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Decreased extrachromosomal fixation of chimeric plasmid in strain N19 of *Haemophilus influenzae* Rd.

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Abstract. Chromosomal DNA carrying closely-linked genetic marker alleles $str^r nov^r$ produces roughly equivalent number of Str^R (streptomycin-resistant) and Nov^R (novobiocin-resistant) transformants from wild type strain but widely differnt ratios (Str^R50: Nov^R1) from a mutant strain N19. Reduction in Amp^R (vector marker) extra chromosomal transformants is observed in N19 with chimeric plasmids carrying chromosomal DNA inserts from *nov* region. Thus, Amp^R transformants with chimeric plasmid DNA pJ1-8N2 and pJ1-8N19 are two to three orders of magnitude lower in N19 than in wild type. However, pJ1-8Nal^R33, pKuvr1, p3 and p10 DNA transform N19 and Rd with near-equal efficiency. Reduction in Amp^R transformants is intermediate (30 to 100-fold lower) in strain N19 with pJ18Str^R38 and pD7. These data are interpreted to suggest the presence of a small "aberration" in the *nov* region.

Keywords. *Haemophilus influenzae*; strain N19; differential transformation; *nov* chimeric plasmid; low extrachromosomal fixation.

1. Introduction

N19 was isolated as a UV-sensitive, differentially transformable strain of *H. influenzae*. Whereas a wild type Rd strain is transformed with equal efficiency for the linked marker alleles str^r and nov^r, transformation for nov^r marker is particularly depressed in N19 (Notani *et al* 1971). Integration of a chromosomal marker (Setlow *et al* 1981) and extrachromosomal fixation of chimeric plasmids carrying chromosomal insert requires *rec*1 and *rec*2 gene expression (Setlow *et al* 1981; Notani 1981; Joshi *et al* 1984). Several genes have now been self-cloned in *H. influenzae* including alleles of *nov, str, nal,* and *uvr*1. It has been proposed that recombinational events *per se* between homologous segments of the resident chromosome and the transforming chimeric plasmid DNA are required for assisting entry of the incoming DNA and/or its extra chromosomal fixation (Joshi *et al* 1984; Kahn and Smith 1984; Barouki and Smith 1985). Depression in Nov^R transformants selectively in N19 and other observations (Notani *et al* 1975) were indicative of at least a minor aberration in the vicinity of the *nov* region in this strain. Accordingly, it seemed of interest to study the effects on fixation of several of the DNA clones that carry genes linked to the *nov* gene and others unlinked from it in the strain N19. Our observations lead us to conclude that extrachromosomal fixation of nov^r or str^r allele-carrying plasmids is depressed in N19 because of requirements for recombinational events which are not met in N19.

2. Materials and methods

2.1 Bacterial strains and plasmids

H. influenzae strains and plasmids used in this study are listed in table 1. Bacterial strains were grown in 2·5% Difco brain heart infusion (BHI broth) supplemented with 10 μg/ml hemin and 2 μg/ml NAD (nicotinamide adenine dinucleotide). All the strains were maintained with 15% glycerol at −70°C. Competent cells were prepared by the aerobic-anaerobic-aerobic method of Goodgal and Herriott (1961). Competent cells transformed at 1 to 5% level for the chromosomal marker (nov^r) when exposed to saturating concentrations (~ 1 μg/ml) of chimeric plasmid DNA.

2.2 Isolation of DNA

Plasmid DNA was isolated by the method of Hirt (1967) with minor modifications (Notani 1981). Plasmid DNA was purified from cleared lysates by CsCl-ethidium bromide equilibrium density-gradient centrifugation (Radloff *et al* 1967) at 40,000 r.p.m. for 60 h in an SW65 rotor in a Beckman L8 ultracentrifuge. Bacterial DNA was isolated according to the method of Marmur (1961).

2.3 ^{32}P-labelling by nick translation of plasmid DNA

Nick translations of pJ1-8N2 and pJ1-8N19 DNA were done as described in Maniatis *et al* (1982). Nick translation kit (Amersham) and α^{32} P dCTP (BARC) were used to carry out the reactions. ^{32}P-labelling of plasmid DNA for uptake study was done as described by Kahn *et al* (1983). Ligation of nick-translated DNA was done since intact DNA is required for efficient transformation. Unincorporated ^{32}P-label was removed by chromotography using Sephadex G-50 preequilibrated column (bed volume 5 ml). This DNA was used as a probe in Southern hybridization experiments following heat denaturation. Labelled DNA was used to study the uptake and transformation efficiency in wild type and strain N19.

