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Effect of the *Streptococcus pneumoniae* MmsA Protein on the RecA Protein-promoted Three-strand Exchange Reaction

IMPLICATIONS FOR THE MECHANISM OF TRANSFORMATIONAL RECOMBINATION*

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Streptococcus pneumoniae **is a naturally transformable bacterium that is able to incorporate DNA from its environment into its own chromosome. This process, known as transformational recombination, is dependent in part on the *mmsA* gene, which encodes a protein having a sequence that is 40% identical to that of the *Escherichia coli* RecG protein, a junction-specific DNA helicase believed to be involved in the branch migration of recombinational intermediates. We have developed an expression system for the MmsA protein and have purified the MmsA protein to more than 99% homogeneity. The MmsA protein has DNA-dependent ATP hydrolysis and DNA junction-helicase activities that are similar to those of the *E. coli* RecG protein. The effect of the MmsA protein on the *S. pneumoniae* RecA protein-promoted three-strand exchange reaction was also investigated. In the standard direction (circular single-stranded (ss) DNA + linear double-stranded (ds) DNA → linear ssDNA + nicked circular dsDNA), the MmsA protein appears to promote the branch migration of partially exchanged intermediates in a direction opposite of the RecA protein, resulting in a nearly complete inhibition of the overall strand exchange reaction. In the reverse direction (linear ssDNA + nicked circular dsDNA → circular ssDNA + linear dsDNA), however, the MmsA protein appears to facilitate the conversion of partially exchanged intermediates into fully exchanged products, leading to a pronounced stimulation of the overall reaction. These results are discussed in terms of the molecular mechanism of transformational recombination.**

Streptococcus pneumoniae is a naturally transformable bacterium that is able to take up DNA from its environment (in the form of ssDNA)[1] and incorporate this DNA into its chromosome (1, 2). It has been proposed that this process, known as transformational recombination, has evolved as a general mechanism that allows *S. pneumoniae* to change its genetic composition in response to environmental changes and stresses (3). For example, transformational recombination is believed to have contributed to the recent emergence of penicillin-resistance in clinical isolates of *S. pneumoniae* (4, 5).

Genetic studies have shown that transformational recombination is dependent on the presence of the *recA* gene, which encodes a DNA recombinase analogous to the RecA protein from *Escherichia coli* (2, 6). We recently developed an expression system and purification protocol for the *S. pneumoniae* RecA protein (7). The purified *S. pneumoniae* RecA protein (RecA(Sp)) has an ATP-dependent three-strand exchange activity that is generally similar to that of the *E. coli* RecA protein (RecA(Ec)) (7). In the standard three-strand exchange reaction, a circular ssDNA and a homologous linear dsDNA are recombined to form a nicked circular dsDNA and a linear ssDNA. This reaction proceeds in three phases. In the first phase, RecA protein polymerizes onto the circular ssDNA (1 RecA monomer/3 nucleotides of ssDNA), forming a helical nucleoprotein filament known as the presynaptic complex. In the second phase, the presynaptic complex interacts with a homologous linear dsDNA, and pairing between the circular ssDNA and the complementary strand from the linear dsDNA is initiated. In the third phase, the complementary linear strand is completely transferred to the circular ssDNA by unidirectional branch migration (in the 5′-3′ direction relative to the circular ssDNA) to yield the nicked circular dsDNA and displaced linear ssDNA products (8, 9).

The three-strand exchange activity of the RecA(Ec) protein is stimulated by the *E. coli* SSB protein, a homotetrameric, nonsequence-specific, single-stranded DNA-binding protein that is involved in many aspects of DNA biochemistry (10). It is believed that *E. coli* SSB protein (SSB(Ec)) stimulates strand exchange both presynaptically (by facilitating the binding of RecA(Ec) protein to the circular ssDNA substrate), and postsynaptically (by binding to the displaced linear strand that is generated when the circular ssDNA invades the linear dsDNA substrate) (8, 9). In our initial characterization of the RecA(Sp) protein, we found that its three-strand exchange activity was also strongly stimulated by SSB(Ec) protein (7). More recently, we isolated the SSB protein from *S. pneumoniae* (SSB(Sp)) and showed that this protein stimulates the RecA(Sp)-promoted three-strand exchange reaction in a manner similar to that of the SSB(Ec) protein (11).

Genetic studies have shown that transformational recombination is also dependent, at least in part, on the *mmsA* gene (12). The *mmsA* gene encodes a protein of 671 amino acids (75,188 Da) having a sequence that is 40% identical to the *E. coli* RecG protein (693 amino acids, 76,438 Da), a protein believed to be involved in the branch migration of recombinational intermediates (12). On the basis of this similarity, it has been proposed that the *S. pneumoniae* MmsA protein (MmsA(Sp)) may be involved in the branch migration of three-

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ϕX, bacteriophage ϕX174; SSB protein, single-stranded DNA-binding protein; DTT, dithiothreitol.

stranded intermediates formed by the RecA(Sp) protein during transformational recombination (12). To directly evaluate its mechanistic role in transformational recombination, we have developed an expression system and purification protocol for the MmsA(Sp) protein. These procedures, the biochemical properties of the purified MmsA(Sp) protein, and the effects of the MmsA(Sp) protein on the RecA(Sp) protein-promoted three-strand exchange reaction are described in this report.

