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Physical Size of the Donor Locus and Transmission of *Haemophilus influenzae* Ampicillin Resistance Genes by Deoxyribonucleic Acid-Mediated Transformation

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The properties of donor deoxyribonucleic acid (DNA) from three clinical isolates and its ability to mediate the transformation of competent Rd strains to ampicillin resistance were examined. A quantitative technique for determining the resistance of individual *Haemophilus influenzae* cells to ampicillin was developed. When this technique was used, sensitive cells failed to tolerate levels of ampicillin greater than 0.1 to 0.2 μg/ml, whereas three resistant type b β-lactamase-producing strains could form colonies in 1- to 3-μg/ml levels of the antibiotic. DNA extracted from the resistant strains elicited transformation of the auxotrophic genes in a multiply auxotrophic Rd strain. For two of the donors, transformation to ampicillin resistance occurred after the uptake of a single DNA molecule approximately 10^4-fold less frequently than transformation of auxotrophic loci and was not observed to occur at all with the third. The frequency of transformation to ampicillin resistance was two- to fivefold higher in strain BC200 (Okinaka and Barnhart, 1974), which was cured of a defective prophage. All three clinical ampicillin-resistant strains were poor recipients, but the presence of the ampicillin resistant genes in strain BC200 did not reduce its competence. Sucrose gradients of DNA from ampicillin-resistant transformants of BC200 and from the original ampicillin-resistant strains showed that: (i) all the DNA preparations had high molecular weights; (ii) donor activity for ampicillin resistance sedimented heterogeneously and in parallel with genome DNA up to the highest molecular weights observed (100×10^6 to 200×10^6); and (iii) genetic transformation of ampicillin resistance from strain BC200-amp90383 required the physical integrity of a linearly integrated segment of DNA having a molecular weight of about 25×10^6 to 30×10^6.

The recent discovery of true β-lactamase production by strains of *Haemophilus influenzae* type b that are implicated in life-threatening diseases raises many clinically important questions and concerns. Other questions, which are dealt with in this paper, concern the genetic transmission of resistance among *H. influenzae* populations and its relevance to the probable origin of resistant strains.

The presence of β-lactamase genes in plasmids carried by gram-negative bacteria is common (5), and the transmission of such plasmids has resulted in the rapid dissemination of antibiotic resistance. The apparently abrupt appearance of ampicillin resistance due to a β-lactamase in a bacterium that over the last decade has shown little tendency to develop truly resistant strains provides reason to speculate that β-lactamase genes of *H. influenzae* are plasmid related. A β-lactamase-bearing plásmid might have originated from at least one such plasmid having made the "jump" between species and having established itself in *H. influenzae*. Given that such an event can and presumably has happened to produce the three ampicillin-resistant strains I have investigated, I have posed the following three questions. (i) Can the β-lactamase gene be transmitted to other strains of *H. influenzae* by deoxyribonucleic acid (DNA)-mediated transformation? (ii) Are the ampicillin-resistant clinical strains capable of becoming genetically transformed as a result of accepting genetic markers borne by DNA from other *H. influenzae*? (iii) Are the genetic determinants for β-lactamase integrated into the bacterial genome of the three clinical isolates examined here, or do they reside on an unintegrated plasmid?

MATERIALS AND METHODS

Bacterial strains. Strains 74-64148, 74-71518, and 74-90383 are ampicillin-resistant *H. influenzae* type b obtained from Clyde Thornsberry, The Center for Disease Control (CDC), Atlanta, Ga., and are so designated by the CDC. See Catlin (3) for other details concerning the origin of these strains.

Strain Rd is a transformable strain of *H. influenzae*. It is the noncapsulated derivative of strain d originally isolated by Leidy and Alexander (4). The strain used in these studies was obtained from B. W. Catlin (4).

Stain BC200 was obtained from B. J. Barnhart, Los Alamos Scientific Laboratory, and is a variety of Rd that has been cured of its defective prophage (14, 16).

Strain A8 was obtained from J. Michalka and S. H. Goodgal (12) and contains eight auxotrophic mutations which span the *H. influenzae* genome (4, 12).

