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Restriction/Modification Systems of Pneumococci: Why Two Methylases in the DpnII System?

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The systems for DNA restriction and modification in Streptococcus pneumoniae are unusual in three respects. First, the DpnI restriction endonuclease, which is present in some strains of S. pneumoniae (formerly called Diplococcus pneumoniae), only acts on a methylated site in DNA (8). Typically, bacterial restriction endonucleases act on unmethylated sites in DNA, and the cells that produce them protect their own DNA by methylating the recognition site. Since DpnI attacks only methylated DNA, cells that make it do not require modification of their DNA. Second, strains of S. pneumoniae that do not harbor DpnI contain a complementary restriction system, that of DpnII (16). The DpnI and DpnII systems are mutually exclusive because DNA from a DpnI strain, which is not methylated at 5'-GATC-3' sites, is susceptible to DpnII, and DNA from a DpnII strain, which contains 5'-GmeATC-3', is susceptible to DpnI (9). Third, genetic and biochemical analysis of the DpnI and DpnII systems (4,5,10) revealed an additional, unexpected protein in each of the restriction systems. In the DpnI system an 18-kDa protein of unknown function is produced, as well as the endonuclease. In the DpnII system three proteins are produced, whereas in a typical restriction system, which like DpnII cleaves at unmethylated sites, only two proteins are generally found--the restriction endonuclease and a modification methylase (21). The function and significance of the additional protein in the DpnII system will be a major focus of this chapter.

Genetic Basis of the DpnI and DpnII Systems

The DpnI and DpnII systems are encoded by genetic cassettes alternatively located at the same position in the pneumococcal chromosome

(10). As shown in Fig. 1, the cassettes are bordered by the same genes, orfL and orfR, on either side. This cassette form of chromosomal localization assures the presence of DpnI genes only in DpnI cells and DpnII genes only in DpnII cells, an arrangement required by the mutually exclusive nature of the two systems. It also allows the exchange of one system for another by bacterial transformation, which is the natural mode of genetic exchange in S. pneumoniae, by providing homologous regions adjacent to the cassettes.

The DpnI cassette contains two genes. The first, dpnC, encodes a 30-kDa polypeptide corresponding to the DpnI endonuclease (5). The second, dpnD, produces a polypeptide of 18 kDa. When hyperexpressed in Escherichia coli, the latter product is found in a structure ten times this size. Nothing is known about the aggregation state of the protein in S. pneumoniae or what its function may be in the restriction system.

A laboratory strain of S. pneumoniae, called Rx, contains neither restriction endonuclease. This null strain apparently was derived from a DpnI strain by a single mutation in the dpnC gene. It is missing a single T:A base pair in a run of eight T:A base pairs at positions 565 to 572 (as numbered in ref. 10). This deletion terminates the DpnC polypeptide at one-third its normal length.

The DpnII cassette contains three genes (Fig. 1). The first (dpnM) and last (dpnB) genes encode the expected modification methylase (DpnM) and restriction endonuclease (DpnB or DpnII) that normally comprise such a typical Type II restriction system. However, a middle gene, dpnA, was found to encode another methylase, DpnA, which like DpnM methylates adenine at 5'-GATC-3' sequences in DNA, but which differs from DpnM in several properties (1).

Examination of the nucleotide sequences of the Dpn cassettes indicates the presence of a single promoter at the start of each cassette and a transcription terminator at each end (5,10). However, such a simple mode of transcription could not allow the regulation required to establish a DpnII system in a null cell, which was shown to occur readily (16). It is possible either that the initial transcripts are processed or that other transcripts are synthesized. Translational control mechanisms may also intervene. Although, as indicated in Fig. 1, some of the genes display normal ribosome binding sites of the Shine-Dalgarno type (18), the DpnM and DpnA methylases appear to be translated from atypical ribosome binding sites (4). These sites have been tentatively identified in the DNA as 5'-AATTTCT-(4 or 5 nucleotides)-TATA-(9 or 10 nucleotides)-ATG-3' (4,14). Their use may be related to translational control of DpnII gene expression. The slight overlapping of the open reading frames in both cassettes, which is evident in Fig. 1, may provide translational coupling in synthesis of the proteins.

Properties of DpnI and DpnII Endonucleases

Physically, the two methylases and the endonuclease of the DpnII system appear to be dimers of the corresponding polypeptides (4). Although the evidence for dimeric structure, from gel filtration and sedimentation behavior, is unequivocal for DpnA and DpnB, DpnM gave values intermediate between monomer and dimer. Conceivably, the DpnM protein is in equilibrium between the two forms. Interestingly, the symmetry shown by crystals of DpnM is indicative of a monomeric protein in the crystal structure. When sedimented in 0.5 M NaCl, the DpnI endonuclease behaved a monomer.

