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Transformation of Restriction Endonuclease Phenotype in *Streptococcus pneumoniae*

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The genetic basis of the unique restriction endonuclease *Dpn*I, that cleaves only at a methylated sequence, 5′-GmeATC-3′, and of the complementary endonuclease *Dpn*II, which cleaves at the same sequence when it is not methylated, was investigated. Different strains of *Streptococcus pneumoniae* isolated from patients contained either *Dpn*I (two isolates) or *Dpn*II (six isolates). The latter strains also contained DNA methylated at the 5′-GATC-3′ sequence. A restrictable bacteriophage, HB-3, was used to characterize the various strains and to select for transformants. One laboratory strain contained neither *Dpn*I nor *Dpn*II. It was probably derived from a *Dpn*I-containing strain, and its DNA was not methylated at 5′-GATC-3′. Cells of this strain were transformed to the *Dpn*I restriction phenotype by DNA from a *Dpn*I-containing strain and to the *Dpn*II restriction phenotype by DNA from a *Dpn*II-containing strain. Neither cross-transformation, that is, transformation to one phenotype by DNA from a strain of the other phenotype, nor spontaneous conversion was observed. Extracts of transformants to the new restriction phenotype were shown to contain the corresponding endonuclease.

The restriction endonuclease *Dpn*I was first found in derivatives of a common laboratory strain of *Streptococcus pneumoniae*, R6 (8). This enzyme is unique among restriction endonucleases discovered so far in that it cleaves only at a methylated DNA sequence, 5′-GmeATC-3′ (9, 21). Another strain, 649, originally thought to be derived from a *Dpn*I-producing strain (9), contains a complementary restriction endonuclease, *Dpn*II, that cleaves at the same sequence, 5′-GATC-3′, but only when it is unmethylated (8, 9, 21). Cells of the latter phenotype also contain a DNA methylase, which methylates adenine in the 5′-GATC-3′ sequences of cellular DNA (9). In the present work a number of independently isolated strains of *S. pneumoniae* were surveyed for the presence of either the *Dpn*I or *Dpn*II phenotype.

Bernheimer found that bacteriophage restriction correlated with the *Dpn*I or *Dpn*II restriction enzyme phenotype of the phage donor and host cells (1). The present results confirm the roles of *Dpn*I and *Dpn*II in phage restriction. In this work, phage HB-3 was developed as a selective and screening agent for the restriction endonuclease phenotype. Unlike phage infection, genetic transformation is not affected by the presence or absence of methylation in the donor DNA and *Dpn*I or *Dpn*II in the recipient cells (8, 11, 12, 21). This observation was attributed to the single-strand and heteroduplex structure of the donor DNA after entry, inasmuch as such forms are not susceptible to the enzymes (9, 10).

The possibility of spontaneous conversion from one restriction phenotype to the other was suggested by the complementary nature of the restriction enzymes and the obscure origin of strain 649. A possible role for DNA methylation in determining two such phenotypic states was proposed (9). No evidence for these possibilities was obtained in the present work, but it was shown that a recipient strain could be transformed by DNA to either the *Dpn*I or *Dpn*II phenotype. These results represent the beginning of a genetic analysis of the complementary restriction system of *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and phages. The strains of *S. pneumoniae* that were used are listed in Table 1. Strain R6 was obtained from R. Hotchkiss, and strain Rx1 was obtained from W. Guild. All other strains, except for derivatives and strain 649, which were isolated here, were generously provided by H. Bernheimer, who also provided bacteriophage HB-3. Other phages or their DNA (or both) were gifts of W. Guild (ω3, PG24) and

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TABLE 1. Strains of *S. pneumoniae* used

Strain name[a]	Serial no.[b]	Synonyms	Capsule type[c]
R36NC[d]	707		2
R6[d]	529	R36A	2
R6end1	593		
R6end1exo3noz19	641		
T4FS	224		
HB661[e]	675		3
A66R2[e]	765		3
Rx1[f]	676	Rx	
RxH4	761		
RxH4end1	762		
HB264	670	8R1	8
HB264FS	698		
HB264end1S	694		
HB264end14	679		
HB649	672	SVIR3	1
I41R	685		1
IILederleR	686		2
IIIHR	688		3
VIIIR13	687		8
649	649		

[a] Derivative strains are shown indented below parent.

[b] Serial numbering of this laboratory.

