Biochemical Properties and Roles of DprA Protein in Bacterial Natural Transformation, Virulence, and Pilin Variation

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ABSTRACT Natural transformation enables bacteria to acquire DNA from the environment and contributes to genetic diversity, DNA repair, and nutritional requirements. DNA processing protein A (DprA) receives incoming single-stranded DNA and assists RecA loading for homology-directed natural chromosomal transformation and DNA strand annealing during plasmid transformation. The *dprA* gene occurs in the genomes of all known bacteria, irrespective of their natural transformation status. The DprA protein has been characterized by its molecular, cellular, biochemical, and biophysical properties in several bacteria. This review summarizes different aspects of DprA biology, collectively describing its biochemical properties, molecular interaction with DNA, and function interaction with bacterial RecA during natural transformation. Furthermore, the roles of DprA in natural transformation, bacterial virulence, and pilin variation are discussed.

KEYWORDS DprA/SmfA, natural transformation, competence, extracellular DNA, DNA uptake sequence, RecA-DprA interaction, genetic competence

Natural transformation (NT) involves the uptake of extracellular DNA (eDNA) from the environment and its integration into the bacterial genome (1, 2). eDNA constitutes a large gene pool outside the cell, either liberated from dead organisms or secreted by living cells through type IV and type II secretion systems (T2SSs) (1, 2). More than 80 bacterial species determined to have natural transformation potential express a conserved macromolecular protein complex and host-specific factors for the uptake and integration of eDNA into their genome (3–5) (Fig. 1). Bacterial species such as *Deinococcus* and *Neisseria* remain naturally competent during the growth phase (6, 7). However, in *Bacillus subtilis* and *Streptococcus pneumoniae*, the natural transformation system is not constitutively active; instead, its competence development, synthesis, and assembly of a macromolecular protein complex are transient and inducible, with very tight genetic regulation (8–10). The uptake of eDNA by the NT system is non-selective to the origin of eDNA, with some exceptions. For example, in *Neisseria gonorrhoeae*, uptake is restricted to its own DNA or an eDNA with a 10-nucleotide (nt) long (5′-GCCGTCTGAA-3′) DNA uptake sequence (DUS) (11). Similarly, *Haemophilus influenzae* can take up eDNA with a 9-nt long DNA sequence (AAGTGCGGT) with a 4-nt conserved GCGG motif in its core region (12).

The primary mechanism of natural transformation begins with the binding of a double-stranded eDNA to a long filament called type IV pilus (Tfp) or a transformation-specific pilus/pseudopilus through its nonspecific DNA binding activity (Fig. 1). The existence of Tfp has been reported in both Gram-negative and Gram-positive bacteria, including diverse phyla such as Deinococcus-Thermus (*Thermus thermophilus, Deinococcus geothermalis*), Firmicutes (*Ruminococcus albus, Clostridium perfringens*), and Cyanobacteria (*Nostoc punctiforme, Microcystis aeruginosa, Synechocystis* spp.) (13). The Tfp/pseudopilus uses the mechanical power to pull bound DNA through the outer membrane/peptidoglycan layer and delivers it to conserved translocase machinery located at the cytoplasmic membrane (2)
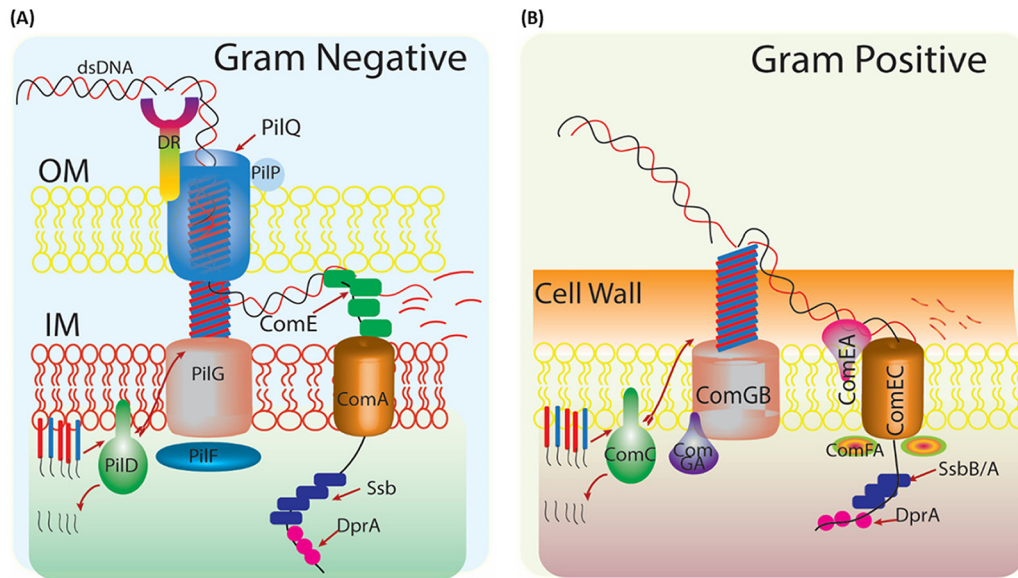
FIG 1 Typical natural transformation machinery of Gram-negative and Gram-positive bacteria. (A) Schematic model of DNA uptake in *Neisseria gonorrhoeae* (Gram-negative). Cleavage of the minor pilin (PilE; red) and major pilin (PilV; blue) by pilin peptidase (PilD), assembled and recruited with the help of polytypic membrane protein (PilF) and traffic NTPase (PilG) to pilus fiber. DNA uptake sequence (DUS) in exogenous/incoming DNA recognized by its unidentified receptor, DR (DNA receptor). Secretin (PilQ) forms a channel with the help of pilot protein (PilP) through double-stranded DNA cross the outer membrane. In periplasm, DNA binding protein (ComE) is involved in uptake and delivery of single-stranded DNA (ssDNA) to cytoplasmic membrane protein (ComA). (B) Schematic model of DNA uptake in *Bacillus subtilis* (Gram-positive). The pseudopilus is composed of the major pseudopilin (ComGC; blue) and minor pseudopilin (ComGD, GE, and GC) and is processed by prepilin peptidase (ComC). The traffic NTPase (ComGA) and polytypic membrane protein (ComGB) are also involved in this process. Pseudopilus allows dsDNA to interact with a membrane-bound receptor (ComEA), which delivers DNA to cytoplasmic membrane protein (ComEC). An ATP binding protein (ComF) helps transport DNA across the membrane. Single-stranded DNA enters the cytoplasm, where SsbB/A and DprA protect it from nuclease action, and with the help of RecA, incoming DNA is integrated into the host genome.

(Fig. 1). Before entering into the cytoplasm, the incoming double-stranded DNA (dsDNA) is converted into single-stranded DNA (ssDNA) by a secretory nuclease (EndA) (2, 14). The conversion of dsDNA to ssDNA occurs at the outer membrane of Gram-negative bacteria and at the cell membrane of Gram-positive bacteria (Fig. 1A and B). The newly generated incoming ssDNA enters the cytoplasm with the help of Com transporter proteins. Due to the danger of degradation by nucleases, DNA processing protein A (DprA/SmfA/CilB) in different bacteria (here referred to as "DprA") and RecA proteins together ensure the functional integrity of incoming DNA (15). At the final stage of integrating incoming DNA, DprA, along with accessory proteins, helps RecA to form nucleoprotein filament with incoming ssDNA and search for unique homologous sequences followed by the DNA strand exchange (DSE) reactions.

DprA was first identified in *H. influenzae* through mutagenesis, where the mutation did not affect plasmid DNA uptake, possibly due to illegitimate translocation of plasmid DNA, but abolished chromosomal natural transformation (16). A null *dprA* mutant of *Campylobacter jejuni* lost ~100-fold chromosome transformation efficiency (17), and the absence of *dprA* in *Helicobacter pylori* severely impaired natural transformation (18). In *B. subtilis*, lack of *dprA/smfA* affected plasmid DNA transformation rather than chromosomal DNA transformation (19). DprA is a mediator for RecA loading and homology-directed RecA-mediated chromosomal transformation in chromosomal DNA transformation. The DprA and RecO proteins, along with single-strand annealing proteins, promote DNA strand annealing for the pairing and circularization of complementary strands coated by SsbA or SsbB during plasmid transformation or viral transfection (20, 21). RecO may compete with DprA for plasmid transformation (7, 22). In *Neisseria meningitidis* and *N. gonorrhoeae*, the *dprA* null mutant is deficient in genetic transformation (23). In *S. pneumoniae*, DprA plays a vital role in competence shutoff (24). The

Escherichia coli dprA/smf gene could partially restore the transformation deficiency in a *Haemophilus influenzae dprA* mutant. Thus, it is apparent that DprA plays a vital role in natural transformation for most bacterial species.