However, the DpnI protein was not examined under physiological salt conditions, where it may exist as dimer. The enzymatic properties of both the DpnI and DpnII endonucleases would appear to require a dimeric structure.

The enzymatic properties of Dpn system endonucleases are crucial for their apparent roles in nature. Neither enzyme can act on single-stranded DNA, whether it is methylated or not (20). When the substrate is double-stranded, both DpnI and DpnII will cleave at the palindromic 5'-GATC-3' sites only when both DNA strands are methylated or unmethylated on the N_6 of adenine, respectively (20). Hemimethylated DNA, in which one strand is methylated and the other is not, is not cleaved by either enzyme on either strand. Simultaneous recognition of two unmethylated or two methylated sites would to require a double recognition site or a dimeric enzyme.

Restriction of Viral Infection

The primary biological purpose of the Dpn systems appears to be the restriction of viral infection. Bacteriophage grown on a DpnI strain is reduced almost a million-fold in its infectivity for a DpnII cell, and the converse is also true (16). The unmethylated phage DNA in the first case, and the methylated DNA in the second case, being double-stranded when injected into the cell, is readily degraded by the DpnII and DpnI endonucleases, respectively.

The presence of complementary restriction systems in the S. pneumoniae species also supports a major role for the systems in protecting the bacteria from viruses (11). It is assumed that natural populations of pneumococci contain cells of both restriction phenotypes. Initiation of a

viral epidemic in such a population would perforce begin with a single infecting phage particle. If that phage had grown on a DpnI host, it could attack a DpnI cell, giving rise to more phage with unmethylated DNA, which would wipe out the DpnI portion of the population. However, the DpnII-containing cells in the population would restrict the phage DNA and survive. If the initial phage had grown on a DpnII host, the DpnI-containing cells would survive. Thus, the presence of complementary restriction systems serves the species by allowing a remnant of a population to survive a viral epidemic.

Effect of Restriction Systems on Bacterial Transformation

Chromosomal transformation is not affected by Dpn restriction enzymes in the recipient cell. Cells are transformed with respect to a chromosomal marker at the same frequency, whether or not the donor DNA is methylated, in both DpnI- and DpnII-containing recipients (8,12). This lack of restriction effect presumably reflects the molecular fate of DNA in transformation. In the transformation of S. pneumoniae and other gram-positive bacterial species, donor DNA is processed during its uptake by the recipient cell (7). The double-stranded donor DNA suffers single-strand breaks when it is bound to the cell surface, and one strand is degraded to oligonucleotides as the complementary strand segment enters the cell. The single-strand segment is subsequently integrated into the recipient cell chromosome. Neither the single strands that enter the cell nor the hemimethylated heteroduplex DNA that results from integration into the chromosome is susceptible to the Dpn restriction enzymes.

Because a linear single strand of a plasmid that enters a cell of S.

pneumoniae via the transformation pathway cannot by itself circularize and replicate, two complementary plasmid strands that enter separately must interact to establish a plasmid (17). Since both donor strands that reconstitute the plasmid would be methylated or not, as the case may be, greater susceptibility of plasmid transfer than of chromosomal transformation to Dpn restriction would be expected. However, plasmid establishment is only mildly restricted by the DpnI and DpnII systems, with transfer reduced to approximately 40% in the cross-transformation (12). In the case of methylated donor plasmids transferred to DpnI-containing cells, this result can be explained by the considerable new synthesis needed to repair gaps in the reconstituted structure (17). Those reconstituted plasmids in which the 5'-GATC-3'sites were located only in regions of repair synthesis would show no susceptible sites (12). However, in the case of transfer of an unmethylated plasmid to a DpnII-containing recipient, repair synthesis would create susceptible sites. Therefore, it was puzzling that the restriction effect observed on unmethylated plasmid transfer to a DpnII-containing strain was so slight.

Behavior of Mutants in the dpnA Gene

A deletion mutation within the dpnA gene, called dpnA275, was constructed in a recombinant plasmid by removal of a DraI segment, and the mutation was introduced into the chromosome of a DpnII strain (1). An isogenic pair of strains carrying the wild-type and dpnA275 mutant DpnII gene cassettes in their chromosomes was compared with respect to restriction of plasmid establishment and of chromosomal transformation (1). The plasmids used, pJS3, pMV158, and pLS70, contained 2, 8, and 11 5'-GATC-

3' sites, respectively. Comparison with results for transformation of the null restriction strain as a control afforded a measure of possible variation in quality or quantity of the donor DNA.

