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Differential Behavior of Plasmids Containing Chromosomal DNA Insertions of Various Sizes During Transformation and Conjugation in *Haemophilus influenzae*

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Plasmids with chromosomal insertions were constructed by removal of a 1.1-kilobase-pair piece from the 9.8-kilobase-pair vector plasmid pDM2 by *Eco*RI digestion and inserting in its place various lengths of chromosomal DNA (1.7, 3.4, and 9.0 kilobase pairs) coding for resistance to novobiocin. A fourth plasmid was constructed by insertion of the largest piece of chromosomal DNA into the *Sma*I site of pDM2. The plasmids without inserts were taken up poorly by competent cells and thus were considered not to contain specific DNA uptake sites. The presence of even the smallest insert of chromosomal DNA caused a large increase in transformation of Rec$^+$ and Rec$^-$ strains. The frequency of plasmid establishment in Rec$^+$ cells by transformation increased exponentially with increasing insert size, but in Rec$^-$ cells there was less transformation by the larger plasmids. Conjugal transfer of these plasmids was carried out with the 35-kilobase-pair mobilizing plasmid pHD147. The frequency of establishment of plasmids by this method not only was not markedly affected by the presence of the insertions, but also decreased somewhat with increase in insert size and was independent of *rec-1* and *rec-2* genes. Recombination between plasmid and chromosome was readily detected after transformation, but could not be detected after transconjugation even when the recipient cells were Rec$^+$ and made competent. These data suggested that there is a special processing of plasmid DNA that enters the competent cells in transformation that makes possible recombination of homologous regions of the plasmid with the chromosome and pairing with the chromosome that aids plasmid establishment.

Much is known about the transfer of chromosomal genes by transformation in *Haemophilus influenzae* (3, 9, 14, 15, 23). The transfer of plasmid genes by a process resembling conjugation has also been described for this species (7, 25, 26). Recently evidence for non-plasmid-mediated chromosomal gene transfer has been obtained in this species by a mechanism involving cell-to-cell contact (1).

In the present work, we have studied the behavior and fate of mobilizable plasmids containing various amounts of chromosomal DNA and taken up into a recipient by conjugation, and we have compared the behavior of the same plasmids in transformation. Earlier work (21) showed evidence for two types of Rec$^+$-dependent recombination between a plasmid containing a chromosomal insertion and the chromosome of the recipient cell after transformation. Genetic information could either be transferred from chromosome to plasmid or from plasmid to chromosome, but apparently not in the same recombination event. We have now found that neither of these types of recombination took place after conjugal transfer. Furthermore, whereas increased size of the chromosomal insert caused a substantial increase in the probability of establishment of the plasmid in transformation of Rec$^+$ cells, there was no such size dependence in conjugal plasmid transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *H. influenzae* strains Rd and BC200, both wild type, and derivatives *rec-1* and *rec-2* (4, 19, 20) were grown in brain heart infusion (Difco Laboratories) supplemented with hemin (Eastman Kodak Co.) at 10 μg/ml and NAD (Sigma Chemical Co.) at 2 μg/ml. Three percent Eugonbroth (Difco) was used for dilutions. The mobilizing plasmid pHD147 (7) was obtained from W. L. Albritton. The plasmid pNov1 (21) conferred resistance to 5 μg of ampicillin per ml and 25 μg of novobiocin per ml, and pDM2 (11) conferred resistance to 4 μg of chloramphenicol per ml and 5 μg of ampicillin per ml. Cleared lysates and purified plasmids were prepared as previously described (16). The mobilizing plasmid was introduced into various strains by conjugation, and pDM2 and its derivatives were introduced by transformation.

Transformation and conjugation. Transformation was carried out with the MIV method of Steinhart and Herriott (24). Conjugation was done on membrane filters on hemoglobin agar as previously described (2).