[c] Refers to progenitors of strains indicated, all of which are uncapsulated (R) mutants.

[d] R36NC and R36A were derived from the same original isolate; R6 is a direct descendant of R36A.

[e] HB661 and A66R2 were both derived from the same isolate, A66.

[f] Rx1 was derived from a genetic cross between ancestral strains of R6 and HB661 (17).

R. Lopez (Dp-4, Cp-1). Markers for nuclease deficiency (*end*, *exo*, *noz*), which are indicated in the strain names, and the procedures for their introduction into cells, were previously described (7, 10). The suffix S indicates the presence of a streptomycin resistance marker.

Bacteriophage preparation and assay. Media used were previously described (6). For growth of phage HB-3, the semisynthetic medium was supplemented with sucrose at 0.2% and a 1:50 dilution of fresh yeast extract. Cultures of the host bacteria were diluted to an optical density at 650 nm (OD_{650}) of 0.04 and inoculated with 10^5 to 10^6 PFU of phage per ml. On incubation at 37°C the culture grew to an OD_{650} of ~0.5 and then lysed, generally within 3 to 5 h from the time of inoculation. If growth exceeded an OD_{650} of 0.8 the culture was diluted fourfold. Crude lysates gave 10^8 to 10^9 PFU/ml.

Phage was purified and concentrated by first centrifuging the crude lysates at 10,000 × *g* for 15 min at 0°C. Solid NaCl was dissolved in the supernatant fluid to 0.5 M. Then solid polyethylene glycol (PEG-6000; Union Carbide) was added and dissolved to give a final concentration of 10% (wt/wt). The mixture was centrifuged as before. The pellets (from a 400-ml culture) were extracted three times with 2-ml portions of 10 mM Tris-hydrochloride (pH 8)–50 mM NaCl–5 mM $MgCl_2$; the suspension was centrifuged at 12,000 × *g* for 10 min at 0°C each time. Phage concentrations in the extracts were ~10^{10} PFU/ml. Crude lysates and purified preparations were sterilized with $HCCl_3$ and stored at 4°C.

The plaque assay was carried out as follows. Basal plates contained 30 ml of medium with 1% agar. Soft agar containing 1.2 ml of medium, 0.1 ml of defibrinated sheep blood (Colorado Serum Co.), and 0.6 ml of 1.5% agar was held at 42°C. A 0.1-ml phage sample, diluted in medium, was mixed with 0.1 ml of a lawn culture grown to an OD_{650} of ~0.2, and the mixture was incubated for 15 min at 37°C. The soft agar solution was added, and the entire mixture was spread on a basal plate. After hardening, this layer was covered with 7 ml of 0.75% agar in medium. Plates were incubated at 37°C, except with Rx1 derivatives serving as the lawn, in which case incubation at 30°C gave more distinct plaques. Plaques were counted after 16 h.

Transformation procedures. Transforming DNA was prepared as previously described (4). Recipient cultures were grown to an OD_{650} of ~0.2 and diluted 1:20 into medium containing 2% (vol/vol) fresh yeast extract, 0.2% glucose, 0.2% bovine serum albumin, and 0.1 mM $CaCl_2$. After incubation at 30°C for 20 min, 1.0-ml portions were added to 2 μg of the donor DNA. Incubation was continued at 30°C for 1 h, and then the cultures were transferred to 37°C, incubated for 1 h, diluted 1:10, and incubated at 37°C for 2.5 h more. Samples were plated in appropriate media (6) for determining streptomycin-resistant transformants and total colony-forming units. The cultures were conserved for subsequent selection of phage-resistant transformants by addition of 12% (vol/vol) glycerol, holding for 10 min at 37°C, and freezing at −70°C.

Transformation of the restriction phenotype was examined with recipient cells that contained neither *Dpn*I nor *Dpn*II. To select for either restriction phenotype, a sample of the transformed culture, containing ~10^5 colony-forming units in 0.1 ml, was mixed with 20 μl of HB-3 phage, containing ~5 × 10^8 PFU, grown in a strain of phenotype alternative to the one under selection. [The phage was designated HB-3(I) when grown in cells containing *Dpn*I and HB-3(II) when grown in cells containing *Dpn*II.] Mixtures were incubated at 37°C, and samples were plated from controls without phage and selection mixtures after 0, 1.5, and 3 h for viable counts. Survival at the later times was generally between 0.4 and 2.0%.

