JOURNAL OF BACTERIOLOGY, Dec. 1981, p. 812-816
0021-9193/81/120812-05$02.00/0

Vol. 148, No. 3

Transformation of *Haemophilus influenzae* by Plasmid RSF0885

NIHAL K. NOTANI, JANE K. SETLOW,* DAVID McCARTHY, AND NANCY-LEE CLAYTON

Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

Received 6 April 1981/Accepted 11 August 1981

Plasmid RSF0885, which conferred ampicillin resistance, transformed competent *Haemophilus influenzae* cells with low efficiency (maximum, less than 0.01%). As judged by competition experiments and uptake of radioactivity, plasmid RSF0885 deoxyribonucleic acid was taken up into competent *H. influenzae* cells several orders of magnitude less efficiently than *H. influenzae* chromosomal deoxyribonucleic acid. Plasmid RSF0885 transformed cells with even lower efficiency than could be accounted for by the low uptake. Transformation was not affected by *rec-1* and *rec-2* mutations in the recipient, and strains cured of the plasmid did not show increased transformation. Plasmid molecules cut once with a restriction enzyme that made blunt ends did not transform. Transformation was favored by the closed circular form of the plasmid.

It has been known for some time that foreign DNA is usually taken up poorly by competent *Haemophilus influenzae* cells(16, 17). Efficient uptake of DNA by these cells requires that the DNA contains a specific sequence of 11 base pairs (21), which is usually not present in foreign DNAs. In this work we studied plasmid RSF0885 (5), which has a molecular weight of 3.7×10^6 (20) and is transformed poorly in *H. influenzae* strain Rd. Our data indicated that there was poor uptake of this plasmid by competent *H. influenzae* cells; in addition, the establishment of this plasmid in cells was apparently not nearly as efficient as transformation by chromosomal DNA. We also found that the blunt-end linear form of the plasmid did not transform at all and that the closed circular form was somewhat better for transformation than open circular molecules.

MATERIALS AND METHODS

Microorganisms. Wild-type *H. influenzae* strain Rd, the mutant derivatives *rec-1* (DB117) and *rec-2*, and the media used have been described previously (18, 19). Strain BC200 (1) lacks an inducible defective prophage (2). Phage HP1 *c1* and the lysogenization methods which we used have also been described previously (18). A strain containing plasmid RSF0885 was obtained from Marilyn Roberts; this plasmid confers resistance to ampicillin. Plasmid RSF0885 was transferred into strain Rd for the experiments described here.

Transformation. We tried all previously described methods for making *H. influenzae* cells competent in an effort to obtain transformation that was as good as possible with plasmid DNA. These included the anaerobic-aerobic method (6), a modification of the diluted medium method (11) of Stuy (24), the $CaCl_2$ method (25), and the MIV method (22). The MIV method gave by far the best transformation frequencies, so this method was used for all of the studies described below. We attempted unsuccessfully to improve plasmid transformation by slightly altering the MIV procedure; the modifications which we tried are listed here as a help to other investigators. If the competent cells were chilled for 10 min before the DNA was added, the level of transformation decreased about a factor of two. Centrifuging the competent cells and resuspending them in warm growth medium before the DNA was added eliminated almost all transformation. Heat shock might be used to increase transformation by decreasing restriction. However, competent cells heated for 30 or 60 s at 50°C were transformed less than control cells. Similarly, a series of small doses of UV radiation (0.5, 1.0, and 1.5 J/m^2 at 254 nm) to the competent cells just before the plasmid DNA was added decreased transformation, although the viability of the cells was not affected. (The idea behind this attempt was that a temporary cessation in host DNA synthesis might help the plasmid become established.) Extending the time of expression of the transformants beyond the usual 90 min before the addition of a layer of agar containing 10 μg of ampicillin per ml also did not increase the observed number of transformants. Competent cells exposed to the plasmid had to be diluted by a factor of at least 100 in order to observe ampicillin transformants. When transformation was low, 10 or more plates were used to obtain adequate numbers of transformants.