Construction of derivatives of pDM2 containing chromosomal insertions pDM2s, pMB1, and pMB2. Purified preparations of pNov1 and pDM2 were individually digested by restriction endonuclease *Eco*RI (Bethesda Research Laboratories) by following the manufacturer's protocol. Figure 1 shows simplified restriction maps of these plasmids. Both pNov1 and pDM2 have two *Eco*RI sites, and the sizes of the smaller fragments obtained by digestion as determined by gel electrophoresis were 1.7 kilobase pairs (kbp) (the chromosomal portion of pNov1) and 1.1 kbp (pDM2). The digested plasmids were subjected to electrophoresis on a 0.5% preparative agarose gel containing 0.5 μg of ethidium bromide per ml. The small fragment of pNov1 and the 8.7-kbp piece of pDM2 were recovered by electroelution into troughs as described before (10). The eluent was phenol extracted and ethanol precipitated, and the dried pellet was suspended in 10 mM Tris–0.1 mM EDTA (pH 7.8). These two DNAs were mixed in the ratio of 1:5 (pDM2 to pNov1 pieces) and ligated with T4 DNA ligase (Bethesda Research Laboratories) for 4 h at 12°C followed by 4°C overnight.

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Another ligation was carried out with the 8.7-kbp fragment alone. The resulting plasmid was designated pDM2s (Fig. 1).

Transformants from the ligated mixture of pDM2 and pNov1 fragments are shown in Fig. 1, designated pMB1 (containing the single 1.7-kbp fragment) and pMB2 (containing two copies of the 1.7-kbp fragment).

pMB3. Digestion of pNov1 with *Pvu*II was followed by purification of the resulting 9.0-kbp chromosomal portion on a preparative gel as above. The plasmid pDM2s was digested with *Eco*RI, and the cohesive ends were digested with S1 nuclease (Boehringer Mannheim Biochemicals) as previously described (17). The pDM2s DNA was then treated with calf intestine alkaline phosphatase (Bethesda Research Laboratories) by the method of Maniatis et al. (10) to prevent any self-ligation of the vector. Equal amounts of vector and chromosomal DNA were ligated with T4 DNA ligase (Fig. 1).

pMB4. Digestion of pDM2 with *Sma*I, inactivating the chloramphenicol resistance marker, was followed by treatment with calf intestine alkaline phosphatase. The 9.0-kbp chromosomal DNA fragment from pNov1 was ligated to the *Sma*I-digested pDM2 (Fig. 1).

Gel electrophoresis. Gel electrophoresis was carried out in a 1% agarose gel horizontal system as described previously (12). Sizing of fragments was by comparison of mobilities with those of *Hpa*I fragments of T7 DNA, kindly donated by F. W. Studier.

Triparental matings. The ratio of donors (containing the mobilizing plasmid pHD147 and pDM2 or its derivatives) to recipients was 10:1. The cultures were grown individually to a cell density of around 5×10^8/ml and washed once with fresh growth medium to eliminate any residual antibiotic in the medium; a total volume of 3 ml of the mixture of mating strains was placed on a filter. Rec^- strains were used as donors to eliminate the possibility of chromosomal transfer from putative recipients to donors (1). Deneer et al. (7) have shown that the presence of the *rec-1* mutation in either the donor or the recipient had no effect on the frequency of mobilization. In all matings involving a Rec^+ recipient, 125 μg of DNase per ml was added to the mating mixture. The plates were incubated for 2 or 3 h before the cells were suspended and plated with antibiotics selective for each of the donors, the recipient and the transconjugants (5 μg of ampicillin per ml, 4 μg of chloramphenicol per ml, 25 μg of