EXPERIMENTAL PROCEDURES

Materials—*S. pneumoniae* RecA protein (7) and *S. pneumoniae* SSB protein (11) were prepared as described. ATP and [α-^{32}P]ATP were from Amersham Biosciences. Circular ϕX ssDNA (+ strand) and circular ϕX dsDNA were from New England Biolabs. The ssDNA oligomers, DNA I (5′-GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTG-CCCACCTGCAGGTTCACCC-3′), DNA II (5′-CAACGGCATAAAGCTT-GACGATTACATTGCTAGGACATGCTGTCTACAGGATCCGACTATC-GA-3′), DNA III (5′-TGGGTGAACCTGCAGGTGGGCAAAGATGTCC-TAGCAATGTAATCGTCAAGCTTTATGCCGTT-3′), and DNA IV (5′-C-AACGGCATAAACGTGACGATTACATTGCTAGGACATCTTTGCCCA-CCTGCAGGTTCACCC-3′), were from Invitrogen.

The three-stranded DNA junction was prepared by annealing DNA I, DNA II, and DNA III as described by Whitby and Lloyd (13). The fully paired dsDNA was prepared by annealing DNA III and DNA IV, and the partially paired dsDNA was prepared by annealing DNA I and DNA III. Linear ϕX dsDNA was prepared from circular ϕX dsDNA by *Pst*I digestion as described by Cox and Lehman (14). Linear ϕX ssDNA was prepared from circular ϕX ssDNA by annealing a complementary DNA oligomer (25 nucleotides) to the region corresponding to the *Pst*I site and then digesting it with *Pst*I. Nicked circular ϕX dsDNA was generated by a RecA protein-mediated strand exchange reaction between circular ϕX ssDNA and linear ϕX dsDNA (using the conditions described in the legend to Fig. 5), and was then purified by agarose gel electrophoresis. Single- and double-stranded DNA concentrations were determined by absorbance at 260 nm using the conversion factors 36 and 50 μg ml$^{-1}A_{260}^{-1}$, respectively. All DNA concentrations are expressed as total nucleotides.

Cloning the S. pneumoniae mmsA Gene—The *S. pneumoniae mmsA* gene was amplified from 2 μl of a saturated culture of R800 *S. pneumoniae* cells (generously provided by Dr. Jean-Pierre Claverys, Université Paul Sabatier, Toulouse Cedex, France) using *in situ* PCR and *Pfu* DNA polymerase as described by the manufacturer (Stratagene). The primers used (5′-GGATGGGAG*CATATG*AATCTACATCAACC-3′ *and* 5′-AAG*GGATCC*TTAGAGAAAGCTTAATCC-3′) corresponded to the 5′ and 3′ ends of the coding sequence (italics) of the *mmsA* gene and also contained the recognition sequences for the restriction enzymes *Nde*I and *Bam*HI (underlined).[2] The DNA product obtained from the polymerase chain reaction was digested with *Nde*I and *Bam*HI and ligated into pET-21a (Novagen) to give the final construct, pETmmsA(Sp). The insert was sequenced by the Johns Hopkins University DNA Sequencing Facility and found to be identical to the *mmsA* gene nucleotide sequence (GenBank™ accession number Z49988) (12).

Expression of S. pneumoniae MmsA Protein— The *S. pneumoniae mmsA* gene was expressed in *E. coli* strain BL21(DE3)pLysS (Novagen). Competent BL21(DE3)pLysS cells were transformed with pETmmsA (Sp) and selected for growth on LB/carbenicillin/chloramphenicol plates. A single BL21(DE3)pLys/pETmmsA(Sp) colony was used to inoculate LB broth (5 ml) containing carbenicillin (50 μg/ml) and chloramphenicol (34 μg/ml), and the resulting culture was incubated overnight at 37 °C. A portion of the culture (2 ml) was then used to inoculate 2 liters of LB broth/carbenicillin (50 μg/ml)/chloramphenicol (34 μg/ml), and the cells were grown at 37 °C to an A_{600} of 0.8. The cells were collected by centrifugation and resuspended in 2 liter of LB broth/carbenicillin (50 μg/ml)/chloramphenicol (34 μg/ml). Isopropyl-1-β-D-thiogalactopyranoside (1 mM) was added to induce expression of the *S. pneumoniae* MmsA protein, and the culture was incubated at 27 °C for 12 h. The cells (10 g) were collected by centrifugation, suspended in 50 ml of 50 mM Tris-HCl (pH 8.0)/20% sucrose/1 mM EDTA, and frozen in liquid nitrogen. The cell suspension was stored at – 80 °C.