The results shown in Table 1 confirm earlier work with a wild-type recipient (12) in showing no restriction of chromosomal transformation and only limited restriction, approximately 50%, of plasmid establishment. Results for the dpnA275 recipient strain were markedly different (Table 1). Establishment of unmethylated plasmids was restricted to a level between 0.2% and 11% of methylated plasmid establishment inversely depending on the number of DpnII restriction sites in the plasmids. Chromosomal transformation was minimally affected in the dpnA275 mutant.

A plausible explanation for the severe restriction of plasmid establishment in dpnA mutant, but not in wild-type recipients, is that the methylation of incoming single-stranded DNA by the DpnA methylase protects the plasmid sites from later attack by the DpnII endonuclease, as illustrated in Fig. 2. In the absence of such methylation, attack by DpnII would follow conversion of the plasmid DNA to a double-stranded unmethylated form by either association with a complementary donor plasmid strand or repair synthesis of unpaired segments after partial plasmid reconstitution. Thus, the biological function of the DpnA methylase may be to allow the transfer of plasmids from DpnI strains of S. pneumoniae or from other bacterial species to DpnII strains.

Properties of DpnM and DpnA Methylases

To test the possible role of the DpnA methylase in protecting incoming plasmid DNA, the methylating activity of purified DpnM and DpnA was

compared with single-stranded and double-stranded DNA substrates unmethylated at 5'-GATC-3' sites (1). Both methylases were active on double-stranded DNA, although DpnM gave a higher specific activity. DpnA was highly active in methylating the single-stranded DNA of phage M13, whereas DpnM showed no detectable activity on M13 DNA. Thus, the protection for incoming plasmid DNA can be provided only by DpnA and not by DpnM. DpnM is presumably better suited for the maintenance of cellular DNA methylation.

In addition to acting on single-stranded DNA, DpnA differs from DpnM in its less stringent requirement for the 5'-GATC-3' target sequence, as first shown by its in vitro ability to methylate DNA already methylated at 5'-GATC-3' sites (4). By varying single-stranded oligonucleotides in a single base of the recognition sequence, it was found that recognition of ATC in the sequence was critical for methylation, but variation of the first base, G, was tolerated. The significance, if any, of this degeneracy is unknown.

The amino acid sequence of DpnA is distinct from DpnM (10); only three small boxes of similarity, common to a variety of DNA adenine methylases are evident. DpnM was found to be homologous to the Dam methylase of E. coli (14), as shown in Fig. 3. DpnA, as indicated in Fig. 4 is similar in sequence to the HinfI methylase of Haemophilus influenzae (3), which methylates adenine in 5'-GANTC-3'; 40% of the DpnA residues are identical to the 255 amino-terminal residues of the HinfI methylase (1). Despite their adjacent location in the DpnII gene cassette, dpnA and dpnM did not arise by gene duplication but rather by derivation from different ancestral prototypes of DNA-adenine methylase genes.

Structural Studies on Dpn Restriction System Enzymes

There is virtually no significant amino acid sequence similarity between any pair of the DpnI or DpnII system proteins. This is particularly interesting because all three of the DpnII system proteins recognize the same 5'-GATC-3' sequence in DNA, and the DpnI endonuclease recognizes a slight modification of that sequence. The basis for DpnI binding to its recognition site may be a zinc finger structure (5). A zinc finger motif--CPNCGNNPLNHFENNRPVADFYCNHC--is present between amino acid residues 34 and 59 in the protein. Multiple fingers with such motifs have been found in eucaryotic regulatory proteins that bind to DNA at specific sites (15,19). DpnI may be an example of how zinc fingers were used individually in ancestral bacteria for ~~protein~~ DNA recognition and binding.

Aside from a few small boxes of similar amino acid residues, which are common to DNA adenine methylases in general (4,13), the two DpnII system methylases exhibit no homology. As indicated above, they even stem from different ancestral methylase genes. The fact that both the DpnM and DpnA methylases and also the DpnII endonuclease differ in primary structure makes it more intriguing that they recognize, nevertheless, the same sequence, 5'-GATC-3'. They must achieve this recognition by different three-dimensional structures. We hope to determine these structures by x-ray diffraction. To this end we have succeeded in crystallizing the DpnM methylase (2) and the DpnII endonuclease (unpublished results). The methylase crystals exhibited very good diffraction. Their unit cell dimensions and space group, $P2_12_12_1$, indicate that the methylase is present as a monomer in the crystal (2).