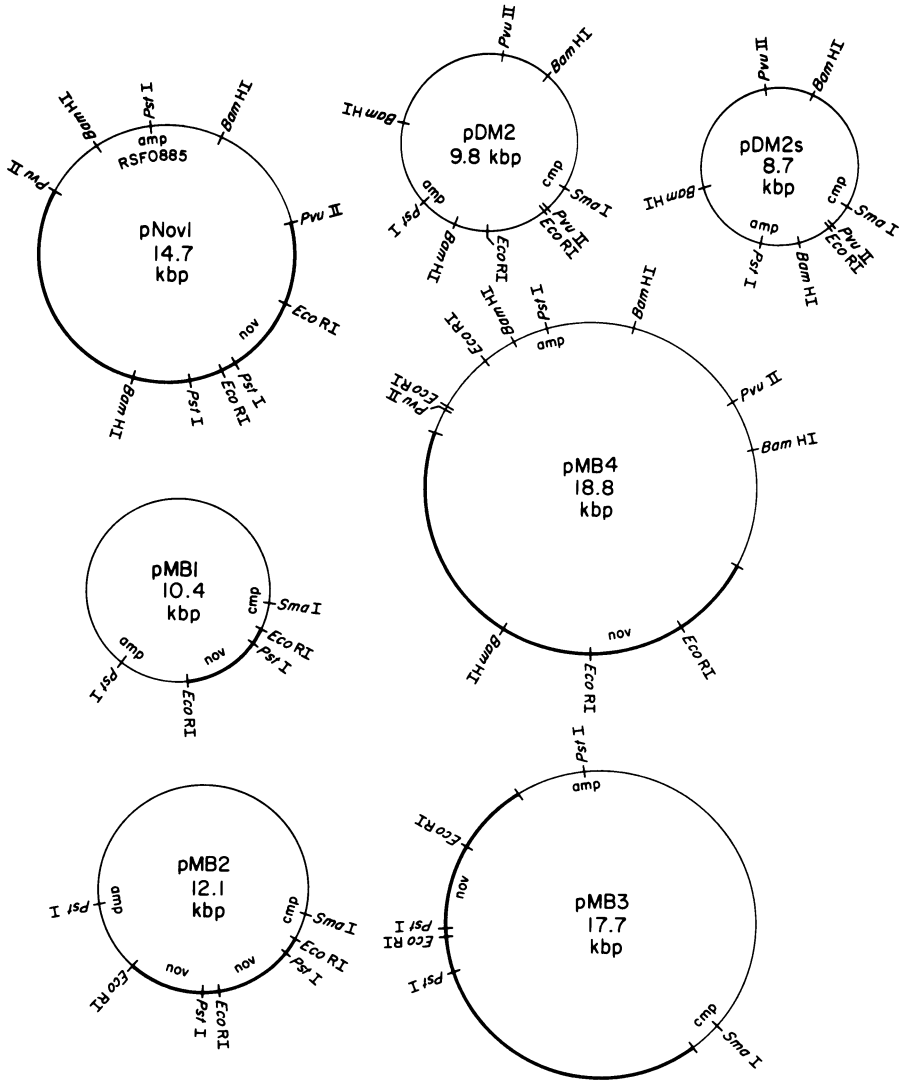

FIG. 1. Plasmids used in this study, drawn to scale. Thick lines represent DNA derived from the chromosome.

novobiocin per ml, 250 μg of streptomycin per ml, 7 μg of kanamycin per ml). The frequency of conjugative transfer was expressed as the number of transconjugants per recipient. Control matings without the mobilizing plasmid were always performed along with the triparental matings.

RESULTS AND DISCUSSION

Lack of expression of novobiocin resistance in pMB1 and pMB2. Since the 1.7-kbp piece of pNov1 had been shown to transform for resistance to 25 μg of novobiocin per ml (Griffin and Setlow, unpublished data), we expected pDM2 derivatives containing this fragment to be able to transform sensitive Rec$^-$ cells to novobiocin resistance, even though there would be negligible recombination of homologous portions of the plasmid with the Rec$^-$ recipient chromosomes. However, although transformants of *rec-1* with the ligated mixture of *Eco*RI-digested pDM2 and pNov1 fragments were resistant to chloramphenicol and ampicillin, we could not detect novobiocin-resistant transformants. These data suggested that the novobiocin resistance in the plasmids was not expressed, possibly because the entire gene was not present in the smaller plasmids. Proof that the novobiocin resistance marker was present was obtained by transforming novobiocin-sensitive wild-type cells to novobiocin resistance with cleared lysates of the transformants.