Purification of S. pneumoniae MmsA Protein—All purification steps were carried out at 4 °C. The frozen BL21(DE3)pLys/pETmmsA(Sp) cell suspension (50 ml) was thawed on ice overnight; the cells lysed upon thawing because of the constitutive expression of T7 lysozyme from the pLysS plasmid (Novagen). The thawed suspension was then centrifuged at 100,000 × *g* for 60 min. The pellet was discarded, and 0.9 ml of Polymin-P (5%, pH 7.9) was added to the supernatant (35 ml). The suspension was mixed for 15 min and then centrifuged at 15,000 × *g* for 15 min. The supernatant was discarded, and the pellet was suspended in 35 ml of R buffer (20 mM Tris-HCl (pH 7.5)/10% glycerol/7 mM β-mercaptoethanol)/1.0 M NaCl. The suspension was centrifuged as described above. Ammonium sulfate (0.42 g/ml final concentration) was added to the supernatant, and the mixture was stirred for 1 h. The mixture was then centrifuged at 15,000 × *g* for 30 min. The pellet was dissolved in 8 ml of R buffer, and the mixture was dialyzed against 2 liters of R buffer/0.5 M NaCl. The dialyzed fraction was diluted with R buffer to a final NaCl concentration of 0.1 M (40 ml final volume) and then loaded onto a DEAE-Sepharose column (30 ml, Sigma) that had been equilibrated with R buffer/0.1 M NaCl. The column was then washed with R buffer/0.1 M NaCl. The protein-containing fractions (Bradford assay) were pooled and then loaded onto an SP-Sepharose column (30 ml; Sigma) that had been equilibrated with R buffer/0.1 M NaCl. The column was washed with 3 column volumes of R buffer/0.1 M NaCl and then eluted with a 100-ml linear gradient of R buffer/0.1–1.0 M NaCl. The protein-containing fractions (centered at 0.4 M NaCl) were pooled and then loaded onto a Sephacryl S-300 HR column (200 ml; Sigma) that had been equilibrated with R buffer/1 M NaCl. The fractions containing MmsA protein (identified by SDS-polyacrylamide gel electrophoresis) were pooled and dialyzed against 2 liters of R buffer/0.2 M NaCl. The dialyzed fraction was loaded onto a Progel-TSK Heparin-5PW column (Supelco) that had been equilibrated with R buffer/0.2 M NaCl, and it was then eluted with a linear gradient (30 ml) of R buffer/0.2–1.0 M NaCl. The fractions containing MmsA protein were pooled and dialyzed against 2 liters of R buffer (pH 8.5). The dialyzed fraction was loaded onto a MonoQ HR 5/5 FPLC column (Amersham Biosciences) that had been equilibrated with R buffer (pH 8.5), and was then eluted with a linear gradient (30 ml) of R buffer(pH 8.5)/0–1.0 M NaCl. The fractions containing MmsA protein (centered at 0.4 M NaCl) were pooled and dialyzed against 2 liters of storage buffer (20 mM Tris-HCl (pH 7.5)/20% glycerol/1 mM DTT/0.1 mM EDTA) to yield the final fraction (2 mg) of highly purified *S. pneumoniae* MmsA protein (Fig. 1).

The concentration of the purified *S. pneumoniae* MmsA protein was determined by UV absorbance at 280 nm using the extinction coefficient of 54,740 M^{-1}cm^{-1}, which was calculated from the amino acid sequence (12) using the formula of Gill and von Hippel (15). Amino-terminal protein sequencing of the purified *S. pneumoniae* MmsA protein was carried out by the Johns Hopkins Protein/Peptide Sequencing Facility. Matrix-assisted laser desorption ionization mass spectrometric analysis was carried out by the Applied Biosynthesis Mass Spectrometry Facility at the Johns Hopkins School of Medicine.

RESULTS

Preparation of the S. pneumoniae MmsA Protein—To develop a purification procedure for the MmsA(Sp) protein, we first used the polymerase chain reaction to amplify the *mmsA* gene from wild-type R800 *S. pneumoniae* cells. The sequence of our amplified gene was identical to that reported previously for the *S. pneumoniae mmsA* gene (12). We then cloned the *mmsA* gene into a pET21a expression vector and expressed the protein in *E. coli* strain BL21(DE3)pLysS. With this expression system, the MmsA(Sp) protein corresponded to ~5–10% of the total protein in the crude cell extract, and we were able to obtain 2 mg of highly purified protein (more than 99% homogeneity) from 10 g of cells (Fig. 1). The mobility of the purified MmsA(Sp) protein during polyacrylamide gel electrophoresis was consistent with a protein with a molecular mass of ~75,000 Da (Fig. 1). Mass spectrometric analysis of the purified MmsA(Sp) protein yielded a molecular mass of ~75,100 Da, in excellent agreement with the molecular mass of 75,188 Da predicted by the protein sequence (12). Furthermore, there was no indication of a peak at the position corresponding to the molecular mass of the *E. coli* RecG protein (76,438 Da; Ref. 16), demonstrating that the MmsA(Sp) protein preparation contained no detectable *E. coli* RecG protein. Amino-terminal protein sequencing confirmed that the preparation corresponded to the MmsA(Sp) protein.

[2] *S. pneumoniae* genome sequence data were obtained from the Institute for Genomic Research Web site at www.tigr.org.

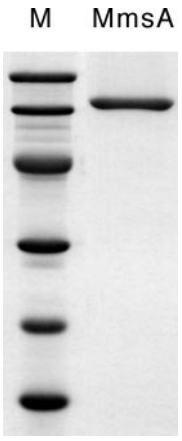

FIG. 1. **SDS-polyacrylamide gel electrophoresis of purified MmsA(Sp) protein.** The *gel lanes* contained purified MmsA(Sp) protein or molecular mass standards (*M*), as indicated. The acrylamide concentration was 5% in the stacking gel and 13% in the separating gel. The gel was stained in 0.1% Coomassie Brilliant Blue R-250.

MmsA(Sp) Protein-catalyzed DNA-dependent ATP Hydrolysis—The purified MmsA(Sp) protein was analyzed for DNA-dependent ATP hydrolysis activity at pH 7.5 and 37 °C. The initial set of reactions was carried out at a fixed concentration of MmsA(Sp) protein (0.3 μM) and ATP (4 mM), in the presence of a variety of oligomeric DNA effectors (1–75 μM). The oligomers that were used to construct these DNA effectors are described under "Experimental Procedures."