Strain JC9 was obtained from John Clark, Queens College, Belfast, and was isolated from a clinical source in Ireland. JC9 is the only strain in this collection that is sensitive to bacteriophage N3 (15).

MAP8(S2) is a strain constructed by the author out of strain Rd. Strain Rd was first lysogenized with bacteriophage S2 (1) and then, by a series of genetic transformations, eight antibiotic resistance genes were introduced. Since transfections by prophage occur at least 10^3-fold less frequently than transformations by the antibiotic resistance genes, the presence of prophage S2 does not interfere when this strain is used as a donor of any of its antibiotic resistance genes.

BC200-amp90383 was constructed from strain BC200 by transforming it to ampicillin resistance with DNA prepared from strain 74-90383. Like strain 74-90383, strain BC200-amp 90383 was found to be a producer of β-lactamase.

Media. MIV (8) was modified by the addition of hypoxanthine, biotin, and tryptophan. These compounds are required by strain A8 for growth (12) and for the development of competence when the distilled water used in the preparation of synthetic competence development medium is rigorously maintained sterile. If precautions were not taken, contamination of freshly distilled water occurred, and bacterial populations of more than 10^6 colony-forming units/ml were found in this water after only a few days. Although medium prepared from such distilled water is sterilized, it often permits competence of strain A8 to develop without the addition of one or more of the above supplements (J. W. Bendler, in preparation).

SSC (or standard saline-citrate) medium consists of 0.15 M sodium chloride + 0.015 M sodium citrate.

Supplemented brain heart infusion (sBHI) medium consists of 3.7% (wt/vol) Difco brain heart infusion (BHI) to which was added 10 μg of hemin per ml and 2 μg of nicotinamide adenine dinucleotide (NAD) per ml. sBHI agar consisted of BHI broth + 1.2% Difco special Noble agar. Hemin and NAD were added as above to the liquefied agar after it was autoclaved and cooled to about 48 C. Hemin and NAD stock solutions were prepared as described by Herriott et al. and correspond to MIc stock solutions 6 and 7 (9).

Dilution medium consisted of 0.1 M sodium chloride; 0.01 M sodium phosphate; 10^{-3} M calcium chloride; 0.5×10^{-3} M magnesium chloride; and 0.1% (vol/vol) Tween 20 (polyoxyethylene [20] sorbitan monooleate). The pH was adjusted to 7.0 before autoclaving.

Determination of levels of resistance to ampicillin in H. influenzae strains. Strains 74-64148, 74-71518, and 74-90383 were inoculated into sBHI broth and simultaneously streaked out on BHI agar, some of which contained supplementary hemin and NAD and some of which did not. All the strains formed colonies in the sBHI broth and agar, and no growth occurred on BHI agar from which either hemin or NAD was omitted.

The three CDC strains were diluted and inoculated into melted sBHI agar so as to give ~200 cells per plate. A similar broth culture of the ampicillin-sensitive strain, Rd, was used as a control.

When the agar solidified, the plates were incubated at 36.5 C for exactly 1 h and then overlaid with an equal volume of sBHI agar containing various concentrations of ampicillin. One set of plates was not overlaid to determine the number of viable cells plated. Approximately equal numbers of colonies were found on plates containing 0.04, 0.08, 0.16, and 1.0 μg of ampicillin per ml (final concentrations) as on the non-overlaid plate for strains 74-64148 and 74-90383. Strain Rd gave as many colonies on the 0.04-μg/ml plate as on the non-overlaid plate, a few colonies on the 0.08-μg/ml plate, and no colonies on the plates with higher concentrations. Strain 74-71518 gave approximately equal numbers of colonies on the 0.04-μg/ml plate as on the control plate, but only half as many colonies were formed on media containing higher concentrations of ampicillin, suggesting that 50% of the cells in the population were sensitive. Independent tests of single-colony isolates from this population by Catlin (3) also indicated that the original culture of strain 74-71518 was a mixture of approximately equal numbers of resistant and sensitive cells and that single-colony, ampicillin-resistant isolates of this strain were unstable and continued to segregate sensitive cells after growth in liquid medium. Ampicillin resistance described elsewhere in this report was defined as ability to form colonies in pour plates overlaid with an equal volume of sBHI agar containing 2 μg of ampicillin per ml after 1 h of preincubation at 36.5 C.

