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Expression and purification of the SsbB protein from *Streptococcus pneumoniae*

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Abstract

The Gram positive bacterium, *Streptococcus pneumoniae*, has two genes, designated *ssbA* and *ssbB*, which are predicted to encode single-stranded DNA binding proteins (SSB proteins). We have shown previously that the SsbA protein is similar in size and in biochemical properties to the well-characterized SSB protein from *Escherichia coli*. The SsbB protein, in contrast, is a smaller protein and has no counterpart in *E. coli*. This report describes the development of an expression system and purification procedure for the SsbB protein. The *ssbB* gene was amplified from genomic *S. pneumoniae* DNA and cloned into the *E. coli* expression vector, pET21a. Although, we had shown previously that the SsbA protein is strongly expressed from pET21a in the *E. coli* strain BL21(DE3)pLysS, no expression of the SsbB protein was detected in these cells. However, the SsbB protein was strongly expressed from pET21a in the Rosetta(DE3)pLysS strain, a derivative of BL21(DE3)pLysS which supplies the tRNAs for six codons that are used infrequently in *E. coli*. The differential expression of the two SSB proteins in the parent BL21(DE3)pLysS strain was apparently due to the presence of two rare codons in the *ssbB* gene sequence that are not present in the *ssbA* sequence. Using the Rosetta(DE3)pLysS/pETssbB expression system, a protocol was developed in which the SsbB protein was purified to apparent homogeneity. DNA binding assays confirmed that the purified SsbB protein had single-stranded DNA binding activity. The expression and purification procedures reported here will facilitate further investigations into the biological role of the SsbB protein.

Keywords: *Streptococcus pneumoniae*; SSB protein; Single-stranded DNA binding protein; Natural transformation; SsbB protein

Streptococcus pneumoniae is a naturally transformable Gram positive bacterium that is able to take up DNA from its environment and incorporate this exogenous DNA into its chromosome. This process, known as transformational recombination, serves as a general mutational mechanism that allows *S. pneumoniae* to change its genetic composition in response to environmental changes and stresses [1]. The transformational recombination reaction involves the assimilation of a single-stranded form of the exogenous DNA into a homologous region of the *S. pneumoniae* chromosome [1]. An inspection of the genome sequence indicates that *S. pneumoniae* has two genes, designated *ssbA* and *ssbB*, which appear to encode single-stranded DNA binding (SSB)[1] proteins (Fig. 1) [2,3]. The SsbA protein (17,350 Da; 156 amino acids) is similar in size and sequence to the extensively studied SSB protein from *Escherichia coli* (18,874 Da; 178 amino acids), a non-sequence-specific single-stranded DNA binding protein that is involved in many aspects of DNA metabolism in *E. coli* [4]. The SsbB protein (14,926 Da; 131 amino acids), in contrast, is a much smaller protein that is induced during natural transformation in *S. pneumoniae* [5]. These results suggest that the SsbA protein may be a

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[1] *Abbreviations used:* SSB, single-stranded DNA binding; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol.

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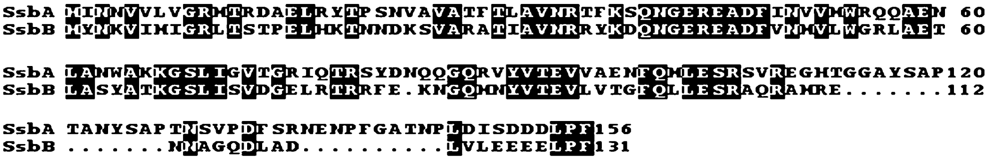

Fig. 1. Sequence comparison of the *S. pneumoniae* SsbA and SsbB proteins. The amino acid sequence of the *S. pneumoniae* SsbB protein (GenBank Accession No. AAL00527.1) is aligned with that of the *S. pneumoniae* SsbA protein (GenBank Accession No. AAL00199.1). Identical residues are highlighted in black.

general SSB protein involved in routine DNA functions (analogous to the *E. coli* SSB protein), and that the SsbB protein may be a specialized SSB protein used primarily during transformational recombination. This idea is consistent with a recent analysis of the genome sequences of 69 different bacterial species which revealed that those naturally transformable Gram positive bacteria that are closely related to *S. pneumoniae* (e.g., *Bacillus subtilis*) generally contain two paralogous *ssb*-like genes, whereas the non-naturally transformable Gram negative bacteria related to *E. coli* (e.g., *Salmonella typhimurium*) contain only a single *ssb* gene [6].