As shown in Fig. 2, the MmsA(Sp) protein exhibited ATP hydrolysis activity in the presence of each of the DNA effectors that was examined. The best effector was a partially paired dsDNA, with a maximal rate of ATP hydrolysis of ~4500 mol ATP hydrolyzed/min/mol MmsA protein being reached at DNA concentrations of 30 μM or higher (Fig. 2). The reaction was less efficient when either a fully paired dsDNA or a ssDNA oligomer was provided as the effector (Fig. 2). There was no detectable ATP hydrolysis by the MmsA protein in the absence of DNA. The preference of the MmsA(Sp) protein for the partially paired dsDNA effector is similar to the DNA specificity that has been reported for the *E. coli* RecG protein (17), and suggests that the MmsA(Sp) protein may interact favorably with ssDNA/dsDNA junctions.

The dependence of the MmsA(Sp) protein-catalyzed ATP hydrolysis reaction on ATP concentration was determined at a fixed concentration of MmsA(Sp) protein (0.3 μM) and a saturating concentration of the partially paired dsDNA effector (50 μM). The maximal rate of ATP hydrolysis under these conditions was ~4500 mol ATP hydrolyzed/min/mol MmsA protein, and the apparent K_m for ATP was ~0.7 mM (Fig. 3).

MmsA(Sp) Protein-promoted Unwinding of a Three-stranded DNA Junction—The MmsA(Sp) protein was examined for DNA junction-helicase activity at pH 7.5 and 37 °C, using an oligomeric three-stranded DNA junction substrate that was used previously with the *E. coli* RecG protein (13). In this three-stranded junction, two noncomplementary single-stranded oligonucleotides are each paired with a third single-stranded oligonucleotide; the 5′-half of the third strand is complementary to one of the noncomplementary strands and the 3′-half is complementary to other noncomplementary strand. The central 12 nucleotides of the third strand are complementary to both noncomplementary strands, thus allowing the three-stranded junction to branch migrate through this region. The oligomers that were used to construct the three-stranded junction are described under "Experimental Procedures."

The reaction that occurred when MmsA(Sp) protein (0.5 nM) was added to the three-stranded junction (500 nM) in the presence of ATP (5 mM) is shown in Fig. 4. In this reaction, the central strand of the three-stranded junction was ^{32}P-end labeled. The MmsA(Sp) protein was able to unwind the three-stranded junction to form either of the two partially paired dsDNAs (^{32}P-labeled) and the corresponding displaced linear ssDNAs (unlabeled) as reaction products. Neither the partially paired dsDNA nor a fully paired dsDNA substrate of the same

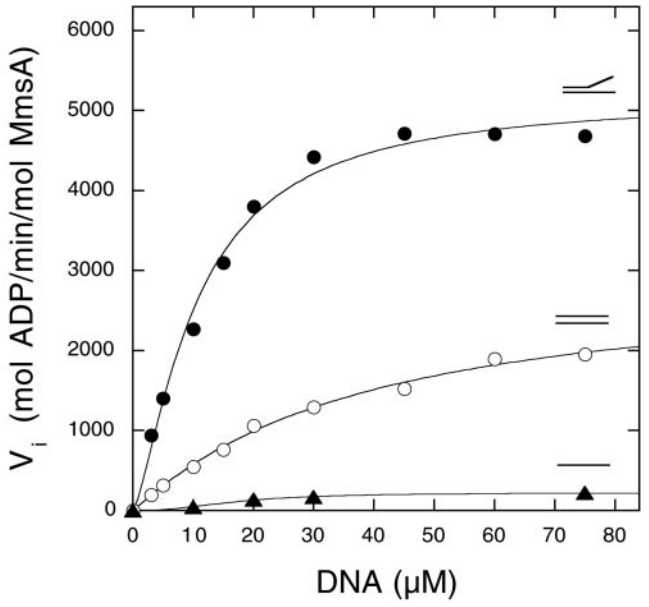

FIG. 2. **DNA-dependent ATP hydrolysis by the MmsA(Sp) protein.** The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.3 μM MmsA(Sp) protein, 4 mM [α-^{32}P]ATP, and the indicated concentrations of linear ssDNA (DNA I, *closed triangles*), fully paired dsDNA (DNA III·DNA IV, *open circles*), or partially paired dsDNA (DNA I·DNA III, *closed circles*). The reactions were carried out at 37 °C. ATP hydrolysis was measured using a thin-layer chromatography method as described previously (25). The *points* represent the initial rates of ATP hydrolysis that were measured at the indicated concentrations of DNA.

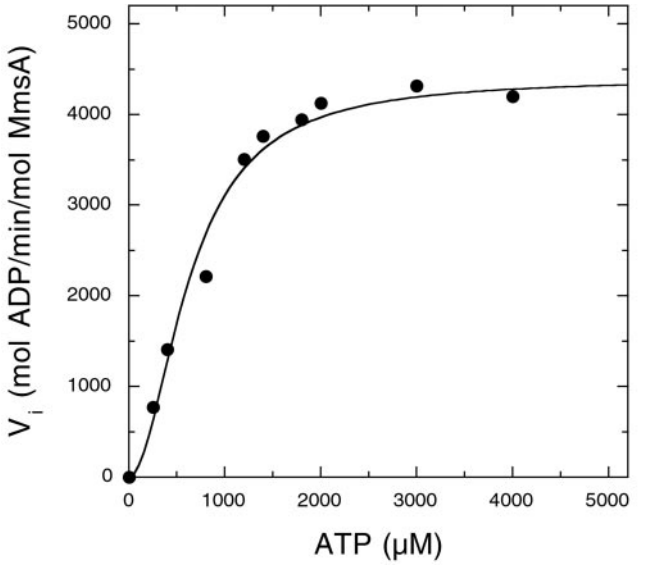

FIG. 3. **Dependence of MmsA(Sp) protein-catalyzed DNA-dependent ATP hydrolysis on ATP concentration.** The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.3 μM MmsA(Sp) protein, 50 μM partially paired dsDNA (DNA I·DNA III), and the indicated concentrations of [α-^{32}P]ATP. The reactions were carried out at 37 °C. ATP hydrolysis was measured using a thin-layer chromatography method as described previously (25). The *points* represent the initial rates of ATP hydrolysis that were measured at the indicated concentrations of ATP.