Preparation of DNA. DNA for transformation studies consisted of crude lysates of concentrated washed cells unless otherwise stated. Cells grown to approximately 5×10^8/ml, as determined by optical density, were washed twice in SSC to which 0.1 M sodium (ethylenedinitrilo)tetraacetate, pH 8.5, was added to a final concentration of 0.01 M. The cells were washed twice more in SSC without sodium (ethylenedinitrilo)tetraacetate, concentrated fivefold in SSC, and lysed by adding lysing agent (1 part 10% sodium dodecyl sulfate and 1 part Sarkosyl) to a final concentration of 0.2% sodium dodecyl sulfate and 2% Sarkosyl. For some experiments the lysates were ex-

tracted with aqueous neutralized phenol by using a modification of the technique by Catlin et al. (4). The use of Sarkosyl permitted efficient phenol extraction even when overnight incubation with Pronase (2) was omitted. When crude DNA from cell lysates was used to initiate genetic transformation, the DNA was diluted by a total of 500-fold or more to avoid toxic effects of the lysing agents on the competent recipient cells. No viable cells remained in any DNA lysates.

Determination of size of DNA segment required to transform recipient cells to ampicillin resistance. The technique for measuring the physical size of DNA segments required for transmission of multiple loci or complex loci by genetic transformation was developed earlier (Bendler, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1968) and was used in other studies (4, 12). The rationale of such measurements may be summarized as follows. A segment of donor DNA that is comprised of $n\mu$ nucleotides and is initially linearly integrated within a much larger strand of DNA will remain intact with a probability given by $P = 1 - (n\mu/n)$ when (i) the larger strand in which it is integrated is broken at random into smaller fragments and (ii) the smaller fragments are fractionated into subsets of molecules consisting of n nucleotides. From this it follows that the relation between the relative number of transformants (i.e., relative to the number of transformants to a point marker) by a genetic marker, μ, whose locus is an extended segment of DNA, versus $1/n$ is graphically represented as a straight line intercepting the $1/n$ axis where $n = n\mu$. Let σ be a "standard" genetic marker whose locus occupies a segment of DNA consisting of $n\sigma$ nucleotides. If the relative number of transformants by σ is measured in each transformation assay in addition to the relative transformations by marker μ, and this measurement is repeated for each size fraction of DNA, a graph of the relative number of transformations by μ *versus* the relative number of transformations by σ will also be a straight line whose intercepts are determined by the relative sizes of σ *versus* μ (i.e., $n\sigma$ *versus* $n\mu$), but which is independent of n. Thus, in making this measurement it is not necessary actually to measure the size of the donor fragments, n, in order to determine the relative sizes of $n\mu$ and $n\sigma$. In the experiments described in this paper, DNA fractions were obtained by partially shearing the donor DNA to a heterogeneous mixture of sizes and fractionating it by ultracentrifugation in a sucrose gradient according to the method given previously (4), except for the modifications described below and in Fig. 2.

Shearing and sedimentation of DNA in sucrose gradients. The DNA preparation was divided into four portions. One portion, which was not further sheared beyond what may have inadvertently occurred during its preparation, contained a substantial amount of DNA in the molecular weight range of 200 $\times 10^6$ or greater. A second portion was lightly sheared by drawing it up and down in a 0.2-ml pipette six times. A third portion was sheared by squirting it forcefully several times out of a syringe having a no. 26 gauge needle. The last portion was sheared by 10 s of sonication, using the microtip attachment of the Branson Sonifier model W185 and a power setting of 4 (~40 W). The four portions were remixed and, using a low-shear technique used previously (4), ~0.05 ml was layered on top of a 5 to 35% sucrose gradient in SSC and sedimented at 35,000 and 20 C for 330 min in the SB283 head of the International B-60 ultracentrifuge. Sedimentation coefficients of DNA in such gradients were calculated by a variation of the technique described by Martin and Ames (11). Molecular weights were then calculated from Studier's equation for double-stranded DNA in neutral solution (17).