To screen the survivors for their restriction phenotype, single colonies were picked into 3 ml of growth medium and distributed into three tubes. One tube received a drop containing ~2 × 10^6 PFU of HB-3(I); another received a similar amount of HB-3(II); the third tube served as control. After incubation at 34°C for 12 to 16 h, the tubes were examined. Control tubes were turbid. Isolates susceptible to the phage either remained clear or lysed soon after becoming turbid. Untransformed parental isolates lysed with both phage types; lysogens were lysed by neither; transformants to *Dpn*I were lysed only by HB-3(I), whereas transformants to *Dpn*II were lysed only by HB-3(II).

A more rapid screening procedure was used in some experiments. Blood agar plates were prepared from reconstituted tryptose blood agar base (Difco Laboratories) containing 0.2% (vol/vol) sucrose and 1.5% (vol/vol) defibrinated sheep blood. Samples containing ~10^7 PFU of phage HB-3(I) and HB-3(II) were

streaked with a wire loop across the upper and lower halves, respectively, of a blood agar plate. The culture to be tested was grown in liquid to an OD_{650} of ~0.2. A loopful was plated across the upper phage streak. Directly opposite, another loopful was plated across the lower streak. Nine isolates could be tested on one plate. The plates were incubated at 30°C for 20 h. The absence of bacterial growth at the intersection indicated susceptibility to the phage.

Analysis of DNA methylation. Methylation of DNA at 5'-GATC-3' sites in a strain was tested indirectly by the susceptibility of phage HB-3 grown in that strain to restriction by a *Dpn*I-containing strain. A phage lysate, initiated by infection of the culture to be tested with HB-3(I), or in the case of *Dpn*II-containing strains with HB-3(II), was subjected to plaque assays on lawns of R36NC (a *Dpn*I producer) and VIIIR13 (a *Dpn*II producer). Absence of plaques on the former indicated methylation of the DNA.

Methylation of DNA was determined directly by the susceptibility of DNA samples to purified *Dpn*I and *Dpn*II in vitro. The preparation of DNA from pneumococcal strains (9) and procedures for purifying the endonucleases (12) have been described. Reaction mixtures were composed, incubated, and subjected to gel electrophoretic analysis as previously described (9).

Restriction endonuclease assay in crude extracts. The presence of *Dpn*I or *Dpn*II could be detected in crude extracts of *S. pneumoniae* provided that the cells were deficient in the major (membrane) endonuclease. Extracts were prepared from 10 ml of culture grown to an OD_{650} of ~0.4. After centrifugation in the cold, the cell pellet was suspended in 0.2 ml of 10 mM Tris-hydrochloride (pH 7.6)–50 mM NaCl. Then 5 μl of Triton X-100 was added, and the mixture was incubated at 30°C for 15 min for cell lysis, after which the extracts were diluted with 0.8 ml of the suspending buffer and stored at −70°C.

Substrate DNA labeled with ^3H was prepared from *Escherichia coli* strain GM28 (*dam*$^+$) and GM33 (*dam-3*) as previously described (9). The specific radioactivity of [^3H]DNA from GM28 (methylated at GATC) was 4.0×10^4 dpm/μg; that from GM33 (unmethylated at GATC) was 1.3×10^4 dpm/μg. Assay tubes contained the following in a volume of 0.2 ml: crude extract (~10 μl) corresponding to 10 μg of protein, 10 mM Tris-hydrochloride (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$, 24 μg of bovine serum albumin, 40 μg of wheat germ RNA (Calbiochem), and 1.0 μg of [^3H]DNA from GM28 or 3.0 μg of [^3H]DNA from GM33. The RNA was added to inhibit residual activity of the membrane nuclease. The tubes were incubated at 37°C for 2 h. The DNA was precipitated by adding 1.0 ml of 95% ethanol and chilling at 0°C for 30 min. After centrifugation the supernatant fluid was discarded, and the precipitate was dried and dissolved in 10 μl of 10 mM Tris-hydrochloride (pH 7.6). The samples were subjected to electrophoresis in 1% agarose as previously described (9). The gels were then treated with scintillant, dried, and exposed for fluorography by the procedure of Laskey and Mills (13).

