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Transfer of Recombinant Plasmids Containing the Gene for *Dpn*II DNA Methylase into Strains of *Streptococcus pneumoniae* That Produce *Dpn*I or *Dpn*II Restriction Endonucleases

SANFORD A. LACKS* AND SYLVIA S. SPRINGHORN

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

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Plasmid transfer via the transformation pathway of *Streptococcus pneumoniae* was weakly restricted by the *Dpn*I or *Dpn*II restriction endonuclease, either of which gave a reduction only to 0.4, compared with phage infection, which was restricted to 10^{-5}. The greater sensitivity of plasmid transfer compared with chromosomal transformation, which was not at all restricted, can be attributed to partially double-stranded intermediates formed from two complementary donor fragments. However, clustering of potential restriction sites in the plasmids increased the probability of escape from restriction. The recombinant plasmid pMP10, in which the gene for the *Dpn*II DNA methylase was cloned, can be transferred to strains that contain neither restriction enzyme or that contain *Dpn*II as readily as can the vector pMP5. Introduction of pMP10 raised the level of methylase by five times the level normally present in *Dpn*II strains. Transfer of pMP10 to *Dpn*I-containing strains was infrequent, presumably owing to the suicidal methylation of DNA which rendered it susceptible to the host endonuclease. The few clones in which pMP10 was established had lost *Dpn*I. Loss of the plasmid after curing of the cell eliminated the methylase but did not restore *Dpn*I. Although this loss of *Dpn*I could result from spontaneous mutation, its relatively high frequency, 0.1% suggested that the loss was due to a regulatory shift.

Streptococcus pneumoniae is unusual in that strains isolated from nature contain one of two complementary restriction systems (3, 18). They contain either *Dpn*I, which acts on the methylated DNA sequence 5′-GmeATC-3′, or *Dpn*II, which cleaves 5′-GATC-3′, and the corresponding DNA methylase (15). Because many of these strains are otherwise genetically very similar, it has been hypothesized that they all contain the genes for both restriction systems but that a regulatory mechanism, possibly involving DNA methylation, allows expression of only one system at a time (15). However, the genetic basis of the restriction phenotypes remains to be explored.

With DNA from strains that make either *Dpn*I or *Dpn*II, it is possible to transform cells of a null mutant strain, which makes neither enzyme, into cells of the donor phenotype (18). Recently, the gene for the *Dpn*II DNA methylase was cloned in *S. pneumoniae* as a 3.7-kilobase(kb) *Bam*HI fragment inserted in both possible orientations in the vector pMP5 to give, respectively, the recombinant plasmids pMP8 and pMP10 (16). The host for the recombinant plasmids was the null strain, 762. The present work examines the transfer of one of these plasmids, pMP10, to strains that produce either *Dpn*I or *Dpn*II.

Before comparing transfer of the methylase plasmid pMP10 with its vector pMP5, it was necessary to examine the effects of restriction on plasmid transfer in general. This was done by determining such effects on the transfer of two plasmids, pMV158 and pLS70, that have no bearing on the restriction phenotype. It was found that plasmid transfer was restricted only to a limited extent. Transfer of the methylase plasmid to a *Dpn*I-containing strain, however, was drastically curtailed. Nevertheless, some of these plasmids did manage to get established, and the nature of such clones was examined.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *S. pneumoniae* and the plasmids that were used in the experiments are listed in Table 1. Their relevant features are indicated in the text and tables. Strains 733 and 736 were derived by transformation of strain 670 (18) with introduction of *malM594* in the former and of *malM567* and *end-1* in the latter case.

Media for growth and selection. Cultures were grown in a semisynthetic medium based on casein hydrolysate (9) and were supplemented with 0.2% sucrose and a 1:50 dilution of fresh yeast extract. Transformed cells were selected in pour plates containing 1% agar. Tetracycline at 1.0 μg/ml was added to select for Tc^r. Streptomycin at 0.1 mg/ml was added to select for Str^r. To select for Mal^+, maltose was substituted for sucrose, and the yeast extract was halved. Plates were incubated at 37°C for 40 h.

Plasmid preparation and analysis. Purified plasmid DNA was made by the method of Currier and Nester (5). Crude plasmid extracts were prepared as previously described (21). The presence and size of the plasmids in crude extracts were determined by agarose gel electrophoresis and staining of the gel with ethidium bromide. Methylation of DNA in these extracts was tested by determining the susceptibility of the DNA to cleavage by *Dpn*II (15).

