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The DpnI/DpnII pneumococcal system, defense against foreign attack without compromising genetic exchange

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Natural genetic transformation and restriction-modification (R–M) systems play potentially antagonistic roles in bacteria. R–M systems, degrading foreign DNA to protect the cell from bacteriophage, can interfere with transformation, which relies on foreign DNA to promote genetic diversity. Here we describe how the human pathogen *Streptococcus pneumoniae*, which is naturally transformable, yet possesses either of two R–M systems, DpnI or DpnII, accommodates these conflicting processes. In addition to the classic restrictase and double-stranded DNA methylase, the DpnII system possesses an unusual single-stranded (ss) DNA methylase, DpnA, which is specifically induced during competence for genetic transformation. We provide further insight into our recent discovery that DpnA, which protects transforming foreign ssDNA from restriction, is crucial for acquisition of pathogenicity islands.

Natural genetic transformation has been proposed as the bacterial equivalent of eukaryotic sexual reproduction, promoting genetic diversity.[1] Transformation involves internalization of foreign DNA in the form of single strands (ss), generated from a double-stranded (ds) substrate, which are recombined into the host genome by homology. Transformation is a widespread process[2] which contributes to genetic diversity in the human pathogen *Streptococcus pneumoniae* (the pneumococcus).[3] Transformation is thus crucial for pneumococcal vaccine escape, promoting switching of capsule loci between isolates,[4,5] where over 90 capsular types exist[6] but only ~10–15% are targeted by current conjugate vaccines. On the other hand, many bacteria possess restriction-modification (R–M) systems, which are suggested to act as analogs of the vertebrate immune system,[7] protecting the cell from attack by foreign DNA. R–M systems, which classically encode a restrictase to degrade foreign DNA and a dsDNA methylase to protect the host genome, are seen as limiting genetic diversity.[8,9] *S. pneumoniae* cells possess one of two R–M systems, DpnI or DpnII, which respectively restrict dsDNA which is me$^+$ is methylated (me$^+$) on the adenine base of the GATC sequence and unmethylated (me^0).[10] The DpnII system, encoded by the *dpnMAB* operon, besides a restrictase (*Dpn*II encoded by *dpnB*) and dsDNA methylase (DpnM), possesses an unusual methylase of ssDNA, DpnA. Re-evaluating the role of this ssDNA methylase in genetic transformation was the focus of our recent study, which led to the discovery that DpnA is crucial for acquisition of me^0 pathogenicity islands by transformation.[11] In this article, we first provide a concise review and discussion of our previous findings. We then genetically investigate the potential effect of the transformation-dedicated ssDNA-binding protein SsbB[12] on DpnA methylation of ssDNA during chromosomal or plasmid transformation. Finally, we evaluate and discuss the activity of both *Dpn*I and *Dpn*II restriction enzymes against transforming plasmid DNA.

The DpnI/DpnII Complementary System Defends Pneumococci Against Phage Attack

The DpnI and DpnII systems protect the cells from me$^+$ or me^0 dsDNA bacteriophage, respectively.[13] This complementary system is believed to be of protective value in the event of phage attack on mixed pneumococcal populations. Thus, DpnII isolates will survive attack by me^0 bacteriophage produced through infection and lysis of DpnI cells (**Fig. 1**). Conversely DpnI isolates would survive an attack by me$^+$ bacteriophage progeny from DpnII cells. As a result, a part of the mixed population will survive either of these bacteriophage attacks. The existence of this complementary R–M system is thus regarded as increasing the likelihood for species survival in phage-containing environment.

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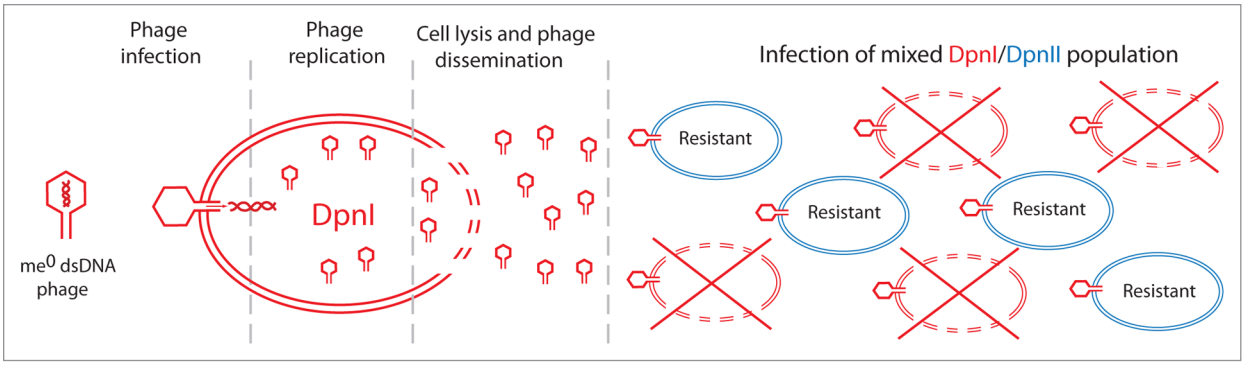