RESULTS

Restriction enzyme phenotype in strains of *S. pneumoniae*. The restriction enzyme *Dpn*I was isolated from derivatives of R6, a typical laboratory strain of *S. pneumoniae* (8). The enzyme *Dpn*II was first isolated from strain 649, which appeared in a culture inoculated with strain 641, probably as an external contaminant. Strain 649 differs from 641 not only in its restriction enzyme phenotype, having *Dpn*II and the corresponding DNA methylase instead of *Dpn*I, but also in its greater resistance to optochin and to elevated temperature, its failure to be lysed by sodium deoxycholate, its non-agglutinability by antibody to R6, and its nontransformability. In cell shape and colony morphology it is identical to R6, and its DNA transforms derivatives of R6, with respect to markers such as Mal$^+$ and Str^r, at frequencies that are ~20% of those obtained with R6 DNA. The properties of strain 649 suggest that it is a close streptococcal relative, but not a derivative of R6. This view is supported by the finding, reported here, of several strains of *S. pneumoniae* that contain *Dpn*II and the methylase, but are very similar to R6 in their other properties.

The restriction enzyme phenotype of a number of independently isolated strains of *S. pneumoniae* was examined by testing the susceptibility of their DNA to *Dpn*I and *Dpn*II. Cleavage by *Dpn*I and not *Dpn*II indicates methylation of adenine at 5'-GATC-3' sites and, hence, the *Dpn*II-DNA methylase phenotype. The converse indicates absence of methylation and the *Dpn*I phenotype. Results for seven strains, in addition to 641 and 649, are shown in Fig. 1. Six of the eight strains of *S. pneumoniae* derived from patients show the *Dpn*II phenotype. These include I41R (Fig. 1, lanes b), IILederle R (lanes c), VIIIR13 (lanes d), IIIHR (lanes e), HB264 (lanes g), and HB649 (lanes h). These results demonstrate only the methylase; however, the *Dpn*II enzyme was observed in a derivative of HB264 and shown to have the same molecular size as the enzyme from strain 649 (12). The only new strain to exhibit the *Dpn*I phenotype of R6 derivatives (Fig. 1, lanes f) was HB661 (lanes a).

TABLE 2. Restriction of phage HB-3

Host strain[a]	*Dpn* type	Phage titer[b]		Cross-restriction[c]
		HB-3(I)	HB-3(II)	
R36NC	I	2.5×10^7	7×10^1	1×10^{-6}
VIIIR13	II	1.2×10^2	8.1×10^7	5×10^{-6}
Rx1	0	1.6×10^7	5.0×10^7	−[d]

[a] Strain used for the lawn in the plaque assay.

[b] Plaques obtained per milliliter of phage preparation. HB-3(I) was produced by strain R36NC; HB-3(II) was produced by strain VIIIR13.

[c] Values give frequency of escape from restriction on host indicated for phage of the opposite type.

[d] −, Small apparent reduction in phage titer was not considered indicative of restriction.

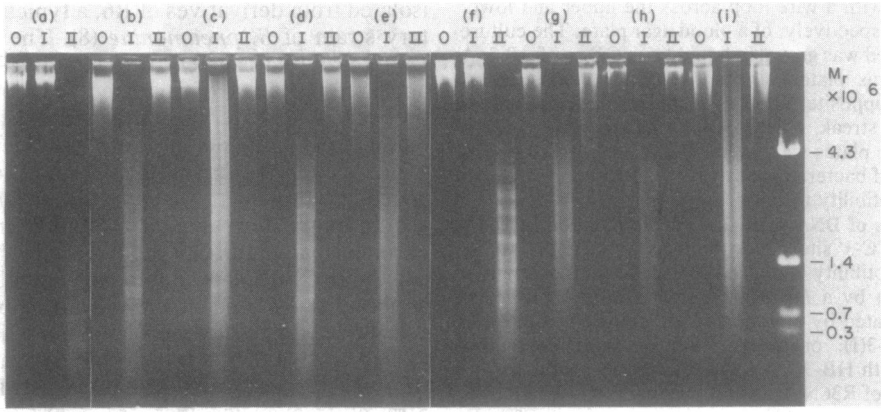

FIG. 1. Methylation of 5'-GATC-3' in the DNA of various strains of *S. pneumoniae*. Samples of DNA from the following strains were treated with methylation-specific restriction enzymes and analyzed by agarose gel electrophoresis: (a) HB661, (b) I41R, (c) IILederleR, (d) VIIIR13, (e) IIIHR, (f) R6end1exo3noz19, (g) HB264, (h) HB 649, (i) 649. 0, Untreated; I, treated with *Dpn*I (specific for methylated sequence); II, treated with *Dpn*II (specific for unmethylated sequence). Fluorescence of gels was photographed after staining with ethidium bromide and exposure to UV light. Molecular weights are shown for *Dpn*I fragments of the coliphage f1 replicative form.