Plasmid transfer. Plasmids were transferred by the process of DNA-mediated transformation. Cultures were grown to competence and treated with DNA as previously described (17). Except for a low frequency ($<10^{-6}$) of clones weakly resistant to tetracycline, which were present also in cultures not treated with plasmid DNA, Tc^r clones always contained plasmids.

Curing of plasmids. Elimination of plasmid pMP10 was accomplished by growing cells in the presence of ethidium bromide (4). Approximately 10^6 CFU of the plasmid-bearing strain was inoculated per ml of medium containing 0.1 μg of

TABLE 1. Strains of *S. pneumoniae* used

Strain		Plasmid	Reference[b]
Number	Name[a]		
213	TM4		9
217	TM1F		9
533	RF46SKNE		14
697	HB264end1S		18
698	HB264FS		18
707	R36NC		18
708	trt1hex4T4		20
733	HB264T4		
762	RxH4end1		18
763	RxH4end1TrI		18
776	HB264end1Y$^+$E7		
781	trt1hex4T4DpnI$^-$ cured		
804	217	pMV158	20
810	217	pLS70	20
829	193	pMP5	6
838	697	pLS70	
839	697	pMV158	
853	762	pMP10	16
855	776	pMP10	
856	708[c]	pMP10	
857	762[c]	pMP10	

[a] For plasmid-containing strains, host strain number is listed.
[b] No reference is indicated for strains derived in the present work.
[c] *Dpn*I-negative derivative.

ethidium bromide per ml. After incubation at 37°C for 8 h, the culture, which grew at half its growth rate in the absence of inhibitor, reached a density of 7×10^7 CFU/ml. The treated culture was then diluted 10-fold into medium without inhibitor and was grown for 2 h. Finally, it was plated in medium with tetracycline at 0.2 μg/ml. Control experiments showed that, with this concentration of drug, cells without the plasmid gave small colonies; those containing the plasmid gave large colonies. With the treated culture, ca. 10% of the colonies on the plates were small. Extracts prepared from these small colonies confirmed their loss of the plasmid.

Phage susceptibility tests. Phage HB-3 was grown on strains 698 and 707 and purified as previously described (18). These phage contained DNA that was methylated or unmethylated, respectively, at 5′-GATC-3′ sites. Three 1-ml samples of the culture to be tested, at 10^5 CFU/ml, were treated in parallel with no phage, unmethylated phage, and methylated phage (2×10^6 PFU per sample). After incubation at 37°C for 7 to 10 h, the turbidity of the sample cultures was checked. Control experiments showed that, for the two phage-treated samples, if the cells contained *Dpn*I, they grew only in the presence of methylated phage; if they contained *Dpn*II, they grew only in the presence of unmethylated phage; if they contained neither enzyme, they did not grow with either phage.

Methylase assay. DNA methylase activity was measured as described elsewhere (16).

RESULTS

Limited restriction effects of the *Dpn* systems on plasmid transfer. Strains of *S. pneumoniae* that contain *Dpn*I or *Dpn*II strongly restrict infection by phage grown on strains of the opposite phenotype. For phage HB-3, plaque-forming frequencies are reduced by 10^{-6} (3, 18). Conjugative transfer of the plasmid pIP501 also appears to be strongly restricted, by 10^{-4} (8). However, DNA-mediated chromosomal transformation is not restricted (10, 12, 14). Examination of DNA-mediated plasmid transfer shows that this process is only affected to a limited extent by either restriction system (Table 2). Similar observations have been made by others (1; W. Guild, personal communication).

Restriction effects were analyzed for plasmids pMV158 and pLS70, the structures of which are illustrated in Fig. 1A. Plasmid pLS70 contains the pneumococcal chromosomal *malM* gene inserted into pMV158 (20). The plasmids were grown on both a *Dpn*I-containing strain and a *Dpn*II-containing strain, and their respective abilities to be transferred to recipients of the two phenotypes were measured. Plasmid preparations from the two sources showed no significant differences in their ability to transfer the plasmid into the null strain 762 (data not shown). The frequency of plasmid establishment was determined from the number of Tc^r transformants obtained. For each combination of source and recipient strains, pLS70 gave 6 to 10 times as much plasmid establishment as pMV158. This is a result of the facilitation of plasmid transfer by the presence of chromosomal homology in pLS70 (17). The Mal$^+$ transformants obtained with pLS70 correspond in vast majority to chromosomal transformation. Although Mal$^+$ transformation reflected the competence of the particular recipient culture, it was not significantly affected by the strain of origin of the pLS70 plasmid (Table 2).