Figure 1. Advantage of the DpnI/DpnII system for survival of mixed pneumococcal population facing phage attack. Infection of a mixed DpnI/DpnII population by me^0 dsDNA bacteriophage. While a DpnII isolate infected by a me^0 phage (red hexagonal shape) survives because it is protected by restriction, infection of a DpnI isolate results in phage replication and cell lysis, releasing me^0 progeny phage (left part of figure). These progeny can infect DpnI (red) or DpnII (blue) cells in the population (right part of figure). DpnI cells will lyse (represented by large red cross) as *Dpn*I cannot restrict me^0 DNA, while DpnII cells will survive due to restriction of me^0 phage dsDNA by *Dpn*II upon infection. Conversely, infection of mixed DpnI/DpnII population by me$^+$ dsDNA bacteriophage leads to killing of DpnII cells and release of me$^+$ progeny phage, while DpnI cells survive (not shown).

The Atypical *Dpn*I Restrictase Intrinsically Provides Defense without Compromising Chromosomal Transformation

The *Dpn*I restrictase is atypical in specifically targeting methylated dsDNA, which is normally the form of DNA offering protection against restriction. As a result, the DpnI system protects the cell from me$^+$ bacteriophage attack. However, the DpnI system does not limit chromosomal transformation, as fully me$^+$ dsDNA is never created in the chromosome. Integration of homologous me$^+$ ssDNA (from DpnII donor) into a DpnI chromosome produces intrinsically resistant me$^{+/0}$ dsDNA. When a heterologous pathogenicity island, which has no homology in the host chromosome, is transferred, its integration occurs via flanking homology. The pathogenicity island DNA thus remains in ss form in the transformation heteroduplex until passage of the chromosomal replication fork, which then produces *Dpn*I-resistant me$^{+/0}$ dsDNA (**Fig. 2A**). On the other hand, integration of me^0 ssDNA (from DpnI donor) produces me^0 dsDNA which is resistant to *Dpn*I (**Fig. 2B**). Thus, DpnI pneumococcal isolates can readily interchange pathogenicity islands by transformation irrespective of the Dpn status of the donor cells.

The DpnII System is Tuned to Avoid Compromising Genetic Exchange

The *Dpn*II restrictase is orthodox in that it restricts me^0 dsDNA. R–M systems, which classically contain a restrictase of me^0 dsDNA and a dsDNA methylase, have been suggested to antagonize genetic exchange.[8,9] During the transformation of a pathogenicity island from me^0 donor DNA, the integrated pathogenicity island sequence is rendered fully me^0 after replication, with neosynthesized me^0 DNA paired with me^0 donor DNA. Once this DNA is produced in the chromosome, the restrictase and methylase compete for access to me^0 sites, with restriction degrading the chromosome and resulting in loss of the transformant cell. However, the DpnII system is unorthodox in that a third enzyme, the unusual ssDNA methylase DpnA, is also expressed.

The main role of DpnA, which is specifically induced during pneumococcal competence for genetic transformation,[14] was suggested to be promotion of plasmid transfer by transformation, as a *dpnA*$^-$ mutant showed a deficit in transformation of me^0 plasmids, but not of an me^0 chromosomal point mutation.[14,15] However, plasmids are rare in *S. pneumoniae*[16,17] and their transfer poorly efficient.[18] Furthermore, recent results clearly indicated that the competence-induced SSB paralogue SsbB, which protects transforming ssDNA and promotes chromosomal transformation, antagonizes plasmid transformation, suggesting that pneumococcal transformation has not been tuned to favor plasmid exchange.[12] These considerations made it unlikely that DpnA was recruited to the competence regulon simply to promote plasmid transfer. We suggested that DpnA should be crucial for acquisition of me^0 pathogenicity islands by DpnII isolates.[3] Logically, DpnA should not be required for transfer of me$^+$ pathogenicity islands, as the transforming DNA is already methylated, protecting the chromosomes of resulting transformants from restriction (**Fig. 2C**). For the transfer of a me^0 pathogenicity island, when DpnA is present, it methylates the transforming ssDNA, so that resulting chromosomal dsDNA is hemimethylated (me$^{+/0}$) post-replication, and thus protected from *Dpn*II (**Fig. 2C and D**). In the absence of DpnA, me^0 pathogenicity islands, which remain in ss form in the heteroduplex, cannot be methylated prior to replication (**Fig. 2D**). After replication, complementary neosynthesized DNA is produced forming fully me^0 dsDNA in the chromosome. This DNA can be degraded by *Dpn*II, destroying the potential transformant (**Fig. 2D**). We used transformation studies to validate this hypothesis, showing that acquisition of foreign me^0 plasticity islands (e.g., switch of capsule locus from DpnI isolates) was severely depleted in a *dpnA*$^-$ mutant, while no effect was observed for acquisition of isogenic me$^+$ islands.[11] By