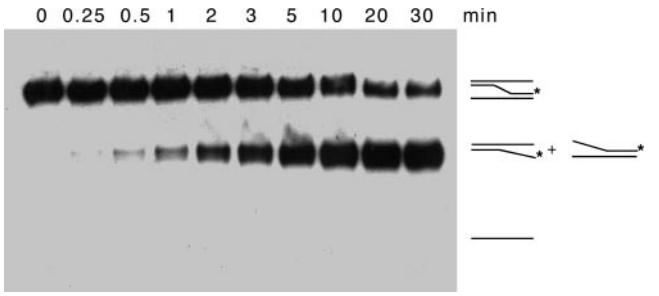

FIG. 4. **MmsA(Sp) protein-promoted unwinding of a three-stranded DNA junction.** The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 5 mM ATP, 500 nM three-stranded DNA junction substrate (^{32}P-end labeled on the central strand), and 0.5 nM MmsA(Sp) protein. The reaction was carried out at 37 °C. At the indicated times, an aliquot (20 μl) was removed from the reaction solution and quenched with SDS (1% final concentration) and EDTA (15 mM final concentration). The quenched aliquots were analyzed by electrophoresis on a 12% polyacrylamide gel using a Tris borate-EDTA buffer system. The substrates and products of the reaction were visualized by autoradiography. The positions of the three-stranded junction substrate and the partially paired dsDNA and linear ssDNA products are indicated.

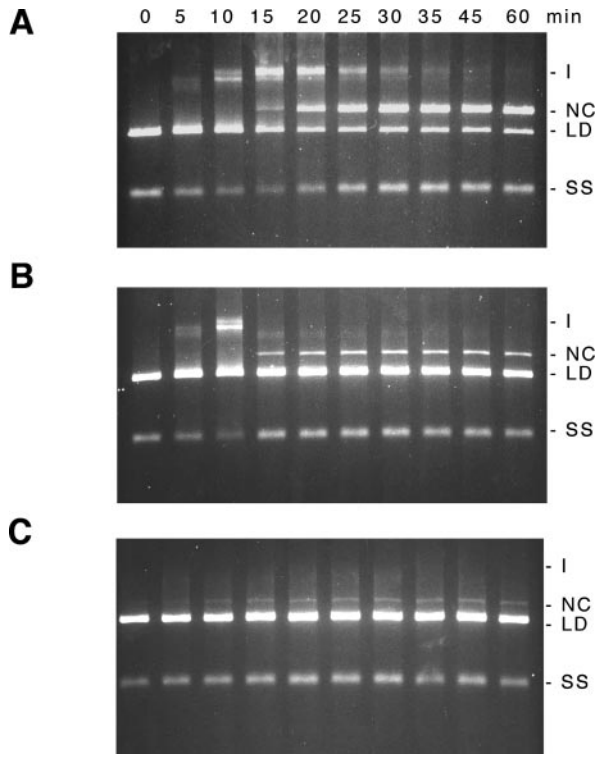

FIG. 5. **Effect of MmsA(Sp) protein on the RecA(Sp) protein-promoted three-strand exchange reaction (standard direction).** The reaction solutions contained 25 mM Tris acetate (pH 7.5), 10 mM Mg(acetate)$_2$, 5% glycerol, 1 mM DTT, 5 μM circular ϕX ssDNA, 15 μM linear ϕX dsDNA, 0.3 μM SSB(Sp) protein, 6 μM RecA(Sp) protein, and either 0 or 1 μM MmsA(Sp) protein. The reaction solutions also contained an ATP regeneration system consisting of 10 units (Sigma)/ml creatine kinase, 12 mM phosphocreatine, and 3 mM potassium glutamate. The reactions were initiated by the addition of ATP and SSB(Sp) protein and were carried out at 37 °C. At the indicated times following SSB(Sp) addition, an aliquot (20 μl) was removed from each reaction solution and quenched with SDS (1% final concentration) and EDTA (15 mM final concentration). The quenched aliquots were analyzed by electrophoresis on a 0.8% agarose gel using a Tris acetate-EDTA buffer system, and the substrates and products of the reactions were visualized by ethidium bromide staining. *A*, no MmsA(Sp) protein added; *B*, MmsA(Sp) protein (1 μM) added immediately following the 10 min time point; *C*, MmsA(Sp) protein (1 μM) added at the 0 min time point. *LD*, linear ϕX dsDNA; *NC*, nicked circular ϕX dsDNA; *SS*, ϕX ssDNA; *I*, partially exchanged DNA intermediates.

length was unwound under these conditions (gel not shown), indicating that the unwinding activity of the MmsA(Sp) protein was specific for the three-stranded junction. No unwinding of the three-stranded junction occurred if ATP was omitted from the reaction solution, indicating that the reaction was dependent on ATP (gel not shown). The ATP-dependent three-stranded junction-helicase activity of the MmsA(Sp) protein is similar to that of the *E. coli* RecG protein (13).