We previously reported the development of an expression system and purification procedure for the *S. pneumoniae* SsbA protein, and showed that the purified SsbA protein had a single-stranded DNA binding activity that was similar to that of the *E. coli* SSB protein [7]. In this report, we describe the expression, purification, and characterization of the *S. pneumoniae* SsbB protein.

Materials and methods

Materials

Streptococcus pneumoniae strain R800 was provided by Dr. Jean-Pierre Claverys (Université Paul Sabatier, Toulouse Cedex, France). Competent *E. coli* BL21(DE3)pLysS and Rosetta(DE3)pLysS cells, and the pET21a expression vector were from Novagen. The Zero Blunt-TOPO PCR Cloning Kit was from Invitrogen. The PCR primers and d(T_{35}) oligomer were from Invitrogen. *Pfu* DNA polymerase was from Strategene and dNTPs were from MBI Fermentas. *Nde*I and *Hin*dIII restriction enzymes, T4 polynucleotide kinase, ΦX single-stranded DNA (ssDNA), and ΦX double-stranded DNA (dsDNA) were from New England Biolabs. Carbenicillin, ampicillin, and chloramphenicol were from Research Products International. Bacto tryptone and Bacto yeast extract were from Becton–Dickinson. The LB and 2× YT bacterial growth media were prepared as described [8]. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Fisher. Dithiothreitol (DTT) was from Roche. Sodium dodecyl sulfate (SDS) was from Life Technologies. Polymin-P was from Sigma. HTP Bio-gel resin was from Bio-Rad. The Hi-Prep 16/10 Heparin FF and MonoQ HR 5/5 columns, and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech. *E. coli* SSB protein was from Promega and the *S. pneumoniae* SsbA protein was purified as described previously [7].

Construction of the pETssbB expression vector

The open reading frame encoding the *S. pneumoniae* SsbB protein was amplified from 2 µl of a saturated culture of R800 *S. pneumoniae* cells using in situ PCR and *Pfu* DNA polymerase as described by the manufacturer (Strategene). The primers used (5′-CCAT*ATGTATAATAAAGTTCATGATTGG*-3′ and 5′-CCAAGC*TTAAAATGGCAATTCTTCCTCTTCC*-3′) were complementary to the 5 ′ and 3′ ends of the coding sequence of the *ssbB* gene (italics), and also contained the recognition sequences for the restriction enzymes *Nde*I and *Hin*dIII (underlined). The DNA product that was obtained from the polymerase chain reaction was ligated into pCR-Blunt II-TOPO as described by the manufacturer (Invitrogen). The resultant plasmid was digested with *Nde*I and *Hin*dIII, and the fragment containing the *ssbB* gene was ligated into pET21a (Novagen) to give the final construct, pETssbB. The insert was sequenced and found to be identical to the *ssbB* sequence that is found in *S. pneumoniae* strain R6 (GenBank Accession No. AAL00527.1) [2].

SsbB protein expression trials

The expression of the SsbB protein was analyzed in the *E. coli* strains, BL21(DE3)pLysS, and Rosetta (DE3)pLysS. The expression levels of the SsbB protein in the two *E. coli* strains were compared to the expression levels of the SsbA protein using the previously constructed pETssbA plasmid [7]. Competent BL21(DE3)pLysS or Rosetta(DE3)pLysS cells were transformed with either pETssbA or pETssbB as described by the manufacturer (Novagen). The resulting transformed cells were selected for growth on LB agar plates supplemented with 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol. An individual colony of each transformed strain was selected from a fresh transformation and used to inoculate 2× YT/ampicillin (50 µg/ml)/chloramphenicol media (10 ml). The culture was incubated at 37 °C with constant shaking overnight. This overnight culture (100 µl) was then used to inoculate fresh 2× YT/ampicillin/chloramphenicol media (10 ml).

The new culture was grown at 37 °C to an A_{600} of 0.6. At this point, 500 μl of the culture was removed to be used as an uninduced sample. The uninduced sample was pelleted and the resultant pellet was resuspended with 30 μl SDS gel loading buffer [100 mM Tris–HCl (pH 6.8)/4% SDS/0.2% bromophenol blue/20% glycerol/0.2 M dithiothreitol]. The remaining culture was induced with a final concentration of 1 mM IPTG. After a 3 h induction, a sample was taken and diluted to match the A_{600} of the uninduced sample (500 μl total volume). This induced sample was centrifuged and the resultant pellet was resuspended in 30 μl SDS loading dye. The uninduced and induced samples (10 μl each) were analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 2).