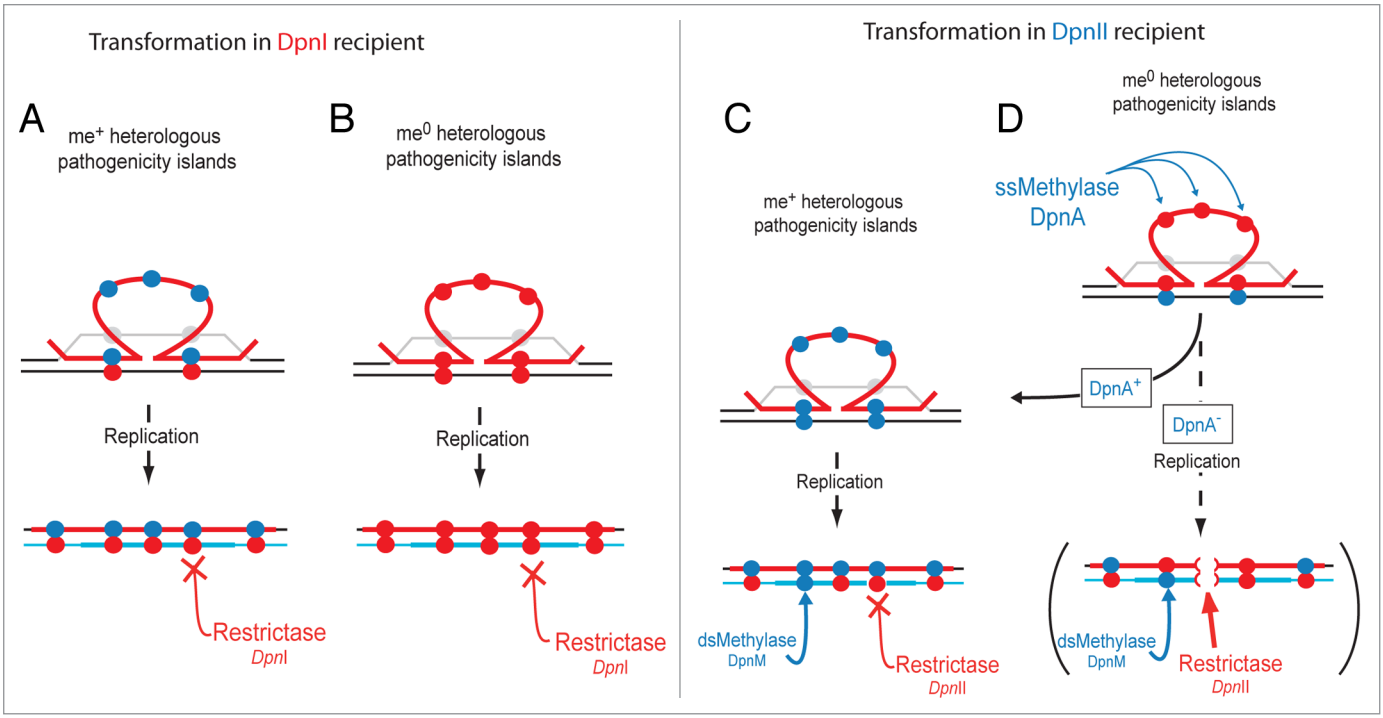

Figure 2. Differential impact of DpnI and DpnII R–M systems on transformation. (**A**) *Dpn*I does not interfere with transformation of a pathogenicity island on me^{+} (closed blue circles) DNA. Transforming me^{+} ssDNA (red line) pairing with homologous DNA on host chromosome (black line) displaces the complementary strand (gray line). The pathogenicity island sequence remains in the form of ssDNA due to lack of homology. After replication, me$^{+/0}$ sites are produced by synthesis of complementary neosynthesized DNA (light blue), which are not sensitive to restriction by the *Dpn*I restrictase. (**B**) *Dpn*I does not interfere with transformation of a pathogenicity island on me^{0} (closed red circles) DNA. DNA identity as in (**A**). (**C**) *Dpn*II does not interfere with transformation of a pathogenicity island on me^{+} DNA. DNA identity as in (**A**). After replication, dsDNA is either fully me^{+} or me$^{+/0}$ depending on the presence and action of methylases, and therefore resistant to *Dpn*II. (**D**) Transformation of a pathogenicity island on me^{0} (closed red circles) transforming DNA into a DpnII strain in the presence or absence of ssDNA methylase DpnA. DNA identity as in (**A**). In the presence of DpnA, the ssDNA of the pathogenicity island in the transformation heteroduplex is methylated, and thus protected from *Dpn*II restriction after replication, as in (**C**) (DpnA^{+} arrow). In the unnatural absence of DpnA, the ssDNA of the pathogenicity island remains me^{0} in the transformation heteroduplex. After replication (DpnA^{-} arrow and brackets), me^{0} sites in the heterologous transforming DNA are paired with me^{0} sites in the neosynthesized DNA. Resulting dsDNA me^{0} sites in the chromosome are sensitive to *Dpn*II, which can restrict the chromosome (red brackets in DNA) and kill the cell.

transforming these strains with heterologous cassettes containing varying numbers of GATC sites, we showed that increasing the number of GATC sites in the heterologous region increased the dependency on DpnA for protection. As well as being induced during competence, DpnA is also expressed constitutively from a promoter upstream of *dpnM*. By mutating the competence-induced promoter in front of *dpnA* (P_{cin}), showed that the majority of DpnA is produced during competence, and this specific induction is crucial for full protection of transforming me^{0} pathogenicity islands. We concluded that the main role of DpnA and of its induction during competence is to promote acquisition of foreign me^{0} pathogenicity islands by transformation. Extrapolating from this, DpnA should be critical to vaccine escape of DpnII isolates, which most likely occurs via exchange of capsule loci by transformation.