DprA shows sequence conservation, and its occurrence in the bacterial genome is ubiquitous irrespective of a bacterium's natural competence ability (4). In Gram-negative bacteria, the pervasive role of DprA in bacterial physiology and species-specific multifunction activity suggested its possible role in bacterial physiology in addition to natural transformation (25). To date, a large volume of research has been published on DprA, and much of it has demonstrated its involvement in molecular events linked to evolutionary consequences in bacteria. However, a comprehensive review describing its biochemical properties, functional interaction with RecA, and roles in natural transformation and bacterial virulence is lacking. This review provides an extensive yet judicious collection of recent findings on DprA and its functions in bacterial transformation, fitness, and virulence.

OCCURRENCE AND GENOMIC ORGANIZATION OF DprA

DprA homologs have been annotated in the genomes of >2,400 bacterial species, while the genomes of *Archaeoglobus fulgidus*, *Rickettsia prowazekii*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, and *Pyrococcus horikoshii* lack *dprA* (26). Archaea such as *Methanococcus voltae*, *Hermococcus kodakarensis*, *Methanothermobacter marburgensis*, and a mutant of *Pyrococcus furiosus* have been classified as naturally competent, and type IV pilus-like structures facilitate this natural competence. However, among the 26 genome sequences available, *Pyrococcus furiosus* (Pfur) is the only archaean in which the *dprA*-like gene has a central Rossmann fold (RF) domain and shows ~33% identity with bacterial DprAs, suggesting that *dprA*-like genes exist outside Eubacteria. Furthermore, the absence of a *dprA*-like gene in natural competence-enabled archaea raises the possibility that these archaea possess DprA orthologues to fulfill the functions of DprA. Thus, the archaeal genome generally lacks *dprA*-like genes. Its occurrence in *P. furiosus* may have been due to horizontal gene transfer from ε-proteobacteria to fulfill a functional requirement other than natural transformation (27). Interestingly, sequence analyses of transformable bacteria do not form clusters but are distributed according to their taxonomic groups (28). A genome-wide sequence analysis suggested that DNA topoisomerases and DNA resolvase genes occasionally co-occur with *dprA*. For instance, in *Neisseria*, *dprA* is co-expressed with *smg* (putative RNA binding translational regulator) and *topA* (type IA topoisomerase) in an operon (6). In *H. pylori* strains, *dprA* is present with the *ilvC*, *minD*, and *minE* genes in upstream and some uncharacterized downstream open reading frames in an operon form (26). In *H. influenzae*, the *dprA* gene is co-transcribed in an operon with *dprB* (encodes RuvC) and an uncharacterized *dprC* (29). The Sxy/cAMP regulon regulates DNA uptake and processing genes, including the *dprA* gene of *E. coli*, *H. influenzae*, and *Vibrio cholerae* (30). The *dprA* gene of *Riemerella anatipestifer* ATCC 11845 presents as a distal gene of a putative operon comprised of RA0C_1074 (group II Hb), RA0C_1075 (RuvC), RA0C_1076 (uncharacterized), and RA0C_1077 (Lrp/AsnC) (31). The *dprA* gene in the radio-resistant bacterium *Deinococcus radiodurans* exists in an operon with elongation factor P and an uncharacterized protein with two tetratricopeptide repeats. In conclusion, genome analysis has suggested that *dprA* gene occurs ubiquitously in bacteria and that archaea usually lack *dprA*-like genes. Furthermore, a causal relationship of *dprA* co-occurrence with DNA topoisomerases and DNA resolvase genes has been observed in some bacteria.

DOMAIN ORGANIZATION IN DprA PROTEIN

The DprA protein varies in size from 240 (*C. jejuni*) to 398 amino acids (*Synechocystis* and *N. gonorrhoeae*) (26). DprA consists of three domains: an N-terminal SAM domain, a central RF domain, and a C-terminal DML1 domain (Fig. 2). DprA proteins from different bacteria essentially have a central RF domain. The N-terminal SAM domain, which participates in protein-protein interaction, is common in most DprA except those of *H.*
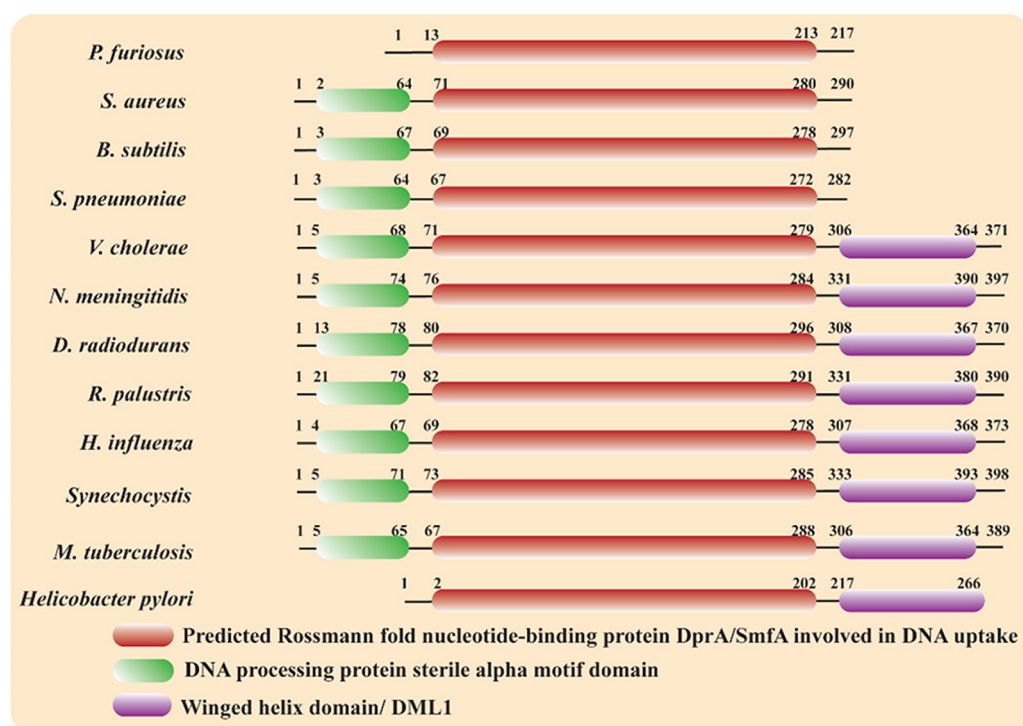
FIG 2 Domain architecture of DprA protein of selected bacteria. Central Rossmann fold (RF) is conserved and present in all DprA proteins, while the N-terminal SAM domain is common in all DprA except those of *Helicobacter pylori* and *Pyrococcus furiosus*. An additional C-terminal DML1 domain is present in the DprA of *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, *Rhodopseudomonas palustris*, *Neisseria meningitides*, *Vibrio cholera*, *Haemophilus influnzae*, and *Synechocytosis* spp.

pylori and *P. furiosus*. DprA proteins from many other bacteria, including *D. radiodurans*, *Mycobacterium tuberculosis*, *Rhodopseudomonas palustris*, *Neisseria meningitides*, *V. cholerae*, *H. influenzae*, and *Synechocytosis* spp., show the presence of the C-terminal DML1 domain (Fig. 2). The crystal structures of DprA from three species, *viz. H. pylori* (DprA$_{Hp}$; PDB ID: 4LJK) (32), *S. pneumoniae* (DprA$_{Sp}$; PDB ID: 3UQZ) (33) and *R. palustris* (DprA$_{Rp}$; PDB ID: 3MAJ) (34) have been reported so far (Fig. 3). The crystal structure of DprA$_{Hp}$ (*H. pylori* DprA) shows that the central RF domain consists of nine α-helices and nine β-strands. All nine strands form an extended structure. The helix flanks both sides of the β-strands to form a sandwiched structure (Fig. 3A). A similar architecture for the central RF is observed in the structures of DprA$_{Sp}$ and DprA$_{Rp}$ (Fig. 3B and C). Structural comparison of DprA$_{Hp}$ with DprA$_{Sp}$ (*S. pneumoniae* DprA) or DprA$_{Rp}$ yields RMSD (root-mean-square deviation) values of 1.9 or 2.0 Å for aligned Ca, atoms, suggesting good similarity among structures (Fig. 3D to F). Only the central RF is visible in the DprA$_{Hp}$ structure. DprA$_{Sp}$ structures show an N-terminal SAM domain consisting of five α-helices along with the central RF, while in the DprA$_{Rp}$ structure, all three domains are visible (Fig. 3).