We have evidence that the entire gene is present in the 9.0-kbp chromosomal piece in pNov1, pMB3, and pMB4, in that the gyrase in cells carrying such plasmids is novobiocin resistant by in vitro assay, even though the chromosome carries the novobiocin-sensitive version of the gyrase gene (22). Thus the novobiocin resistance involves a structural mutation in gyrase.

Transformation by pDM2 and its derivatives. We have measured two types of transformation by the plasmids, (i) that resulting in establishment of the plasmid in the recipient cell as a free plasmid and (ii) novobiocin transformation from the chromosomal inserts. Our justification for equating transformation to chloramphenicol or ampicillin resistance with establishment of free plasmids is that we have examined many hundreds of gels of pDM2- and RSF0885-transformed Rec$^+$ and Rec$^-$ cells as well as gels of transformants by these plasmids carrying chromosomal inserts, and we have always found the plasmid markers to be associated with free plasmids seen on gels. Furthermore when the plasmid resistance marker was lost, the free plasmid was no longer seen on gels. These data have substantiated our original observation of the invariant association of the plasmid antibiotic resistances with visible plasmids (16), based on much smaller numbers of cases.

The plasmid pDM2 and its parent RSF0885 are taken up poorly by competent *H. influenzae*, as judged by radioactive label experiments and by competition with transforming DNA (16), leading to the assumption that RSF0885 and pDM2 lack specific DNA uptake sites (11), and thus pDM2s must also. We define uptake sites here to mean any local configuration of the DNA that can bind specifically to the cell, either the 11-base sequence (6) or the so far undefined regions that also show specific binding in the absence of the 11-base sequence (8). In view of the poor uptake of pDM2 and RSF0885, it is reasonable that both plasmids transformed very poorly (Fig. 2).

Transformation of the BC200 wild-type strain to chloramphenicol resistance by pMB1, pMB2, and pMB3 increased exponentially as a function of insert size, indicating that plasmid establishment took place more readily with larger inserts. This increase was about 2 orders of magnitude higher for pMB3 than for pMB1. We postulated previously (21) that the much higher transformation of Rec$^+$ cells to ampicillin resistance by pNov1 compared with that of the parent plasmid was the result not only of uptake sites, but also of stabilization of the plasmid by pairing of the plasmid with the chromosome at homologous sites. However, the similar transformation of Rec$^-$ strains was orders of magnitude lower (21). A comparable phenomenon was seen in the present results (Fig. 2). The effect of the insert in pMB1 on *rec-2* transformation was somewhat smaller than the corresponding effect on the wild type. Nevertheless, the insert caused an increase in *rec-2* transformation over that of pDM2 of more than 2 orders of magnitude. We presume that this increase is entirely the result of the presence of uptake sites in pMB1. With the large inserts, transformation of *rec-2* decreased, indicating that the increasing size of plasmids was disadvantageous for plasmid establishment in Rec$^-$ cells. Similar results were obtained with *rec-1* recipients (data not shown).