Purification of the SsbB protein

The Rosetta(DE3)pLysS/pETssbB expression system described above was used as the basis for the development of a purification procedure for the SsbB protein. The results from the following expression and purification steps are summarized in Table 1 and in Fig. 3. All protein purification steps were carried out at 4 °C.

Cell growth

Competent Rosetta(DE3)pLysS cells were transformed with pETssbB and selected for growth on LB agar plates supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. An individual Rosetta(DE3)pLysS/pETssbB colony was used to inoculate 60 ml of 2× YT/ampicillin (50 μg/ml)/chloramphenicol media and the culture was incubated overnight at 37 °C with shaking. This confluent culture was used to inoculate 6 L of fresh 2× YT/ampicillin/chloramphenicol media (10 ml overnight culture per liter). The cells were grown at 37 °C to an A_{600} of 0.6 and then IPTG (1 mM final concentration) was added to induce expression of the SsbB protein. After 3 h at 37 °C, the cells (25 g) were collected by centrifugation, suspended in 70 ml of 50 mM Tris–HCl (pH 7.5)/10% glycerol/1 mM EDTA/200 mM NaCl, and frozen in liquid nitrogen. The cells were stored at −80 °C.

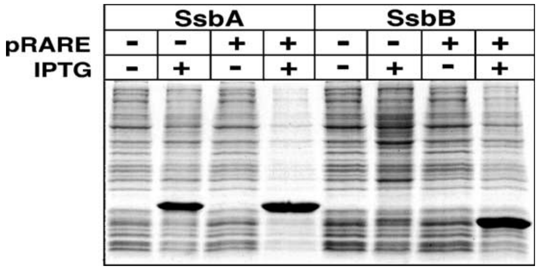

Fig. 2. Expression of the SsbA and SsbB proteins. SDS–polyacrylamide gel electrophoresis analysis of the expression of the SsbA and SsbB proteins from the pETssbA and pETssbB expression vectors in *E. coli* BL21(DE3)pLysS cells (− pRARE) and in Rosetta(DE3)pLysS cells (+ pRARE), both before addition of IPTG (− IPTG), and 3 h after addition of IPTG (+ IPTG). The acrylamide concentration was 5% in the stacking gel and 13% in the separating gel. The gel was stained with 0.1% Coomassie brilliant blue R-250.

Table 1
Purification of *S. pneumoniae* SsbB protein from Rosetta(DE3)pLysS/pETssbB cells

Fraction	Total protein amount (mg)	SsbB purity (%)	Yield of SsbB (%)
Cell lysate	250	10	100
After Polymin-P precipitation	50	25	50
HTP Bio-gel column	32	25	32
Precipitation step	6.4	95	24
Hi-Prep 16/10 Heparin FF column	4.8	>99	19

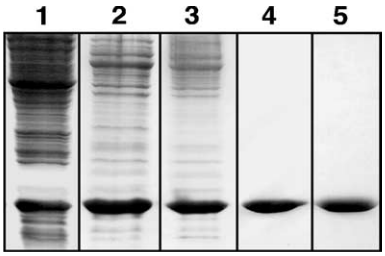

Fig. 3. Purification of the SsbB protein. SDS–polyacrylamide gel electrophoresis analysis of the SsbB protein-containing fractions from the SsbB protein purification procedure (see text for details). Lane 1, soluble fraction after cell lysis; lane 2, high salt fraction from Polymin-P precipitation step; lane 3, pooled fractions from the HTP Bio-gel column chromatography step; lane 4, redissolved precipitate from pooled HTP Bio-gel column fractions; and lane 5, pooled fractions from the Hi-Prep 16/10 Heparin FF column chromatography step. The acrylamide concentration was 5% in the stacking gel and 13% in the separating gel. The gel was stained with 0.1% Coomassie brilliant blue R-250.