DprAs are DNA-binding proteins with a preference for ssDNA (28, 33, 35). Various parameters, including amino acid residues, domains, and the kinetics of their interactions with dsDNA and ssDNA, have been worked out in greater detail for DprA$_{Bs}$, DprA$_{Hp}$, and DprA$_{Sp}$ (32, 33). Wang et al. (32) have obtained a DprA$_{Hp}$ structure bound with 35-bp-long deoxythymine nucleotide (dT$_{35}$) ssDNA. However, only seven dTs could be seen in the structure, as the electron density for the remaining dTs was not observed in the crystal structure. Both hydrophobic interactions and hydrogen bonds are involved in the binding of DNA to HpDprA. The DprA dimer interaction with ssDNA, DNA binding pocket residues, and the residues involved in DprA dimerization are shown in Fig. 4.
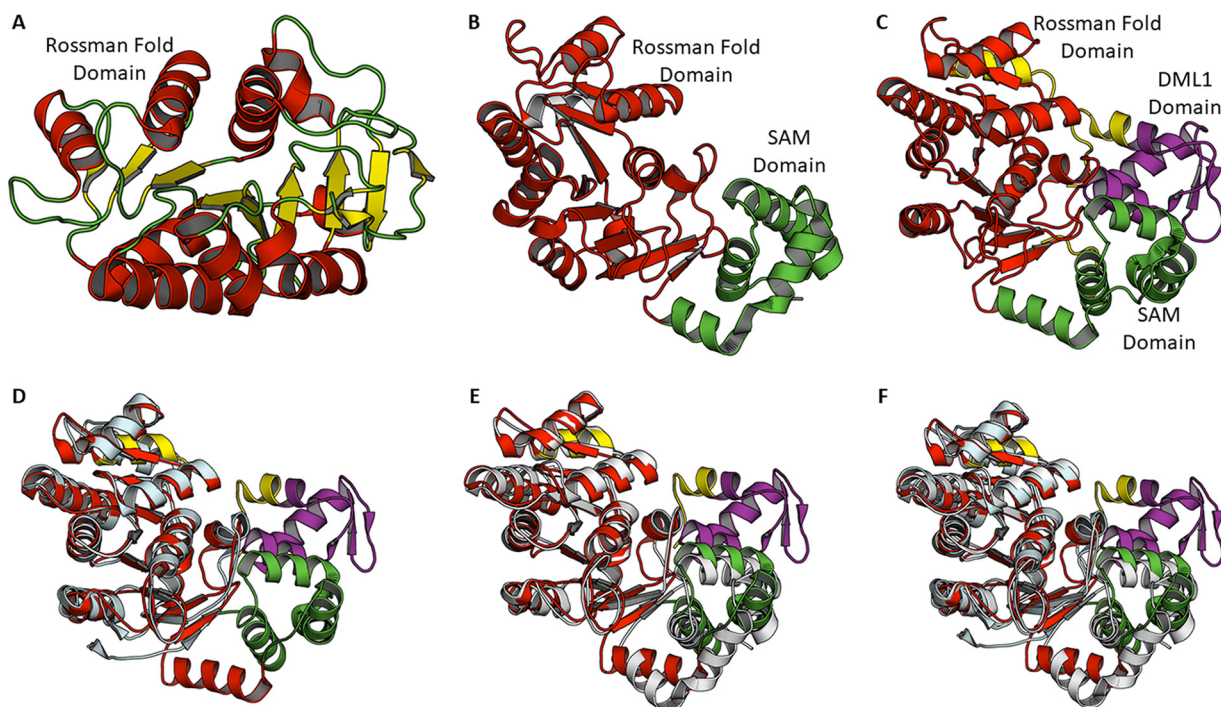
FIG 3 Crystal structures of DprA. (A) Cartoon representation of DprA from *H. pylori* in which only the RF domain is observed. The central β-sheet is sandwiched between α-helices. (B) Crystal structures of DprA from *S. pneumoniae* showing RF domain (red) and N-terminal SAM domain (green). (C) Crystal structures of DprA from *R. palustris* show three domains: N-terminal SAM domain (green), central RF domain (red), and C-terminal DML1 domain (purple). Residues shown in yellow are linker regions. (D) Structural superposition of DprA from *R. palustris* and *H. pylori* (E) Structural superposition of DprA from *R. palustris* and *S. pneumoniae* (F) Structural superposition of DprA from *R. palustris*, *S. pneumoniae* and *H. pylori*.

CELLULAR LOCALIZATION AND OLIGOMERIC PROPERTIES OF DprA

B. subtilis transformation-specific proteins (ComEA, ComEC, ComF, DprA, SsbB, RecA, CoiA, and pilin proteins) are either associated with the membrane or reside in the cytosol and relocalize to a single cell pole which is the preferred site for transforming eDNA uptake during transformation (19, 36). DprA bound with incoming ssDNA facilitates RecA filament formation for recombination during natural transformation (37). Time-lapse microscopy has shown that RecA forms a thread-like structure presumably on eDNA from the cell pole to nucleoid in *B. subtilis* competent cells when it is incubated with eDNA. The DprA of *S. pneumoniae* localizes at a single cell pole along with competence-specific σ factor ($σ^X$), ComW, ComD, ComE, and exogenous competence-stimulating heptadecapeptide (CSP) (38). The competence-dedicated alternative sigma factor $σ^X$ assists DprA and ComD in their polar localization to facilitate competence shutoff (38).

The oligomeric nature of DprA was revealed by crystal structure and biochemical analysis of $DprA_{Hp}$ (32). The $DprA_{Hp}$ residues which form hydrophobic interaction in the dimeric form are Pro183 (P183), Leu196 (L196), and Phe205 (F205), while intermolecular hydrogen bonds form between Arg185 (R185) and Glu188 (E188) and between Tyr57 (Y57) and Leu186 (L186) (Fig. 4C to E). Full-length $DprA_{Hp}$ forms an oligomer in the presence of glutaraldehyde, whereas $DprA_{Hp}$ protein lacking C-terminal domain (RF_{Hp}) remains dimer, suggesting the crucial role of the C-terminal $DML1_{Hp}$ field in the formation of the higher-order oligomeric structure of $DprA_{Hp}$. Furthermore, the structural superimposition of the three DprA structures indicated that the dimeric form is essential for DprA biochemical functions and is a widely adopted quaternary structure (32).

Unlike in $DprA_{Hp}$, the Pro248 (P248), Gly249 (G249), and Ile263 (I263) residues of $DprA_{Sp}$ form tail-to-tail dimers, and this type of dimerization is crucial for nucleoprotein complexes *in vitro* and genetic transformation (33). Interestingly, the DML1 domain at the C-terminal regions of $DprA_{Rp}$ and $DprA_{Ec}$ has been shown not to affect the formation of RF-
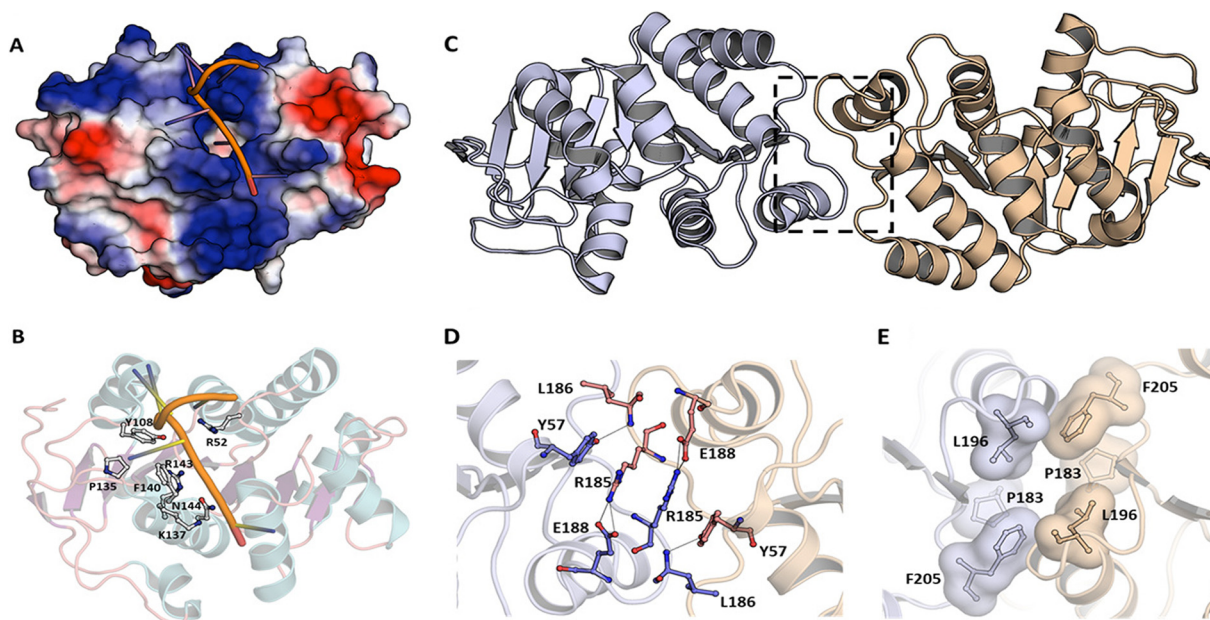
FIG 4 Interaction of *H. pylori* DprA with ssDNA and DprA dimerization organization. (A) Electrostatic surface potential representation with bound ssDNA. (B) Residues involved in the DNA binding are shown in stick and ball model. Dimeric organization of *H. pylori* DprA in crystal structure. (C) Cartoon representation showing dimer interface. The two dimer subunits are shown in different colors. (D) Residues involved in hydrogen bond formation in *H. pylori* DprA dimerization. (E) Hydrophobic residues providing dimer interface. Residues are shown as sticks and their Van der Waals surface is also shown.