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

Strains or chimeric plasmids	Characteristics of the bacterial strain or of the insert DNA	Reference
Strains		
Wild type: Rd	Wild type	S H Goodgal
Mutant strain: N19	Yields differential transformation for *nov* and *str* alleles	Notani *et al* (1971)
Chimeric plasmids		
pJ1-8N2	Contains nov^r allele	Joshi and Notani (1984)
pJ1-8-N19	Contains nov^r allele	Joshi and Notani (1984)
pJ1-8 Str^R38	Contains str^r allele	E B Samiwala (unpublished)
pJ1-8 Nal^R33	Contains nal^r allele	E B Samiwala (unpublished)
p3	Contains unmarked DNA	This paper
p10	Contains unmarked DNA	This paper
pKuvr1	Contains $uvr1^+$ allele	Kanade and Notani (1987)
pD7	Contains unmarked DNA	Notani (1981)

2.4 *Transformation*

Transformation was carried out in a glass test tube containing 0·8 ml BHI broth ± 0·1 ml (6×10^8) competent cells and 0·1 ml DNA. Varying DNA concentrations were used in different experiments. To study the uptake of DNA, 1 ml of competent cells were directly mixed with ^{32}P-labelled DNA. The mixture was incubated for 5 min at 37°C. Uptake of DNA was stopped by addition of 100 μg/ml DNAase and incubating it for 2 min at 37°C, followed by centrifugation and washing (2X) of the cells with cold SSC (saline 0·15 M and sodium citrate 0·014 M). Aliquots of washed cells were spotted on Whatman 3MM paper discs and counted in BBOT toluene-based scintillation fluid in a Packard Scintillation Counter. 0·1 ml of transformation mixture was diluted and plated with BHI agar supplemented with hemin and NAD. Plates were incubated at 37°C for 1·5 h and then challenged with BHI agar containing appropriate antibiotics. Transformants were scored after 40 h incubation at 37°.

2.5 *Southern blotting*

Chromosomal DNA of *H. influenzae* wild type strain or mutant strain N19 and pJ1-8N2 or pJ1-8N19 plasmid DNA were digested with *Eco* RI, *Pst* I or *PvuII* (BRL). Reaction buffers and assay conditions were according to Maniatis *et al* (1982). Digested DNA were electrophoresed through 1% agarose (Sigma) gels to separate the DNA fragments. Blotting of the DNA from the gels was carried out for 10–16 h by using the procedure described by Southern (1975) and Maniatis *et al* (1982). The blots were baked at 80°C in a vacuum oven for 2 h and stored at room temperature. Hybridization was carried out in a plastic box at 68°C, and approximately $2\text{–}5 \times 10^6$ cpm were used per blot. The nitrocellulose blots were washed according to Maniatis *et al* (1982), dried and exposed to X-ray film (Indu) for 24 h at −70°C.

2.6 *Isolation of chimeric plasmids*

p3 and p10 chimeric plasmids were isolated according to the procedure described earlier (Joshi and Notani 1984).

3. Results

N19 cells grow slowly as compared to the wild type. Since the viable count of the competent cells is about 50 to 60% of that of the wild-type strain, double the amount of inoculum was used.

3.1 *Uptake of ^{32}P-labelled DNA*

Data in table 2 show uptake of ^{32}P-labelled pJ1-8N2 DNA by competent Rd and N19 strain. Uptake of DNA per viable cell count is comparable in the two strains. However, Nov^R and Amp^R transformants are lower by more than three orders of magnitude in N19.

3.2 *Transformation efficiency*

The transformation efficiency of competent wild type and N19 cultures with various chimeric plasmids is given in table 3. On the basis of transformation frequencies in

Table 2. Competent wild type and N19 cultures were exposed to ^{32}P-labelled pJ1-8N2 plasmid DNA. DNA concentration used was 5 ng/ml and specific activity of DNA was $1{\cdot}6 \times 10^7$ cpm per μg DNA.

