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An estimate of the physical distance between two linked markers in *Haemophilus influenzae*

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Abstract. Using DNA clones, the physical distance between the linked genes *nov* and *str* in *Haemophilus influenzae* was estimated. Although none of the cloned inserts contained both the markers, pJ1-8Str^R 13 (insert of 18·7 kb) included *str* gene at one end and part of *nov* gene at the other end of the insert. By *Eco*RI restriction analysis and by Southern hybridization, the distance between the two *Eco*RI sites, cutting at which inactivates the two genes, was estimated to be 17·7 kb. A single continuous *Eco*RI fragment (containing 4 *Eco*RI sites within it) carrying both the genes intact would need to be 20·4 kb in size. These estimates were confirmed independently using different clones of *nov*^{r} and *str*^{r} alleles as probes for hybridization with *Bam*HI-digested chromosomal DNA.

Keywords. *Haemophilus influenzae;* linked genes *nov* and *str;* physical distance.

Introduction

High molecular weight DNA extracted from a *Haemophilus influenzae* strain resistant to the antibiotics novobiocin (*nov*^{r}) and streptomycin (*str*^{r}), can co-transform *H. influenzae* strain sensitive to both antibiotics with a relatively high frequency. Although alleles of both these genes have been individually cloned (Setlow *et al.,* 1981; Joshi and Notani, 1984; McCarthy and Cox, 1986; Samiwala, 1987; Goodgal, S. H., unpublished results), the two genes have not been cloned on a single fragment. From transformation studies Bagci and Stuy (1979) estimated the physical distance between *nov* and *str* genes to be 15 kb and Joshi *et al.* (1984), using a *nov* gene clone, estimated the distance to be greater than 8 kb. The high-efficiency vector pJ1-8 (Joshi and Notani, 1983) was used to make a DNA construction which carries *str* ^{r} gene at one end of the insert and part of *nov*^{r} gene at the other. Using the information obtained from the restriction map of pJ1-8Str^R 13 and comparing it with the information available from *str*^{r} and *nov*^{r} plasmids, a new, more accurate estimate of the physical distance between the two genes was obtained. The present report describes the construction of the chimeric DNA and the analysis that enabled us to make the new estimate of the physical distance between *nov* and *str* in *H. influenzae.*

Materials and methods

Bacterial strains and plasmids

H. influenzae strains and plasmids used in the study are listed in table 1.

Media, growth and storage conditions

Cells were grown in 3% Difco Brain Heart Infusion (BHI) broth supplemented with

Table 1. *H. influenzae* strains and plasmids used.

Strain	Phenotype/genotype	Source/reference
Rd	Wild type, rough, serotype d	S. H. Goodgal
Rd Nov^r Str^r	Rd strain carrying linked alleles *nov*^{r} *str*^{r} that confer resistance to 2·5 μg/ml novobiocin and 2000 μg/ml streptomycin	S. H. Goodgal
pJ1-8 plasmid	A DNA cloning vector; resistance to 5 μg/ml (or more) ampicillin; has a single *Eco*RI site outside *amp*^{r} marker	Joshi and Notani (1983)
pJ1-8N19 and pJ1-8N2	Chimeric DNA, carries *amp*^{r} plasmid marker and *nov*^{r} chromosome marker	Joshi and Notani (1984)
pJ1-8Str^R 14	Chimeric DNA, carries *amp*^{r} plasmid marker and *str*^{r} chromosome marker	E. B. Samiwala and N. K. Notani
pJ1-8Str^R 13	Chimeric plasmid, carries *amp*^{r} *str*^{r} and part of *nov*^{r} markers	Present communication

2 μg/ml NAD (nicotinamide adenine dinucleotide) and 10 μg/ml hemin. For solid medium, 1·2% Difco Bacto agar was added to the broth. Media were sterilized at 15 psi for 18 min. Supplements were added to the medium just before use. Plasmid-bearing cultures were grown in the presence of appropriate antibiotics. Antibiotics were used at the following final concentrations: ampicillin, 5 μg/ml; novobiocin, 2·5 μg/ml; and streptomycin, 200 μg/ml. Strains were preserved by freezing exponential-phase cell cultures at – 73°C with 15–20% sterile glycerol.

Extraction of DNA

Chromosomal DNA was isolated according to the method of Marmur (1961). Plasmid DNA was isolated by the method of Hirt (1967), with minor modifications (Notani, 1981). Plasmid DNA was purified by ethidium bromide-cesium chloride (EtBr–CsC1) equilibrium density gradient centrifugation by the method described by Maniatis *et al.* (1982).