Effect of MmsA(Sp) Protein on RecA(Sp) Protein-promoted Three-strand Exchange (Standard Reaction)—The effect of the MmsA(Sp) protein on the strand exchange activity of the RecA(Sp) protein was examined at pH 7.5 and 37 °C, using the standard ATP-dependent three-strand exchange reaction (14). In this reaction, a circular ϕX ssDNA (5386 nucleotides) and a homologous linear ϕX dsDNA (5386 base pairs) are recombined to form a nicked circular ϕX dsDNA and a linear ϕX ssDNA; the substrates and products of this reaction are readily monitored by agarose gel electrophoresis (14). The reactions were carried out in the presence of SSB(Sp) protein, which strongly stimulates the strand exchange activity of the RecA(Sp) protein (11).

The three-strand exchange reaction that was promoted by the RecA(Sp) protein in the absence of MmsA(Sp) protein is shown in Fig. 5*A*. In this reaction, the formation of partially exchanged DNA intermediates was apparent within 5 min and the fully exchanged nicked circular dsDNA product could be detected within 15 min. The nicked circular dsDNA product accumulated to a maximal level after ~30 min. This reaction time course is consistent with our previously reported results (11).

The three-strand exchange reaction that was promoted by the RecA(Sp) protein in the presence of MmsA(Sp) protein is shown in Fig. 5, *B* and *C*. When MmsA(Sp) protein was added to the reaction after the partially exchanged intermediates had formed but before they had been converted into the fully exchanged nicked circular dsDNA product (10 min), the intermediates disappeared rapidly and the formation of the fully exchanged product was strongly inhibited (Fig. 5*B*). When MmsA(Sp) protein was added at the beginning of the reaction, there was no detectable accumulation of intermediates and the yield of the fully exchanged product was reduced further still (Fig. 5*C*). These results suggest that the partially exchanged intermediates that were formed by the RecA(Sp) protein were branch-migrated by the MmsA(Sp) protein in the direction opposite of the RecA protein-promoted strand exchange reaction (*i.e.* in the 3′-5′ direction relative to the circular ssDNA substrate), resulting in the reformation of the circular ssDNA and linear dsDNA substrates. A similar inhibitory effect has been reported for the *E. coli* RecG protein on the RecA(Ec) protein-promoted three-strand exchange reaction (13).

Effect of MmsA(Sp) Protein on RecA(Sp) Protein-promoted Three-strand Exchange (Reverse Reaction)—A consideration of the results described above suggested that the effect of the MmsA(Sp) protein on the three-strand exchange activity of the RecA(Sp) protein might depend on the manner in which the strand exchange reaction is carried out. In particular, we reasoned that if we carried out the reaction in the direction opposite of that of the standard three-strand exchange reaction (*i.e.* by starting with linear ϕX ssDNA and nicked circular ϕX dsDNA as our strand exchange substrates), then a MmsA(Sp) protein-promoted branch migration of the partially exchanged intermediates to form circular ssDNA and linear dsDNA would act to stimulate (rather than inhibit) the overall strand exchange reaction. To test this idea, we examined the RecA(Sp) protein-promoted three-strand exchange reaction between a linear ϕX ssDNA and nicked circular ϕX dsDNA in the pres-

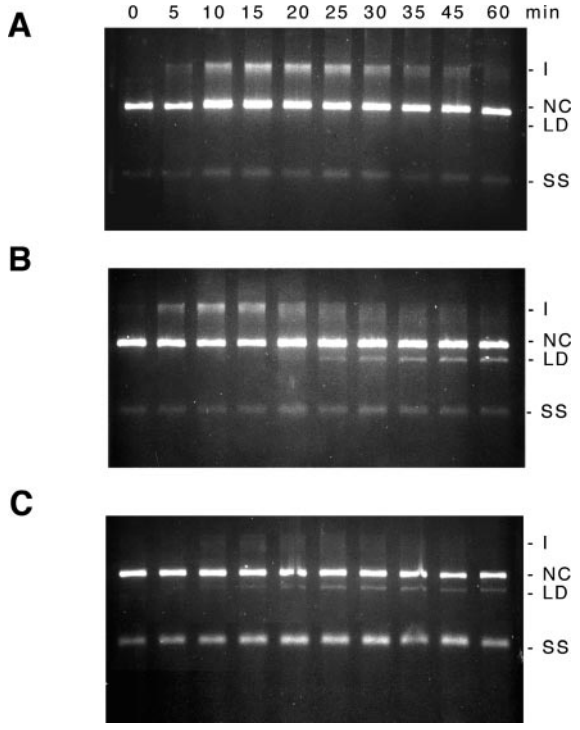

FIG. 6. **Effect of MmsA(Sp) protein on the RecA(Sp) protein-promoted three-strand exchange reaction (reverse direction).** The reaction solutions contained 25 mM Tris acetate (pH 7.5), 10 mM $Mg(acetate)_2$, 5% glycerol, 1 mM DTT, 5 μM linear ϕX ssDNA, 15 μM nicked circular ϕX dsDNA, 0.3 μM SSB(Sp) protein, 6 μM RecA(Sp) protein, and either 0 or 1 μM MmsA(Sp) protein. The reaction solutions also contained an ATP regeneration system consisting of 10 units (Sigma)/ml creatine kinase, 12 mM phosphocreatine, and 3 mM potassium glutamate. The reactions were initiated by the addition of ATP and SSB(Sp) protein and were carried out at 37 °C. At the indicated times following SSB(Sp) addition, an aliquot (20 μl) was removed from each reaction solution and quenched with SDS (1% final concentration) and EDTA (15 mM final concentration). The quenched aliquots were analyzed by electrophoresis on a 0.8% agarose gel using a Tris acetate-EDTA buffer system, and the substrates and products of the reactions were visualized by ethidium bromide staining. *A*, no MmsA(Sp) protein added; *B*, MmsA(Sp) protein (1 μM) added immediately following the 15 min time point; *C*, MmsA(Sp) protein (1 μM) added at the 0 min time point. *LD*, linear ϕX dsDNA; *NC*, nicked circular ϕX dsDNA; *SS*, ϕX ssDNA; *I*, partially exchanged DNA intermediates.

ence and absence of MmsA(Sp) protein (this reaction will be referred to below as the reverse three-strand exchange reaction).