Recipient cells	Number of cells per millilitre	Transformants/ml		Uptake of ^{32}P-labelled pJ1-8N2 DNA (cpm/ml)
		Nov^R	Amp^R	
Wild type Rd	$6{\cdot}4 \times 10^9$	$6{\cdot}7 \times 10^6$	$1{\cdot}4 \times 10^5$	$6{\cdot}4 \times 10^4$
N19	$5{\cdot}4 \times 10^9$	$1{\cdot}0 \times 10^2$	$1{\cdot}0 \times 10^2$	$5{\cdot}0 \times 10^4$

N19, chimeric plasmids are divided into three groups. Chimeric plasmids carrying inserts from *nov* region like pJ1-8N2 or pJ1-8N19 were put in group A. Transformation frequency of *amp*^{r} and *nov*^{r} marker with this group A plasmid DNA is lower by two to three orders of magnitude in N19 as compared to wild-type bacteria. Reduction in transformation frequency with group B plasmid DNA is 30–100 fold. Chimeric plasmids pJ1-8 Str^{R}38 and pD7 were classified as members of group B. Chimeric plasmid pJ1-8 Str^{R}38 carries an insert from *str* region and pD7 from an undetermined region. Transformant frequency in the strain N19 versus wild type is only a little lower with group C chimeric plasmid DNA. We interpret this to mean that chromosomal DNA inserts in these plasmids are not from *nov-str* or near *nov-str* region.

Since the reduction in transformant frequency is maximal with chimeric plasmids carrying *nov* insert, the effect of increasing DNA concentration on the frequency of transformants was studied. Figure 1 shows transformation versus DNA concentration response curve in wild type and N19. It is clear from figure 1 that the nature of the Nov^R and Amp^R curves in the wild type and strain N19 is similar. This indicates that the reduction in transformation efficiency in N19 is independent of DNA

Table 3. Competent culture of *H. influenzae* wild type Rd or mutant strain N19 exposed to chimeric plasmid DNA carrying different chromosomal inserts.

Group	Chimeric plasmid DNA	No. of transformants/10^8 cells/$0{\cdot}1\,\mu$g DNA				
		Vector marker (*amp*^{r})			Insert marker	
		Wild type Rd	Mutant strain N19	Marker	Wild type Rd	Mutant strain N19
A	pJ1-8N2	$5{\cdot}1 \times 10^5$	$2{\cdot}5 \times 10^3$	*nov*^{r}	$9{\cdot}0 \times 10^6$	$1{\cdot}3 \times 10^4$
	pJ1-8N19	$8{\cdot}0 \times 10^5$	$3{\cdot}0 \times 10^3$	*nov*^{r}	$7{\cdot}0 \times 10^6$	$4{\cdot}7 \times 10^3$
B	pJ1-8Str^{R}38	$2{\cdot}1 \times 10^4$	$6{\cdot}0 \times 10^2$	*str*^{r}	$6{\cdot}5 \times 10^5$	$1{\cdot}0 \times 10^4$
	pD7	$4{\cdot}8 \times 10^5$	$5{\cdot}0 \times 10^3$	–	–	–
C	pKuvr1	$1{\cdot}0 \times 10^6$	$6{\cdot}0 \times 10^5$	–	–	–
	pJ1-8Nal^{R}33	$7{\cdot}0 \times 10^5$	$1{\cdot}2 \times 10^5$	*nal*^{r}	$1{\cdot}2 \times 10^7$	$4{\cdot}4 \times 10^6$
	p3	$6{\cdot}0 \times 10^5$	$4{\cdot}5 \times 10^5$	–	–	–
	p10	$3{\cdot}0 \times 10^6$	$7{\cdot}4 \times 10^5$	–	–	–