Our results suggest two important roles for the DpnII R–M system. First, the *Dpn*II restrictase plays an important role in protecting the cell from me^{0} bacteriophage attack, a role which is mirrored by the protection against me^{+} bacteriophage by the complementary *Dpn*I restrictase.[13] Second, the presence of the unusual ssDNA methylase DpnA maintains the plasticity potential of the bacterium by methylating foreign DNA, promoting acquisition of me^{0} pathogenicity islands by protecting transformant chromosomes from *Dpn*II. In the absence of this protection, the DpnII R–M system would limit genetic diversity, as is the case for other R–M systems,[8,9] by degrading the chromosomes of transformant clones. The genetic organization of the *dpnMAB* operon allowing co-expression of the three genes and the co-induction of only *dpnA* and *dpnB* at competence constitutes a remarkably economical and elegant set-up ensuring simultaneously increased protection against bacteriophage throughout the competence window (i.e., during a period when cells are physiologically at risk)[19] and negation of any antagonizing effect on genetic transformation. DpnA-like ssDNA methylases appear rare, although the DpnII locus is also present in the closely-related *Streptococcus mitis* species. Similar methylases are also present in other Streptococci such as *Streptococcus suis*[20] and *Streptococcus mutans*. These enzymes remain uncharacterized, although it is tempting to speculate that they may play a similar role of maintenance of genetic plasticity in these members of the diverse Streptococcal genus.[21-23]

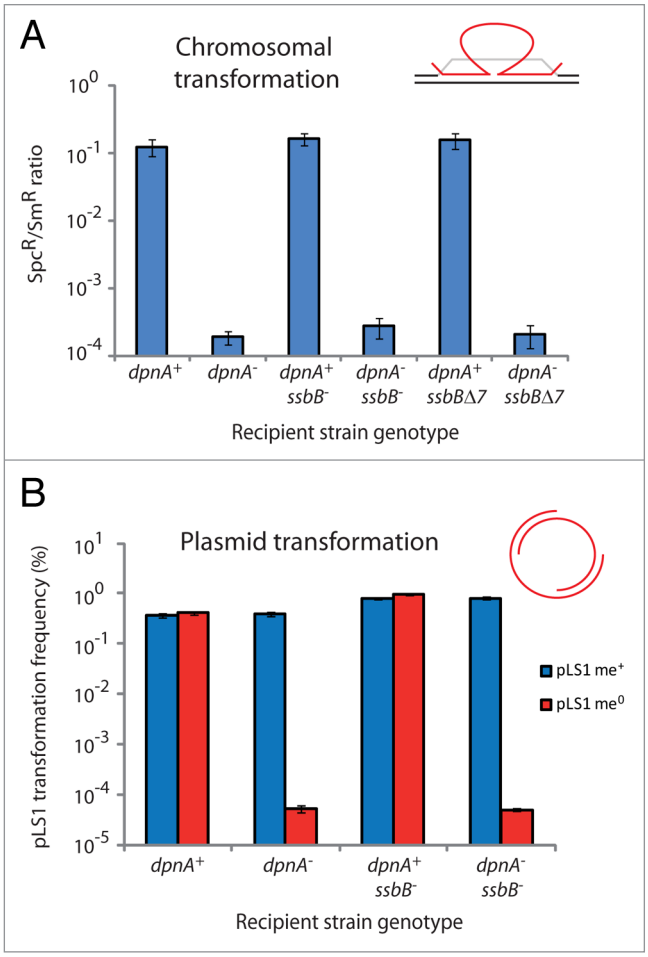

Figure 3. Alteration of SsbB does not affect methylation of chromosomal or plasmid ssDNA by DpnA. (**A**) Transformation efficiency of *ciaR*::*spc22*^{c} cassette into DpnII strains lacking *dpnA* or *ssbB*, or possessing the *ssbBΔ7* mutation. Efficiency represented as a ratio of Spc^{R} transformants to Sm^{R} transformants, selecting the *rpsL41* point mutation present on the same donor DNA (R1173), as described in reference 11. Error bars calculated from triplicate repeats. (**B**) Transformation efficiency of pLS1 plasmids (me^{+} and me^{0}) into same strains as in (**A**), represented as a percentage of Tet^{R} transformants. Red bars, pLS1 me^{0}; blue bars, pLS1 me^{+}. Error bars calculated from triplicate repeats.

