6. Goel, H. C., Surendra Singh, Adhikari, J. S. and Rao, A. R., *Jpn. J. Exp. Med.*, 1985, **55**, 219.
7. Panigrahi, G. B. and Rao, A. R., *Mutat. Res.*, 1983, **122**, 347.

A NOVEL METHOD FOR CLONING CHROMOSOMAL DNA IN *HAEMOPHILUS INFLUENZAE* Rd

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PLASMID pRSFO885, genetically marked with an ampicillin-resistance marker[1], transforms *Haemophilus influenzae* very poorly[2]. However, if sequences homologous to chromosomal DNA are spliced to its only *Pvu*II site, transformation frequency increases a thousand-fold for the *amp*^{r} marker[3]. The enzyme *Pvu*II produces blunt ends in the DNA. Consequently, ligation to DNA segments is not as efficient as with a sticky-end-cut DNA vector and insert. A vector, pJ1–8, has been derived from pD7 (consisting of a chromosomal DNA segment spliced to pRSFO885) which has only one *Eco*R1 site (and no *Pvu*II site)[4]. Like pRSFO885, pJ1–8 gives little or no transformation for the *amp*^{r} marker, but when *Eco*R1-generated segments of chromosomal DNA are spliced to it, the frequency of Amp^R transformants increases by 3–4 orders of magnitude. Using this plasmid, several DNA clones carrying alleles of *nov*, *str*, *nal* and *uvrI* genes have been cloned[5–7]. However, most of the clones (except pKuvr1) are lost in the absence of selection pressure. A similar observation has been made[8] with DNA fragments cloned in pRSFO885. A new type of cloning system was serendipitously discovered. Plasmid pRSFO885 has two *Bam*HI sites, generating fragments designated here as A and B. The *amp*^{r} marker is on fragment A. In an attempt to clone DNA in one of the two sites by partial digestion, a few stable clones were obtained, although at a very low frequency. Analysis of some of these revealed that all such clones should carry a duplication of the 2.45 kb *Bam*HI fragment of pRSFO885. This report describes the construction and isolation of such clones.

A wild-type strain of *H. influenzae* Rd was used. Plasmids used were pJ1-8 (ref. 4), pRSFO885 (ref. 1), pJ1-8Str^{R}38, pJ1-8Str^{R}14 and pJ1-8Nal^{R}33 (ref. 6).

H. influenzae cells were grown at 37°C with aeration in BHI (brain heart infusion, Difco) broth containing 2 μg/ml NAD and 10 μg/ml haemin. Cells were grown to exponential phase, before freezing at −73°C in the presence of 15–20% glycerol.

Cells were made competent by the aerobic–anaerobic method[9]. A transformation mixture normally consisted of 0.1 ml competent cells + 0.1 ml DNA + 0.8 ml BHI broth. DNA uptake was allowed for 10–15 min before plating. Plates were challenged with ampicillin (5 μg/ml) after 1.5–2 h of incubation by overlaying with 10 ml BHI agar containing ampicillin.

Chromosomal DNA was extracted by the method of Marmur[10]. Plasmid DNA was extracted by the method of Hirt[11], with minor modifications. Plasmid DNA was purified by cesium chloride–ethidium bromide equilibrium density gradient centrifugation[12].

For electrophoresis of DNA, 0.7–1.4% agarose gels were used in Tris–acetate buffer (pH 8). Electrophoresis was carried out at 4 V/cm for 4–5 h[13].

Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories and used according to their instructions.

Plasmid loss was measured[8] by growing the cells overnight without ampicillin, and then plating on plates without ampicillin. Colonies were replica-plated on plates with or without ampicillin. Colonies that grew on plates without ampicillin but failed to grow on plates with ampicillin were considered to be those of cured cells.

pRSFO885 DNA has two *Bam*HI sites and it was considered possible that either of the two sites may be used for the cloning of DNA. Accordingly, *Bam*HI-digested chromosomal DNA was spliced with a partial digest of pRSFO885. Upon exposing a competent wild-type cell culture to the ligated DNA, a large number of Amp^R transformants were obtained (approximately 2×10^4 per ml). Five hundred Amp^R colonies were picked and analysed. Transformation using clear lysates obtained from these clones revealed that most of them give little or no transformation for the *amp*^{r} marker (less than 1×10^3 per ml) when reused for transformation. Only four clones were exceptions in that they gave high transformation frequency (table 1). These clones were designated pS43, pS121, pS322 and pS436. Agarose gel electrophoresis of the clear lysates showed that all the plasmids that fail to give *amp*^{r} transformation fall into any of the 3–4 groups of particular sizes (data not shown). Apparently,

Table 1 *Amp^R transformants obtained in Haemophilus influenzae Rd with various chimeric plasmids constructed using pRSFO885 for cloning at either BamHI site*