A small restriction effect was evident for plasmid transfer. This was calculated as the ratio of Tc^r transformants with plasmid grown on the strain having the opposite *Dpn* phenotype to those with plasmid grown on the strain having the same phenotype as the recipient strain. Similar results were obtained in the presence and absence of chromosomal facilitation of plasmid transfer. Each of the four cases studied gave a restriction effect of 0.4 (Table 2), which is significantly greater than the restriction of chromosomal transformation but rather small compared with phage restriction. Despite the fact that pMV158 and pLS70, respectively, contain 8 and 11 *Dpn*I or *Dpn*II restriction sites, transfer of the plasmids is subject to very limited restriction.

The above estimate of the restriction effect on plasmid transfer was made by comparing transfer frequencies of two differently modified plasmids into a single recipient. With the data in Table 2, it is also possible to compare transfers of a single plasmid preparation into recipients with different

TABLE 2. Effect of *Dpn* restriction systems on plasmid transfer and chromosomal transformation

Recipient[a]			Marker	Transformants per milliliter with plasmid grown on strain:		Restriction effect[c]
Strain no.	*Dpn* type	Donor plasmid[b]		217 (*Dpn*I)	697 (*Dpn*II)	
708	I	pMV158	Tc^r	7.4×10^3	2.7×10^3	0.36
708	I	pLS70	Tc^r	6.5×10^4	2.6×10^4	0.40
708	I	pLS70	Mal$^+$	1.6×10^6	2.2×10^6	1.4
733	II	pMV158	Tc^r	6.6×10^3	1.5×10^4	0.44
733	II	pLS70	Tc^r	4.1×10^4	1.1×10^5	0.37
733	II	pLS70	Mal$^+$	7.0×10^6	9.9×10^6	0.71

[a] Strain 708 is derived from R6; strain 733 is derived from HB264; both contain *malM594*, a single-site marker.
[b] Recombinant plasmid pLS70 contains the *malM* gene cloned into pMV158.
[c] Ratio of transformants with plasmid grown on the opposite phenotype to transformants with plasmid grown on the same phenotype as that of the recipient.

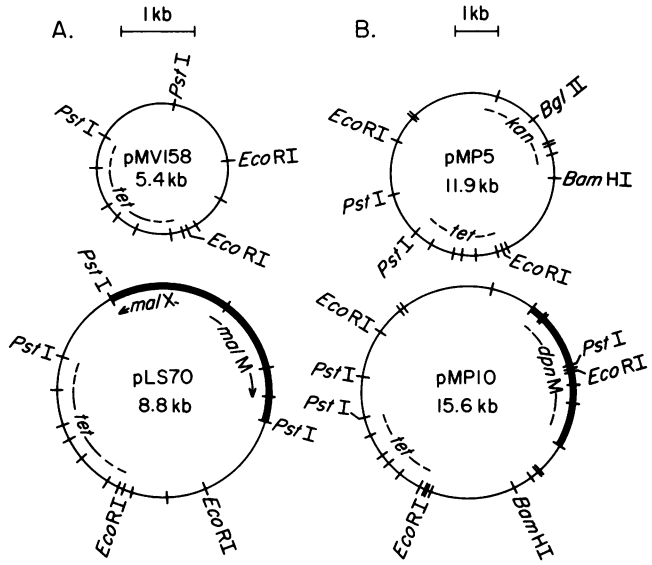

FIG. 1. Maps of plasmids. (A) pMV158 and pLS70. (B) pMP5 and pMP10. Restriction sites are indicated for *Pst*I, *Eco*RI, *Bam*HI, and *Bgl*II. Unlabeled crossmarks correspond to *Dpn*I or *Dpn*II sites. Approximate locations of genes are shown; arrows indicate direction of transcription. In pLS70, the chromosomal *Pst*I-fragment containing the amylomaltase gene was inserted into a *Pst*I site of pMV158; in pMP10, the chromosomal *Bam*HI-fragment containing the methylase gene was inserted into the *Bgl*II site of pMP5. All of the plasmids contain the large *Eco*RI segment (split in pLS70) of pMV158; they are drawn in the same orientation relative to it. The pneumococcal plasmid origin of replication is within the 1-kb *Pst*I segment. (More detailed restriction maps can be found in references 13 and 16.)