RF dimers by these DprAs, and dimerization seems to be similar as known for RF-RF dimers of $DprA_{Sp}$. For $DprA_{Sp}$, no variant(s) supporting N/N-terminal interaction have been isolated, suggesting a less crucial role of N/N-terminal interaction in $DprA_{Sp}$ dimerization. The residues involved in DprA dimerization and RecA interaction have considerable overlap. The DprA residues for RecA interaction appear to be evolutionarily less conserved. Nonetheless, the considerable overlap between DprA dimerization and RecA interaction interfaces has led to the hypothesis that RecA disrupts DprA dimerization, a prerequisite for DprA function, thereby giving RecA access to incoming ssDNA (33). Together, structural comparison of $DprA_{Hp}$, $DprA_{Sp}$, $DprA_{Rp}$, and the 3D models of *E. coli* DprA has revealed (i) the widespread association of a SAM and an RF domain in bacterial DprA, (ii) the evolutionary conservation of DprA dimerization potential and interface residues, and (iii) that the essential residues for self-dimerization of $DprA_{Sp}$ (P248, G249, and I263) are also identical in the $DprA_{Rp}$, $DprA_{Bs}$, and $DprA_{Ec}$ proteins (Fig. 5).

DNA BINDING PROPERTIES OF DprA

DprA receives incoming DNA during natural transformation in its dimer conformation. The DprA of *S. pneumoniae* and its *B. subtilis* homolog (SmfA) bind cooperatively with ssDNA (28). DprA has shown higher affinity toward ssDNA versus dsDNA, except for *D. radiodurans* DprA ($DprA_{Dr}$), which has equal affinity for ssDNA and dsDNA (39). The ability of $DprA_{Dr}$ to bind with dsDNA is an interesting phenotype and suggests a possible role of $DprA_{Dr}$ in DNA metabolism beyond natural transformation. Previously, RecA of *D. radiodurans* was shown to prefer dsDNA over ssDNA and was able to catalyze the inverse strand exchange reaction (40–43). The footing size of bacterial DprA is 30 to 40 bp on ssDNA, and therefore a minimum 50-bp length is essential for a stable nucleoprotein complex (6, 33). DprA has reversible nonspecific ssDNA binding activity and does not require DNA ends to bind. Unlike SSB_{Eco}, which destabilizes the helical structure of DNA, transmission electron microscopy analysis of DprA and ϕX ssDNA has revealed that $DprA_{Sp}$ and $DprA_{Bs}$ maintain or induce the DNA helical structure of DprA to form a tightly packed discrete complex with ϕX ssDNA (28). $DprA_{Sp}$ showed no affinity toward linear and relaxed dsDNA. However, it did show affinity toward
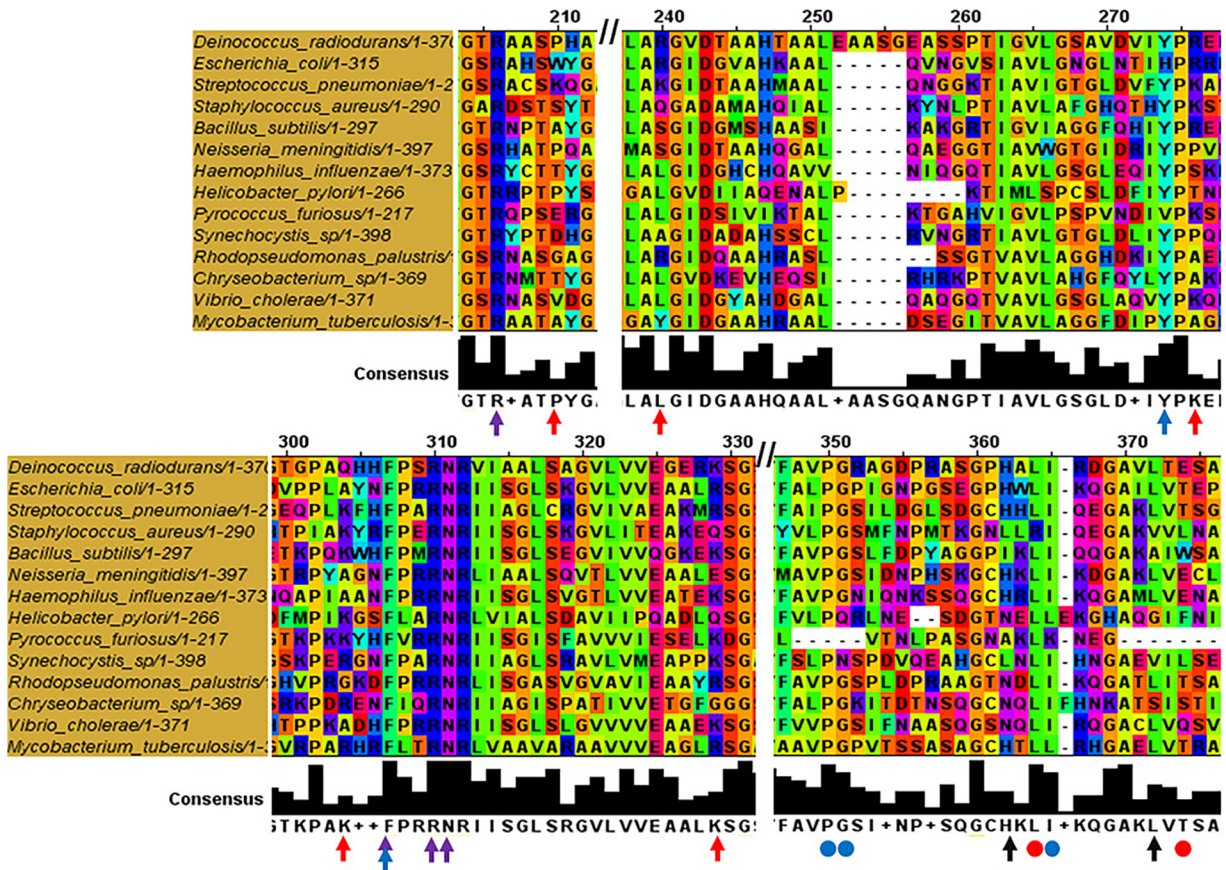
FIG 5 Sequence alignment of DprAs of different species highlighting conserved residues. Multiple sequence alignment was done using MUSCLE (Multiple Sequence Alignment-EMBL-EBI) and view using Jalview. Red arrow represents conserved lysine (K) of *S. pneumoniae* (K119, K144, K175, K202, and K225); purple and blue arrows represent conserved *H. pylori* DprA residues (R52, F-140, R-143, K137, and N-144) responsible for DNA binding; black arrows represent hydrophobic residues of *H. pylori* DprA (H264, and L273) crucial for dimerization. Red dot represents hydrophobic core dimerizing residues of *H. pylori* DprA (L196 and F205); blue dots represent the residues involved in self-dimerization of *S. pneumoniae* DprA (P248, G249, and I263).

negatively supercoiled ϕX DNA. This result was explained by the ability of the DprA protein to interact with ssDNA regions within ϕX DNA molecules and the apparent formation of a sleeve-like structure, which suggests that it can form a bridge or juxtapose two or more DNA molecules (28). Atomic force microscopy of DprA$_{Bs}$ revealed that depending on DprA concentrations, it forms discrete complexes without affecting the DNA helical structure (44).