Plasmid	Amp^R transformants per ml ($\times 10^4$)
pS43	35
pS121	170
pS322	57
pS436	40

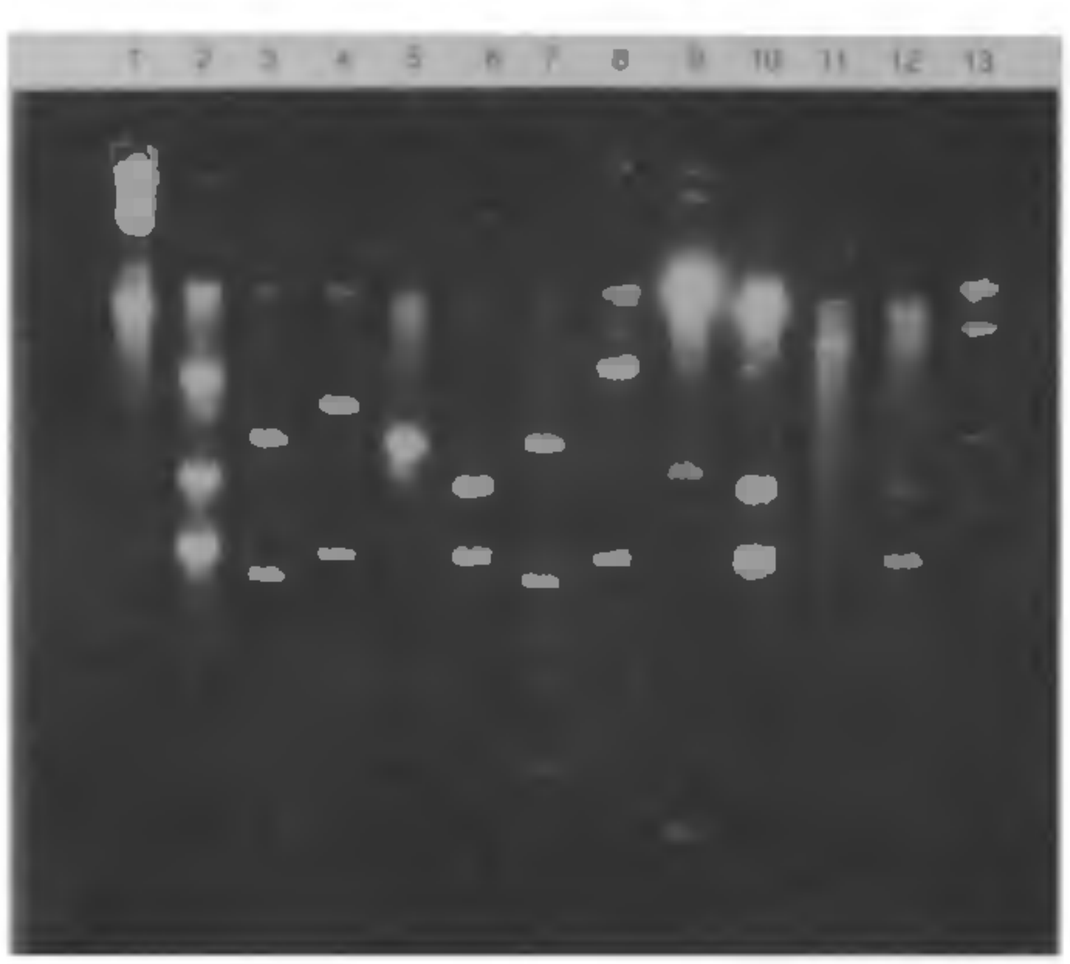

Figure 1. Agarose gel electrophoresis of restriction digests of various plasmids. Lane 1, pS121 digested with *Eco*RI; lane 2, pS121 digested with *Bam*HI; lane 3, pJ1–8Str^{R}14 digested with *Eco*RI; lane 4, pJ1–8Str^{R}14 digested with *Bam*HI; lane 5, pJ1–8 digested with *Eco*RI; lane 6, pRSFO885 digested with *Bam*HI; lane 7, pJ1–8Str^{R}38 digested with *Eco*RI; lane 8, pJ1–8Str^{R}38 digested with *Bam*HI; lane 9, pS322 digested with *Eco*RI; lane 10, pS322 digested with *Bam*HI; lane 11, pS43 digested with *Eco*RI; lane 12, pS43 digested with *Bam*HI; lane 13, *Sau*IIIA digest of T7 DNA (length standards, gift from F.W. Studier). The 2.45 kb band in lanes 2, 10 and 12 contains two fragments of equal size.

these are constructs from one or more combinations of the two *Bam*HI fragments containing a chromosomal insert with uptake sequences[14,15], which is lost following entry. Since the ligated DNA did give transformation initially these transformants may have lost the chromosomal insert present in the plasmids after plasmid entry into the cells. This led to the question of why plasmids pS43, pS121, pS322 and pS436 gave transformation for the *amp*^{r} marker. In the process of determining the molecular size of these four plasmids by the method of McDonell *et al.*[16], it was found that these four plasmids carried