Conclusion

The DpnA methylase is unusual in its ability to methylate single-stranded DNA. It appears to have evolved for this particular function inasmuch as the DpnII restriction system, of which it is a part, already contains a potent methylase for double-stranded DNA, DpnM. Its primary biological role may be to enable plasmid transmission to DpnII-containing cells via the transformation pathway of DNA entry, which introduces DNA in single-stranded form (6). Methylation of the incoming strand would protect the subsequently reconstituted plasmid from DpnII cleavage. The mechanism of chromosomal transformation itself assures its resistance to restriction (20). Thus, the systems of S. pneumoniae that allow the species to benefit from genetic exchange and plasmid transfer are largely immune to restriction, which is presumably directed at the prevention of bacterial virus infection. The fact that the dpnA gene was apparently incorporated and maintained by the DpnII restriction system solely for the purpose of allowing plasmid transfer points up the considerable importance of systems for genetic exchange in the survival of living species.

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Table 1. Effect of *dpnA* mutation on restriction of plasmid transfer[a]

Recipient DpnII genotype	Donor DNA[b]	Marker	Transformants/ml with donor DNA from DpnI strain (unmodified)	DpnII strain (modified)	Ratio I/II
null	pJS3	Cm^r	1,300	800	1.62
	pMV158	Tc^r	2,800	2,600	1.08
	pLS70	Tc^r	6,100	17,000	0.36
	chrom.	Str^r	90,000	100,000	0.90
wild	pJS3	Cm^r	570	740	0.77
	pMV158	Tc^r	4,900	10,000	0.49
	pLS70	Tc^r	59,000	160,000	0.37
	chrom.	Str^r	250,000	260,000	0.96
A275	pJS3	Cm^r	2,400	28,000	0.086
	pMV158	Tc^r	290	35,000	0.0083
	pLS70	Tc^r	230	380,000	0.0006
	chrom.	Str^r	320,000	760,000	0.42

[a] Adapted from data in ref. 1.

[b] Plasmids pJS3, pMV158, and pLS70 carry 2, 8, and 11 *DpnI/II* sites, respectively.

FIGURE LEGENDS

FIG. 1. Restriction gene cassettes of S. pneumoniae and their products. Symbols: thin bar, S. pneumoniae chromosome; thick bar, dpn cassette; open boxes, coding regions of open reading frames; thin arrows, direction of transcription from putative promoters, P, to terminators, T; closed circles, Shine-Dalgarno sites; open circles, putative atypical ribosome binding sites.

FIG. 2. Role of DpnA methylase in enabling unmethylated plasmid transfer into cells containing the DpnII restriction system. On account of the degradative processing of DNA entering the cell by the transformation pathway, plasmid establishment requires the reconstitution of a plasmid from complementary strands that separately enter the cell. In a $dpnA^-$ host, unmethylated plasmid DNA, upon reconstitution to a double-stranded form, is cleaved by the DpnII endonuclease. In a $dpnA^+$ host, the DpnA methylase methylates the single strands upon entry, so that the reconstituted plasmid is protected from the DpnII endonuclease.

FIG. 3. Comparison of DpnM methylase of S. pneumoniae and Dam methylase of E. coli. Amino acid sequences are shown from the amino termini (upper left) to the carboxyl termini (lower right). They are aligned to give maximum correspondence. Dashes indicate gaps produced by this alignment in one or the other sequence. Numbers at right correspond to positions in the individual polypeptide sequences. Dots over the Dpn sequence indicate every 10th residue. In the comparison, two dots indicate identical residues and one dot indicates amino acid residues with similar properties.

FIG. 4. Comparison of DpnA methylase of S. pneumoniae and HinfI methylase of H. influenzae. See legend to Fig. 3.

