Expression, purification and biochemical characterization of *Listeria monocytogenes* single stranded DNA binding protein 1

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**ABSTRACT**

Single-stranded DNA binding proteins play an important role in DNA metabolic processes including replication, recombination, and repair. Here, we report the identification and biochemical characterization of the SSB1 protein from the foodborne pathogen *Listeria monocytogenes*. The *L. monocytogenes* SSB1 share 33% identity and 50.5% similarity with the prototype *E. coli* SSB protein. The electrophoretic mobility shift assay revealed that the purified *L. monocytogenes* SSB1 protein binds to single stranded DNA, including the M13 circular single stranded DNA and oligonucleotide, with high affinity. The plasmid based strand transfer activity showed that, in the absence of the SSB protein, *L. monocytogenes* RecA fails to catalyze the reaction whereas, the *E. coli* RecA protein has shown nicked DNA formation. Interestingly the addition of SSB1 protein stimulates both *L. monocytogenes* and *E. coli* RecA strand transfer activities however, it is sensitive to the order of addition of SSB1 protein. *L. monocytogenes* RecA fails to catalyze the reaction when SSB1 is added prior to RecA; nevertheless, it readily catalyzes the reaction when added after the RecA filament formation. These results suggest that the interaction among of gene product between RecA and SSB1 is required to promote optimum strand exchange activities. Altogether, these studies provide the first functional characterization of the *L. monocytogenes* SSB1 protein and give insights into DNA repair and recombination processes in the gram-positive foodborne pathogen *L. monocytogenes*.

**1. Introduction**

*Listeria monocytogenes* is a gram-positive, rod-shaped, a facultative intracellular foodborne pathogen that causes listeriosis among humans, with an average fatality rate of 20–30% in hospitalized patients [1,2]. *L. monocytogenes* is highly contagious and is mainly transmitted through food such as poultry, meat, and dairy products especially in pre-packaged ready-to-eat products [3]. This pathogen can adapt and grow under different stress conditions such as low pH, high osmolality during food processing and gastrointestinal stress during host infection [4]. The extreme environmental conditions lead to stress-induced DNA damage. Therefore, it is necessary to understand the mechanical aspects of DNA recombination and repair machinery in *L. monocytogenes*.

A key DNA repair mechanism is homologous recombination (HR); it also plays a major role in the maintenance of an organism’s genome [5]. In the initial stage of the HR process, helicase and nuclease enzymes act upon double stranded DNA (dsDNA) to expose single stranded DNA (ssDNA). The transiently exposed ssDNA is highly prone to nuclease attack, which subsequently leads to degradation; they were protected by single stranded DNA binding proteins (SSBs) [6,7]. SSBs are conserved across all kingdoms, and despite their divergent structures, they serve a common function [8,9]. RecA promoted HR involves SSB protein in both the early and later stages in vitro [6]. In the early phase of the HR process, SSB removes the secondary structure of ssDNA and coordinates the assembly of RecA to ssDNA to form a contiguous nucleoprotein filament [10–12]. In the later stage of the HR process, SSB helps in sequestering the displaced ssDNA to prevent its participation in the reverse strand exchange reaction [13]. The structural analysis of the prototype *E. coli* SSB revealed the presence of an N-terminal DNA-binding domain that contains oligonucleotide/oligosaccharide-binding (OB) domains, a C-terminal domain responsible for protein–protein interaction and a non-conserved intrinsically disordered linker (IDL) [8].