restriction enzymes. The analysis is more complex because of the need to take into account variations in competence between the recipients. From the results of the Mal^+ chromosomal transformation, which is not subject to restriction, it is evident that the competence of strain 733 for chromosomal transformation was 4.4 times greater than that of strain 708. However, the relative competence for plasmid transfer does not appear to be identical. This latter ratio can be determined by comparing the transfer of a *Dpn*II-strain-grown plasmid into the *Dpn*II-containing recipient with the transfer of the corresponding *Dpn*I strain-grown plasmid into the *Dpn*I-containing recipient. For example, with pMV158, the ratio is $1.5 \times 10^4/7.4 \times 10^3$, or 2.0. Why the competence of strain 733 for plasmid transfer is only twofold greater than that of strain 708, whereas its competence for chromosomal transformation is fourfold greater, is conjectural. However, a possible explanation is that a higher density of binding sites, at which DNA is degraded during entry (11), allows more DNA uptake but gives fragments of smaller size. The size reduction would be relatively inconsequential for a point transformation of the chromosome but would be more inimical for the establishment of a plasmid replicon. By using the calculated value to correct for the difference in competence between the two strains for plasmid transfer, it is possible to compute restriction effects. For example, with pMV158 grown in strain 217, which gave 7.4×10^3 in strain 708, the expected frequency in strain 733 would be $2.0 \times 7.4 \times 10^3$, or 1.5×10^4; but only 6.6×10^3 was obtained, so the restriction effect appears to be 0.44. Similar calculations for the other three plasmid transfers give 0.31, 0.36, and 0.47, for a mean value of 0.40, which agrees well with the value computed by the previous method.

Transfer of the methylase recombinant plasmid to strains of different restriction phenotypes. When the vector plasmid pMP5 or the recombinant plasmid pMP10 (Fig. 1B) is transferred to strain 762, which produces neither *Dpn*I nor *Dpn*II, approximately equal numbers of Tc^r transformants are obtained (Table 3). These plasmids are transferred to strain 776, which is a *Dpn*II producer, also with equal frequency. In the latter case, the fact that the pMP5 donor DNA was not methylated did not appreciably affect its ability to become established in the *Dpn*II-producing cells, which is consistent with the prior results showing limited restriction effects on plasmid transfer.

When an attempt is made to transfer the methylase plasmid pMP10 to *Dpn*I-containing strains, however, its frequency of establishment is very low compared to pMP5 (Table 3). Whether the recipient was strain 763, which originated as a transformant of strain 762 to *Dpn*I type, or strain 708, which aboriginally was of the *Dpn*I phenotype, the reduction of pMP10 transfer was the same, to a level of 0.002 relative to a non-*Dpn*I recipient. The low level of transfer is readily explained by the suicidal interaction of a plasmid that methylates cellular DNA, thereby rendering it susceptible to a host endonuclease.

In view of the suicidal interaction, it was of interest to elucidate the nature of the relatively few Tc^r transformants obtained. Twenty-one transformant colonies were grown up and examined for plasmid content. Three contained no plasmid and were presumably spontaneous Tc^r chromosomal mutants. Nine contained plasmids smaller than pMP10 that were susceptible to cleavage by *Dpn*II; they apparently resulted from deletions of the original donor plasmid that eliminated the methylase gene function. The clones carrying the deleted plasmids still produced *Dpn*I, inasmuch as three that were tested were shown to be resistant to methylated phage HB-3. Presumably, in this case deletion of all or part of the methylase gene before or during establishment of the plasmid allowed its maintenance in a *Dpn*I background. The remaining nine Tc^r clones contained plasmids the size of pMP10, and both their plasmid and chromosomal DNA were resistant to *Dpn*II.

Analysis of *Dpn*I strain transformants containing pMP10. The restriction endonuclease content of pMP10-containing clones derived from *Dpn*I recipients was examined by the phage sensitivity test. Susceptibility of all the clones tested to both methylated and unmethylated phage HB-3 indicated that the clones contain neither *Dpn*I nor *Dpn*II. Methylation of the recipient chromosomal DNA did not induce synthesis of *Dpn*II. Loss of *Dpn*I must have occurred before or during establishment of pMP10. The frequency of this loss in the population, whether by a genetic mutation or by a regulatory shift, was ca. 0.1%.