Extraction and purification of pJ1-8Str^{R}13 DNA

pJ1-8Str^{R}13 DNA was recovered in somewhat low yields. After isolating the plasmid DNA by the usual methods, the cleared lysate was directly mixed with CsCl and EtBr. CsCl–EtBr equilibrium density gradient centrifugation was performed using a Type 65 rotor in a Beckman L8 ultracentrifuge at 139,500 *g* for 60 h at 20°C. At the end of the run, the tubes showed only one band, which on investigation proved to be the chromosomal DNA band. Solution from the region below the chromosomal band (where plasmid DNA is expected to band) was collected from 6 tubes and pooled together into one tube. Equilibrium density gradient centrifugation was carried out once again. This time a faint lower band was obtained, which was recovered. This consisted of the covalently closed circular (CCC) form pJ1-8Str^{R}13. This was used for all experiments.

^{32}P-Labelling of plasmid DNA

^{32}P-Labelled plasmid DNA was made by the method of Kahn *et al.* (1983).

Restriction digestion and ligation

All restriction enzymes and T4 ligase were purchased from Bethesda Research Laboratories and used according to the instructions provided.

Southern hybridizations

Southern hybridization was carried out as described by Maniatis *et al.* (1982).

Genetic transformation

The transformation mixture consisted of 0·8 ml BHI + 0·1 ml DNA (> 1 μg) + 0·1 ml competent cells (cells made competent by the method of Goodgal and Herriott, l96l). The mixture was incubated for 10–15 min at 37°C for uptake of DNA. Appropriate dilutions were then made in saline and cells were pour-plated with 10 ml BHI agar. After incubation at 37°C for 1·5–2 h, BHI agar (10 ml) containing the appropriate antibiotic(s) was added to the plates.