Though there are few reports on the regulation of SOS response in *L. monocytogenes* [14 and references therein], there is a lack of in-depth studies of the HR process and regulation of the different proteins involved in this pathway. Further unlike the prototype *E. coli* that has only one SSB protein, *L. monocytogenes* has two SSB paralogs: SSB1 and SSB2. Therefore, in the present investigation, we aimed to characterize...
the *L. monocytogenes* SSB1 protein and to understand role of SSB in the mechanistic aspects of DNA strand exchange, which is a central step in the HR processes, promoted by *L. monocytogenes* RecA (LmRecA) in comparison with *E. coli* RecA (EcRecA). Accordingly, the *L. monocytogenes* SSB1 (LmSSB1) protein has been cloned, expressed and purified near to homogeneity. The purified protein robustly binds to ssDNA showing its canonical activity. Further, our findings suggest striking mechanistic differences in the strand exchange promoted by LmRecA and EcRecA in the presence of the LmSSB1 protein. The two prominent differences were observed: First, in the absence of the LmSSB1 protein, LmRecA fails to catalyze the strand exchange reaction whereas EcRecA shown its activity. Second, the addition of LmSSB1 in the strand exchange reaction stimulates the activities of both LmRecA as well as EcRecA but sensitive to the order of addition of the SSB1 protein in the reaction. The stimulation effect of the LmSSB1 protein for strand exchange activity happens when it is added to the reaction after RecA forms the nucleoprotein filament and, not prior to the addition RecA. Together, these results provide insights into the role of the LmSSB1 protein in homologous recombination and its interaction with RecA in foodborne pathogen *L. monocytogenes*.

2. Materials and methods

2.1. Chemicals, enzymes, bacterial strains, and DNA

The routine chemicals and media were obtained from Himedia laboratories, Mumbai, India. The fine chemicals for this study were of analytical grade and procured from Sigma-Aldrich, USA. The restriction enzymes, *pfu* polymerase, and T4 DNA ligase were obtained from New England Biolabs. The genomic DNA kit was obtained from Sigma. The plasmid isolation kit, DNA gel extraction kit, and Ni²⁺-NTA resin were acquired from Qiagen. The agaroose was procured from Lonza. The primers for cloning and oligonucleotides (ODN) used in this study were purchased from Sigma-Genosys. The OND were as follows: 80-mer primer, 5′-ATATGGATCCATGATGAATCGTGTAGTACTTG-3′; 3′; 3′ primer, 5′-ATATAAGCCTTTAGAATGGCAAATCGTC-3′. The PCR product was purified through the PCR clean-up kit and both the plasmid and PCR product were kept for double digestion with restriction enzymes. The digested product was then processed by phenol-chloroform extraction followed by ethanol precipitation of DNA. The 537 base pair *ssb* gene product was ligated into the vector pRSET A, having N-terminus 6xHis-tag in between the two restriction sites BamHI and HindIII. The recombinant plasmid designated as pLMSSB1, was confirmed by restriction mapping and nucleotide sequencing.

2.4. Expression and purification of *L. monocytogenes* SSB1

The pLMSSB1 construct was transformed into the *E. coli* Rosetta2(DE3)pLyS expression host for overexpression of the LmSSB1 protein. The overnight grew culture was inoculated into a freshly prepared LB medium containing ampicillin and chloramphenicol to a final concentration of 100 μg ml⁻¹ and 34 μg ml⁻¹ respectively and then incubated at 37 °C. At *A₆₀₀* = 0.4, Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to the final concentration of 0.5 mM and further incubated for 4 h for the expression of LmSSB1. The cells were harvested by centrifugation at 6000 rpm for 15 min at 4 °C. The pellet was washed with buffer A containing 10 mM Tris hydrochloride (Tris-HCl) (pH 7.5), 100 mM sodium chloride (NaCl) and 1 mM ethylenediaminetetraacetic acid (EDTA). The washed cells were suspended in buffer B containing 50 mM Tris-HCl (pH 8), 0.2 M NaCl, 1 mM EDTA and 10% glycerol; this was then flash frozen and stored at −80 °C until use. For purification of SSB1, the thawed cells were lysed by sonication (Vibra Cell Sonicator, Sonics and Materials Inc, USA) in pulse mode (50% duty cycle) for 30 min. The lysed cells were clarified by centrifugation at 30,000 rpm for 60 min at 4 °C (Beckman counter). The supernatant was loaded onto a Ni²⁺-NTA column (5 ml) and extensively washed with buffer B containing 20 mM imidazole. The protein bound to the matrix was eluted using a linear gradient of 20–500 mM imidazole in buffer B. The active fractions containing LmSSB1 protein were pooled and precipitated with 0.2 g/mL ammonium sulfate. The pellet was collected by centrifugation at 14,000 rpm for 30 min at 4 °C. The protein was dialyzed against buffer B for 18 h with three changes at every 6 h. The dialyzed protein was passed through a Sephacryl S-100 column (GE Healthcare) that was pre-equilibrated with buffer B. The fractions containing highly pure LmSSB1 protein were subjected to precipitation with ammonium sulfate (0.2 g/mL). The pellet was collected by centrifugation at 14,000 rpm for 30 min at 4 °C and dialyzed against the...
storage buffer containing 20 mM Tris-HCl, (pH 8), 0.5 M NaCl, 1 mM EDTA, 50% glycerol and 1 mM 2-mercaptoethanol for 12 h. The concentration of protein was estimated by the Coomassie dye binding method, in which bovine serum albumin (BSA) was used as an internal standard [18]. Small aliquots of the protein sample were stored at −20 °C. The quality of the protein was assessed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. The protein was free from endo- and exonuclease activities.