The phage restriction pattern of HB264 and HB649 has been shown to indicate the presence of *Dpn*II, and that of HB661 has been shown to indicate *Dpn*I (1).

The restriction enzyme phenotype showed no correlation with the capsular type from which the rough strains tested were derived (Table 1). Thus, the *Dpn*I-containing strains originated from capsular types 2 (R6 or R36NC, which came from the same isolate) and 3 (HB661), both of which, in addition to 1 and 8, were associated with *Dpn*II strains. The presence of one or the other restriction phenotype also could not be correlated with the presence of a plasmid. Intensive search for a plasmid in strains 641 (*Dpn*I) and 649 (*Dpn*II) revealed none. The presence of a cryptic plasmid in R36NC (19) and its absence in R6 was apparently unrelated to the *Dpn*I phenotype of these strains.

Selection of restriction phenotype with phage HB-3. An early attempt to use *S. pneumoniae* phage ω3 (20) to select for the restriction phenotype was unsuccessful, since this phage infected both *Dpn*I and *Dpn*II type strains, apparently because its DNA was resistant to both enzymes (9). Similarly, DNAs from phages PG24 (16) and Dp-4 and Cp-1 (14) were also resistant to both enzymes (unpublished data). The lack of susceptibility of these phage DNAs to either enzyme presumably resulted from their content of abnormal bases (14, 16).

A temperate phage of *S. pneumoniae*, HB-3, which was isolated by Bernheimer (1), proved useful for selection of the *Dpn*I or *Dpn*II phenotype. Bernheimer showed that when this phage was grown on R36A it gave no detectable titer on 8R1, and, conversely, when grown on 8R1 it gave no plaques on R36A (1). Such restriction of HB-3 grown in a *Dpn*I strain [designated HB-3(I)] by a *Dpn*II-containing strain is demonstrated in Table 2. In this experiment the escape from restriction was measurable, but only of the order of 10^{-6} in either direction. The powerful cross-restriction was used selectively to enrich cultures containing potential transformants to a particular restriction phenotype. The limiting factor in this selection, aside from phage adsorption, was lysogenization of the population. The frequency of lysogenization varied between 10^{-3} and 10^{-2} (see Table 3). After such selective enrichment, the phage system was used, also, to test individual isolates for restriction phenotype by assay in liquid medium, screening on plates, or formation of plaques.

Restriction of HB-3 by the *Dpn*I and *Dpn*II systems is illustrated in Fig. 2, which shows a plate screening of several strains. Phage HB-3(I) was streaked, left to right, across the upper part of the plate (Fig. 2A), and HB-3(II) was streaked across the lower part (Fig. 2B). Cultures of various strains were then streaked across the phage. As indicated by a clear region at the junction, the *Dpn*I-containing strain R36NC (Fig.2, streak 1) was susceptible to HB-3(I), but it restricted HB-3(II). The *Dpn*II-containing HB264FS (Fig. 2, streak 7) showed the converse pattern. Strain RxH4end1 (Fig. 2, streak 4) was susceptible to both phage preparations. Transformants to either restriction phenotype behaved like their respective prototype.

Recipient strain with null restriction phenotype. It was suggested to us by M. Smith and W.

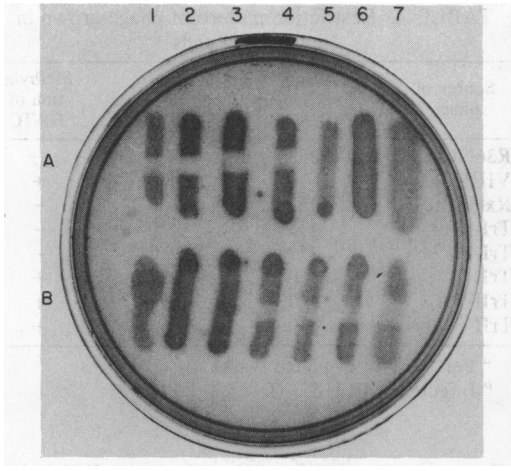

FIG. 2. Susceptibility of pneumococcal strains and transformants to phage HB-3 grown in type I or II hosts. Samples of phage were streaked across the plate: A, HB-3(I) grown on R36NC; B, HB-3(II) grown on HB264FS. Samples of culture were cross-streaked as follows: 1, R36NC; 2, TrI-18; 3, TrI-19; 4, RxH4end1; 5, TrII-28; 6, TrII-68; 7, HB264FS. Strains TrI-18 and TrI-19 were type I transformants; TrII-28 and TrII-68 were type II transformants of RxH4end1. The plate, which contained blood agar medium, was incubated at 30°C for 20 h.