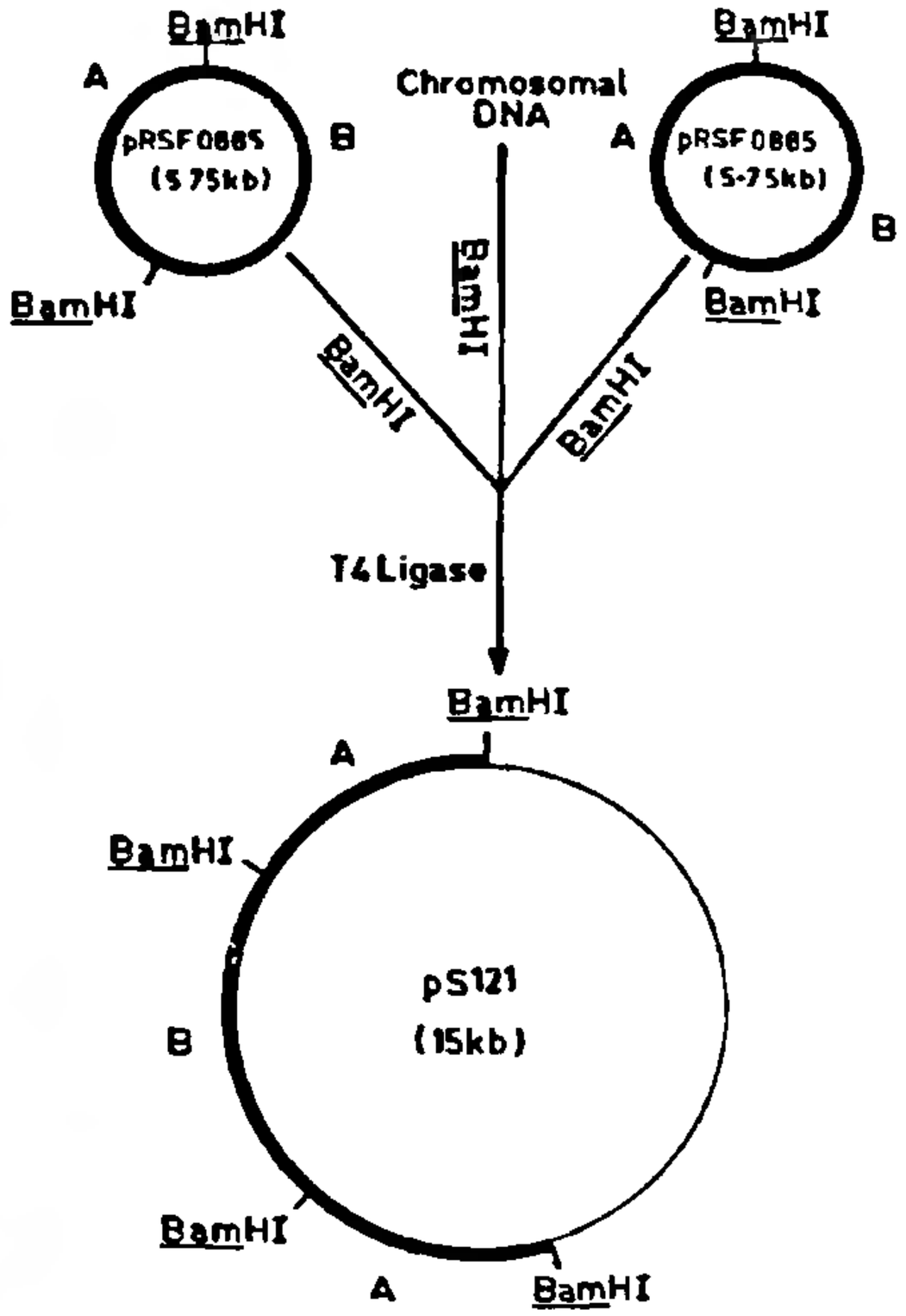

Figure 2. Schematic representation of the structure of pS121 and the orientation in which two copies of the *Bam*HI fragment A (2.45 kb) and one copy of fragment B (3.3 kb) of plasmid pRSFO885 are ligated to allow the insertion of a chromosomal DNA fragment.