Preparation of plasmid DNA. We used a number of different methods, which were based on previously described procedures (8-10, 26). We made small preparations that were not highly purified by growing 6 to 25 ml of cells to early stationary phase, centrifuging the suspension, and resuspending the cells at 1/15 the original concentration in 1× SSC (0.15 M NaCl plus 0.015 M trisodium citrate) containing 1% sodium do-

decyl sulfate; these preparations were incubated for 30 min at 37°C, 1 M NaCl was added, and the mixtures were left at 4°C overnight. They were then centrifuged for 30 min at 13,000 rpm in a Sorvall no. 34 rotor, and the supernatant containing plasmid DNA and only a small fraction of chromosomal DNA could be used without any further manipulation for gel electrophoresis or for the biological assay. Purified preparations were made from much larger amounts of cells (2 liters), which were usually harvested and suspended in 27% sucrose in either 1× SSC or TE buffer (30 mM Tris, pH 7.6, 50 mM NaCl, 5 mM EDTA); these preparations were incubated for 10 min with 0.6% sodium dodecyl sulfate and then centrifuged at 14,000 rpm in the Sorvall no. 34 rotor. The supernatant was subjected to deproteinization and RNase treatment by the Marmur procedure (12) and finally was centrifuged to equilibrium in cesium chloride-ethidium bromide (15), usually twice.

Gel electrophoresis. Gel electrophoresis was carried out in a 1% agarose gel horizontal system, as described previously (13).

Restriction endonuclease treatment of plasmid RSF0885. *Pvu*II was purchased from New England Biolabs. The reaction was carried out for 3 h at 37°C in a total volume of 0.05 ml containing 2.5 U of enzyme, 10 μg of plasmid DNA, 6 mM Tris buffer (pH 7.5), 6 mM $MgCl_2$, 6 mM beta-mercaptoethanol, 100 μg of bovine serum albumin per ml, and 6 mM NaCl.

Electron microscopy. Supercoiled plasmids were relaxed by mixing them with ethidium bromide (300 μg/ml) and then irradiating the mixture with a 100-W incandescent bulb at a distance of 3 inches (7.6 cm) for 24 h. The ethidium bromide was removed with 2-butanol, followed by ethanol precipitation. Samples were spread in 40% (vol/vol) formamide, as described previously (4). The grids were stained with uranyl acetate and shadowed with a Pt-Pd mixture (4:1) at an angle with a tangent of 1/8. The frequencies of multimer circles in plasmid preparations were determined by scanning populations of 4,000 plasmids.

RESULTS

Effect of plasmid DNA concentration on transformation. Figure 1 shows that transformation of competent wild-type cells to ampicillin resistance by RSF0885 DNA was related approximately linearly to plasmid DNA concentration and that transformation began to be saturated at a concentration of about 2 μg/ml. From 1 μg of plasmid DNA per ml approximately 10^4 transformations per ml were obtained. This is more than three orders of magnitude lower than the level of transformation by chromosomal DNA (3). The fact that the efficiency of transformation (number of transformants per microgram of DNA) did not increase with increasing concentration suggested that no more than one molecule was required for each successful transformation.

Rec independence of transformation. Table 1 shows that transformation by plasmid RSF0885 was not depressed in either the *rec-1* mutant or the *rec-2* mutant, although chromosomal transformation in these strains was 10^{-6} (*rec-1*) and 10^{-7} (*rec-2*) of the wild-type frequency and transfection by phage DNA was depressed profoundly in these strains (14).

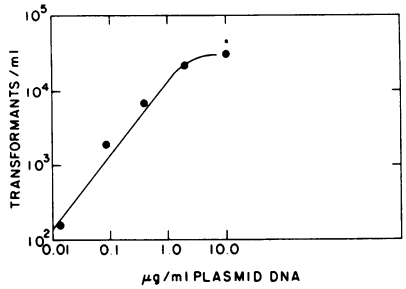

FIG. 1. *Transformation of strain Rd as a function of RSF0885 DNA concentration.*

TABLE 1. *Transformation of Rec⁻ and Rec⁺ strains by 0.4 μg of RSF0885 per ml*

Strain	No. of transformants per ml	No. of viable counts per ml	Frequency
Rd	9.9×10^3	9.6×10^8	10×10^{-6}
rec-1	3.8×10^3	4.3×10^8	9×10^{-6}
rec-2	18.7×10^3	5.5×10^8	34×10^{-6}

Cells transformed by plasmid RSF0885 are not a genetically special fraction of the population. One possible explanation for the low efficiency of transformation by plasmid RSF0885 DNA was that only a small fraction of the cells could be transformed by the plasmid, perhaps because only a small fraction of the cells did not restrict plasmid DNA. To test this hypothesis, we attempted to obtain cured strains that had lost the plasmid originally obtained by transformation. The plasmid was extraordinarily stable even when cells were grown without ampicillin. For example, after many generations of growth without the antibiotic, no ampicillin-sensitive single-colony isolates were found among 62 tested. Plasmid-bearing cells were also grown overnight in a high concentration of acridine orange(83 μg/ml); 5 ampicillin-sensitive isolates were found among 756 isolates tested from three different treated cultures. Transformation of these strains, which did not contain plasmids, as determined by their gel patterns, was indistinguishable from transformation of the wild type (data not shown).