Guild that strain Rx1 may not contain either *Dpn*I or *Dpn*II. This was in fact demonstrated by the phage susceptibility of Rx1 and its derivatives (Fig. 2, Table 2). These strains were unable to restrict phage of either type. The absence of *Dpn*I or *Dpn*II activity in extracts is documented below.

The DNA methylase of the II phenotype is not present in Rx1. This is evident from the absence of modification of phage grown in an Rx1 derivative that would allow it to grow in a *Dpn*II-containing strain (see Table 4). The absence of 5'-GATC-3' methylation suggests that Rx1 originated from a strain of phenotype I, with loss of *Dpn*I by mutation. This is consistent with the history of the strain (17), which was derived by transformation of R36A with DNA from the progenitor of HB661, both of which are *Dpn*I-containing strains. That Rx1 may have undergone considerable mutation in its history is supported, also, by its apparent loss of the *hex* function for mismatch repair (7).

The absence of both *Dpn*I and *Dpn*II made Rx1 a convenient recipient to test for the transformation of the restriction phenotype. To increase its utility the H4 mutation (*malM558*) and *end-1*, which gives a partial deficiency of the major endonuclease (10), were introduced. The latter mutation does not interfere appreciably with transformation, but it reduces the major nuclease activity sufficiently to allow detection of the restriction endonuclease in crude extracts.

DNA-mediated transformation of restriction phenotype. Cultures of RxH4 and RxH4end1 were each transformed with DNA from a type I (T4FS) and a type II (HB264end1S) strain. The results are shown in Table 3 for each stage of the selection and screening process. Frequencies of transformation of the restriction phenotype were compared with a standard streptomycin resistance marker. The frequencies of transformation to the *Dpn*I phenotype were relatively high and comparable to the Str^r frequency. An occasional *Dpn*I transformant was observed even in the

TABLE 3. Transformation of restriction enzyme phenotype

Recipient strain	Donor DNA type[a]	% Survival on selection with phage[b]		Survivors tested[c]					Transformation frequency (%)[d]		
		I	II	Total	Parental	Lysogen	Type I	Type II	I	II	Str^r
RxH4	I	1.2		38	20	17	1	0	2.9	<0.03	2.0
			4.6	20	8	3	9	0	2.1		
RxH4	II	1.5		40	29	6	0	5		0.2	1.0
			1.5	94	67	27	0	0	<0.02		
RxH4end1	I	1.2		153	48	104	1	0	0.6	<0.01	0.45
			1.3	38	25	4	9	0	0.31		
RxH4end1	II	1.4		78	44	30	0	4		0.07	0.35
		0.4[e]		42	25	15	0	2		0.02	
			1.4	39	19	20	0	0	<0.04		
			0.7[e]	39	5	34	0	0	<0.02		

[a] The sources of type I and II DNAs were strain T4FS and HB264end1S, respectively.
[b] Relative to untreated control; incubation for 90 min unless indicated.
[c] Screening in liquid medium as described in the text.
[d] For selected type, product of percent survival and proportion of type among survivors tested; for unselected type, latter proportion only.
[e] Incubation for 180 min.

absence of selection for it (Table 3). The frequencies observed are characteristic of transformation for markers corresponding to single-site mutations. This finding is compatible with the possibility that Rx1 lost *Dpn*I by mutation. Transformation to the *Dpn*II phenotype also occurred, but at a lower frequency, about one-fifth that of Str^r. Such a lower frequency would be consistent with the introduction of a nonhomologous DNA segment larger than a single gene (6, 23). Interestingly, only DNA from a *Dpn*I strain could transform the recipient to the I phenotype, and only DNA from a *Dpn*II strain could transform to the II phenotype. No transformation to the *Dpn*I phenotype by DNA from a *Dpn*II-containing strain, or vice versa, was observed. Such cross-transformation would have been detected if it were 10% of the direct transformation (Table 3).