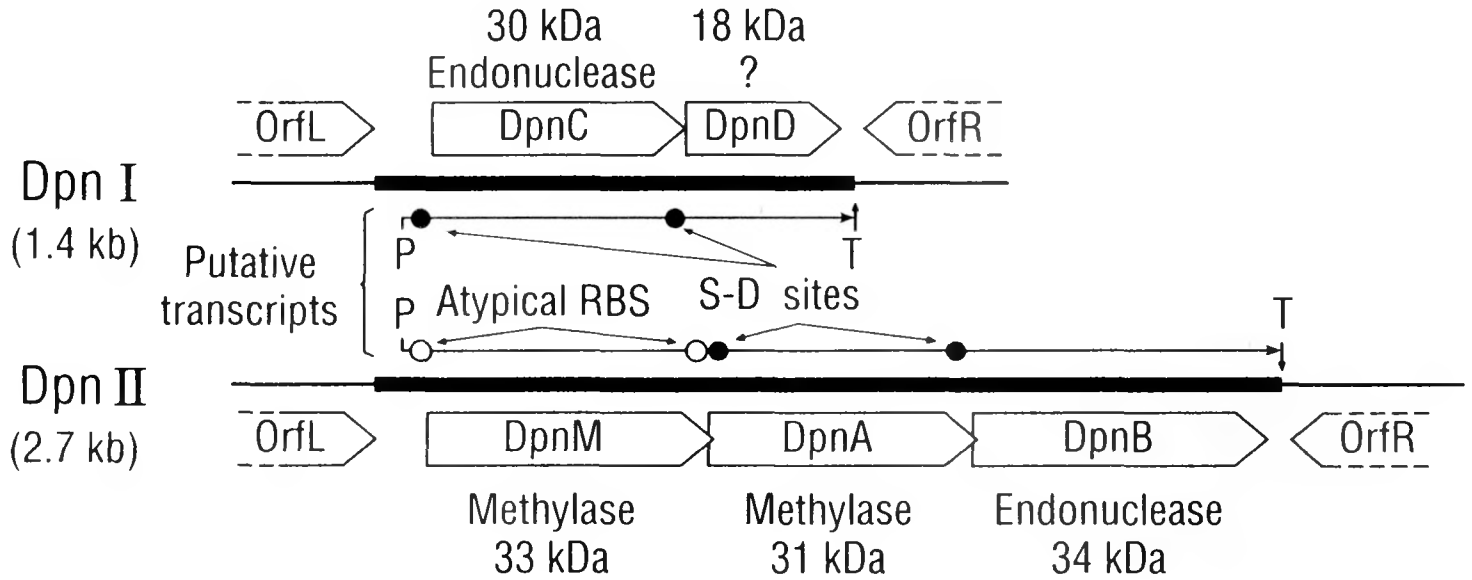
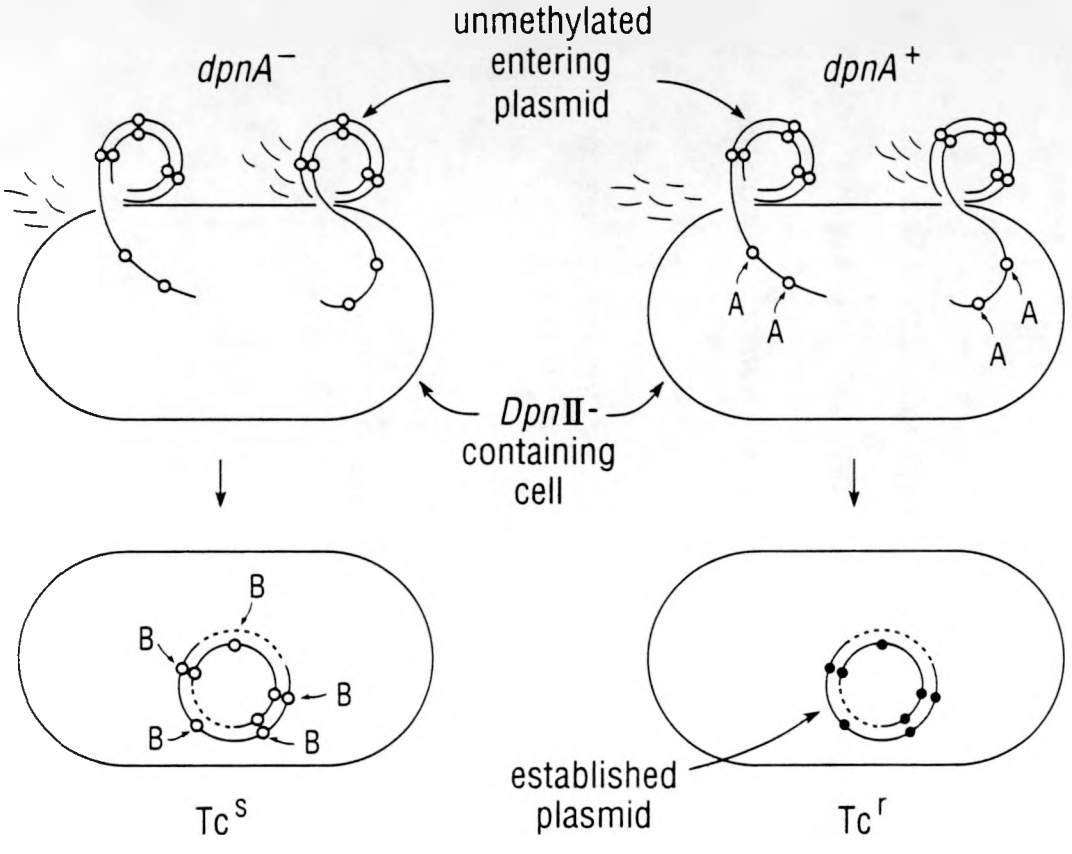