Interplay between DpnA and SsbB in the Processing of Internalized ssDNA

As described, transformation proceeds through internalization and integration of ssDNA into the host chromosome via flanking homology. Directly after uptake, exogenous ssDNA is presumably coated by tetramers of the ssDNA-binding protein SsbB.[24] This paralogue of the essential house-keeping SSB, SsbA, is specifically induced during competence,[25] and creates a reservoir of transforming ssDNA, protected from degradation by endogenous nucleases.[12] SsbB-coated ssDNA was thus shown to be resistant to nuclease digestion in vitro.[26,27] SsbB could therefore compete with DpnA for access to transforming ssDNA. Alternatively, SsbB may actively recruit DpnA to transforming DNA, promoting methylation. This hypothesis is based on the observation that SsbB possesses a carboxy terminus enriched in acidic amino acids, reminiscent of the acidic tail of *Escherichia coli* and *Bacillus subtilis* SSB proteins, which is the site for specific protein–protein interactions with various partners involved in DNA metabolism and enables their recruitment to the replication fork.[28] To investigate the interplay between SsbB and DpnA, we explored the effect of the inactivation of the *ssbB* gene as well as of the deletion of the 7 acidic carboxy terminal amino acids of SsbB (*ssbBΔ7* mutant).[12] Neither the lack of SsbB nor the absence of its acidic tail altered chromosomal transformation of plasticity islands (**Fig. 3A**), suggesting that the ssDNA-binding protein neither competes with or recruits DpnA to internalized ssDNA in the reservoir.

This conclusion was further confirmed through testing of the effect of *ssbB* inactivation on replicative plasmid transformation in the presence or absence of DpnA. Essentially no difference was observed between transformation efficiency of me^{+} or me^{0} plasmids into *ssbB*$^{+}$ or *ssbB*$^{-}$ strains, whether they be *dpnA*$^{+}$ or *dpnA*$^{-}$ (**Fig. 3B**). An increase in transformation efficiency was observed in *ssbB*$^{-}$ strains, as previously observed for high plasmid concentrations.[12] However, there was no other difference in transformation efficiencies between the tested strains, suggesting that although SsbB antagonizes plasmid transformation,[12] it does not alter the ability of DpnA to methylate transforming plasmid DNA.

Where Does Methylation of Foreign DNA by DpnA Occur?

One aspect not discussed in our previous study was the location of ssDNA methylation by DpnA. This may occur at two distinct stages during genetic transformation. First, DpnA may be able to methylate me^{0} ssDNA within the SsbB-protected reservoir (**Fig. 4A**). Second, after formation of the transformation heteroduplex in the host chromosome, heterologous DNA such as a pathogenicity island remains in ss form due to lack of homology (**Fig. 2**). DpnA may access the ssDNA present in the heteroduplex and methylate it here, prior to replication (**Fig. 4B**), thus maintaining protection of resulting transformants from *Dpn*II restriction (**Fig. 2D**).

The simplest interpretation of the observation that DpnA is equally efficient in protecting plasmid and chromosomal DNA (**Fig. 3**) is that DpnA methylates internalized ssDNA in the reservoir. This would imply that DpnA has the ability to displace SsbB to methylate GATC sites, despite the previously documented effect of SsbB binding resulting in protection of ssDNA from DNase I, Neurospora endonuclease, nuclease P1, and pneumococcal

EndA nuclease.[26] If this is the case, the displacement of SsbB by DpnA does not depend on the acidic tail of SsbB, which is thought to recruit functional partners of SSB proteins (**Fig. 3A**).[12] Could there be a utility for homologous transformation in methylation of ssDNA by DpnA in the reservoir, since such DNA could be methylated by DpnM in the form of dsDNA once integrated into the heteroduplex? This could be useful during transformation of homologous DNA with high numbers of methylation sites. In this situation, were the replication fork to pass over the $me^{+/0}$ heteroduplex before DpnM had rendered every site me^{+}, me^{0} dsDNA could be produced prompting restriction of the newly replicated transformant chromosome. Thus, any prior methylation of transforming ssDNA by DpnA would lighten the workload of DpnM after heteroduplex formation, and could thus also favor acquisition of point mutations in this situation.

However, the alternative possibility that DpnA cannot displace SsbB and therefore does not access ssDNA in the reservoir can by no means be excluded. As concerns chromosomal transformation, DpnA should then methylate ssDNA in the transformation heteroduplex, i.e., in the heterologous ssDNA loop (**Fig. 2D**). This hypothesis necessarily implies that SsbB is not covering the ssDNA loop, possibly because it is displaced during heteroduplex formation. It is of note that such a mechanism would reduce the load on DpnA activity as after heteroduplex formation, only heterologous me^{0} sites remain in the form of ssDNA, whereas if acting on ssDNA in the reservoir, DpnA should methylate me^{0} sites on all internalized ssDNA molecules. For plasmid transformation, which does not involve heteroduplex formation, DpnA should act at some stage during reconstitution of the plasmid replicon, which presumably occurs through annealing of partially overlapping complementary ssDNA strands (**Fig. 3B**).[18] This hypothesis would also imply that SsbB is displaced during the annealing reaction to provide access to DpnA for methylation of me^{0} ssDNA regions before repair/replication restores a fully ds plasmid molecule that would be sensitive to *Dpn*II.