When phenotype II was transferred, the transformed cells, in addition to displaying the phage restriction pattern diagnostic of *Dpn*II, also synthesized the DNA methylase. This can be seen by the ability to modify phage grown in the transformants so that the phage are susceptible to *Dpn*I and therefore cannot form plaques on R36NC (Table 4). Transformants that made *Dpn*I, as well as the recipient Rx1-derived strains, did not methylate 5'-GATC-3' sites.

Restriction endonuclease in transformed clones. The presence of *Dpn*I or *Dpn*II in the corresponding transformants was demonstrated directly in crude extracts. The ability to degrade either DNA methylated at 5'-GATC-3' (from wild-type *E. coli*) or unmethylated DNA (from an *E. coli dam-3* mutant) to lower-molecular-weight forms that migrated faster in gel electrophoresis served as the basis of the test (Fig. 3). In the first gel, four transformants to restriction phenotype I (Fig. 3, lanes 2 through 5) were compared with the prototype I (lanes 6) and II (lanes 1) strains. They showed only *Dpn*I activity. In the second gel, four transformants to phenotype II (lanes 9 through 12) were compared with the same I (lanes 13) and II (lanes 8) prototypes; they all gave only *Dpn*II activity. Extracts of RxH4end1 (lanes 7 and 14) showed no restriction endonuclease action on either methylated (lane a) or unmethylated (lane b) DNA.

TABLE 4. Restriction pattern of phage grown in transformed cells

Source of phage	Plaques[a] produced on:		Methylation of GATC
	R36NC	VIIIR13	
R36NC	253	0	−
VIIIR13	0	6,400	+
RxH4end1[b]	165	0	−
TrI-18	145	0	−
TrI-19	252	0	−
TrII-2	0	287	+
TrII-9	0	52	+
TrII-68	0	53	+

[a] Per 10^{-6} ml of phage lysate.
[b] Infected with HB-3(I).

DISCUSSION

Eight independent isolates of *S. pneumoniae* all showed one or the other of the complementary restriction phenotypes, with two containing *Dpn*I and six containing *Dpn*II. Only the laboratory strain Rx contained neither enzyme. This strain, which has passed through several laboratories, originated as a spontaneous rough mutant of a smooth transformant of strain R36A by DNA from strain A66, the smooth progenitor of A66R2 and HB661 (17). Both R36A and A66R2 are *Dpn*I strains. During the course of its passage, Rx may have undergone mutations in genes not relevant to the laboratory environment, such as *hex* (7) and the structural gene for *Dpn*I.

What could be the function under natural conditions of the complementary restriction system? The *Dpn*I and *Dpn*II endonucleases are able to restrict phage and, hence, to prevent cell death by viral infection. They have no effect on transforming DNA in vivo, so that the protection afforded the cell from viruses does not interfere with the normal process of genetic exchange. Natural populations of *S. pneumoniae* could contain cells of both *Dpn* phenotypes. Infection of such populations by a phage particle, originating perforce on a host of one or the other phenotype, would result in death of only cells of that phenotype. Cells of the complementary phenotype would restrict the phage and remain viable, so that any viral epidemic of the *S. pneumoniae* population would leave a surviving bacterial remnant.

What light do the present results throw on the genetic basis of the restriction phenotype? Strains of *S. pneumoniae* isolated from patients exhibit a variety of capsular types. Transformation to a new capsular type apparently requires the transfer of a large segment of DNA (2). There was no correlation of capsular type to restriction phenotype, however, so it is unlikely that the restriction enzyme genes are located in the capsular gene segment. Neither does the restriction phenotype have a plasmid basis. The frequencies of transformation of Rx to a particular restriction phenotype are informative. The relatively high frequency of transformation to the *Dpn*I phenotype is consistent with the transformation of a single-site mutation in the *Dpn*I structural gene. The relatively low frequency of