Competition between plasmid DNA and chromosomal DNA. Figure 2 shows that chromosomal DNA competed very effectively with plasmid DNA, so that when the concentration of each DNA was 10 μg/ml, plasmid transformation was not observed. Chromosomal DNA

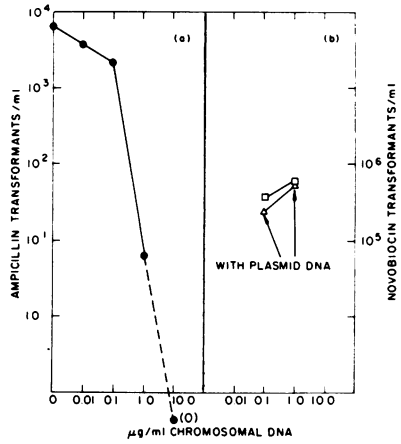

FIG. 2. *(a) Transformation of strain Rd to ampicillin resistance by 10 μg of RSF0885 DNA per ml in the presence of different amounts of chromosomal DNA. (b) Transformation of strain Rd to novobiocin resistance by chromosomal DNA at two concentrations in the presence or absence of 10 μg of RSF0885 DNA per ml.*

exerted a profound effect on plasmid transformation even at a concentration 100 times lower than the concentration of plasmid DNA. The curve appeared to have two parts, an initial less steep portion and a later precipitous drop. The DNA concentration curve for the chromosomal DNA used in this experiment began to be saturated at the concentration (0.1 μg/ml) where the drop began (data not shown). Our data indicated that chromosomal DNA competed about 100 times more effectively than plasmid DNA.

The converse experiment (Fig. 2) showed that plasmid DNA at a concentration of 10 μg/ml had a small (but reproducible) depressing effect on transformation by chromosomal DNA at lower concentrations.

Uptake data obtained with radioactively labeled plasmid DNA confirmed the conclusion from the competition experiment that chromosomal DNA was taken up by competent cells several orders of magnitude more efficiently than plasmid DNA. Only a few percent of the counts in [^{3}H]thymidine-labeled plasmid DNA were bound under conditions such that almost all of chromosomal DNA was bound (the specific activity of the plasmid DNA was around 10^5 cpm/μg, and the number of input plasmid molecules was less than 10^8 with 2×10^9 to 3×10^9 cells).

Kinetics of uptake of chromosomal DNA and plasmid DNA. Figure 3 shows that plasmid DNA and chromosomal DNA in the same preparation were taken up at different rates, as indicated by the effect on transformation of the addition of DNase at different times after competent cells and DNA were mixed. In this experiment the amount of chromosomal DNA, which was estimated to be less than 1 ng/ml, was too low to have an appreciable effect on plasmid transformation; in addition, the transformation from this DNA was the residual activity after heat denaturation.

Differential effects of elevated temperature during uptake on transformation by chromosomal DNA and plasmid DNA. Table 2 shows that when competent cells were exposed to chromosomal and plasmid DNAs at 37 or 41.5°C, the decrease in transformation at the higher temperature was greater for the plasmid DNA than for the chromosomal DNA under conditions that did not appreciably alter cell viability. Our data suggested that the uptake of plasmid DNA occurred by a mechanism different from that of uptake of chromosomal DNA.

Form of the plasmid that is biologically active for transformation. The restriction endonuclease *Pvu*II makes a single cut in RSF0885, as shown by gel analysis (20). Such treatment

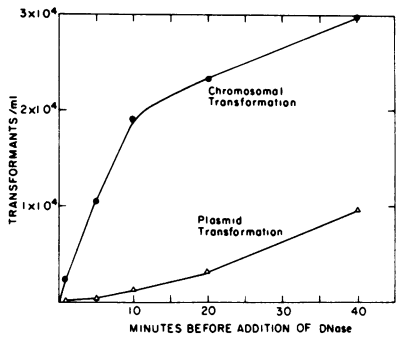

FIG. 3. *Transformation by plasmid DNA and chromosomal DNA as a function of time of addition of DNase after the competent cells and DNA were mixed. The cells were plated after 40 min. The DNA was from a crude preparation of plasmid DNA that was heat denatured by boiling for 10 min just before use.*

TABLE 2. *Effect of temperature during uptake on transformation of strain Rd by chromosome DNA and plasmid DNA*

Temp (°C)	No. of chromosome transformants per ml[a]	No. of plasmid transformants per ml[b]	No. of viable counts per ml
37[c]	1.4×10^4	1.2×10^4	6.5×10^8
41.5	4.1×10^3	1.1×10^3	6.0×10^8

[a] Transformants to streptomycin resistance.
[b] Transformants to ampicillin resistance.
[c] The ratios of transformants at 37°C to transformants at 41.5°C were 3.4 for chromosome transformants and 11 for plasmid transformants.

totally eliminated the transforming activity (no transformants were detected from the cut molecules, representing less than 1% of the transformation from untreated plasmid DNA). Thus, linear plasmid DNA, which had blunt ends, could not transform for the plasmid marker, presumably because of the inability of the DNA to circularize after it entered a cell. In contrast, RSF0885 converted to the linear form by restriction endonucleases that make sticky ends was active in transforming *Haemophilus parainfluenzae* (7).