2.5. Electrophoretic mobility shift assay

The DNA binding experiment was performed with both the longer plasmid-based substrate as well as the shorter oligonucleotide. The assay was performed as previously described [19,20]. Briefly: The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl or 200 mM NaCl, 3 μM of 6-FAM labelled oligonucleotide (34-mer or 80-mer) or 10 μM of M13 ctsDNA and increasing concentrations of the LmSSB1 protein. The reaction mixture was incubated at 37 °C for 10 min and 30 min with the oligonucleotide and M13 ctsDNA respectively. The oligo-based assay was subjected to 8% native polyacrylamide gel in 89 mM Tris/borate buffer (pH 8.3) at 150 V for 3 h.
The gels were directly visualized using the Typhoon FLA-9500 phosphorimager (GE Healthcare). The bands were quantified using Gene tools from Syngene software and plotted using GraphPad Prism (ver.6.0). The M13 plasmid-based reaction was electrophoresed on ethidium bromide (EtBr) free 0.8% agarose gel in 89 mM Tris/borate buffer (pH 8.3) at 2 V/cm for 4 h. The gels were stained in EtBr solution (0.2 μg/mL) and visualized using the Uvitec Cambridge gel documentation unit.

2.6. DNA three-strand exchange reaction

The DNA three-strand exchange reaction was carried out as previously described [21]. Briefly: The reaction mixture (40 μl) contained 20 mM Tris-HCl (pH 7 for LmRecA, 7.5 for EcRecA), 3 mM adenosine triphosphate (ATP), 10 mM MgCl2, 5 mM phosphocreatine, 10 U/ml phosphocreatine kinase, 1 mM DTT, 10 μM M13 cssDNA and 5 μM LmRecA or EcRecA. The reaction mixture was incubated at 37 °C for 5 min followed by the addition of 0.66 μM LmSSB1, and the incubation was continued for an additional 5 min. The strand exchange reaction was initiated by the addition of 10 μM M13 linear dsDNA and incubated for 120 min at 37 °C. The reaction was stopped by the addition of 4 μl 5X stop buffer (5% SDS and 100 mM EDTA) and deproteinized using proteinase K (0.2 mg/mL) at 37 °C for 15 min. The sample was electrophoresed in EtBr-free 0.8% agarose gel in 89 mM Tris/borate buffer (pH 8.3) at 2 V/cm for 11 h. The gel was stained in EtBr solution (0.2 μg/mL) and visualized through the gel documentation unit. The bands were quantified using the Gene tools from Syngene software.