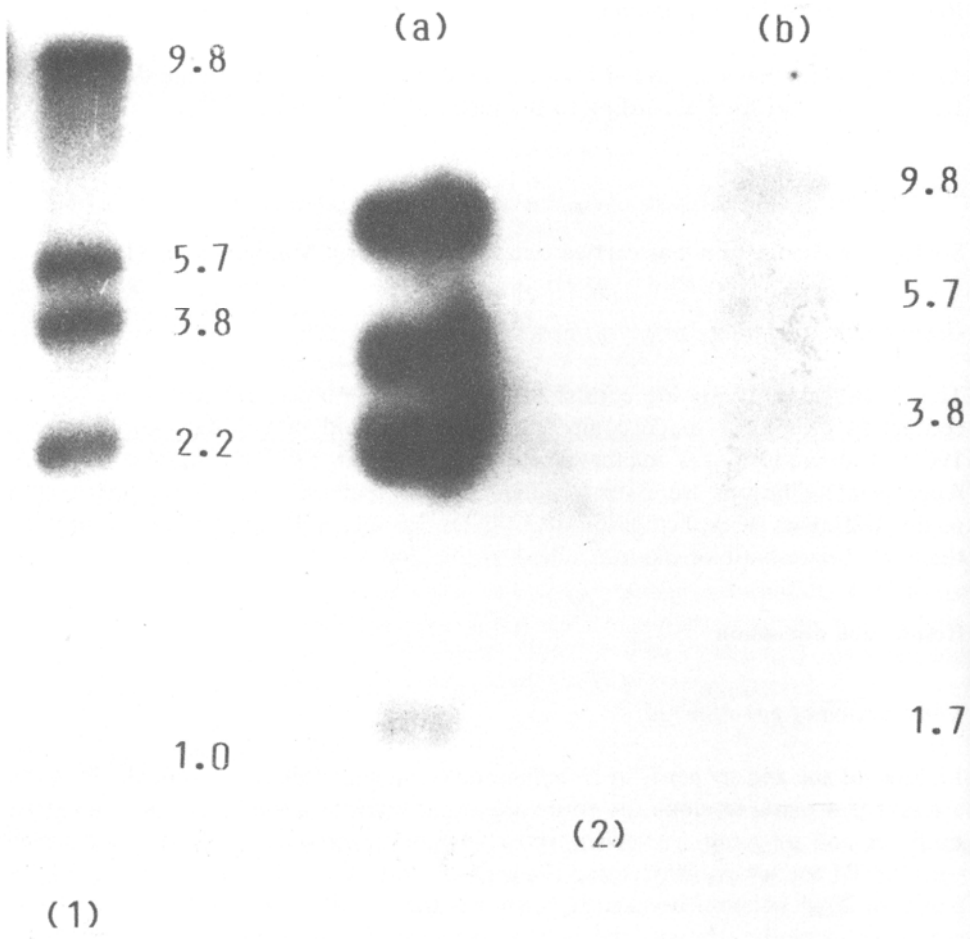
Results and discussion

Construction of pJ1-8Str^R 13

Linkage of *nov* and *str* genes in *H. influenzae* is known (Voll and Goodgal, 1961). An attempt was made to clone the entire segment of chromosomal DNA which carries both *nov* and *str* genes. For this purpose, *nov*^{r} *str* ^{r} chromosomal DNA was digested with *Eco*RI for a very short time (1 μg DNA was digested with 1 unit *Eco*RI for 5 min in 20 μl volume) because it is known that *Eco*RI inactivates both *nov*^{r} and *str*^{r} genes as well as breaks the linkage between the two genes (Samiwala, 1987). Inactivation of *nov*^{r} and *str*^{r} marker transformation activity by the treatment used was only 13 and 28% respectively (data not shown). This DNA was ligated to *Eco* RI-digested pJ1-8 DNA (which carries the *amp*^{r} marker). Transformation of competent wild-type Rd cells with ligated DNA failed to yield any Nov^{R}Str^{R}Amp^R colonies but one of the Str^{R}Amp^R colonies was found, by hybridization with the pJ1-8 N 19 probe, to be carrying part of *nov*^{r} allele along with the entire intervening sequence and the *str*^{r} gene on the plasmid. This plasmid was designated pJ1-8Str^R 13.

Determination of the size of plasmid pJ1-8Str^R 13

Restriction analysis of this plasmid was carried out using ^{32}P-labelled DNA. ^{32}P-Labelled pJ1-8Str^{R}13 DNA was digested with *Eco*RI and electrophoresed on agarose gels. Autoradiography of the gels revealed that pJ1-8Str^{R}13 consists of 5 *Eco*RI fragments (figure 1). Two of the fragments are the 2·2 kb and 1 kb fragments which carry the *str*^{r} allele. The other 3 are 9·8, 5·7 and 3·8 kb fragments. When a Southern blot of *Eco*RI-digested pJ1-8Str^R 13 was probed with labelled pJ1-8N19 DNA, it was found that the 9·8 kb fragment carries part of the *nov* gene. The 5·7 kb fragment flanks the *nov* gene and the 3·8 kb fragment was a pJ1-8 vector fragment



Figures 1 and 2. 1. Restriction pattern obtained by digesting ^{32}P-labelled pJ1-8Str^{R}13 DNA with *Eco*RI. Numbers indicate DNA fragment size in kb. **2.** Southern hybridization of *Eco*RI digested pJ1-8N19 (lane a) and pJ1-8Str^{R}13 (lane b) DNAs with ^{32}P-labelled pJ1-8N19 probe. The 1·7kb *Eco*RI fragment is missing in the case of pJ1-8Str^{R} 13. Numbers indicate DNA fragment size in kb.

(figure 2). Since pJ1-8Str^{R}13 does not carry the 1·7 kb *Eco*RI fragment which carries the rest of the *nov* gene (Joshi and Notani, 1984), it fails to transform cells to the Nov^{r} phenotype. Based on these data, a physical and genetic map of the pJ1-8Str^{R}13 insert was prepared and compared with those of the pKLT1 (McCarthy and Cox, 1986), pJ1-8Str^{R}14, pJ1-8N19 and pJ1-8N2 inserts (figure 3). The size of pJ1-8Str^{R}13 was estimated to be 22·5 kb. The physical distance between the two *Eco*RI sites, cutting at which inactivates the *nov*^{r} and *str*^{r} genes, is 17·7 kb. The physical distance between the intact *nov*^{r} and *str*^{r} genes is estimated to be less than 20·4 kb.

Confirmation of the physical distance between the two genes

The above estimate of physical distance is based on the assumption that the chromosomal insert in pJ1-8Str^{R}13 is a continuous one. The physical distance