It has been found that plasmids from competent cells are much more likely to be present as multimer circles than plasmids from exponentially growing cells (McCarthy, unpublished data). To determine whether plasmid transformation was favored by multiple forms, we compared two plasmid preparations which had approximately equal concentrations; one of these was from competent cells containing 2.0% dimers, as determined by electron microscopy, and the other was from exponential cells containing no detectable dimers. There were 3.2×10^3 ampicillin transformants per ml from the competent cell preparation and 1.3×10^3 ampicillin transformants per ml from the other preparation. These data suggested that plasmid transformation was not exclusively from multiple forms, but that multiple forms may have been favored.

In another experiment transformation from a plasmid preparation (10 μg in 0.5 ml) nicked with 5 ng of pancreatic DNase I (15 min at 37°C) was compared with an unnicked control. There was about twice as much transformation from the unnicked preparation, suggesting that whereas both open and closed forms could transform, the closed circular molecules were somewhat more efficient (the difference in the ratio of open and closed molecules in the two preparations as seen on a gel was much greater than a factor of two).

Resident plasmid RSF0885 does not interfere with phage development or the frequency of chromosomal transformation. Two strains of BC200 lysogenic for phage HP1 *c*1, one containing RSF0885 and one without resident RSF0885, were induced to produce phage with mitomycin C, as previously described (18). There was no difference in the phage yield. Also, chromosome transformation frequencies were not changed by the presence of the plasmid.

DISCUSSION

Gromkova and Goodgal (7) reported that plasmid RSF0885 grown in *H. parainfluenzae* was taken up efficiently into *H. influenzae* Rd cells made competent by the MIV method, but that the plasmid was not biologically active in this system. We found that strain Rd may be transformed by RSF0885 grown in *H. influenzae*, although this transformation is inefficient (Fig. 1). This difference may be due to restriction in *H. influenzae* of plasmids coming from *H. parainfluenzae*. However, the concentration of plasmids used by Gromkova and Goodgal was similar to the concentration of the least concentrated DNA used in the experiment shown in Fig. 1. We needed a large number of plates to detect transformation from this amount of plasmid. Since these authors did not say how many plates were used for their determination of no transformants, it is possible that their data and ours are not in disagreement. However, there is a clear disagreement concerning the efficiency of uptake into MIV-competent cells. Efficient uptake of RSF0885 into *H. influenzae* has not been observed either in our laboratory or in that of Hamilton Smith (personal communication). It is unlikely that the discrepancy results from the different sources of the plasmid, since it is known that *H. influenzae* does not discriminate against *H. parainfluenzae* DNA (23).

The poor uptake of plasmid RSF0885 into cells reflects a lack of the specific base sequence on the plasmid DNA (David Danner and Hamilton Smith, personal communication). This is not necessarily the case for other *H. influenzae* plasmids, since it has been found that the large plasmid p2265 DNA competes effectively for chromosomal DNA and thus must contain the specific sequence (W. L. Albritton and J. K. Setlow, unpublished data). The difference in kinetics of uptake of plasmid DNA and chromosomal DNA probably also reflects a difference in mechanism. The difference in the effects of an elevated temperature during the uptake period on transformation by plasmid DNA and chromosomal DNA (Table 2) may also result from the differing types of uptake mechanisms; however, this could also result from a heat-induced alteration in physiological state of the cells that might affect plasmid establishment.

The dose-response data in Fig. 1 show that at a concentration higher than about 3×10^{11} plasmid molecules per ml there is no further increase in transformation (calculated from the plasmid molecular weight of 3.7×10^6, as shown by measurements in the accompanying paper [20]). Thus, even considering the low efficiency of uptake (around 1 in 100 molecules taken up), there should be more than one plasmid taken up per cell. However, only about 1 cell in 10,000 is transformed. These data suggest that establishment of the plasmid is a very inefficient process.

ACKNOWLEDGMENT

This research was carried out at Brookhaven National Laboratory under the auspices of the Department of Energy.