3. Results

3.1. Bioinformatics analysis of L. monocytogenes SSB1 protein

A BLAST (basic local alignment search tool) search showed the presence of two putative SSB proteins, in L. monocytogenes, namely, SSB1 (178 amino acids) and SSB2 (160 amino acids). The amino acid sequence alignment of the L. monocytogenes SSB1 protein shares 33.0, 33.0, 59.6 and 69.1% identity and 50.5, 50.5, 72.7 and 78.5% similarity with E. coli, Shigella flexneri, Staphylococcus aureus, and Bacillus cereus respectively (Fig. S1). A close inspection of the length of amino acids show that LmSSB1 possesses same length as that of E. coli SSB; i.e., 178 residues, whereas LmSSB2 has 160 residues. The sequence identity and similarity between E. coli SSB and LmSSB1 is as indicated above, whereas between E. coli SSB and LmSSB1 is 29.8% and 49.4% respectively. L. monocytogenes SSB1 and SSB2 share 71.0% identity and 78.1% similarity in their amino acid sequence alignments (Fig. S2). Hence, it would be appropriate to characterize LmSSB1 protein.

3.2. Gene cloning and purification of L. monocytogenes SSB1

To investigate the functional characteristics of the LmSSB1 protein, the plasmid pLMSSB1 bearing the ssb gene was transformed in E. coli Rosetta2 (DE3)pLysS cells. The SSB protein was overexpressed by the addition of IPTG as described in ‘Materials and methods’. The protein was purified using Ni2+–NTA affinity and size exclusion chromatography. The purified protein migrated as a single band in 12.5% SDS-PAGE (Fig. 1A) and its corresponding molecular weight (19,493 Da) was calculated from the protein sequence. The purified protein yield was in the range of 2–2.5 mg from 1 liter of culture. Further, the identity of the protein was confirmed by immunoblot analysis (Fig. 1B) using anti-His antibody.

3.3. DNA binding properties of L. monocytogenes SSB1 protein

The DNA binding activity of the LmSSB1 protein was analyzed by using the electrophoretic mobility shift assay. M13 cssDNA and linear dsDNA were incubated with an increased concentration of LmSSB1
protein as described in ‘Materials and methods’. The LmSSB1 clearly binds to ssDNA (Fig. 2A); it was seen that the DNA protein complex migrates in agarose gel slower than the free DNA with increasing concentrations of protein; however, it fails to bind with the duplex (Fig. 2B). Further, to check the binding mode of LmSSB1, fixed concentrations of oligonucleotides of different lengths (34-mer and 80-mer) was incubated with increasing concentrations of protein in different salt concentrations. As seen in Fig. 2C, E, in the presence of different salt concentrations (100 mM NaCl and 200 mM NaCl), the binding affinity of LmSSB1 is concentration-dependent and exhibits saturable protein binding with both 34 and 80-mer oligonucleotides. The apparent $K_D$ value was 0.13 μM and 0.20 μM with the 34-mer oligonucleotide in 100 mM and 200 mM NaCl respectively (Fig. 2D). The observations are consistent with earlier studies with the E. coli SSB protein under different salt concentrations [22].

3.4. Effect of L. monocytogenes SSB1 on DNA three-strand exchange activity

To understand the mechanistic aspects of the effect of LmSSB1 protein on LmRecA-promoted strand transfer activity in vitro, we performed a chemical forward reaction, the three strand exchange assay using M13 cssDNA and linear dsDNA as substrates. The RecA-promoted strand exchange would result in the generation of joint molecule intermediates and the formation of nicked DNA. The scheme of the experimental approach is shown in Fig. 3A.

Three independent experiments were designed: a. in the absence of the LmSSB1 protein, b. LmSSB1 added prior to the RecA protein and c. LmSSB1 added after the RecA protein. In the absence of LmSSB1, LmRecA failed to catalyze the reaction; on the other hand, EcRecA effectively catalyzed the reaction with almost 48% nicked DNA formation after 2 h of incubation (Fig. 3B and C).

Further, in the second experiment, the effect of LmSSB1 addition to the reaction prior to the RecA protein was examined. The experiment was done as described in ‘Materials and methods’ with a minor modification in experimental conditions: initially, M13 ccssDNA was incubated with the LmSSB1 protein followed by the addition of RecA in the reaction (Fig. 4A). As seen in Fig. 4B EcRecA effectively catalyzed the reaction with 48% nicked DNA formation whereas the LmRecA activity was reduced and it did not show any nicked DNA formation as shown in Fig. 4C. The alleviation of inhibition may be due to the partial displacement of the SSB protein from ssDNA by the RecA protein. It has been shown that in vivo the RecFOR complex replaces the SSB protein and stabilizes the RecA nucleoprotein filament for strand transfer activity [21].