TABLE 3. Transfer of methylase recombinant and vector plasmids to strains that do or do not produce *Dpn*I

Recipient strain	*Dpn* type	Transformants per milliliter with DNA[a]			pMP10/pMP5 ratio
		Chromosomal[b] (Str^r)	pMP5[c] (Tc^r)[d]	pMP10 (Tc^r)	
762	0	9.3×10^5	3.9×10^4	5.0×10^4	1.2
763	I	8.6×10^5	1.9×10^5	2.9×10^2	0.0015
708	I	1.8×10^5	9.9×10^4	2.5×10^2	0.0025
776	II	3.6×10^3	2.4×10^3	2.5×10^3	1.0

[a] Chromosomal DNA at 500 ng/ml; plasmid DNA at 50 ng/ml.
[b] From strain 533.
[c] From strain 829.
[d] Tc^r transformants measure plasmid transfer.

TABLE 4. DNA methylase activity in strains of *S. pneumoniae* in the presence and absence of plasmid pMP10

Host strain	*Dpn* type	Plasmid	DNA methylase[a] (pmol/h per mg of protein)
762	0	None	<20
762	0	pMP10	1,150
776	II	None	220
776	II	pMP10	1,370
708	I	None	<20
708	0[b]	pMP10	1,000
708	0[b]	None[c]	<20

[a] Substrate DNA from strain 213, a *Dpn*I-producer.
[b] Lost ability to make *Dpn*I during or before transfer of pMP10.
[c] Strain cured of plasmid pMP10.

To see whether the absence of *Dpn*I was related to the presence of the methylase plasmid, we attempted to eliminate the plasmid by growth in ethidium bromide. Cells of one clone were cured in this manner, and four plasmid-free isolates were examined. They were sensitive to tetracycline, and their chromosomal DNA was now susceptible to *Dpn*II. However, they still lacked *Dpn*I, as indicated by their continued susceptibility to methylated phage. Apparently, the loss of *Dpn*I was permanent, and it did not result simply from methylation of chromosomal DNA.

Methylase content of *S. pneumoniae* strains with and without pMP10. Table 4 lists the DNA methylase activity observed in crude extracts of various strains with DNA from an R6 derivative as the substrate. Similar results (data not shown) were obtained with thymus DNA. Neither DNA is methylated at 5′-GATC-3′ sites (15). The presence of the multicopy plasmid pMP10 in strain 762 results in approximately five-times-greater specific activity of the enzyme compared with the specific activity of the enzyme in strain 776, which contains a single chromosomal copy of the gene. Introduction of pMP10 into the latter strain increased its methylase activity sixfold. Strain 708, which originally produced *Dpn*I, lost that ability and produced the methylase when pMP10 was introduced into it. Curing of the plasmid from strain 708(pMP10) eliminated its methylase activity.

DISCUSSION

Compared with the restriction of phage infection, the restriction of plasmid transfer is quite limited. It is, however, significantly greater than the restriction of chromosomal transformation, which is not detectable. The susceptibility of phage DNA can be attributed to its introduction into the cell in duplex form. Transforming DNA is not cleaved because it enters as a single strand and, on integration, gives a hemimethylated product; neither the single strand nor its product serves as a substrate for *Dpn*I or *Dpn*II (23). Plasmid DNA also enters as single strands, but, as shown by Saunders and Guild (19), complementing strand fragments from two separately entering molecules interact to allow circularization and establishment of the plasmid. Such interactions are illustrated in Fig. 2 for pMV158 and pLS70. In the regions of donor strand overlap, DNA strands would be susceptible to cleavage by restriction enzymes.