LITERATURE CITED

1. **Barnhart, B. J., and S. H. Cox.** 1968. Radiation-sensitive and radiation-resistant mutants of *Haemophilus influenzae*. J. Bacteriol. **96**:280–282.
2. **Boling, M. E., D. P. Allison, and J. K. Setlow.** 1973. Bacteriophage of *Haemophilus influenzae*. III. Morphology, DNA homology, and immunity properties of HP1c1, S2 and the defective bacteriophage from strain *Rd*. J. Virol. **11**:585–591.
3. **Boling, M. E., J. K. Setlow, and D. P. Allison.** 1972. Bacteriophage of *Haemophilus influenzae*. I. Differences between infection by whole phage, extracted phage DNA and prophage DNA extracted from lysogenic cells. J. Mol. Biol. **63**:335–348.
4. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21D**:413–428.
5. **Elwell, L. P., J. de Graaff, D. Seibert, and S. Falkow.** 1975. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type *b*. Infect. Immun. **12**:404–410.
6. **Goodgal, S. H., and R. M. Herriott.** 1961. Studies on transformations of *Hemophilus influenzae*. I. Competence. J. Gen. Physiol. **44**:1201–1227.
7. **Gromkova, R., and S. Goodgal.** 1981. Uptake of plasmid deoxyribonucleic acid by *Haemophilus*. J. Bacteriol. **146**:79–84.
8. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
9. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
10. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. Methods Enzymol. **68**:268–280.
11. **Kooistra, J., and J. K. Setlow.** 1976. Similarity in properties and mapping of three Rec mutants of *Haemophilus influenzae*. J. Bacteriol. **127**:327–333.
12. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**: 208–218.
13. **McDonell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
14. **Notani, N. K., and J. K. Setlow.** 1974. Mechanism of bacterial transformation and transfection. Prog. Nucleic Acid Res. Mol. Biol. **14**:39–100.
15. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514–1520.
16. **Schaeffer, P., R. S. Edgar, and R. Rolfe.** 1960. Sur l'inhibition de la transformation bactérienne par des désoxyribonucléates de compositions variées. C. R. Acad. Sci. **154**:1978–1983.
17. **Scocca, J. J., R. L. Poland, and K. C. Zoon.** 1974. Specificity in deoxyribonucleic acid uptake by transformable *Haemophilus influenzae*. J. Bacteriol. **118**: 369–373.
18. **Setlow, J. K., M. E. Boling, K. L. Beattie, and R. F. Kimball.** 1972. A complex of recombination and repair genes in *Haemophilus influenzae*. J. Mol. Biol. **68**:361–378.
19. **Setlow, J. K., D. C. Brown, M. E. Boling, A. Mattingly, and M. P. Gordon.** 1968. Repair of deoxyribonucleic acid in *Haemophilus influenzae*. I. X-ray sensitivity of ultraviolet-sensitive mutants and their behavior as hosts to ultraviolet-irradiated bacteriophage and transforming deoxyribonucleic acid. J. Bacteriol. **95**: 546–558.
20. **Setlow, J. K., N. K. Notani, D. McCarthy, and N.-L. Clayton.** 1981. Transformation of *Haemophilus influenzae* by plasmid RSF0885 containing cloned segments of chromosomal deoxyribonucleic acid. J. Bacteriol. **148**:804–811.
21. **Sisco, K. L., and H. O. Smith.** 1979. Sequence-specific DNA uptake in *Haemophilus influenzae*. Proc. Natl. Acad. Sci. U.S.A. **76**:972–976.
22. **Steinhart, W. L., and R. M. Herriott.** 1968. Fate of recipient deoxyribonucleic acid during transformation in *Haemophilus influenzae*. J. Bacteriol. **96**:1718–1724.
23. **Steinhart, W. L., and R. M. Herriott.** 1968. Genetic integration in the heterospecific transformation of *Haemophilus influenzae* cells by *Haemophilus parainfluenzae* deoxyribonucleic acid. J. Bacteriol. **96**:1725–1731.
24. **Stuy, J. H.** 1960. Studies on the radiation inactivation of microorganisms. VI. X-ray-induced breakdown of deoxyribonucleic acid in *Haemophilus influenzae* and in other bacteria. J. Bacteriol. **79**:707–715.
25. **Stuy, J. H.** 1979. Plasmid transfer in *Haemophilus influenzae*. J. Bacteriol. **139**:520–529.
26. **Zasloff, M., G. D. Ginder, and G. Felsenfeld.** 1978. A new method for the purification and identification of covalently closed circular DNA molecules. Nucleic Acids Res. **5**:1139–1152.