While these experiments were in progress, it was noted that a 5 to 20% gradient would have been more suitable where the Hershey equation (10) was to be used for comparing the molecular weights of different DNAs run in the same gradient, since the sedimentation velocity of DNA in a 5 to 20% gradient remains more nearly uniform while the DNA traverses the gradient in the IEC swinging bucket head used. However, since the calculated 15 to 50% variation in sedimentation velocity with position was unimportant to any of the calculations actually used, all of the gradients used were 5 to 35% (wt/vol) to facilitate direct comparisons among gradients.

Genetic transformations. Transformation experiments were carried out by using previously described methods (4), except for the modifications in the competence development medium, MIV, as noted above. In general, the procedure followed consisted of the addition of 0.10 ml of the diluted DNA to 2.0 ml of a 10-fold dilution (in 0.1 M NaCl) of the competent bacteria. This suspension was incubated at 37 C for 30 min, diluted in dilution medium, and pour plated by mixing 0.1 ml of the diluted cells in the appropriate liquefied agar medium.

RESULTS

Ampicillin-resistant clinical strains as donors of genetic markers. DNA from each of the clinical strains elicited transformation of the multiple auxotrophic strain A8 (Table 1). The *ala*, *bio*, and *leu* markers were transformed to prototrophy at similar frequencies by each DNA. In more extensive tests carried out with

TABLE 1. *Transformation of the multiply auxotrophic strain A8 to prototrophy by DNA preparations from ampicillin-resistant clinical isolates*

DNA donor strain	No. of transformant colonies[a]		
	Ala$^+$	Leu$^+$	Bio$^+$
74-64148	1.1×10^6	1.0×10^6	1.1×10^6
74-71518	0.9×10^6	0.92×10^6	0.49×10^6
74-90383	0.6×10^6	1.2×10^6	0.7×10^6
MAP8(S2)[b]	2.07×10^6		2.18×10^6

[a] A8 recipient population, 10^9 colony-forming units/ml, was treated with ~1.5 μg of each DNA per ml.

[b] Average of four replicas in a separate, similar experiment.

donor DNA from strain 74-90383, seven out of seven of the auxotrophic markers in strain A8 were transformed to prototrophy by this DNA.

Transformation to ampicillin resistance occurred in the Rd-derived strains, A8 and BC200, and in strain JC9, when strains 74-64148 and 74-90383 or strain BC200-amp90383 was used as donor (Table 2). Strain BC200 was the best recipient for ampicillin resistance donated by strain 74-90383, yielding about three to five times as many ampicillin-resistant transformants as the other two. Donor DNA obtained from BC200-amp90383-resistant cells proved to be a 10- to 20-fold more efficient donor of ampicillin resistance than the original donor of its ampicillin resistance gene, strain 74-90383.

The BC200 ampicillin-resistant transformants were picked, streaked onto sBHI agar, tested by the procedure described by Catlin (3), and found to be positive for β-lactamase activity.

Relative sensitivities to UV. The ultraviolet light (UV) sensitivities of Rd, BC200, and BC200-amp90383 were compared to determine whether the presence of the ampicillin resistance marker increased the UV sensitivity of BC200. It was earlier recognized that BC200 is more resistant to UV than is strain Rd (14). Cultures of BC200, Rd, and BC200-amp90383 were grown to densities corresponding to about 10^9 bacteria/ml. The cells were washed once and resuspended in dilution medium to their original concentration, and 5 ml was placed in a 10-cm-diameter petri dish. The dish was rotated during irradiation with UV from a 3.5-W germicidal lamp at approximately 50 cm. Samples of the irradiated cells were taken at intervals between 1 and 8 min, diluted, and plated in sBHI agar. The relative survival of colony-forming units obtained from these experiments (not shown) indicated that strain BC200-amp90383 had a sensitivity to UV radiation that was intermediate to that of Rd and BC200 and suggested that the ampicillin resistance gene(s) may be linked to a UV-inducible agent that is capable of killing its host, but less efficiently than the defective prophage carried by strain Rd.