In a parallel experiment, when LmSSB1 was added after the addition of LmRecA protein in the reaction (Fig. 5A), the activities of both LmRecA, as well as EcRecA proteins, were effectively enhanced. As seen in Fig. 5B EcRecA in non-cognate presence of LmSSB1 showed 100% nicked DNA formation within 30 min of the incubation period whereas, LmRecA with LmSSB1 generated joint molecules in the initial stage of the reaction with 71% nicked DNA formation after 2 h of incubation as shown in Fig. 5C, producing strand exchange products. The above results suggest that LmRecA-promoted strands transfer requires the specific interaction with LmSSB1 in vitro for optimum activity. However, the possibility that different experimental conditions are needed to detect the consequence of LmRecA and LmSSB1 interaction on strand transfer activity cannot be excluded. Further, there is a possibility that
the specific interaction between LmRecA and its cognate SSB plays an important role in the early stage of the HR process in L. monocytogenes.

4. Discussion

In all DNA metabolic processes, the SSB protein is responsible for recruiting a number of DNA binding proteins to ssDNA and for initiating replication, recombination, and repair [22]. The E. coli SSB was one of the first SSB proteins that was extensively studied functionally as well as structurally and became the prototype to study other proteins in this class [24–26]. Over time, a large family of SSBs has been characterized however a very few studies are available on foodborne pathogens. It has been shown that there are three SSB paralogs present in the foodborne pathogen S. aureus (SSB1, SSB2, and SSB3) [26] whereas, in L. monocytogenes, there are only two SSBs paralogs (SSB1 and SSB2). The amino acid sequence alignment that was performed in the present study shows that L. monocytogenes SSB1 shares 30–70% identity and 50–70% similarity with the prototype E. coli SSB and other foodborne pathogens such as S. aureus, B. cereus, and S. flexneri. The primary activity of SSB is to bind to the ssDNA during DNA metabolism with high affinity and low sequence specificity. The E. coli SSB can bind to ssDNA in at least three distinct binding modes (SSB)n, where the SSB tetramer can bind to n = 35, 56, and 65 nucleotides [7]. The binding is also influenced by the temperature of the solution, pH, and presence of monovalent and divalent cations, anions and free SSB concentration. There are also two different positive cooperative bindings of SSB to ssDNA that have been observed which correlates with different salt concentrations.

The prototype E. coli RecA can promote strand exchange reactions in vitro in the presence of cognate and non-cognate SSB to perform strand transfer activities [21]. The Streptococcus pneumoniae RecA shows a very weak strand transfer activity in the absence of the SSB protein [29]. In the present study, we observed that the activity of LmRecA in plasmid-based strand transfer assay depends on the presence of the LmSSB, suggesting that the SSB protein might remove the secondary structure of DNA, and allow the formation of contagious RecA.
nucleoprotein filaments that lead to RecA promoted strand transfer activity. The earlier, studies suggested that in the presence of the SSB protein, the RecA nucleoprotein filaments that are formed are more stable, remain active and extended comparatively to those formed with RecA alone. It has been also shown that the highly acidic C-terminus of E. coli SSB not only interacts with other DNA binding proteins but also regulates the RecA filament assembly [30]. The truncation mutation at the C-terminus of EcSSB (SSBΔC) decreases the efficiency of the RecA assembly to ssDNA [31]. Considering the experimental observations, we were suggesting that LmSSB1 may regulate the nucleoprotein filament formation of the LmRecA protein, and thereby promote the RecA driven recombination and repair functions. Altogether, in the current study, we demonstrated the identification and functional characterization of the LmSSB1 protein in conjunction with the cognate RecA protein provides an insight into the specific interaction to facilitate the RecA promoted recombination process in the deadly foodborne pathogen, L. monocytogenes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2019.04.007.

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