Preparation of cell lysate

The frozen Rosetta(DE3)pLysS/pETssbB cell suspension was thawed on ice overnight; the cells lysed upon thawing due to the constitutive expression of T7 lysozyme from the pLysS plasmid. The lysed cell suspension was then centrifuged at 168,000*g* for 60 min. The soluble fraction that was obtained after the insoluble pellet was discarded is shown in Fig. 3 (lane 1); the SsbB protein corresponded to approximately 10% of the total protein in this fraction (Table 1). Next, 3.3 ml Polymin-P (10% w/v, pH 6.9) was added to the soluble supernatant fraction (78 ml). After incubating the sample on ice with stirring for 1 h, the suspension was centrifuged at 168,000*g* for 30 min. This supernatant was discarded and the pellet was homogenized in 20 ml T buffer [20 mM Tris–HCl (pH 7.5)/10% glycerol/1 mM DTT] and 0.4 M NaCl. The suspension was centrifuged at 12,000*g* for 15 min and the supernatant was then discarded. The pellet was homogenized in 20 ml T buffer/1.0 M NaCl and kept on ice with stirring for 1 h. The suspension was centrifuged as before and the pellet was discarded. The supernatant was dialyzed overnight against 2 L of P buffer [25 mM potassium phosphate (pH 7.0)/10% glycerol/1 mM EDTA] and 1.0 M NaCl. The dialyzate was clarified by centrifugation (12,000*g* for 10 min). This fraction is shown in Fig. 3 (lane 2); the SsbB protein corresponded to approximately 25% of the total protein in this fraction (Table 1).

Bio-gel HTP column chromatography

The clarified dialyzate was loaded onto a Bio-gel HTP column (40 ml bed volume; Bio-Rad) that had been equilibrated with P buffer/1.0 M NaCl. The column was washed with 3 column volumes of P buffer/1.0 M NaCl and then eluted with a 200-ml linear gradient of potassium phosphate (0.025–0.5 M, pH 7.0)/1.0 M NaCl. The fractions containing the SsbB protein (centered at 0.060 M potassium phosphate/1.0 M NaCl) were pooled to give the combined fraction shown in Fig. 3 (lane 3); the SsbB protein corresponded to approximately 25% of the total protein in this fraction (Table 1). The pooled fractions were dialyzed against 2 L T buffer/0.025 M NaCl overnight. This dialysis step led to the precipitation of the SsbB protein from the solution. This mixture was centrifuged at 12,000*g* for 15 min and the SsbB protein-containing pellet was suspended in 20 ml T buffer/10% glycerol/0.025 M NaCl (for a total of 20% glycerol). The sample was then dialyzed overnight against 2 L T buffer/10% glycerol/0.025 M NaCl. The resulting fraction is shown in Fig. 3 (lane 4); the SsbB protein corresponded to approximately 95% of the total protein in this fraction (Table 1).

Hi-prep 16/10 Heparin FF chromatography

The fraction from the previous step was clarified by centrifugation (12,000*g* for 10 min) and the resulting supernatant was loaded onto a Hi-Prep 16/10 Heparin FF column (20 ml bed volume; Amersham Pharmacia Biotech) that had been equilibrated with T buffer/10% glycerol/0.025 M NaCl. The column was washed with 5 column volumes of T buffer/10% glycerol/0.025 M NaCl and then eluted with a 200-ml linear gradient of T buffer/10% glycerol/(0.025–1.0 M NaCl). Although the SsbB protein was greater than 99% pure after the Hi-Prep 16/10 Heparin FF column chromatography step (Fig. 3, lane 5 and Table 1), it eluted in a broad peak (centered at 0.42 M NaCl). To concentrate the SsbB protein, the fractions were pooled and dialyzed overnight against 2 L T buffer/10% glycerol/0.025 M NaCl. The dialyzate (20 ml) was loaded onto a MonoQ HR 5/5 column (1 ml bed volume; Amersham Pharmacia Biotech) that had been equilibrated with T buffer/10% glycerol/0.025 M NaCl. The column was washed with 5 column volumes of T buffer/10% glycerol/0.025 M NaCl and then eluted with a 30-ml linear gradient of T buffer/10% glycerol/(0.025–1.0 M NaCl). The protein-containing fractions (centered at 0.38 M NaCl) were pooled and dialyzed overnight against 2 L of storage buffer (T buffer/10% glycerol/1 mM EDTA/0.025 M NaCl) to yield the final fraction of highly purified *S. pneumoniae* SsbB protein (3 ml) (Fig. 4). The SsbB protein corresponded to greater than 99% of the total protein in this fraction. The concentration of the purified SsbB protein was determined by UV absorbance at 280 nm using the extinction coefficient 10,810 $M^{-1} cm^{-1}$, which was calculated from the predicted amino acid sequence using the formula of Gill and von Hippel [9].