The amino acid residues responsible for ssDNA binding are Arg52, Phe140, Arg143, and Asn144 for DprA$_{Hp}$ (32) (Fig. 5, Fig. 4A and B). Multiple sequence alignment showed two motifs in DprA family proteins which are essential for ssDNA binding. The first "G-S/T/A-R" motif is located in a loop (b3 to a3) region, and the corresponding residues are Gly50 to Arg52 in DprA$_{Hp}$; while the second "F/L/Y-X-X-R-N/D" motif is located in helix α6 and corresponds to residues Phe140 to Asn144 in DprA$_{Hp}$ (32). The R123E mutant of DprA in *R. anatipestifer* cannot form nucleoprotein complexes and is deficient in transformation (31). Thus, RF plays an important role in DNA binding and other functions of DprA.

Together, DprA structure-function studies and biochemistry have suggested the following conclusions: (i) DprA binding with ssDNA is nonspecific; (ii) a dimer is required for optimal binding to ssDNA; (iii) the stable oligomeric complex of ssDNA-DprA dimer is observed at 1:1 stoichiometry (for DprA$_{Hp}$), and the ssDNA should be long enough (>50 nt) so that it can effectively be docked onto two specific binding pockets of DprA dimer (32); (iv) the C-terminal DML1-like domain contributes higher-order oligomer
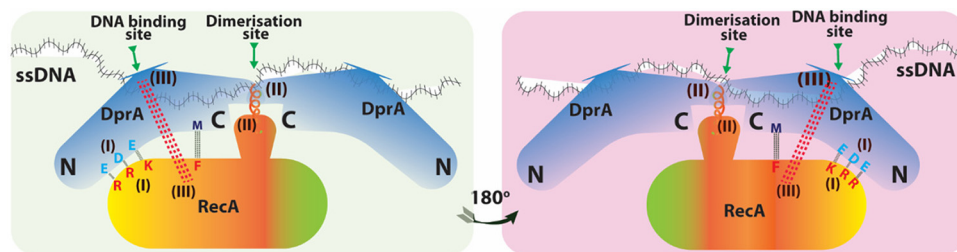
FIG 6 Residues important for RecA-DprA interaction. Three regions are responsible for DprA$_{Hp}$ and RecA interaction. Region I represents DprA$_{Hp}$ E235, D243, and E265 (EDE) triad interacting with R189, R226, and K267 (RRK) triad of RecA$_{Hp}$, respectively. Involvement of RecA$_{Hp}$ F230 and DprA$_{Hp}$ M238, which is present near EDE domain of DprA$_{Hp}$. Region II shows DprA$_{Hp}$ dimerization domain with RecA$_{Hp}$ N-terminal α-helix. Region III shows RecA$_{Hp}$ polar loop 2 motifs interacting with DprA$_{Hp}$ DNA binding site. Regions I and II are only accessible for the first RecA monomer binding to DprA and are entirely inaccessible for RecA oligomers. Region III has loop 2, responsible for the loading of RecA on ssDNA, as it competes with the basic patch of DprA to facilitate the transfer of ssDNA from DprA to RecA. Regions II and III are responsible for destabilization/disruption of the DprA dimer and transfer process.

formation with the increased protein concentration because it possesses a weak secondary ssDNA binding site (45).

FUNCTIONAL INTERACTION OF DprA WITH OTHER GENOME INTEGRITY PROTEINS

RecA plays a vital role in natural transformation-mediated horizontal gene transfer, and DprA is a crucial protein associated with this process. During transformation, RecA loading onto incoming ssDNA is a multistep process (46–49). Various ssDNA binding proteins (SsbA, SsbB, and DprA) assist in this process (25, 44, 46, 50, 51). The interaction of DprA with RecA was initially recognized by FRET-based co-localization studies in *B. subtilis* (36). More recently, DprA interaction with RecA has been shown in many bacterial species, such as *S. pneumoniae*, *R. palustris*, and *B. subtilis* (24, 28, 33, 35, 44). DprA-RecA interactions have been shown by TAP-tag experiments, electron microscopy experiments *in vitro*, and Y2H (yeast two-hybrid) screening *in vivo*. RecA broadly interacts with two types of protein groups: (i) mediators which assist in RecA loading on ssDNA and (ii) modulators which are required for homology search and DNA strand exchange reaction. DprA belongs to the mediator group of RecA partners (46–49). RecAs of *B. subtilis*, *D. radiodurans*, and *S. pneumoniae* have higher activity with dATP. The RecA-dATP complex could assist DNA strand exchange without accessory factors such as DprA, SsbA, RecO, and SsbB (41, 52–54). On the other hand, the RecA·ATP complex cannot compete with a saturating concentration of SSB proteins. Therefore, the DprA protein helps the RecA.ATP to nucleate and polymerizes on the SsbA·ssDNA complex more efficiently than on protein-free ssDNA. The DprA of *B. subtilis* can facilitate active nucleoprotein filaments formation by RecA.ATP on ssDNA preincubated with SsbA, but not when bound with SsbB (46). The DprA$_{Sp}$ protein and its ortholog in *B. subtilis* may bind ssDNA cooperatively and have the ability to self-interact with RecA (24, 44). The DprA$_{Sp}$-RecA-ssDNA filaments may catalyze the homology-dependent formation of joint molecules and limits the SSB barrier function during natural bacterial transformation (46).

The acidic residues E235, D243, and E265 (EDE triad) in DprA$_{Sp}$ are crucial for DprA$_{Sp}$-RecA interaction, and mutations in these residues lead to a loss of/weakened interaction with RecA (33). RecA residues necessary for DprA interaction have also been identified, and it has been shown that EDE-triad and methionine-238 residues of DprA$_{Sp}$ interact with R189/R226/K267 (RRK triad) and phenylalanine-230 residues at the N terminus of RecA, respectively (33) (Fig. 6). Additionally, the fact that the RecA$_{Sp}$ loop 2 region has a nonpolar motif (V212/G213/V214/M215/F216) suggests its role in RecA-DprA interaction, as the mutants of these residues decreased interaction activity. Because DprA and RecA can form self-oligomers, the heterogenic interaction of individual homopolymeric proteins is difficult to interpret. When RecA is present in an oligomeric form, the interacting region of this protein with DprA is largely inaccessible. Quevillon et al. (33) showed that DprA$_{Sp}$ binds with
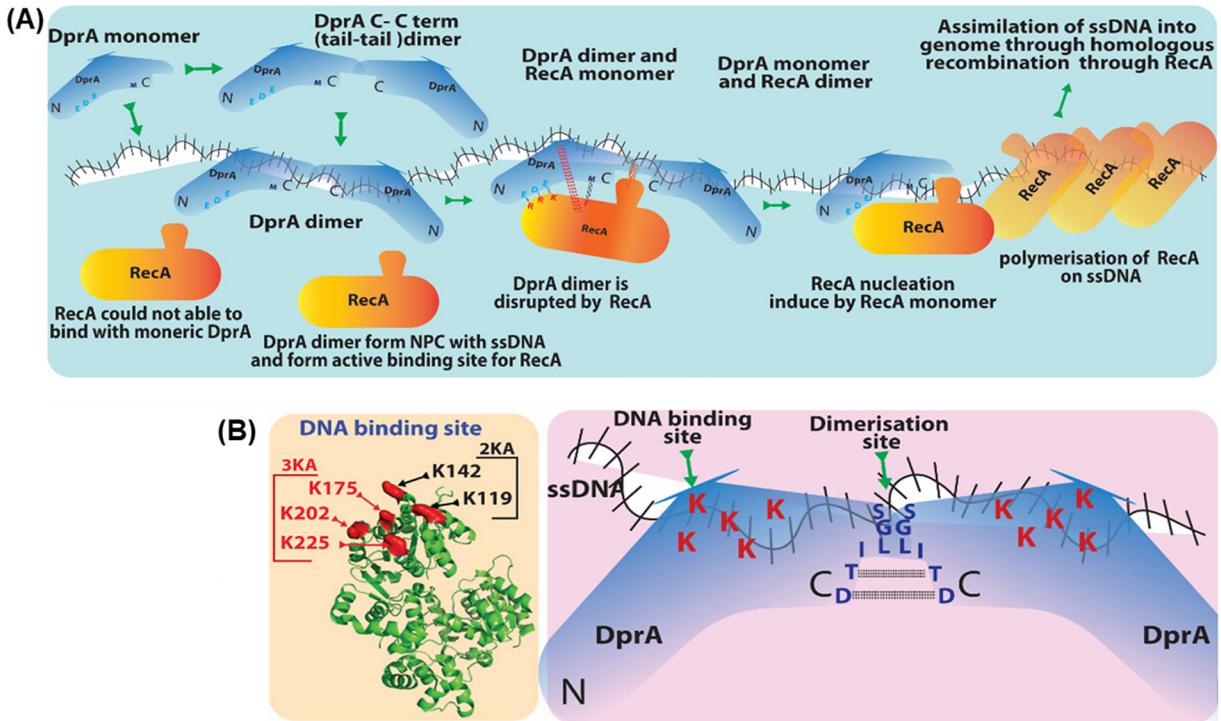
FIG 7 (A) DprA-mediated loading of RecA on ssDNA. DprA$_{Sp}$ dimer (C-C term) forms a nucleoprotein complex with ssDNA. DprA$_{Sp}$ dimer bound to ssDNA offers the best suitable platform for RecA nucleation. After ssDNA binding of DprA$_{Sp}$ dimer conformation change takes place, and sites of DprA$_{Sp}$-RecA interaction open up (EDE triad of DprA$_{Sp}$ interacts with RRK triad of RecA). Once DprA$_{Sp}$-RecA interaction is established, DprA$_{Sp}$ is converted to a monomer. This change allows RecA loading on ssDNA, followed by RecA nucleation, continued polymerization, and DprA$_{Sp}$ leaving the ssDNA. (B) Residues important for *S. pneumoniae* DprA protein DNA binding and dimerization. Five lysine (K119/K144/K175/K202/K225) residues on the basic face of DprA$_{Sp}$ are essential for DNA binding activity. DNA binding activity is completely abolished when lysine is mutated to alanine, whereas the partial patch mutation 3KA and 2KA produces a similar effect equal to that of R115A single mutant. This basic patch is localized opposite the RecA binding site.