Ability to be transformed by DNA from strain Rd. To test whether any of the three CDC strains was itself capable of accepting DNA from other *H. influenzae* strains, these strains were carried through the Herriott competence regimen, using MIV medium modified as above. To 2.0-ml portions of these cells was added 0.05 ml of a phenol-extracted preparation of DNA from strain MAP8(S2), which contains resistance markers for eight antibiotics, including the Str 2000 streptomycin resistance marker. In assays where saturating concentrations of this donor DNA were used, recipient Rd strains typically yielded about 1×10^5 to 20×10^5 streptomycin transformants from 2×10^8 cells. The number of transformations observed for strain BC200 fell within this range (Table 3). Thus, transformation was at least 10^3-fold less frequent in one of the three CDC strains and at least 10^5-fold less frequent in the other strains under these conditions.

Transformations to ampicillin resistance by a single uptake event. A lysate of strain BC200-amp90383 was prepared as in Materials and Methods and, after an initial 100-fold dilution, was diluted serially by factors of 2.

TABLE 3. *Transformation of ampicillin-sensitive and -resistant strains to streptomycin resistance by DNA from Rd-Str 2000*

Recipient strain	No. of transformants[a]
BC200	1.13×10^5
BC200-Amp-r	3.90×10^5
74-90383	5.1×10^2
74-71518-R-s[b]	0×10^1
74-64148	0×10^1

[a] Transformation conditions identical to those in Table 1.

[b] Noncapsulated, ampicillin-sensitive strain.

TABLE 2. *Transformations of A8 and BC200 to ampicillin resistance by DNA preparations from ampicillin-resistant clinical isolates and ampicillin-resistant BC200*

DNA donor strain	Recipients strains[a]		
	A8[b]	JC9	BC200[b]
74-64148	5×10^2	3×10^2	6×10^2
74-71518	$0 (<0.2) \times 10^2$	$0 (<0.2) \times 10^2$	$0 (<0.2) \times 10^2$
74-90383	2×10^2	5×10^2	16×10^2
BC200-Amp-r	10^3	8×10^2	308×10^2

[a] Transformation conditions were identical to those for Table 1.

[b] These strains were derived from strain Rd.

Aliquots of this serially diluted lysate (DNA) were used to transform strain BC200 to ampicillin resistance.

The number of transformants to ampicillin resistance decreased linearly with dilution of donor DNA (Fig. 1). Thus, transformation to resistance required the uptake of only a single fragment of donor DNA. This result is consistent with the inference that in the donor strain the presence of a single genetic locus specifying the enzyme, β-lactamase, is entirely responsible for the observed resistance and that resistance does not require two or more widely separated genetic determinants. This result does not, however, preclude the possibility that the resistance determinant requires the transfer and integration of a considerable portion of DNA, as is the case for the genetic locus determining production of the b-type capsule (4). In fact, the relatively low transformation efficiency to ampicillin resistance, by analogy with the low efficiency observed for the transformation of the b-type capsule, suggests that an extensive segment of DNA must be transferred intact in order to accomplish this transformation. The next two sections of this report deal with the question of the size of this DNA locus.

Sucrose gradient sedimentation of donor DNA. To determine whether ampicillin resistance donor activity cosedimented with high-molecular-weight genome DNA or as a smaller, homogeneous molecule, DNA was prepared from the three CDC ampicillin-resistant strains