The proposed mechanism accounts for the sensitivity of plasmid transfer relative to chromosomal transformation, but why, with plasmids containing 8 to 11 potential restriction sites, is the restriction effect so limited? The explanation may be the clustering of *Dpn*I or *Dpn*II sites in these plasmids. pMV158 has a 3.1-kb stretch, over half of the plasmid, which is free of these sites, and pLS70 has two such stretches, of 3.1 and 2.1 kb, which total to over half of the plasmid length of 8.8 kb (Fig. 2). Another plasmid for which minimal *Dpn*I restriction effects were observed (1), pFB9, also has clustered *Dpn*I-sensitive sites, with 6 of the 7 sites within 2.9 kb of the 6.6-kb plasmid and two stretches of 1.7 and 2.0 kb which are free of *Dpn*I sites (2). These vacant stretches give considerable opportunity for overlapping of fragments to escape restriction. Examples of restricted and nonrestricted interaction of fragments of pMV158 and pLS70 are depicted in Fig. 2. (For the latter plasmid, the interacting fragments are taken to be 5.3 kb, which corresponds to the average segment length found for the circular, covalently closed PM2 DNA, [which is about the same size as pLS70], when it is bound to cells of *S. pneumoniae* [11].) This model fully explains the extent of restriction in a *Dpn*I host, but, on replicative completion of the circular strands in a *Dpn*II host, the new synthesis would leave duplex unmethylated sites susceptible to restriction. A possible mechanism for averting such restriction, based on a suggestion by W. Guild (personal communication), is methylation of sites in the single-stranded-DNA portion of the plasmid intermediate. Another possibility is that methylation follows but is closely coupled to the replicative synthesis.

The drastic reduction in plasmid transfer when the methylase plasmid pMP10 was introduced into *Dpn*I-containing cells was presumably not due to restriction but to destruction of the host cell. About half of the instances of successful plasmid establishment represented deletions of the methylase gene. The other half represented establishment of pMP10, but in cells that no longer produced *Dpn*I. Three possibilities for loss of this enzyme can be considered: (i) mutants in the structural gene for *Dpn*I preexist in the culture; (ii) regulatory variants in which the *Dpn*I gene was turned off preexist in the population; (iii) DNA methylation blocks *Dpn*I synthesis, but preexisting enzyme destroys most of the transformants. Arguing against the last possibility is the finding that cells cured of the methylase plasmid did

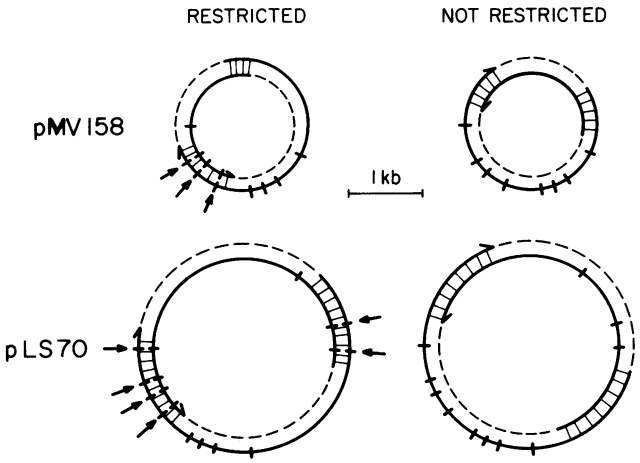

FIG. 2. Model to explain limited restriction effect on plasmid transfer. Hypothetical intermediates shown represent interaction of monomer single-stranded fragments after they have separately entered the cell. Heavy lines depict donor-derived material. Half arrows indicate strand polarity. Light hatching identifies double-stranded regions. Heavy crossmarks are recognition sites for *Dpn*I or *Dpn*II. Arrows point to sites susceptible to cleavage. Dashed lines represent subsequent synthesis to complete the plasmids. Clustering of *Dpn*I or *Dpn*II sites allows frequent escape from restriction.

not revert to *Dpn*I production. If the first possibility is correct, the frequency of such mutants (1.0×10^{-3}) is much higher than values previously found for forward mutations in Hex^- strains of *S. pneumoniae*, which range from 1.4×10^{-7} for *rif* (22) to 2.6×10^{-5} for *amiA* (7). At this juncture, the second possibility seems most attractive.

When pMP10 was transferred into strain 762, which makes none of the restriction system enzymes, the methylation of the host DNA did not turn on any latent *Dpn*II gene. So far, no regulatory role for methylation has been demonstrated in this system. There also has been no direct evidence for the presence of structurally intact, functionally repressed restriction genes. Nevertheless, the possibility remains open that regulatory effects independent of DNA methylation allow a transition from one system to another. The transition may occur through an intermediate null state, exemplified by the *Dpn*I-negative cells that allowed establishment of pMP10.

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