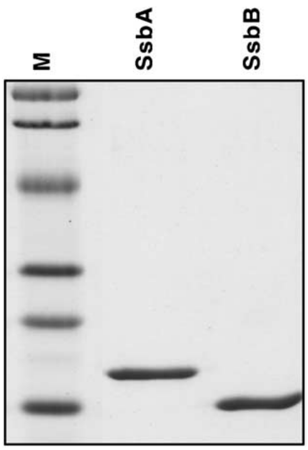

Fig. 4. SDS–polyacrylamide gel electrophoresis of purified SsbA and SsbB protein. The gel lanes contain purified *S. pneumoniae* SsbA protein, *S. pneumoniae* SsbB protein, and molecular mass standards, as indicated. The acrylamide concentration was 5% in the stacking gel and 13% in the separating gel. The gel was stained with 0.1% Coomassie brilliant blue R-250.

Analysis of purified SsbB protein

Amino-terminal protein sequencing of the purified SsbB protein was carried out by the Johns Hopkins Protein/Peptide Sequencing Facility. MALDI mass spectroscopic analysis of the SsbB protein was carried out by the Applied Biosynthesis Mass Spectrometry Facility at the Johns Hopkins School of Medicine. The purified SsbB protein was also analyzed for the presence of contaminating nucleic acids, and for contaminating DNA nuclease activity.

Nucleic acid analysis

The purified SsbB protein (10 μl; 0.6 μg/μl) was mixed with 5 μl gel loading solution (0.25% bromophenol blue, 40% sucrose), and analyzed by electrophoresis on a 0.8% agarose gel using a Tris–acetate–EDTA (40 mM Tris–acetate, 1 mM EDTA) buffer system. The gel was then stained with ethidium bromide to visualize any contaminating nucleic acids present in the preparation. No contaminating nucleic acid was detected in the SsbB protein preparation using this assay.

DNA nuclease analysis

The reaction solutions contained 25 mM Tris–acetate (pH 7.5), 10 mM $Mg(acetate)_2$, 5% glycerol, 1 mM DTT,

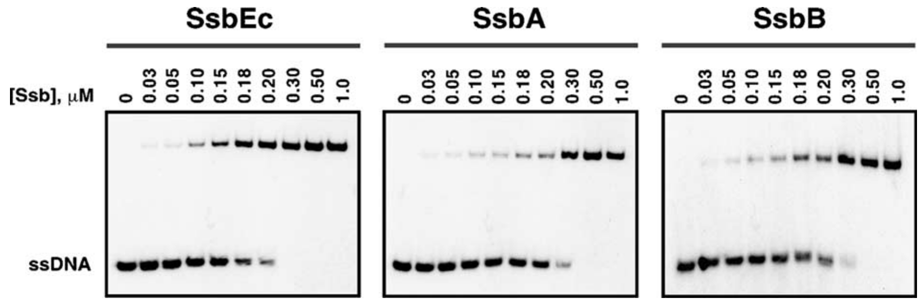

Fig. 5. Binding of the SsbB protein to d(T_{35}). The reaction solutions contained 5.3 µM d(T_{35}) (^{32}P-end-labeled) and the indicated concentrations of the *S. pneumoniae* SsbB protein, *S. pneumoniae* SsbA protein, or *E. coli* SSB protein (0–1.0 µM tetramer). The reaction solutions were incubated at 37 °C for 15 min and then analyzed by non-denaturing polyacrylamide gel electrophoresis. The bands corresponding to unbound d(T_{35}) (ssDNA) and the various SSB–d(T_{35}) complexes were visualized by autoradiography.

0.3 µM SsbB protein, and either 5 µM circular ΦX ssDNA, linear ΦX ssDNA, circular ΦX dsDNA, or linear ΦX dsDNA. The reaction solutions were incubated at 37 °C for 2 h. An aliquot (20 µl) was removed from each reaction solution and added to 5 µl of quench solution (70 mM EDTA/6% SDS/0.12% bromophenol blue/20% sucrose). The quenched aliquots were then analyzed by electrophoresis on a 0.8% agarose gel using a Tris–acetate–EDTA (40 mM Tris–acetate, 1 mM EDTA) buffer system. The DNA was visualized by ethidium bromide staining. No degradation of any of the DNA substrates was apparent in these assays, indicating that the SsbB protein preparation contained no detectable single and double-stranded DNA endonuclease or exonuclease activity.