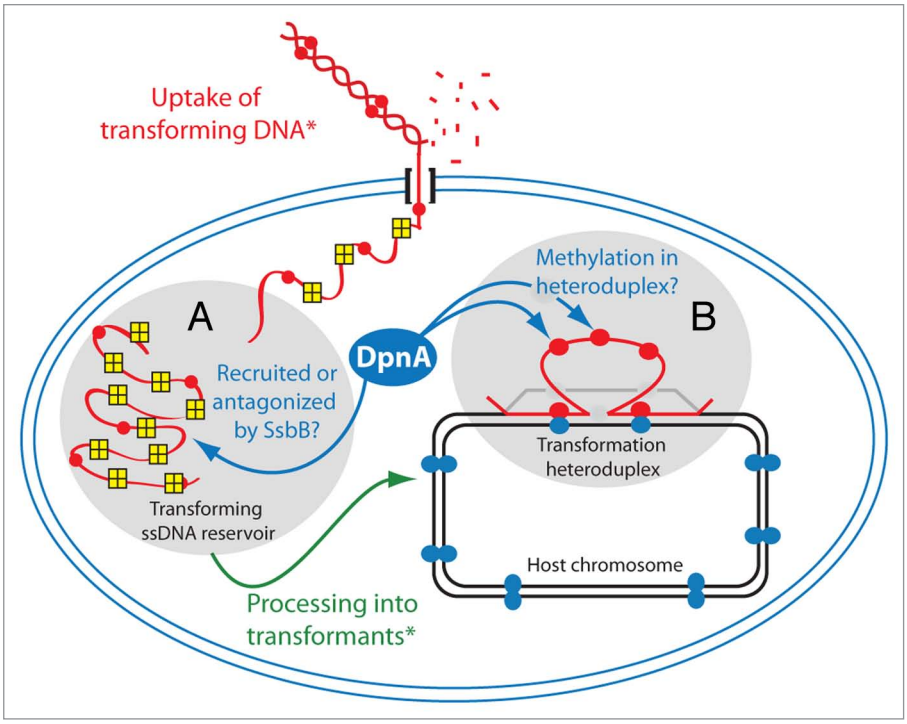

Figure 4. Potential subcellular sites of methylation of me^{0} heterologous transforming ssDNA by DpnA. Transforming me^{0} dsDNA (red line with red circles) is internalized in ss form through the DNA entry pore*, with one strand of the dsDNA substrate degraded outside of the cell (short red lines). SsbB tetramers (yellow squares) coat internalized ssDNA and protect it from degradation, producing a reservoir for transformation (gray oval **A**). Processing of ssDNA into transformants* (green arrow) involves formation of a transformation heteroduplex (as in **Fig. 2**), with the ss loop maintained in the heteroduplex until replication (see **Fig. 2**). During this process, DpnA (blue oval) could methylate transforming ssDNA in the reservoir (gray oval **A**) or in the heteroduplex of transformation (gray oval **B**). *For a review of transformation processes and proteins involved in *S. pneumoniae*, see reference [28].

Re-Evaluating Efficiency of Restriction by *Dpn*I and *Dpn*II During Plasmid Transfer

While methylation status of transforming ssDNA integrated into the chromosome is irrelevant in DpnI isolates (see above), the situation differs for transformation of me^{+} plasmids as strands of ssDNA will be internalized, with dsDNA formed by annealing of complementary molecules[18] to form a molecule composed of both ssDNA and dsDNA components, with ds me^{+} sites in the overlapping dsDNA regions (**Fig. 3B**, diagram). In an attempt to directly compare the restrictive activity of *Dpn*I and *Dpn*II on plasmids, we compared efficiency of transformation of isogenic me^{+} or me^{0} replicative plasmids into wild-type DpnI or DpnII *dpnA*-recipient cells. Since DpnI strains do not have an equivalent to DpnA to protect internalized ssDNA, comparison of transformation efficiency between DpnI and DpnII *dpnA*$^{-}$ strains gives a truer comparison of the activity of the restriction enzymes on plasmid transfer. As expected, a large loss of efficiency was observed for me^{0} plasmids in a DpnII *dpnA*$^{-}$ strain (**Fig. 5A**), mirroring results observed previously[15] and confirming that methylation of internalized ssDNA by DpnA is important for plasmid transfer. In comparison, me^{+} plasmids transformed into DpnI strains with only 10-fold less efficiency than isogenic me^{0} plasmids (**Fig. 5A**). No difference in transformation efficiency was observed in control DpnII or Dpn0 strains (results not shown). Plasmid transfer in Dpn strains was previously explored in two studies.[15,29] We observe very similar results to those published for the DpnII *dpnA*$^{-}$ strain. However, we first show a greater loss of me^{+} plasmid transfer in DpnI strains (~10-fold compared with ~3-fold). This could be due to