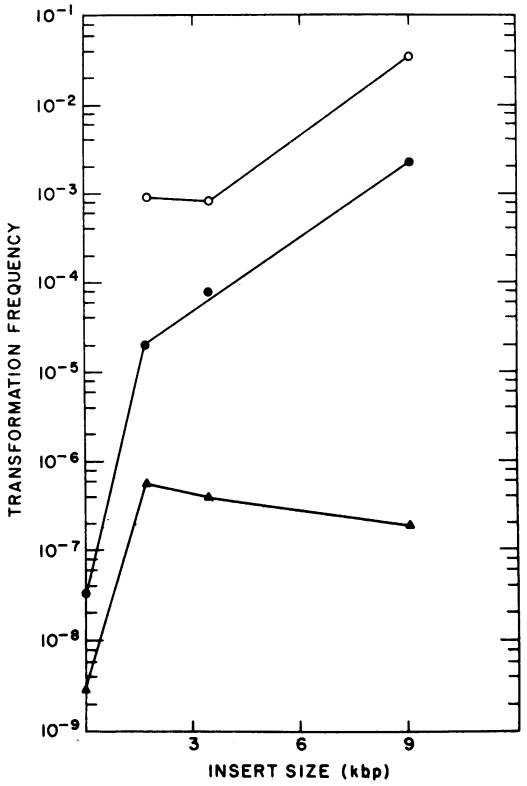

FIG. 2. Transformation of BC200 (○, ●) and *rec-2* (▲) by pDM2 and its derivatives pDM2s, pMB1, pMB2, and pMB3, to novobiocin resistance (○) and chloramphenicol resistance (●, ▲). BC200 transformation to chloramphenicol resistance was the same for pDM2 and pDM2s. The concentration of the plasmid DNA with the competent cells was 0.3 μg/ml.

Transformation to novobiocin resistance could only be measured in the Rec$^+$ cells. The dependence of this transformation on insert size followed a slightly different pattern from that of transformation to chloramphenicol resistance in Rec$^+$ cells (Fig. 2). The plasmids pMB1 and pMB2 gave rise to approximately the same number of novobiocin-resistant transformants, although there were substantially more chloramphenicol transformants by pMB2 than by pMB1. The orientation of the 1.7-kbp chromosomal inserts in the two plasmids was the same, as seen by restriction analysis (data

TABLE 1. Recombination between plasmids and chromosomes in transformation

Plasmid	No. of plasmid-containing clones tested	% Recombination[a]
pMB1	20	15
pMB2	20	5
pMB3	84	38
pMB4	83	71

[a] The percentage of novobiocin-sensitive, plasmid-containing clones (for pMB1 and pMB2 this was the percentage of clones not containing DNA capable of transformation to novobiocin resistance).

not shown). The fact that transformation to novobiocin resistance was independent of whether there were one (pMB1) or two (pMB2) copies, whereas the plasmid marker transformation was greater by about a factor of 4 when there were two copies, suggests that the pairing leading to insertion of homologous plasmid DNA into the chromosome (resulting in transformation to novobiocin resistance) is different from the pairing that helps to establish the plasmids. The tandem repeat itself showed considerable instability. Analysis of plasmids from pMB2 transformants of wild-type cells selected for chloramphenicol resistance revealed that the majority (16 of 20) contained plasmids the same size as pMB1.

Chromosome-to-plasmid gene transfer resulting from transformation. After transformation by pMB3 and pMB4, chloramphenicol-resistant (pMB3) or ampicillin-resistant (pMB4) colonies were picked, grown in the respective antibiotic and individually spotted on chloramphenicol or ampicillin plates and plates containing novobiocin as well as chloramphenicol or ampicillin, to measure the transfer of novobiocin sensitivity from the chromosome to the plasmid (Table 1). The recombination of the chromosomal insert in pMB3 is only about half that in pMB4, presumably because of the different positions of the 9.0-kbp insert in pDM2. Since similar measurements could not be done with pMB1 and pMB2, because the inserted portions did not express the novobiocin resistance marker, a different approach had to be used. Chloramphenicol-resistant transformants of BC200 by pMB1 and pMB2 were picked and grown, and lysates were made as described previously (5). The presence or absence of the novobiocin resistance marker was determined by transformation with these lysates. The probability of acquisition of chromosomal genes by plasmids was considerably lower in the case of the plasmids with smaller inserts, presumably because the probability of pairing would be expected to be lower where the homologous region is shorter.