Table 2 *Stability of plasmids derived from pRSFO885 and pJ1–8*

Plasmid	Per cent Amp^R stable
pS43	60.0 (145)*
pS121	72.0 (189)
pS322	93.3 (163)
pS436	86.1 (130)
pJ1–8	6.2 (193)
pRSFO885	96.4 (110)
pJ1–8Str^{R}38	3.8 (183)
pJ1–8Str^{R}14	19 0 (189)
pJ1–8Nal^{R}33	35 0 (177)

*Figures in parentheses are numbers of clones checked.

the small *Bam*HI fragment (2.45 kb) of pRSFO885 in duplicate (as indicated by the relative intensity of the band on agarose gels). Figure 1 shows the agarose gel electrophoresis of *Eco*RI and *Bam*HI

digests of 3 of the 4 plasmids. Also shown are restriction digests of pRSFO885 and other plasmids. It was found that the molecular weights estimated from the *Bam*HI fragments were lower than the estimates obtained by using the *Eco*RI fragments. This discrepancy could be resolved if the small *Bam*HI fragment of pRSFO885 was considered to be present in duplicate (data not shown). Duplication of this fragment is required probably because both the *Bam*HI sites in pRSFO885 are in vital segments of plasmid DNA and the only way to hold an insert stably at a *Bam*HI site would be by having a fragment duplicated such that both sites are left intact and a third new site is available for insertion of foreign DNA. A schematic representation of this is shown in figure 2. It should be noted that the three *Bam*HI fragments have to be in the correct orientation for an extra *Bam*HI site to be available for insertion of foreign DNA in the presence of two functional *Bam*HI sites.

The four plasmids, and a few others, were tested for their stability. As shown in table 2, the four chimeric plasmids derived from *Bam*HI-cut pRSFO885 are more stable than the plasmids derived from pJ1–8 (with the exception of pKuvr1)[17]. The fact that all four plasmids carried only the small fragment in duplicate may be purely a coincidence.

A new principle of cloning DNA emerges from this i.e. even when the cutting site (of *Bam*HI) in the DNA may be in a gene with a vital function, the problem can be overcome by duplication of the intervening plasmid segments in the construct.

The help of Dr V. P. Joshi on several occasions is gratefully acknowledged.

16 January 1989

1. DeGraff, J., Elwell, L. P. and Falkow, S., *J. Bacteriol.*, 1976, **126**, 439.
2. Notani, N. K., Setlow, J. K., McCarthy, D. and Claylon, N., *J. Bacteriol.*, 1981, **148**, 812.
3. Setlow, J. K., Notani, N. K., McCarthy, D. and Clayton, N. L., *J. Bacteriol.*, 1981, **148**, 804.
4. Joshi, V. P. and Notani, N. K., *J. Biosci.*, 1983, **5**, 339.
5. Joshi, V. P. and Notani, N. K., *Indian J. Exp. Biol.*, 1984, **22**, 625.
6. Samiwala, E. B. and Notani, N. K., Manuscript under preparation.
7. Kanade, R. P. and Notani, N. K., *J. Biosci.*, 1987, **12**, 115.
8. Setlow, J. K., Spikes, D. and Ledbeter, M., *J. Bacteriol.*, 1984, **158**, 872.
9. Goodgal, S. H. and Herriott, R. M., *J. Gen. Physiol.*, 1961, **44**, 1201.
10. Marmur, J., *J. Mol. Biol.*, 1961, **3**, 208.
11. Hirt, B., *Mol. Biol.*, 1967, **26**, 365.
12. Radloff, R., Bauer, W. and Vinograd, J., *Proc. Natl. Acad. Sci. USA*, 1967, **57**, 1514.
13. Maniatis, T., Fritsch, E. F. and Sambrook, J., In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory, New York, 1982, p. 93.
14. Sisco, K. L. and Smith, H. O., *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 972.
15. Danner, D. B., Deich, R. A., Sisco, K. L. and Smith, H. O., *Gene*, 1980, **11**, 311.
16. McDonell, M. W., Simon, M. N. and Studier, F. W., *J. Mol. Biol.*, 1977, **110**, 119.
17. Kanade, R. P., *Cloning of DNA Repair Gene(s) in Haemophilus influenzae*, Ph.D. thesis, University of Bombay, 1986.

TRANSFER OF SALINE TOLERANCE FROM ONE STRAIN OF RICE TO ANOTHER BY INJECTION OF DNA

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SALINITY and drought are regarded as the major problems facing rice production in the world. The moderately saline-tolerant variety of rice, Pokkali, is widely cultivated in the brackish water areas of Kerala, India and the sensitive variety of rice IR-20, is cultivated in the irrigated areas of the country. The tall and long-duration rice variety Pokkali cannot be adopted for cultivation under upland conditions.

Direct injection of genomic DNA of Pokkali into developing floral tillers of variety IR-20 produced transgenic seeds that were similar to Pokkali in husk colour, germinated well in 0.2 M NaCl, and had a 4–6-fold higher proline content. Transgenic seeds expressing chimeric genes have practical use in plant breeding to improve the quality and yield of cereal crops.

Genomic DNA (75 μg) isolated from rice variety Pokkali[1] and purified by gel filtration through a