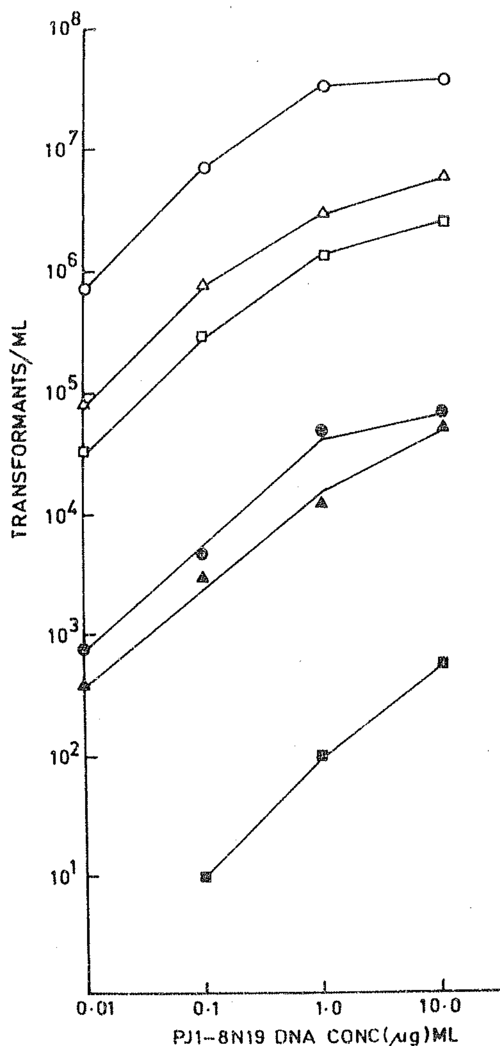

Figure 1. Competent *H. influenzae* Rd and N19 cultures were exposed to various concentrations of pJ1-8N19 DNA. Nov^R (○) Amp^R(△) and $Amp^R Nov^R$ (□) indicate transformants, open symbols Rd and filled symbols N19.

concentration used. Moreover, there is reduction in transformation efficiency even at saturating DNA concentration.

It should be noted that fixation of a chromosomal (insert) marker is more efficient than the vector marker during transformation with chimeric plasmid DNA in the wild type strain. Transformation for both the markers indicates extrachromosomal fixation of the chimeric plasmid(s). The ratios of vector marker: insert marker and vector marker: two markers (vector marker plus insert marker) are calculated and given in table 4. The data in table 4 gives the Amp^R: Nov^R and Amp^R: Str^R ratios, which are wider in the wild type than in mutant strain N19, indicating efficient recombination between incoming chimeric plasmid DNA and resident chromosome in wild type but not in strain N19. On the other hand, ratios of Amp^R:$Amp^R Nov^R$ and Amp^R:$Amp^R Str^R$ are wider in strain N19 than in wild type. However, such ratios obtained with pJ1-8Nal^R33 plasmid are comparable in both the strains.

Table 4. Ratios of transformants obtained from wild type Rd and mutant strain N19 following exposure of competent cells to chimeric plasmid DNA. Vector marker is *amp*^{r} and insert marker is *nov*^{r}, *str*^{r} or *nal*^{r}

Host strain	Chimeric plasmid and transformant ratio		
	pJ1-8N19 Amp^R:Nov^R:Amp^{R}Nov^R	pJ1-8Str^{R}38 Amp^R:Str^R:Amp^{R}Str^R	pJ1-8Nal^{R}33 Amp^R:Nal^R:Amp^{R}Nal^R
Wild type Rd	1:9:0·38	1:31:0·35	1:135:1
N19	1:1·2:0·003	1:17:0·05	1:325:1

3.3 *Southern hybridization of nov probes with wild type and N19* DNA

The results obtained from transformation studies indicated the possibility of an aberration in the *nov*/*str* region in the chromosome of N19. Therefore attempts were made to detect the aberration by using the Southern hybridization technique. *Eco*RI *Pvu*II or *Pst*I-digested wild type or N19 chromosomal DNA were used to prepare the blots. The blots were then hybridized with ^{32}P-labelled pJ1-8N19 or pJ1-8N2 probe. No aberration could be detected by this method. The possibility remains that a very small aberration or inversion of a big fragment in this region is involved but could not be detected.

4. Discussion

The most striking observation reported here is that in the mutant strain N19, Amp^R transformants are lower by about three orders of magnitude when the chimeric plasmid carries an insert from the *nov* region. All the chimeric plasmids were divided into three groups on the basis of reduction in Amp^R transformation. Reduction in transformation, thus, has at least some dependence on the insert.

Although the N19 cells grow somewhat slowly, competence development is not significantly affected (table 2). Uptake of DNA is comparable in strain N19 and wild type. Thus, the reduction in transformation efficiency observed is not due to slow growth or lack of competence. Group C chimeric plasmid DNA are found to transform competent N19 cells with efficiency almost equal to that of wild type Rd. These results indicate that the overall recombination mechanism in this strain is not affected, and that the disturbance in the strain N19 is in the vicinity of the *str*/*nov* region.

The expected disturbance could be a small deletion in this region or an inversion of a segment. This kind of lesion may reduce the levels of recombination in this region. However, our Southern hybridization results could not detect this with the enzymes used.

Earlier we had tried to induce *nov*^{r} mutation in the mutant strain N19 (*nov*^{s}) with N-methyl-N-nitro-N-nitrosoguanidine as a mutagenic agent. No Nov^R mutants could be obtained (Notani *et al* 1975). These observations also support the idea of at least a multisite mutation or a small deletion in the *nov* region which is not detected with the Southern hybridization technique.

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