Polyacrylamide gel electrophoresis mobility shift assays

The reaction solutions contained 25 mM Tris–acetate (pH 7.5), 10 mM Mg(acetate)$_2$, 5% glycerol, 1 mM DTT, 5.3 µM d(T_{35}) (5′-end-labeled using [γ-^{32}P]ATP and polynucleotide kinase), and the concentrations of the various SSB proteins given in the legend to Fig. 5. Reaction solutions were incubated at 37 °C, as indicated in the figure legend. Aliquots (20 µl) were removed from each reaction solution and added to 2 µl of gel loading solution (0.25% bromophenol blue, 40% sucrose). The aliquots were analyzed by electrophoresis on a 5% native polyacrylamide gel using a Tris–borate–EDTA buffer system (45 mM Tris–borate, 1 mM EDTA). The bands corresponding to unbound and SSB-bound d(T_{35}) oligomers were visualized by autoradiography.

Results

Construction of the pETssbB expression vector

The open reading frame encoding the *S. pneumoniae* SsbB protein was amplified from *S. pneumoniae* R800 cells using in situ PCR. A DNA fragment containing the *ssbB* gene was isolated and then ligated into the *Nde*I and *Hin*dIII cloning site of the expression vector pET21a to produce the construct pETssbB. In the pETssbB construct, the expression of the native form of the SsbB protein is under the control of the bacteriophage T7 transcription and translation signals that are built into the pET21a vector.

Expression of the S. pneumoniae SsbB protein

We previously cloned the *S. pneumoniae ssbA* gene into the pET21a expression vector to produce a pETssbA construct analogous to the pETssbB construct described above. We then transformed pETssbA into the *E. coli* strain BL21(DE3)pLysS to produce an IPTG-inducible expression system for the SsbA protein (BL21(DE3)pLysS carries a chromosomally encoded IPTG-inducible T7 RNA polymerase which can drive expression of the target genes in the pET21a vector that is under the control of the T7 RNA polymerase promoter). With the BL21(DE3)pLysS/pETssbA expression system, the SsbA protein corresponded to approximately 25% of the total protein in the crude cell extract after induction with IPTG (Fig. 2). We were able to use this expression system to develop a purification protocol in which the SsbA protein was purified to greater than 99% homogeneity [7]. Since this expression system was so successful for the SsbA protein, we transformed BL21(DE3)pLysS with the pETssbB vector to produce an analogous expression system for the SsbB protein. However, when we attempted to use this system for the expression of SsbB protein, we found that there was no detectable expression of the SsbB protein even after induction with IPTG (Fig. 2).

We then explored the expression of the SsbA and SsbB proteins in the BL21(DE3)pLysS derivative strain, Rosetta(DE3)pLysS. This strain contains the pRARE plasmid which supplies the tRNAs for six codons that are rarely used in *E. coli* (AUA, AGG, AGA, CUA, CCC, and GGA). When we transformed Rosetta(DE3)pLysS with pETssbA and then examined the IPTG-induced expression of the SsbA protein, we found that the SsbA protein was strongly expressed at levels similar to that obtained previously with the BL21(DE3)pLysS strain (Fig. 2). Moreover, when we transformed Rosetta(DE3)pLysS with pETssbB and then examined the IPTG-induced expression of the SsbB protein, we found that the SsbB protein was also strongly expressed in this strain. The expression of the SsbB protein in the Rosetta(DE3)pLysS strain corresponded to approximately 25% of the total protein in the crude cell extract after IPTG induction (Fig. 2). This level of SsbB protein expression in the Rosetta(DE3)pLysS strain was comparable to the expression that was observed for the SsbA protein.