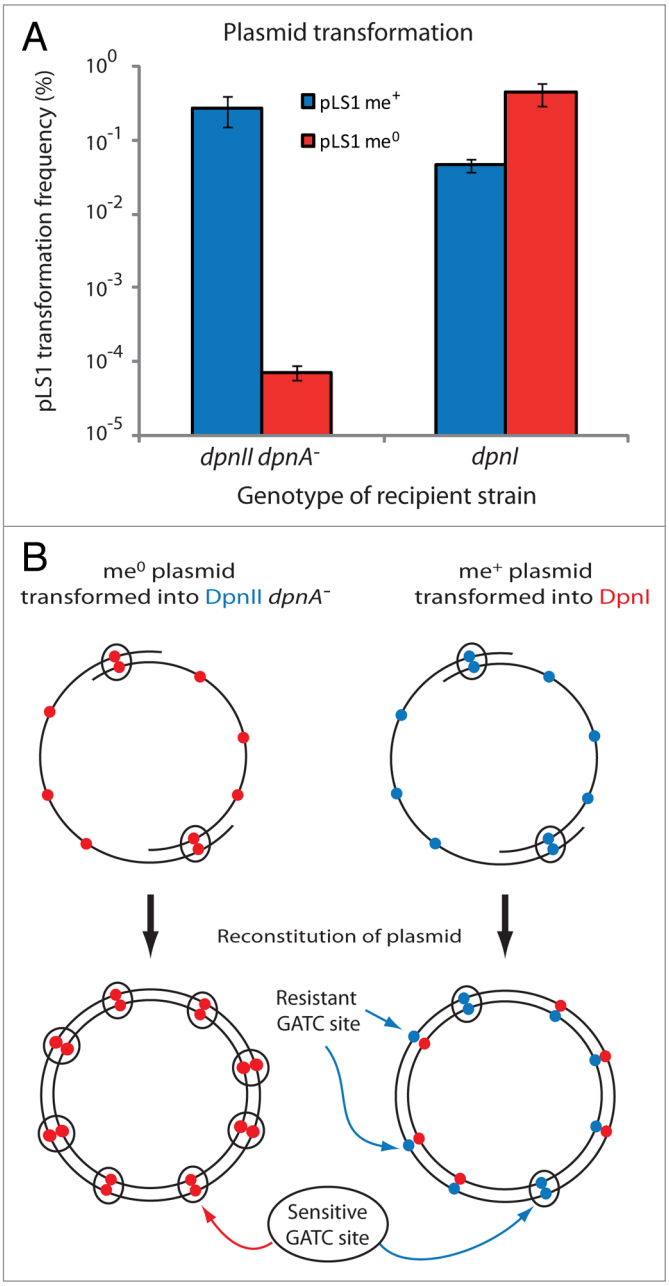

Figure 5. Comparing restrictive activity of *Dpn*I and *Dpn*II on pLS1 plasmid transformation. (**A**) Transformation of pLS1 plasmid (me$^+$ and me^0) into DpnII *dpnA*$^-$ and DpnI strains, represented as a percentage of Tet^R transformants. Red bars, pLS1 me^0; blue bars, pLS1 me$^+$. Error bars calculated from triplicate repeats. (**B**) Schematic representation of reconstitution of me^0 and me$^+$ plasmid replicons during transformation, showing regions of plasmid susceptible to *Dpn* restriction enzyme during and after reconstitution. Closed red circles, me^0 GATC sites; closed blue circles, me$^+$ GATC sites. Single circle, ssDNA; circle pairs, dsDNA. GATC sites sensitive to restriction are enclosed in black circles in each case.

the number and position of GATC sites within the plasmids used. Second, while we observed no difference in efficiency of plasmid transfer in DpnII strains, authors observed losses of me$^+$ plasmid efficiency ranging from ~3-fold ~25-fold in different experiments. Oddly, such differences were also observed for transfer into a Dpn0 strain containing neither DpnI nor DpnII system, which should readily accept both plasmids. These differences may result from the non-isogenic nature of the donor strains used. Our study employs more precise transformation conditions, and isogenic donor and recipient strains giving more precise and reproducible ratios of transformation efficiency.

Unprotected plasmids were transformed with over 100-fold more efficiency in DpnI than DpnII *dpnA*$^-$ strains (**Fig. 5A**), suggesting that restriction of reconstituted plasmids by *Dpn*II is more efficient than by *Dpn*I. We suggest that the explanation for this difference lies in the nature of the reconstitution process of plasmid replicons. Upon annealing between two linear plasmid molecules to reconstitute the plasmid, only regions of annealing between the two ssDNA plasmid molecules will initially be dsDNA and thus sensitive to restriction (**Fig. 5B**). However, neosynthesis of complement to the ssDNA to create a fully ds plasmid molecule has differing outcomes for sensitivity to *Dpn*I or *Dpn*II (**Fig. 5B**). In the case of *Dpn*II, neosynthesis creates fully me^0 GATC sites in the plasmid and *Dpn*II can restrict any GATC site on the plasmid, in competition with the dsDNA methylase DpnM. In the case of *Dpn*I, the donor plasmid is me$^+$ while the neosynthesized complement is me^0, creating resistant me$^{+/0}$ dsDNA. As a result, me$^+$ DNA is only found at the regions of initial annealing between the two ssDNA molecules, limiting the number of target GATC sites for *Dpn*I, and likely producing a fraction of fully resistant plasmids where no GATC sites are present in this region. This could explain why *Dpn*I appears less able to restrict plasmids than *Dpn*II. On the other hand, it is possible that the *Dpn*II restrictase may simply be more efficient than *Dpn*I at restricting GATC sites in vivo. This could be due to the specific co-induction of *dpnB* (encoding *Dpn*II) with *dpnA* during competence, which may lead to *Dpn*II concentration increase in competent cells similar to that observed for DpnA.[11] Alternatively, access of restriction enzymes to reconstituted plasmid replicons may differ, with *Dpn*II readily able to access the me^0 GATC sites, and *Dpn*I less so.

