carry plasmids either smaller or bigger than pJ1. Thus we conclude that at least about 70% of the plasmids are other than pJl



Figures 2 and 3. 2. Electrophoresis patterns of pJl DNA, lane A–two prominent bands show CCC form of plasmid DNA (the fastest moving) and OC form (the slowest moving). *Eco*RI-digested pJ1 lane B–two bands which correspond to the 1st and the 4th slowest bands of *Hpa*-I digested T7 DNA standards in lane C (McDonnel *et al.*, 1977) with molecular weights of 3.9×10^6 and 2.5×10^6 respectively. **3.** Electrophoresis patterns of *Eco*RI-cut pJ1-8, pJ1-26 and pJ1 DNA (lanes A, C, E respectively) and uncut pJ1-8, pJ1-26 and pJ1 (lanes B, D, F respectively).

Table 2. Binding of *Eco*RI-cut pJl DNA label by competent *H. influenzae* Rd cells.

Fragments	cpm/0.1 ml (acid insol.)		Transformants (amp^r)
	Input	Uptake	
Big fragment			
Experiment I	300	239	0
Experiment II	436	116	0
Small fragment			
Experiment I	120	14	0
Experiment II	211	10	0

whereas all of the 10 *amp*^{r} clones from self-ligated plasmids DNA showed the presence of a plasmid of the same size as the original pJ1.

Three of the 19 recombinant plasmids were studied further. These three plasmids were designated as pJ1-8, pJ1-18 and pJ1-26. pJ1-26 is the largest of the three (figure 3 lane D) and transforms most efficiently (table 3). pJ1-8 gives little or no [^{3}H]-DNA binding to the competent cells as well as no transformation (table 3)

Table 3. Efficiency of newly-isolated, chimeric plasmid DNA's to transform *H. influenzae* cells.

DNA source	Uptake of DNA cpm/0.1 ml	*amp*^{r} transformants/ 10^{-4} ml
pJ1	1116	18
pJ1-18	1902	58
pJ1-26	1184	84
pJ1-8	10	0 (10^{-2})

and is the smallest in size (figure 3 lane B). Upon splicing with *H. influenzae* chromosomal DNA *amp*^{r} transformants could be scored by plating even at 10^{-4} dilution of the transformation mixture. pJ1-18 and pJ1-26 are both bigger than pJ1 and pJ1-8 is smaller than pJ1. Molecular weight of pJ1-8 was estimated to be about $2.5\times l0^6$. Figure 3 shows *Eco*RI-cut pJ1, pJ1-8 and pJ1-26 electrophoresed through agarose gels. pJ1 and pJ1-26 each yield 2 bands but pJ1-8 gave only a single band. Both pJ1 and pJ1-26 have one band corresponding in size to pJ1-8.

Discussion

Data in tables 1 and 3 indicate that there is variability in transformation efficiency amongst different plasmids. This could be due to the size of the chromosomal insert, since *rec* 1 and *rec* 2 gene expression is required for transformation with chimeric plasmids (Setlow *et al.*, 1981). pJ1-8 gives little or no transformation but upon splicing with *H. influenzae* chromosomal DNA the increase is substantial. Thus it fulfils the main criterion of efficient and facile screening for plasmid carrying inserts as it was in the case of RSF0885 cut with *pvu*II (Setlow *et al.*, 1981). Moreover, it is cut only once with *Eco*RI which produces 'sticky' ends unlike *Pvu*II which cuts once into RSF0885 but produces blunt ends. It may be anticipated that ligation would be more efficient with sticky ends.

Molecular weight of RSF0885 is 3.7×10^6 whereas that of pJ1-8 is only2.5×10^6. it is apparent that RSF0885 has suffered a deletion in the non-essential region. *Eco*RI site, not present in the original plasmid RSF0885 may have been generated with the deletion or more likely a bit of chromosomal DNA with *Eco*RI site may still be present. From low transformation (nearly zero) it may be deduced that pJ1-8 does not have an uptake site. At any rate, pJ1-8 works as a very efficient vector for screening and cloning *H. influenzae* chromosomal DNA inserts.

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References

Goodgal, S. H. and Herriott, R. M. (1961) *J. Gen. Physiol.,* **44,** 1, 201.
Hirt, B. (1967) *J. Mol Biol.,* **26,** 365.
Marmur, J. (1961) *J. Mol Biol.,* **3,** 208.
McDonell, M. W., Simon, M. N. and Studier, F. W. (1977) *J. Mol.Biol.,* **110,** 119.
Notani, N. K. (1981) *J. Biosci.,* **3,** 431.
Notani, N. K., Setlow, J. K., McCarthy, D. and Clayton, N. L. (1981) *J. Bacteriol.,* **148,** 812.
Radloff, R., Bauer, W. and Vinograd, J. (1967) *Proc. Natl. Acad. Sci. USA,* **57,** 1514.
Schleif, R. F. and Wensink, P. C. (1981) in *Practical Methods in Molecular Biology,* Sponsoring editor Philip Manor (New York, Heidelberg and Berlin: Springer-Verlag) p. **90,** 220.
Setlow, J. K., Notani, N. K., McCarthy, D. and Clayton, N. L. (1981) *J. Bacteriol.,***148,** 804.
Sisco, K. L. and Smith, H. O. (1979) *Proc. Natl. Acad. Sci. USA,* **76,** 1972.