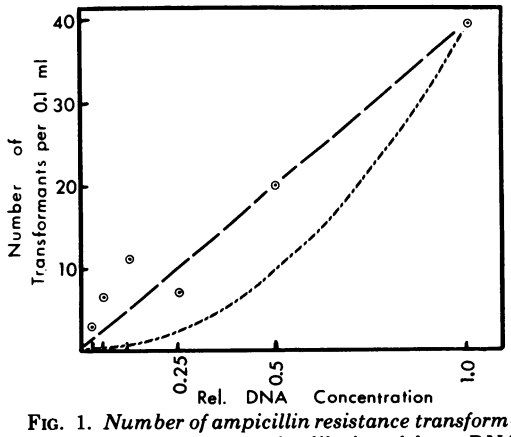

FIG. 1. *Number of ampicillin resistance transformants of strain BC200 versus the dilution of donor DNA (strain BC200-amp90383). The solid line is an arbitrary straight line whose slope corresponds to a linear decrease in transformants with DNA dilution. The dashed line illustrates the slope of a hypothetical curve that would result if transformation to ampicillin resistance required the uptake of exactly two independent fragments of DNA.*

and from the Rd strain, BC200-amp90383, as described in Materials and Methods, except that this DNA was not deliberately sheared. Approximately 5 μg of the DNA was carefully pipetted onto the top of an 11.0-ml, 5 to 35% gradient prepared in SSC. After sedimentation at 35,000 rpm and 20 C for the times indicated, the bottom of the tube was punctured and sufficient drops were collected to produce 0.5-ml fractions. Each fraction was used as donor in genetic transformation assays in which A8 was used as recipient. Each assay scored the number of A8 cells transformed to biotin and alanine prototrophy and to ampicillin resistance.

Figure 2 shows the results obtained for BC200-amp90383 donor. There is no indication that ampicillin resistance donor activity sedimented as a homogeneous fragment, that is, in a manner suggesting a different size distribution than that of the bacterial genome.

Similarly, the sedimentation profiles for donor activity of DNA from strains 74-71518 and 74-90383 were obtained (not shown). The size distribution profile for genome markers was indistinguishable from that obtained for strain BC200-90383 DNA. No donor activity was found for ampicillin resistance in the gradient of 74-71518 DNA, but 22 transformations to ampicillin resistance were scored in the assay of 74-90383 DNA. The distribution of this small number of ampicillin resistance donor units did not appear to differ significantly from the distribution observed for BC200-90383 and suggests that the gene for at least this β-lactamase is linearly integrated into the *H. influenzae* genome. Therefore, additional determinations were undertaken to confirm this.

Size of the DNA segment required for transmission of ampicillin resistance by genetic transformation. The following experiments were designed (i) to demonstrate that donor activity for ampicillin resistance was linearly integrated into the bacterial genome, and (ii) to measure the size of the segment of DNA required for transformation of a recipient to ampicillin resistance. The linked pair of bacterial markers conferring resistance to streptomycin and to novobiocin, respectively, was used as "standard" extended marker (see Materials and Methods for rationale).

Extensive measurements of the size of this pair of markers indicated that they are separated by 18,000 to 23,000 nucleotides, corresponding to a segment of DNA of molecular weight 12×10^6 to 15×10^6 (Bendler, Ph.D. thesis). The Str-Nov pair of markers was incor-

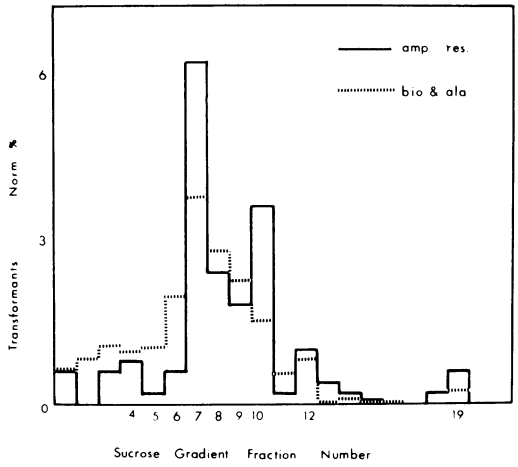

FIG. 2. *Sedimentation behavior of ampicillin resistance donor activity and of the average of alanine and biotin transformants versus sucrose gradient fraction. DNA from BC200-amp90383 was prepared and sedimented in a 5 to 35% sucrose gradient as described in the text. Care was taken to avoid shear throughout. Fractions were assayed by genetic transformation for donor activity of bacterial genome markers (biotin and alanine) and for ampicillin resistance donor activity. Ampicillin donor activity parallels the activity of genome markers even at the peak, which represents DNA of approximately 200 × 10^6 g/mol. To obtain the ordinate (transformants, norm %), the number of transformants for the locus in question was divided by the total number of transformants in the entire gradient, and this ratio was multiplied by 100. This unit facilitates comparison of the size distribution among different genetic markers whose efficiencies as transformation donors may differ greatly.*