DpnA and the Raison d'être of DNA Uptake in *S. pneumoniae*

There has long been debate as to the reason that bacteria actively take up

Table 1. Strains and plasmids used in this study

S. pneumoniae strain	Genotype/description	Ref.
R246	*R800 hexA*::*ermAM, dpnI*; Ery^R	36
R1173	R800 Δ*comC, rpsL1*, *ciaR*::*spc119*^{A}, *endA*::*kan6*^{C}, *dpnI*; Sm^R, Spc^R, Kan^R	11
R2888	R800 *dpnC*::Janus *(dpn0)*, *rpsL1*; Kan^R, Sm^S	11
R3087	R800 *hexA*::*ermAM*, *dpn*II; Ery^R	11
R3088	R800 *hexA*::*ermAM*, *dpn*II, *dpnA*$^-$; Ery^R	11
R3089	R800 *hexA*::*ermAM*, *ssbB*::*kan*, *dpn*II; Ery^R, Kan^R	This study
R3090	R800 *hexA*::*ermAM*, *ssbB*::*kan, dpn*II, *dpnA*$^-$; Ery^R, Kan^R	This study
R3091	R800 *hexA*::*ermAM*, *ssbB*Δ*7*, *dpn*II; Ery^R, Cm^R	This study
R3092	R800 *hexA*::*ermAM*, *ssbB*Δ*7*, *dpn*II, *dpnA*$^-$; Ery^R, Cm^R	This study
Plasmid	**Identity; isolation**	
pLS1 me^0	Multicopy pneumococcal plasmid; unmethylated plasmid purified from R246 DpnI strain; Tc^R	37
pLS1 me^+	As above; methylated plasmid purified from R3087 DpnII strain; Tc^R	This study

$^{S/R}$Sensitivity/Resistance; Cm, chloramphenicol; Ery, erythromycin; Kan, kanamycin

exogenous DNA.[19,30-32] Two credible suggestions have emerged, suggesting that internalized ssDNA is used for genome maintenance via template-directed repair, and for genetic diversity via chromosomal integration of exogenous DNA. Template-directed repair involves the repair of dsDNA breaks in the chromosome by recombination, thus requiring specifically homologous transforming DNA as a template. Conversely, promotion of genetic diversity involves acquisition of heterologous, foreign DNA sequences, although these must be flanked by homologous sequence to allow classic recombination to occur. By uncovering the role of DpnA in me^0 pathogenicity island transfer, we show that protection of foreign, heterologous DNA is a mechanism programmed by the host cell. The only reason we can see to have such a programmed mechanism of protection of foreign DNA is to promote genetic diversity, as foreign, heterologous DNA should be of no use for genome maintenance. Our results thus provide the first concrete evidence that *S. pneumoniae* takes up DNA with the specific goal of promoting genetic diversity.

How do R–M Systems Antagonize Transformation in Other Species?

Although a number of studies had shown previously that R–M systems were capable of antagonizing genetic transformation,[8,9] the mechanisms involved had remained unclear. The enigma was that transforming DNA is in the form of ss, while restriction enzymes tend to act exclusively on dsDNA. As a result, authors were unable to provide a concrete hypothesis as to the mechanisms involved, suggesting that restriction may occur after integration into $me^{+/0}$ DNA transiently produced immediately downstream of the replication fork[8] or even extracellularly prior to conversion of dsDNA template to transforming ssDNA.[33] The first of these hypotheses appears far-fetched, as to prevent the acquisition of heterologous cassettes in this manner, a transformation heteroduplex would need to form, and since the heterologous region lacks complement in the genome and thus remains ss, the homologous flanking regions would need to be restricted. As admitted by the authors, the large deficit in transformation observed would necessitate that almost every heteroduplex formed downstream of the replication fork and involved only the transiently me^0 newly replicated strand, which seems highly unlikely.[8] The second hypothesis is also unlikely, since nothing exists to suggest that restriction enzymes are present extracellularly. Recently, the situation has been further complicated by model figures in large audience reviews showing restriction enzymes attacking transforming DNA immediately after internalization, when it is in ss form and thus resistant to most restriction enzymes.[33,34] The models elaborated in our study provide a simple explanation for the observed antagonization, suggesting that the restrictases do not act on transforming ssDNA per se, but rather on the post-replicative transformant chromosomes themselves, where fully me^0 methylation sites will be present (in the absence of a DpnA analog, **Fig. 2B**). Our results are fully consistent with these models, and thus solve a long-standing conundrum in the field.

Materials and Methods

Bacterial strains, plasmids, growth and transformation conditions

S. pneumoniae strain growth and transformation were performed as described.[35] Strain and plasmid information can be found in **Table 1**. Recipient strains were rendered *hex*$^-$ by insertion of the *hexA*::*ermAM* cassette as described,[36] negating any effect of the mismatch repair system on transformation efficiencies.[38] Antibiotics were used at the following concentrations; Spectinomycin (Spc) 200 μg ml^{-1}, Streptomycin (Sm) 200 μg ml^{-1}, Tetracycline (Tc) 1.5 μg ml^{-1}.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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