30 kDa
Endonuclease
18 kDa
?
OrfL
DpnC
DpnD
OrfR
Dpn I
(1.4 kb)
Putative
transcripts
P
T
P
Atypical RBS
S-D sites
T
Dpn II
(2.7 kb)
OrfL
DpnM
DpnA
DpnB
OrfR
Methylase
33 kDa
Methylase
31 kDa
Endonuclease
34 kDa

FIG. 1



Key: ○ = unmethylated GATC site
● = methylated GATC site
A = DpnA Methylase
B = *Dpn* II Endonuclease

FIG. 2

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DpnM: MKIKEIKKVTLQPFTKWTGGKRQLLPVIRELIPKTYNRYFEPFVGGGALFFDLAPKDAVINDFNAELINCYQQIKDNPQE  80
Dam:      MKKNRA--FLKWAGGKYPLLDDIKRHLPKG-ECLVEPFVGAGSVFLNTDFSRYILADINSDLISLYNIVKMRTDE  72

DpnM: LIEILKVHQEYNSKEYYLDLRSADRDERIDMMSEVQRAARILYMLRVNFNGLYRVNSKNQFNVPYGRYKNPKIVDEELIS 160
Dam: YVQAARELFVPETNCAEVYYQF--REEFNKSQDPFRRAVLFLYLNRYGYNGLCRYNLRGEFNVPFGRYKKPYFPEAELYH 150

DpnM: AISVYINNNQLEIKVGDFEKAIVDVRTGDFVYFDPPYIPLSETSAFTSYTHEGFSFADQVRLRDAFKRLSDTGAYVMLSN 240
Dam: FAEKAQNAFFYCESYADS-MARADDASV--VYCDPPYAPLSATANFTAYHTNSFTLEQQAHLAEIAEGLVERHIPVLISN 227

DpnM: SSSALVEELYKDFNIHYVEATRTNGAKSSSRGKISEIIVTNYEK 284
Dam: HDTMLTREWYQRAKLHVVKVRRSISSNGGTRKKVDELLALYKPGVVSPAKK 278
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FIG. 3

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DpnA: MKNNEYKYGGVLMTKPYYNKNKMILVHSDTFKFLSKMKPESMDMIFADPPYFLSNGGISNSG-GQVVSVDKGDWDKISSF  79
HinF:             MMKENINDFLNTILKGDCIEKLKTIPNESIDLIFADPPYFMQTEGKLLRTNGDEFSGVDDEWDKFNDF  68

DpnA: EEKHEFNRKWIRLAKEVLKPNGTVWISGSLHNIYSVGMALEQEGFKILNNITWQKTNPAPNLSCRYFTHSTETILWARKN 159
HinF: VEYDSFCELWLKECKRILKSTGSIWVIGSFQNIYRIGYIMQNLDFWILNDVIWNKTNPVPNFGGTRFCNAHETMLWCSKC 148

DpnA: DKKARHYYNYDLMKELNDGKQMKDVWTGSLTK-----KVEKWAGKHPTQKPEYLLERIILASTKEGDYILDPFVGSGTTG 234
HinF: -KKNKFTFNYKTMKHLNQEKQERSVWSLSLCTGKERIKDEEGKKAHSTQKPESLLYKVILSSSKPNDVVLDPFFGTGTTG 227

DpnA: VVAKRLGRRFIGIDAEKEYLKIARKRLEAENETN 268
HinF: AVAKALGRNYIGIEREQKYIDVAEKRLREIKPNPNDIELLSLEIKPPKVPMKTLIEADFLRVGQTLFDKNENAICIVTQD 307

DpnA: GNVKDNEETLSIHKMSAKYLNKTNNNGWDYFYLFRNNNFITLDSLRYEYTNQ 359
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FIG. 4