incoming ssDNA through its C/C dimer, followed by the nucleation of RecA on ssDNA. RecA interaction with DprA$_{Sp}$ destabilize the DprA$_{Sp}$ dimer interface, resulting in ssDNA transfer from DprA to RecA (Fig. 7A). The strong hydrophobic character of the loop 2 region of RecA might further facilitate the weakening of DprA homodimer binding to ssDNA, making DprA exit from ssDNA and concurrently loading RecA of DNA (33). Independently, Lisboa et al. (35) showed that a region in DprA$_{Sp}$ consisting of R115 and five basic residues (K119/K144/K175/K202/K225) surrounding it (Fig. 7B) was positioned at the opposite side of RecA interacting motif, close to its loop 2 region. Therefore, it is likely that the loop 2 region of RecA competes with the basic patch of DprA and paves the way to transfer ssDNA from the DprA dimer to RecA (35). Thus, the regulation of structural dynamics of RecA and DprA upon interaction for their functions would be an example of how two proteins with different oligomeric properties interact in heterogenic interaction for their functions.

Mismatch repair protein (MutS) may regulate recombination during *B. subtilis* horizontal gene transfer via natural chromosomal transformation. In *B. subtilis*, it has been shown that the natural chromosomal transformation frequency decreased exponentially with increased sequence divergence up to 15% in wild-type cells or cells lacking MutS2 or mismatch repair proteins (MutL, MutS, or both). However, if sequence divergence is more than 15%, the chromosomal transformation efficiency is ~100-fold higher in Δ*mutS* and Δ*mutSL* than in wild-type cells and Δ*mutS2*. Thus, transformation frequency decreased in a biphasic manner with increasing sequence difference of incoming DNA. The addition of MutS overcame the heterologous barrier between incoming DNA and dsDNA, possibly through the modulation of RecA filament on bidirectional ssDNA (55). RecA-mediated DSE in the presence of DprA, RecO, and SsbA proteins reconstituted *in vitro* led to the observation that DNA sequence

divergence halts RecA strand exchange in a log-linear fashion and showed a minor priority for the 3′ end of duplex DNA (48). Natural chromosomal transformation (CT) in *B. subtilis* is governed by sequence divergence (SD) of up to 15%, and integration occurs through homology-directed CT.

In contrast, the interspecies boundaries prevail beyond 23% SD or more, and CT frequency decreases log-linearly. The interspecies CT is RecA-dependent, and the activity of the *dprA*, *radA*, *recJ*, *recX*, or *recD2* genes is suggested to be crucial for interspecies CT, the generation of genetic diversity, and speciation (20). In addition to DprA's native role in natural transformation to promote RecA-mediated bidirectional or reciprocal recombination, DprA may also promote RecA-catalyzed unidirectional gene conversion and produce pilin variation in *Neisseria* (see below, "DprA role in the regulation of bacterial virulence").

DprA can protect ssDNA from nucleolytic degradation by ExoT (3′ to 5′ exonuclease), RecJ (5′ to 3′ exonuclease), and Mung Bean endonuclease *in vitro* and reverse the inhibitory effect of RecX and RecU on RecA-mediated DNA strand exchange (21, 56). The restriction-modification systems (RM systems) in bacteria discriminate self and non-self DNA and help maintain the genome integrity of an organism by reducing horizontal gene transfer in bacteria (29, 57). Dwivedi et al. (45) showed that DprA$_{Hp}$ can protect dsDNA substrate from type II restriction endonucleases and stimulate methyltransferase activity *in vitro*. However, the involvement of type II RM systems in natural transformation by interacting with DprA is less likely *in vivo* because the incoming ssDNA during natural transformation was shown to be a poor substrate for type II restriction endonucleases (58).

The radiation-resistant bacterium *D. radiodurans* is naturally transformable, and various factors (PilQ, PilD, type IV pilins, PilB, PilT, ComEC-ComEA, and ComF) responsible for DNA uptake and translocation have been recently identified (7). Cells devoid of DprA have severely abolished transformation, and the absence of either *recF*, *recO*, or *ddrB* in a *dprA*⁻ genetic background completely negates transformation (7). *In vitro* studies have suggested that DprA$_{Dr}$ has equal affinity binding for ssDNA and dsDNA and protects ssDNA from nucleolytic degradation. Furthermore, it has been suggested that the oligomerization and DNA binding properties of DprA$_{Dr}$ are essential to support RecA-catalyzed strand exchange reactions (SER) *in vitro* (39). Ithurbide et al. (7) did not observe a role of DprA in gamma radiation resistance. However, our current work on DprA's role in gamma radiation resistance and DNA repair suggests that *dprA* plays an important role in gamma irradiation and showed faster DNA double-strand break repair kinetics and elevated rifampicin resistance mutation frequency (D.K.S., H.S.M., and Y.S.R., unpublished data).

The roles of DprA in replication and cell division are not apparent. However, indirect evidence has shown that lower DNA content in a *dprA* mutant of *N. meningitides* post-antibiotic treatment highlights impaired DNA replication in *dprA* mutant cells (6). Thus, different findings have suggested some direct or indirect functional interaction of DprA with many proteins involved in genome integrity and metabolism, which would be worth monitoring in the future.