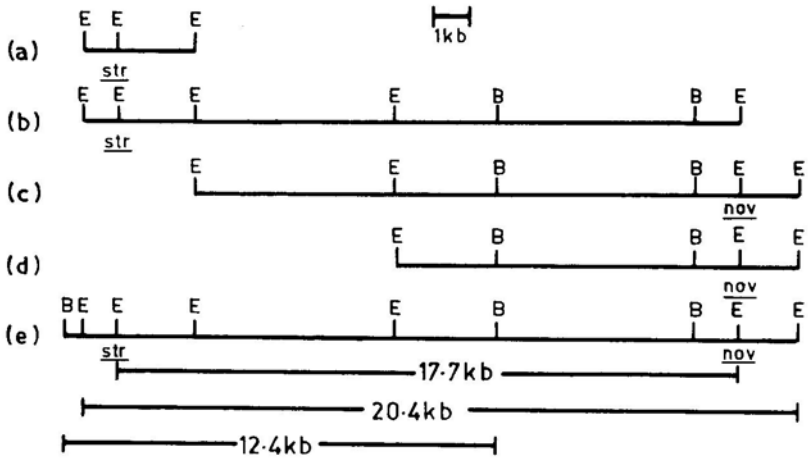


Figure 3. Restriction and genetic maps of chromosomal DNA inserts of (**a**) pJ1-8Str$_R$14; (**b**) pJ1-8Str$_R$13; (**c**) pJ1-8N19 and (**d**) pJ1-8N2. (**e**) Chromosomal map deduced from the information available from (**a**) to (**d**) and McCarthy and Cox (1986). E, *Eco*RI site; B, *Bam*HI site.

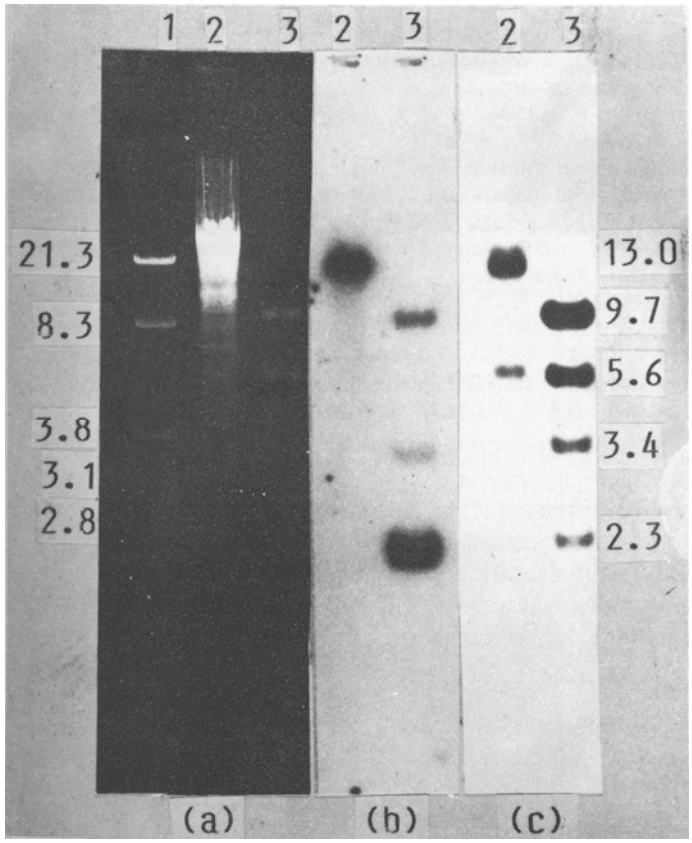


Figure 4. (a) Agarose gel (1%) electrophoresis of *Bam*HI digested *H. influenzae* chromosome (lane 2) and pJ1-8N19 (lane 3), and *Hpa*I-digested T7 DNA as standard (lane 1). Numbers indicate DNA fragment size in kb. (**b**) Southern blot of gel in (**a**) probed with nick-translated pJ1-8Str^{R}14 DNA. (**c**) Southern blot of gel in (**a**) probed with nick-translated pJ1-8N19 DNA.

between *nov* and *str* genes was confirmed by independent means. According to McCarthy and Cox (1986), a *Bam*HI site lies to the left of *str* gene (0·6 kb away from the left-most *Eco*RI site, figure 3a). Another *Bam*HI site is on the 9·8 kb *Eco*RI fragment (figure 3b) which carries part of the *nov* gene. The *Bam*HI fragment is thus 12·4 kb in size. If this estimate is correct, then *Bam*HI-digested chromosomal DNA should show a fragment which hybridize with both nov^r (pJ1-8N19) and str^r (pJ1-8str^R14) probes and this fragment should be 12–13 kb in size. This was indeed found to be true (figure 4): the *Bam*HI fragment which hybridizes with both nov^r and str^r probes was found to be 13 kb in size. This estimate is somewhat higher than the estimates made earlier. The estimate of Joshi *et al.* (1984) of greater than 8 kb was constrained by the length of the nov^r alone available at that time. Bagci and Stuy's (1979) estimate may have limitations in the use of uncloned DNA for transformation. However, the latter is underestimated only by about 5 kb. Using uncloned DNA, an average size of the insert (2 crossovers) during transformation was estimated at about 9 kb (Notani and Goodgal, 1966). Thus, if exchanges were purely random and without interference, roughly 4 exchanges would occur between *nov* and *str* genes.

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