porated into strain BC200-amp90383 by genetic transformation. Unpurified, high-molecular-weight DNA was prepared from strain BC200-amp90383, Str-Nov. The DNA was sheared and layered onto a sucrose gradient as described in Materials and Methods. Gradient fractions were collected as previously described. Approximately 0.05 ml from each fraction was pipetted by a technique found to minimize shear (4) into culture tubes where the genetic transformation assay was carried out. The transformation assay for each fraction measured the number of cells transformed to streptomycin resistance (total number) [Str], streptomycin-novobiocin double transformants [Str-Nov], and ampicillin-resistant transformants [Amp]. The brackets are used to denote the number of transformants by the marker contained within. The ratios [Str-Nov]/[Str] and [Amp]/[Str] were then calculated and plotted. Figure 3 shows the results of this operation. These results are to be compared with a theoretical model in which (i) the points fall along a straight line if the segment of DNA giving rise to ampicillin-resistant transformants is linearly integrated into the *H. influenzae* genome; (ii) the line will terminate at a unique point that corresponds to the "maximum" values of [Str-Nov]/[Str] and [Amp]/[Str], achieved when the molecular weight of the donor DNA is (effectively) infinite; and (iii) the line will intercept the axis corresponding to the smaller of the two segments of DNA (i.e., the segment required for transformation of the marker) at a point that is related to the relative sizes of the markers, R_{xy}, as follows: $R_{xy} = N_x/N_y = 1 - (y_i/y_m)$ (assuming y_i, the y-intercept, is positive), where N_x is the size of the larger segment, N_y is the size of the smaller segment, and y_m is the value of the y coordinate at the maximum point.

Although the results of the experiment illustrated in Fig. 3 display considerable scatter, the points approximate a straight line. The linear regression line for these points intercepts the y-axis (corresponding to the pair of markers

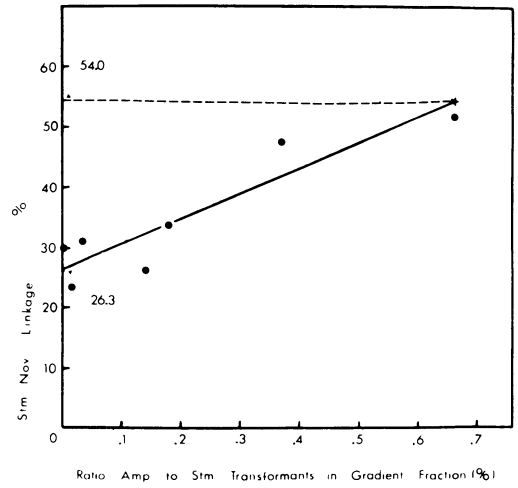

FIG. 3. *Results of a series of transformation assays using donor DNA sheared as described in the text and fractionated by sedimentation in a sucrose gradient as described in the text. In the assay for each fraction, donor activity was determined for total streptomycin resistance transformations, joint streptomycin-novobiocin resistance transformations, and ampicillin resistance transformations. Then the streptomycin-novobiocin "linkage" (i.e., the ratio of the number of double transformants to the total streptomycin transformants) and the ampicillin "activity" (i.e., the ratio of ampicillin to streptomycin transformants) were calculated and plotted for each fraction in the lower portion of the gradient where ampicillin activity was declining to zero.*

Str-r and Nov-r) at 26.3, and the y coordinate for the maximum point is 54.0; thus, y/y_m = 0.49. It follows from the theory that the segment required for transformation to ampicillin resistance is twice the size of the Str-Nov segment, or about 25×10^6 to 30×10^6 (36×10^3 to 46×10^3 nucleotides). The actual figures calculated for this and other experiments range from 22×10^6 to 30×10^6, assuming 12×10^6 and 15×10^6, respectively, as the molecular weight of the segment of DNA spanned by the Nov-Str segment.