DprA FUNCTIONS IN NATURAL TRANSFORMATION AND REGULATION OF COMPETENCE

DprA function is necessary for the natural transformation of chromosomal and plasmid DNA in *R. anatipestifer*, *N. meningitidis*, *N. gonorrhoeae*, *D. radiodurans*, and *S. pneumoniae* (6, 7, 15, 17, 23). However, in *H. influenzae*, DprA is needed for chromosomal DNA transformation but not for plasmid DNA transformation (16). The non-requirement of DprA for plasmid DNA transformation was an intriguing observation. Experimental evidence suggested that different topological forms of plasmid DNA may allow DNA translocation as intact dsDNA without degradation. This DNA uptake mode is called illegitimate transformation (59). Bacteria such as *D. radiodurans* and *B. subtilis* have redundant pathways for chromosomal DNA transformation, in which the RecO and DprA proteins are functionally redundant (7, 44, 60). The role of DprA in shutting off competence has been reported in *S. pneumoniae* (24).
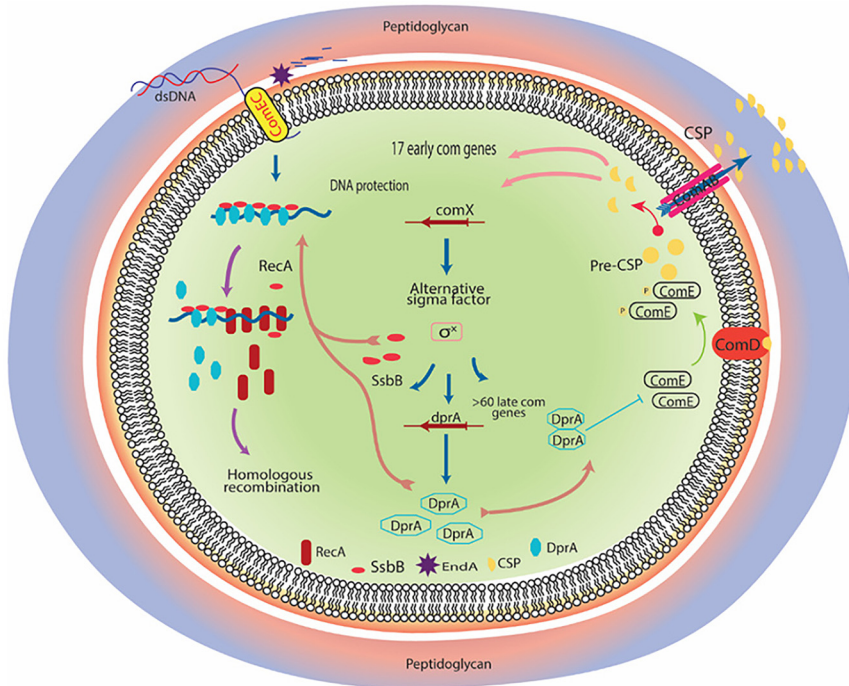
FIG 8 Regulation of competence in *Streptococcus pneumoniae*. Dynamicity of competence pathway in *S. pneumoniae* is regulated through competence-stimulating peptide (CSP). CSP is processed from pre-CSP (precursor form) and encoded by *comC*. ComAB is an ABC-type membrane transporter that converts pre-CSP into CSP and exports it; ComDE is a two-component signal transduction system (TCSs); and ComD (histidine kinase) binds with CSP to trigger its autophosphorylation (ComD~P) followed by transphosphorylation of its cognate response regulator molecule (ComE) by transfer of a phosphate group. ComE~P activates 17 early *com* genes, including *comX*, which encodes a competence-specific alternate sigma transcription factor (σ^X). *dprA*, *ssbB*, and more than 70 genes are regulated through σ^X alternate transcription factor. After internalization of DNA, elevated level of DprA sequester the ComE~P and consequently shuts off the competence pathway.

About ~80 bacterial species are capable of natural transformation (3). Among these, many naturally occurring species, such as *Deinococcus* and *Neisseria*, remain competent during their growth phase. However, *B. subtilis* and *S. pneumoniae* adopt a transient phase of competence to take up environmental DNA. Nutrition deprivation activates the competence of the *B. subtilis* bacterium and acquired DNA used for carbon sources during the stationary phase (60). DprA plays a more crucial role in regulating competence in *S. pneumoniae* (Fig. 8). The competence or X-state of *S. pneumoniae* is activated by competence-stimulating heptadecapeptide, encoded by the *comC* gene (9). CSP binding to ComDE (bacterial two-component system) can activate the classical mode of activation in which autophosphorylation of a sensor histidine kinase (ComD) and transphosphorylation of a response regulator (comE~P) activate the *comAB*, *comCDE*, and *comX* operon genes, which in turn activates a positive feedback loop of CSP (10, 38, 61) (Fig. 8). These changes lead to a sudden rise in CSP levels, rendering all cells X-state (competent). *comX* gene (master regulator of X-state) encodes competence-specific σ factor (σ^X) (Fig. 8). The genes which come under the control of σ^X are late *com* genes, including DNA uptake machinery genes, *dprA*, *ssbB*, and other recombination mediator proteins (62, 63). DprA$_{Sp}$ shuts down competence by suppressing *comX* expression (Fig. 8). DprA$_{Sp}$ shifts the concentration of phosphorylated ComE toward unphosphorylated ComE. These changes lead to the suppression of comX promoter activity and, consequently, no activation of *comX* and *comW* genes (61). Together, these changes result in diminished or inhibited activation of σ^X and subsequent suppression of late competence genes, including *dprA*, which leads to shutoff of X- or competence state (Fig. 8). Johnston et al. (38) investigated the chronology of pneumococcal competence development and shutoff through spatiotemporal localization dynamics of the key regulators (DprA, σ^X, ComW, ComD, ComE, and exogenous CSP) at the single-cell level and suggested that two intertwined and transient transcription waves regulate

S. pneumoniae competence. The stress-inducible phosphor-relay between ComD and ComE constitutes the first wave, while the second wave controls expression of an alternative sigma factor σ^X, regulating the expression of DprA which turns off competence through interaction with phosphorylated ComE. Experimental evidence has also suggested that σ^X physically conveys DprA next to ComD when all these proteins co-localize at one pole in competent cells. Through the polar DprA targeting function, σ^X mediates the timely shutoff of the pneumococcal competence cycle, preserving cell fitness. Thus, DprA-mediated regulation of competence is crucial for *S. pneumoniae* to switch swiftly between the competence and rapid growth phasse according to environmental conditions, nutritional state, and host factors (10, 38, 61). Moreover, in *Acinetobacter baylyi*, DprA-led shutoff of competence and regulation of natural transformation was shown to effectively reduce the cost of transformation if internalized DNA was utilized for the generation of the nucleotide pool and incorporated into DNA biosynthesis pathways. On the contrary, using internalized DNA for recombination and repair may have costly consequences because recombination with transforming DNA dramatically reduces transformant survival and induces DNA strand breaks (64).

Transformation efficiency in *S. pneumoniae* is not dependent on the abundance of DNA; instead, it is entirely reliant on the level of cellular DprA. It has been estimated that approximately ~650 to ~2,250 DprA molecules per cell are required for maximum transformation efficiency, while the presence of fewer than 650 DprA molecules results in a rapid loss of transforming ssDNA. Interestingly, higher levels of DprA dimer (~4,000 molecules per cell) dictate competence shutoff in *S. pneumoniae* by either dephosphorylation or sequestering of ComE~P dimmers. In addition, a *dprA* null mutant of *S. pneumoniae* showed rapid cell lysis compared to the wild type due to a nonfunctioning shutoff pathway, indicating that the cell's inability to shut off competence hurts cell viability and physiology (3).

DprA$_{Cj}$ (*C. jejuni* DprA) is involved in the natural transformation pathway of *C. jejuni*, affecting efficiency for both plasmid and chromosomal DNA. A *dprA* null mutant exhibited reduced transformation efficiency (2-log) for chromosomal DNA while altogether abolishing plasmid DNA electrotransformation (17, 65). This loss in transformation could be trans-complemented by wild-type DprA from *H. pylori* and *H. influenza*, suggesting the evolutionary conservation of DprA function across species.

DprA ROLE IN THE REGULATION OF BACTERIAL VIRULENCE

The absence of the *dprA* gene negatively impacts *S. pneumoniae* growth and virulence. This phenotype is due to DprA's contribution to shutting off the competence state, but not to its role in DNA uptake and integration. A Δ*dprA* mutant of *S. pneumoniae* showed a growth defect when stimulated with CSP and had difficulty quitting the competence state. CSP-activated ComE positively regulates 24 early competence genes, including alternative sigma factor σ^X. The σ^X binds to the "com-box" in the promoter and regulates the expression of ~80 late competence genes. Among these genes, 16 are essential for competence and genetic transformation, and 14 late genes are necessary for bacterial fitness during bacteremia infection in the host (66, 67). Among these 14 late genes, *cbpD*, *cibAB*, and p1 promoter-regulated genes have roles in bacterial virulence by enhancing the release of pneumolysin and expression of *lytA*, *radC*, *spd_0030*, *spd_0031*, *spd_0981*, *spd_1778*, and *spd_1828*.