Conjugal transfer of pDM2 and its derivatives. Transconjugation of pDM2 and its derivatives was totally dependent on the presence of the strain carrying the mobilizing plasmid (Table 2). Figure 3 shows the frequencies of conjugal transfer of pDM2 and derivatives. The proper control plasmid for this experiment is pDM2s, because the other plasmids (with inserts) were also missing the 1.1-kbp fragment of the original pDM2. The frequency of transconjugation of pDM2s is lower than that of pDM2 by about a factor of 30. McNicol et al. (13) have shown a positive correlation between homology with the conjugative plasmid and transconjugation frequency with the mobilizing plasmid pHD147. Thus the missing 1.1-kbp *Eco*RI fragment of pDM2s may have some homology with the conjugative plasmid. Since the addition of a 1.7-kbp chromosomal fragment to replace the 1.1-kbp fragment of pDM2 appeared to restore the transconjugation frequency (Fig. 3), it is also possible that the decrease in this frequency in pDM2s is caused by an unfavorable close juxtaposition of two regions in the plasmid, such as the origin of transfer and the origin of replication. An insertion would then restore the favorable configuration.

Unlike the case for transformation (Fig. 2), there was some decrease in transconjugation frequency with increasing size of the chromosomal insert. Thus, the larger the insert, the smaller the probability of plasmid establishment. Also, unlike transformation, transconjugation frequency was Rec^+ independent, in accord with previous results (7). The transconjugation results for pDM4 were similar to those for pMB3 (data not shown), indicating that the different sites of insertion did not affect conjugal transfer.

Lack of recombination of plasmid and recipient chromosmes resulting from transconjugation. Gel electrophoresis of crude lysates of transconjugants always showed plasmids the same size as the plasmids in the donor cells. Although pMB1 and pMB2 did not express the novobiocin resistance marker, if plasmid to chromosome gene transfer took place, we would expect to find novobiocin-resistant transconjugants. How-

TABLE 2. Typical mating experiment[a]

Mating cells	Total no. of cells in the suspension			
	Donors	Cells containing mobilizing plasmid	Recipients	Transconjugants
rec-2(pHD147) *rec-1*(pMB3) Rd (wild type)	1.6×10^9	2.4×10^9	1.2×10^9	1.2×10^5
rec-1(pMB3) Rd	2.2×10^9		8.8×10^8	0[b]

[a] After the cells were incubated on a filter for 2 h, they were suspended in a volume of 2 ml and plated with appropriate dilutions and antibiotics. The donors, *rec-1*(pMB3), were resistant to novobiocin and chloroamphenicol; the cells containing the mobilizing plasmid, *rec-2*(pHD147), were resistant to kanamycin; and the recipients, Rd, were resistant to streptomycin. Transconjugants were resistant to streptomycin, chloramphenicol, and novobiocin.

[b] Less than 1 in 4×10^6 recipients.

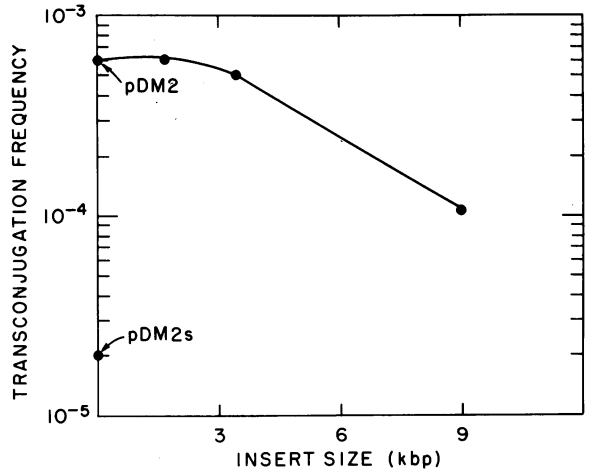

FIG. 3. Transconjugation of pDM2 and derivatives with the aid of the mobilizing plasmid pHD147 into Rd and *rec-1*. The data were averaged from two or more determinations of each type, and there was 30% or less deviation between individual determinations of the same type. There was no appreciable difference between the values for the two recipients.

ever, such was not the case (Table 3). Transformation by these two plasmids resulted in a considerable amount of transfer of the novobiocin marker to the chromosome (Fig. 2), but no such recombination could be observed after conjugal plasmid transfer.