DISCUSSION

Two of the three strains of ampicillin-resistant *H. influenzae* I have examined could donate their ampicillin resistance markers to other strains of competent *H. influenzae* by a single DNA uptake event. The third strain either was unable to donate ampicillin resistance at all or did so with an efficiency that was too low to detect (less than one transfer per 10^9 recipient cells at saturating DNA concentrations).

The best recipient for the ampicillin resistance marker was strain BC200, a strain that has been cured (14) of the defective prophage reported by Stachura et al. (16). Compared with other recipients, this strain was transformed to ampicillin resistance two- to fivefold more frequently at saturating concentrations of DNA. In other experiments (not described here) the presence of a (defective) prophage of S2 makes no measurable difference to the efficiency of transformation. The defective prophage that is missing in strain BC200 may carry a restriction-modification system not found in the ampicillin resistance donors. If strain Rd possess a restriction system not found in BC200, its effects are not discernible in transformations involving point mutations between these two strains. At least one host cell restriction system of *H. influenzae* is known for which there is little, if any, effect on the transformation efficiency of point markers (7).

A 5- to 10-fold increase in the efficiency of transformation of BC200 occurs when BC200-amp90383 is used as the donor of ampicillin resistance in place of 74-90383. This further improvement presumably results from the elimination of other restriction incompatibilities between BC200 and 74-90383. Nevertheless, even in this case the efficiency of transformation is at best 100-fold less than the efficiency of transformations involving genetic markers that span only one or a few nucleotides.

The locus of the genetic information of the β-lactamase carried by strains BC200-90383 and 74-90383 has not yet been mapped. Attempts to map this locus are in progress. The experiments reported here show that it is linearly integrated into the genome of the bacteria.

The segment of DNA required to confer on another cell the genetic capability to produce β-lactamase is much larger than that required to encode a single enzyme but is in good agreement with the 30×10^6 molecular weight of the larger ampicillin resistance plasmid found by Elwell et al. (6). If strains 74-90383 and BC200-amp90383 were carrying such a plasmid in an integrated state (that is, as an episome), the transformation of the β-lactamase gene would require that the whole episome be transferred intact to provide the required regions of homology at each end of the otherwise heterologous segment of DNA bearing the episome.

Donor activity residing on a separate plasmid was never found in these experiments. Since the β-lactamase genes are unstable in strain 74-71518 (3), this strain would appear to be the best candidate for a plasmid-associated β-lactamase. However, no donor activity for ampicillin resistance was ever detected in DNA prepared from this strain and, therefore, it remains a moot question as to whether plasmid-borne markers can ever serve as effective donors for genetic transformation.

There is no a priori reason to believe that plasmid DNA, per se, has a configuration that does not permit genetic transformation, however. The ability of circular forms of S2 and HP1-c1 DNA to initiate transfection (13) suggests that circular nonhomologous DNA can penetrate into competent cells in a biologically active form. Other alternatives that could account for the failure of this particular donor include the possible existence of additional restriction incompatibility or the presence in our recipient strain of a silent plasmid (or episome) that is incompatible with the ampicillin plasmid in strain 74-71518.

The latter two possibilities would also be expected to interfere with plasmid transfer by conjugation. Although failures to detect conjugal transfer have been reported (6), Thorne and Farrar (18) have apparently succeeded in transferring ampicillin resistance plasmids by conjugation in the strains they have examined. Using a variety of techniques, I have not successfully transmitted ampicillin resistance from any of the three donor strains examined to any of the recipients by conjugation, but such negative experiments are not adequate support for any model.

These experiments do not support the contention that genetic transformation is more likely than conjugation to have been the means by which the original ampicillin-resistant strains acquired their β-lactamase genes in the first place. Strain 74-71518, as well as its noncapsulated, ampicillin-resistant derivatives, appears to be untransformable and yet carries a β-lactamase gene for which no donor activity has yet been demonstrated, even in genetic transformation experiments involving other strains that are recipients for the other donor markers.

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