The importance of DprA in *S. pneumoniae* virulence during the infection cycle was tested in model in which the abilities of wild-type *S. pneumoniae* (D39) and a Δ*dprA* mutant to cause bacteremia and acute pneumoniae, respectively, were evaluated in a CD1 mouse model. *dprA* deletion severely affects the virulence and infectivity of *S. pneumoniae* and competitiveness to cause bacteremia, nasopharyngeal infection, and acute pneumonia infection (68) (Fig. 9A, B). Thus, DprA's functional role in *S. pneumoniae* fitness during infection by regulating the competence state rather than by processing incoming DNA has been suggested. The inability of Δ*dprA* mutants to exit the competence state has contributed to attenuated virulence, perhaps by creating an imbalance in energy utilization by *S. pneumoniae* in host cells and excess energy use to maintrain of competence (3) (Fig. 9C).
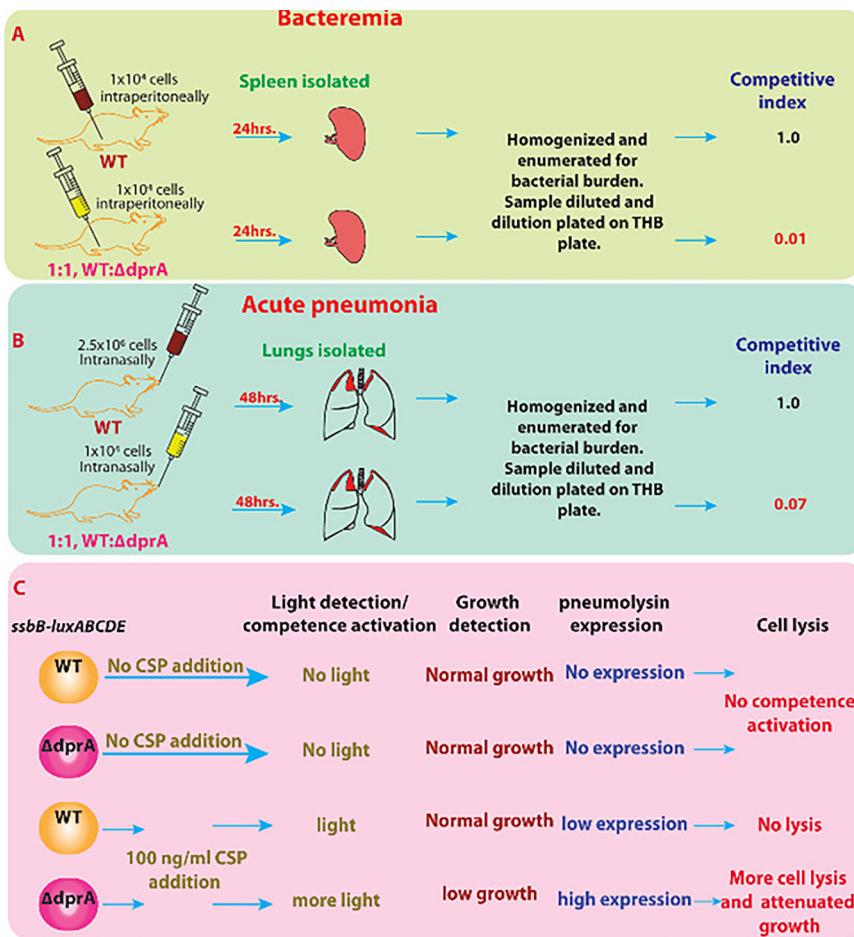
FIG 9 DprA involvement in *Streptococcus pneumoniae* virulence and pathogenicity. *Streptococcus pneumoniae* D39 (WT) and Δ*dprA* mutant were used to evaluate bacteremia and acute pneumonia in CD1 mice. (A) For the bacteremia mouse model, 1×10^4 CFU bacteria were injected intraperitoneally. After 24 h, spleens were isolated from euthanized mice, homogenized dilutions were plated, and pneumonia burdens were calculated. (B) For the acute pneumonia mouse model, animals were anesthetized using isoflurane and intranasally administered 5×10^6 CFU pneumococci. After 48 h, mice were euthanized, lungs were removed, and bacterial burdens were calculated. The competitive index (CI) is the ratio of mutant versus wild type in the output sample divided by the input ratio of mutant versus wild type provided in the sample. A CI of >0.7 is defined as attenuated. (C) DprA is required to shut off competence and regulate ~100 "early" and "late" competence genes. Overexpression of early and late competence genes causes cellular stress and excess energy consumption (energy-deprived condition) in *dprA* mutant. Here, *ssbB* (late competence gene) is translation-fused with firefly luciferase gene to generate translation fusion construct *SsbB-luxABCDE* and measure the overexpression of late competence gene after competence activation through the addition of CSP1. A Δ*dprA* mutant of *S. pneumoniae* cannot shut off the competence pathway activated by CSP1 addition, leading to more energy consumption and causing unwanted cellular stress and energy deprivation. The Δ*dprA* mutant is susceptible to low CSP1 concentration for prolonged competence activation (more light), resulting in overexpression of allolytic factors LytA, CbpD, and CibAB. It also induces the fratricide immunity protein ComM. Combined action of low energy and excessive overexpression of allolytic enzyme, followed by the release of pneumolysin, potentially leads to bacterial death and attenuated virulence.

DprA ROLE IN BACTERIAL PILIN VARIATION

The pilus plays crucial roles in cellular adherence, bacterial aggregation, twitching motility, and natural DNA transformation in *Neisseria* (69–72). Pilin protein constitutes a major subunit of the *Neisseria* type IV pilus system (73–75). Variation in pilin antigenic determinants creates genetic diversity and plays vital roles in virulence, survival, cell adhesion, and escape from the host immune response in *Neisseria* and *Streptococcus*. Pilin variation is a convenient mechanism which allows an organism to introduce a high rate of recombinations without deleterious effects on genome function. Two mechanisms mediate pilus phase variation: a RecA-independent mechanism in which slippage and mispairing of the *pilC* gene strands lead to subunit variation in pilin, and RecA-dependent

high-frequency gene conversion that promotes non-reciprocal DNA recombination events between multiple *pilS* loci and the *pilE* expression locus (72, 76–78). *N. gonorrhoeae* and related pathogens such as *N. meningitidis* use minor pilus protein (ComP) to take up DNA with DUS (DNA uptake sequence) and induce efficient DNA uptake to the periplasm (79–82). A novel function of DprA beyond transformation was shown for pilin protein variation in *Neisseria* (23). It was observed that *dprA* mutants show increased RecA-dependent pilin variation compared to isogenic parental strains ($P < 0.05$). However, this was restored to the parental level when DprA was complemented in *trans*. Using *recA* and a *dprA* double mutant further confirmed that DprA affects only RecA-dependent variation in pilus (23). Pilus variation in *Neisseria* invokes numerous recombination proteins (RecA, RecX, RecR, RecO, RecQ, RecG, and RuvABC) and mismatch repair proteins to offer gene conversion by non-reciprocal recombination process (83).

Pilin variation-related recombination events are unidirectional gene conversion, while natural transformation-led recombination is bidirectional or reciprocal. Although the role of DprA in bidirectional recombination is known, its role in unidirectional gene conversion was unclear. It is possible that DprA promotes RecA-catalyzed unidirectional gene conversion and produces pilin variation in *Neisseria* (23). The mechanisms which can explain DprA's role in unidirectional gene conversion owed to its ability to load RecA on ssDNA, influencing and increasing DSE activity (23). Besides this, it is also likely that DprA binds with small single-stranded fragments generated during double-strand breaks at the pilus locus, which eventually load RecA and may stimulate invasion of the *pilS* locus (84).

CONCLUDING REMARK

The competence state enables bacteria to take up extracellular DNA from the environment. The natural transformation process involves the uptake of DNA followed by its processing and integration into the bacterial genome. The role of DprA is indispensable in natural transformation. It facilitates RecA functions during the integration of incoming ssDNA into the genome. Structure-function studies have identified important residues for homotypic and heterotypic interactions of DprA and RecA. DprA can alleviate the inhibitory effects of SSB, RecX, and RecU on RecA-mediated DNA strand exchange by directly antagonizing the inhibitory impact of RecX. *dprA* has been reported to occur in most bacterial species. However, many bacteria do not encode the other components of natural transformation machinery. This observation suggests a strong possibility of DprA having roles in other molecular events in bacterial physiology, possibly beyond natural transformation, such as regulation of virulence, pilin variation, and genome integrity maintenance. The molecular mechanisms underlying DprA's role in the regulation of RecA function have been studied in a few bacteria, but are lacking in most bacteria which have DprA with complete competence machinery or DprA alone. A DprA$_{sp}$-mediated shift in the concentration of phosphorylated ComE and its cascade leads to competence shutoff and energy conservation in *S. pneumoniae*, leading to speculation on this protein's role as a stress-response regulator of DNA metabolism. The wide distribution of DprA in most bacteria, irrespective of their natural transformation ability, highlights possibly novel roles of DprA beyond natural transformation in bacterial physiology that would be worth exploring in the future.

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