Transconjugants of the larger plasmids (pMB4) were grown in the appropriate antibiotics and spotted individually on plates containing ampicillin and novobiocin plus ampicillin. In addition, novobiocin-resistant transconjugants of pMB3 and pMB4 were selected and tested in the same way on chloramphenicol or ampicillin. All of the plasmid-containing transconjugants retained the novobiocin resistance marker, and all of the novobiocin-resistant transconjugants retained the plasmid (Table 4). Thus, there was again no observable recombination between plasmids and chromosomes, even when the recipient cells were made highly competent.

Another way to measure plasmid-to-chromosome gene transfer is to examine the phenotype of a transformant or transconjugant after it has been cured of the plasmid. When this is done after transformation, some cured clones contain the chromosomal marker that came from the plasmid (21). The strain Rd transconjugants of pMB4 were not so easily cured as pNov1, used in the earlier study. When the Rd strain containing pNov1 is grown as dispersed single colonies on the surface of plates, around 95% of the colonies are without plasmids, because the chromosomal insert in pNov1 codes for a gyrase, which causes induction of defective phage in that strain, and thus there is selection for plasmid-free cells (22). A similar treatment of pMB4 resulted in only 17% of plasmid-free colonies (18 of 104). All 18 of the cured clones had also lost the novobiocin resistance marker. Thus, there had been no recombination between the plasmid and the chromosome. The difficulty in curing the cells of pMB4 may have resulted from a low copy number. It was previously observed (22) that a low-copy-number plasmid containing a gyrase gene caused much less selection against the plasmid. The low frequency of establishment of pMB4 in transformation (data not shown) may also have resulted from aberrant copy number control, possibly involving the *Sma*I site of the chromosomal insert.

It has been shown that there is very rare or no recombination between pNov1 that is established and the chromosome, even when the cells are made competent (21). We have also presented evidence that there is no such recombination during conjugal transfer, but recombination occurs readily in transformation. This suggests that there is a special processing of DNA when it enters the cell by transformation, possibly the formation of single-stranded ends as observed for transforming DNA (3, 18).

TABLE 3. Mating involving plasmids that do not express the novobiocin resistance marker unless there is integration of plasmid DNA into the chromosome[a]

Mating cells	No. of cells/ml of suspension			
	Donors	Recipients	Recipients containing plasmids	Novobiocin-resistant recipients
rec-2(pHD147) *rec-1*(pMB1) Rd	4.8×10^8	3×10^8	5×10^5	0[b]
rec-2(pHD147) *rec-1*(pMB2) Rd	6×10^8	5×10^8	6×10^5	0[c]

[a] The cells were mixed and incubated overnight on hemoglobin agar. Some were then scraped off, suspended, and plated, after appropriate dilution, with antibiotics to distinguish between the strains. Resistance of the strains was the same as in Table 2, except that *rec-1*(pMB1) and *rec-1*(pMB2) were resistant to chloramphenicol only.

[b] Less than 1 in 3×10^7 recipients.

[c] Less than 1 in 5×10^7 recipients.

TABLE 4. Lack of recombination between plasmids and chromosomes in transconjugants[a]

Plasmid	State of recipient	No. of plasmid-containing clones tested	% Recombination[b]	No. of novobiocin-resistant clones tested	No. of chloramphenicol- or ampicillin-sensitive clones[c]
pMB4	Noncompetent	82	0	61	0
pMB4	Competent	48	0	44	0
pMB3	Noncompetent			60	0

[a] The recipients in the matings were all wild type and originally streptomycin resistant and novobiocin sensitive. Donors and mobilizing plasmid strains were Rec$^-$ and streptomycin sensitive.

[b] Percentage of novobiocin-sensitive plasmid containing clones.

[c] Among the novobiocin-resistant clones.

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