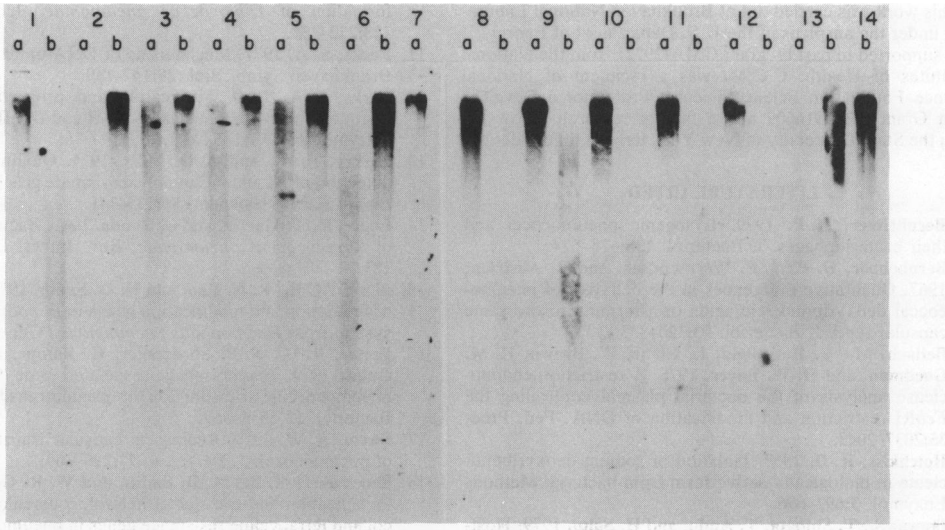

FIG. 3. Restriction endonuclease activity of transformed clones. Crude extracts of the parental strains or transformants were incubated with (a) [^{3}H]DNA from *E. coli* GM28 (methylated at GATC) or (B) [^{3}H]DNA from *E. coli* GM33 (unmethylated at GATC). DNA in the mixtures was subjected to electrophoresis in 1% agarose gels. The gels were soaked in scintillant, dried, and exposed to film for 2 weeks at −65°C. Photographs of the fluorograms are shown. 1, HB264end1S; 2, TrI-18; 3, TrI-19; 4, TrI-28; 5, TrI-32; 6, R6end1; 7, RxH4end1; 8, HB264end1S; 9, TrII-2; 10, TrII-9; 11, TrII-28; 12, TrII-68; 13, R6end1; 14, RxH4end1.

transformation to *Dpn*II is consistent with the transfer of a larger segment of DNA, which could be either a new cluster of genes or a rearranged segment of DNA. Transformation frequencies of *mal* deletion mutants were shown to be inversely correlated with the length of the DNA segment (6). Introduction of new genetic material such as capsular gene clusters (2) or chromosomal drug resistance elements (18) by transformation generally occurs at low frequency.

The absence of cross-transformation may be significant. DNA from *Dpn*I-containing strains failed to transform Rx to *Dpn*II and vice versa. This does not support the hypothesis that genes for both enzymes are always present but turned on or off by methylation of the DNA. Particularly if the absence of *Dpn*I in Rx is due to a simple mutation, it would be expected that DNA from a *Dpn*II strain (that carried the unexpressed *Dpn*I gene) could transform the *Dpn*I mutation in Rx without also introducing the *Dpn*II system. Furthermore, the absence of apparent cross-transformants showed that spontaneous conversion of restriction phenotype must be infrequent (<0.02%), if it occurs at all. It is still possible that genes for both restriction systems are present but that a DNA rearrangement, such as the inversion which controls flagellar antigen synthesis in *Salmonella typhimurium* (24), prevents cross-transformation. For example, inverted regions adjacent to the *Dpn*I or *Dpn*II structural genes could prevent homologous pairing and integration of those genes.

The introduction of a new restriction enzyme, as in the transformation of Rx to *Dpn*II production, entails the problem of modifying the DNA before the restriction endonuclease can act on it. Other cases of introduction of a gene coding for a restriction endonuclease, along with the corresponding DNA methylase, have been reported. These include introduction into *E. coli* of the plasmids coding for *Eco*RI (3), the cloning in *E. coli* of *Hha*I (15) and *Pst*I (22), and the transformation of *Bacillus subtilis* to various restriction enzyme phenotypes (5). In all of these cases the methylase is presumably synthesized first, and it modifies the DNA before the new endonuclease is made. Although such an arrangement could allow the transformation of the null strain, Rx, it would not permit the spontaneous conversion of the restriction phenotype or the transformation of a *Dpn*I- or *Dpn*II-producing strain by DNA from the complementary type. This latter transformation has not yet been tried. However, the present results indicate that the restriction phenotype of a cell can be transformed to the complementary type at least through the intermediate of a null stage.

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