The reverse three-strand exchange reaction that was promoted by the RecA(Sp) protein in the absence of MmsA(Sp) protein is shown in Fig. 6*A*. In this reaction, the formation of partially exchanged intermediates was apparent within 5 min and these intermediates accumulated to a maximum level after ~15 min. In contrast to the results obtained in the standard reaction, however, the partially exchanged intermediates were not converted to fully exchanged products in the presence of RecA(Sp) protein alone (Fig. 6*A*). When MmsA(Sp) protein was added to the reaction after the partially exchanged intermediates had formed (15 min), however, the intermediates disappeared rapidly, and the formation of the fully exchanged linear dsDNA product was apparent within 10 min after MmsA(Sp) protein addition (Fig. 6*B*). When MmsA(Sp) protein was added at the beginning of the reaction, there was no detectable accumulation of intermediates, but the formation of linear dsDNA was still detected (Fig. 6*C*). No strand exchange products were formed in the presence of MmsA(Sp) protein if RecA(Sp) protein was omitted from the reaction solution (gel not shown). These results suggest that the partially exchanged intermediates that were formed by the RecA(Sp) protein were branch-migrated by the MmsA(Sp) protein in the direction opposite of the RecA protein-promoted strand exchange reaction (*i.e.* in the 3′-5′ direction relative to the linear ssDNA substrate) to form the linear dsDNA product.

DISCUSSION

The MmsA(Sp) protein has DNA-dependent ATP hydrolysis and DNA junction-helicase activities that are similar to those of the RecG protein from *E. coli*. These findings provide biochemical support for the previous proposal (based on sequence similarity and genetic evidence) that the MmsA(Sp) protein is an *S. pneumoniae* homolog of the *E. coli* RecG protein (12). Recent studies suggest that the *E. coli* RecG protein may be involved in the recombinational repair of stalled replication forks (18), and it is possible that the MmsA(Sp) protein will be found to play a similar role in *S. pneumoniae*, although this has not yet been investigated. It has been shown, however, that a null mutation in the *mmsA* gene leads to marked reduction in

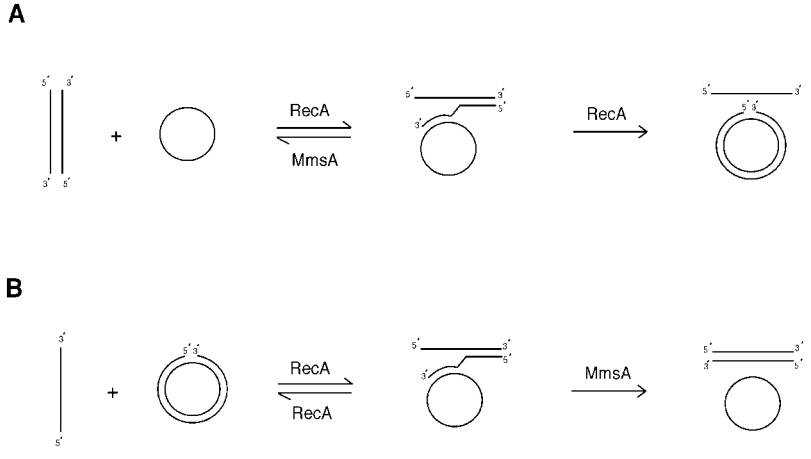

FIG. 7. **Hypothetical representations of the standard and reverse three-strand exchange reactions.** *A*, standard three-strand exchange reaction. A circular ssDNA and a homologous linear dsDNA are initially paired by the RecA(Sp) protein (via the 3′-end of the complementary strand of the linear dsDNA) to form a three-stranded intermediate. The three-stranded intermediate can then be branch-migrated in the 5′-3′ direction (relative to the circular ssDNA) by the RecA(Sp) protein to form the nicked circular dsDNA and linear ssDNA products or in the 3′-5′ direction by the MmsA(Sp) protein to reform the circular ssDNA and linear dsDNA substrates. *B*, reverse three-strand exchange reaction. A linear ssDNA and a homologous nicked circular dsDNA are initially paired by the RecA(Sp) protein (via the 3′-end of the linear ssDNA) to form a three-stranded intermediate. The three-stranded intermediate can then be branch-migrated in the 3′-5′ direction (relative to the linear ssDNA) by the MmsA(Sp) protein to form the circular ssDNA and linear dsDNA products or in the 5′-3′ direction by the RecA(Sp) protein to reform the linear ssDNA and nicked circular dsDNA substrates.

transformational recombination in *S. pneumoniae*, indicating that the MmsA(Sp) protein plays a direct role in this process (12).

The first step in transformational recombination involves the binding of exogenous dsDNA to one of the specific uptake sites on the surface of a *S. pneumoniae* cell (19). The DNA is initially cleaved into fragments (several kb in size) by a surface-bound nuclease. One strand of the dsDNA fragment is then degraded further into smaller oligonucleotides, whereas the complementary linear ssDNA is transported through the cell wall and membrane into the cell cytosol (19). It is presumed that the RecA(Sp) protein will then mediate the assimilation of this ssDNA into a homologous region of the double-stranded *S. pneumoniae* chromosome (20).

The means by which the MmsA(Sp) protein would facilitate the RecA(Sp) protein-mediated transformational recombination reaction has not been clear inasmuch as it has been reported that the *E. coli* RecG protein inhibits the three-strand exchange activity of the RecA(Ec) protein (12–13, 20). Although our results indicate that the MmsA(Sp) protein can similarly inhibit the three-strand exchange activity of the RecA(Sp) protein, this inhibitory effect was observed only when the strand exchange reaction was carried out in the standard manner, with a circular ssDNA and a linear dsDNA as the starting substrates (Fig. 7*A*). In this direction, the circular ssDNA substrate can pair with the 3′-end of the complementary strand of the linear dsDNA substrate. The branch migration activity of the RecA(Sp) protein can then extend this three-stranded junction in the 5′-3′ direction (relative to the circular ssDNA substrate) to form the nicked circular dsDNA and linear ssDNA products (8, 9). When MmsA(Sp) protein is included in the reaction, however, the initial partially exchanged intermediates may be branch-migrated in the direction opposite of the RecA(Sp) protein to regenerate the starting circular ssDNA and linear dsDNA substrates (Fig. 7*A*). In contrast, if the three-strand exchange reaction is carried out in the reverse direction, with a linear ssDNA and a nicked circular dsDNA as the starting substrates, a MmsA(Sp) protein-promoted branch migration of the partially exchanged intermediates to form circular ssDNA and linear dsDNA would have the effect of stimulating (rather than inhibiting) the overall reaction (Fig. 7*B*).

It has been reported that the RecA(Ec) protein was unable to promote a strand exchange reaction between linear ssDNA and gapped circular dsDNA substrates that were similar to those used in our reverse strand exchange reactions (21). Our results are consistent with this earlier report in that they show that although the RecA(Sp) protein is able to form initial pairing intermediates between a linear ssDNA and nicked circular dsDNA, it is unable to readily extend these intermediates to form fully exchanged products. The incomplete reaction that was observed in the reverse direction may be due to the linear nature of the ssDNA substrate. Because RecA protein polymerizes on ssDNA in the 5′-3′ direction and dissociates from the 5′-end of the RecA-ssDNA filament, the 3′-end of the linear ssDNA substrate will be preferentially covered with RecA protein and will therefore be more likely to invade the nicked circular dsDNA (8, 9). If the 3′-end of the linear ssDNA invades the dsDNA, however, the branch migration activity of the RecA protein (which is 5′-3′ relative to the linear ssDNA) will be unable to extend this heteroduplex junction (Fig. 7*B*). When the MmsA(Sp) protein is included in the reaction, however, it appears to facilitate the branch migration of these junctions in the 3′-5′ direction (relative to the linear ssDNA), leading to the formation of the fully exchanged linear dsDNA product (Fig. 7*B*).

Because transformational recombination is believed to involve the recombination of a linear ssDNA into the double-stranded *S. pneumoniae* chromosome (19), the reverse three-strand exchange reaction may be more appropriate than the standard reaction as a model reaction for this process. In fact, the sequential reaction that we describe here, in which an initial RecA(Sp) protein-mediated invasion of a linear ssDNA into a homologous dsDNA is followed by a MmsA(Sp) protein-mediated branch migration of the three-stranded intermediate, is consistent with a hypothetical model for transformational recombination that was envisioned by Claverys and colleagues (12, 20). However, although our results suggest that the RecA(Sp) and MmsA(Sp) protein can act together to carry out a complete strand exchange reaction between a polymeric linear ssDNA and homologous dsDNA, the efficiency of this coupled reaction is relatively low under our present reaction conditions. In particular, a comparison of the reaction time courses in Figs. 5 and 6 indicates that the yield of initial pairing intermediates is lower in the reverse reaction than in the standard reaction. This is likely due to an inefficient assembly of RecA(Sp) protein on the linear ssDNA substrate. The three-strand exchange activity of the RecA(Sp) protein is strongly stimulated by SSB(Sp) protein (11) and SSB(Sp) protein was included in the reactions shown in Figs. 5 and 6. The RecA(Sp) protein differs from the RecA(Ec) protein, however, in that its ssDNA-dependent ATP hydrolysis activity is completely inhibited by SSB(Sp) protein, apparently because SSB(Sp) protein displaces RecA(Sp) protein from ssDNA (22). These results indicate that in contrast to the mechanism that has been established for the RecA(Ec) protein, SSB(Sp) protein does not facilitate the formation of a presynaptic complex between the RecA(Sp) protein and the ssDNA substrate. Instead, the stimulatory effect of SSB(Sp) protein in the RecA(Sp) protein-promoted strand exchange reaction may be due entirely to the postsynaptic binding of the displaced single strand that is generated when the ssDNA substrate invades the homologous linear dsDNA. The competing displacement of RecA(Sp) protein from the ssDNA substrate by SSB(Sp) protein, however, will reduce the efficiency of strand invasion and intermediate formation (22). These findings suggest that the efficient assembly of RecA(Sp) protein on the linear ssDNA substrate may require additional recombination accessory proteins, perhaps analogous to the *E. coli* RecO and RecR proteins, which have been shown to facilitate the assembly of the RecA(Ec) protein on SSB(Ec) protein-covered ssDNA (23, 24). A search for these putative recombination accessory proteins as well as studies aimed at more clearly defining the mechanistic nature of the interaction between the RecA(Sp) and MmsA(Sp) proteins at the three-stranded junction are in progress.

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