To understand the basis for the difference in the expression patterns for the SsbA and SsbB proteins in the BL21(DE3)pLysS and Rosetta(DE3)pLysS cells, we examined the nucleotide sequences of the *ssbA* and *ssbB* genes [2,3]. This revealed that the *ssbB* sequence contained two codons, AGA (Arg 56) and CUA (Leu 53 and Leu 57), that are recognized by the tRNAs encoded by the pRARE plasmid. These codons are not present in the *ssbA* sequence. Thus, it is likely that the presence of the AGA and CUA codons in the *ssbB* gene sequence prevented the SsbB protein from being expressed in the BL21(DE3)pLysS cells, and that the tRNAs for these codons which are supplied by the pRARE plasmid allowed the SsbB protein to be efficiently expressed in the Rosetta(DE3)pLysS strain. Since the *ssbA* gene sequence did not contain either of these codons (or any other rare codons that are not present in the *ssbB* sequence), the SsbA protein was expressed with similar efficiencies in BL21(DE3)pLysS and in Rosetta(DE3) pLysS cells.

Purification of the S. pneumoniae SsbB protein

The Rosetta(DE3)pLysS/pETssbB expression system described above was used as the basis for the development of a purification procedure for the SsbB protein. The expression and purification steps are summarized in Table 1 and in Fig. 3.

Rosetta(DE3)pLysS/pETssbB cells were grown to log phase in 2× YT media (containing ampicillin and chloramphenicol), and the expression of the SsbB protein was induced by the addition of IPTG. The cells were collected by centrifugation and then lysed by freezing in liquid nitrogen and thawing on ice. The insoluble material from the lysis mixture was removed by centrifugation to yield a soluble fraction which contained most of the SsbB protein (Fig. 3, lane 1). The soluble fraction was treated with Polymin-P to precipitate any remaining nucleic acids. Most of the SsbB protein also precipitated from the solution when Polymin-P was added and was extracted from the Polymin-P pellet with a high salt buffer wash (T buffer/1.0 M NaCl) (Fig. 3, lane 2).

The high salt fraction was dialyzed into P buffer/1.0 M NaCl, loaded onto the Bio-gel HTP column (equilibrated in P buffer/1.0 M NaCl), and the SsbB protein was eluted with a linear potassium phosphate gradient. The SsbB protein eluted at a low potassium phosphate concentration (0.06 M) and was separated from a number of minor protein contaminants (Fig. 3, lane 3). When the pooled SsbB protein-containing fractions were dialyzed against a low salt buffer (T buffer/25 mM NaCl), most of SsbB protein precipitated from solution. The SsbB protein was resolubilized in this same low salt buffer by increasing the glycerol concentration from 10 to 20%. The SsbB protein was approximately 95% pure at this point, indicating that the precipitation and resolubilization steps had resulted in a significant purification of the SsbB protein (Fig. 3, lane 4). The glycerol concentration was maintained at 20% for the remainder of the purification steps.

To remove the remaining minor contaminants, the resolubilized SsbB protein fraction was loaded onto a Hiprep 16/10 Heparin FF column (equilibrated in T buffer/25 mM NaCl) and eluted with a linear NaCl gradient. Although the SsbB protein was greater than 99% pure after this step, it eluted in a broad peak (centered at 0.42 M NaCl). To concentrate the SsbB protein, the pooled fractions (20 ml) were dialyzed against low salt buffer (T buffer/25 mM NaCl), loaded onto a Mono Q HR 5/5 FPLC column, and eluted with a linear NaCl gradient. The final fraction of SsbB protein was greater than 99% pure as judged by SDS–polyacrylamide gel electrophoresis (Fig. 3, lane 5). This SsbB protein preparation was free of contaminating nucleic acids and contained no detectable ssDNA or dsDNA nuclease activity.

Characterization of the purified S. pneumoniae SsbB protein

The mobility of the purified SsbB protein during polyacrylamide gel electrophoresis was distinctly different from that of the SsbA protein and was consistent with a monomeric molecular mass of approximately 15,000 Da (Fig. 4). Mass spectrometric analysis of the purified SsbB protein yielded a molecular mass of 14,927 Da, in excellent agreement with the molecular mass of 14,926 Da predicted by the protein sequence [2,3]. Amino-terminal protein sequencing confirmed that the preparation corresponded to the SsbB protein.

Single-stranded DNA binding activity of the S. pneumoniae SsbB protein

The purified SsbB protein was analyzed for single-stranded DNA binding activity. In these experiments, a fixed concentration of d(T_{35}) was incubated with various concentrations of SSB protein and the resulting complexes were analyzed by non-denaturing polyacrylamide gel electrophoresis. As shown in Fig. 5, the amount of d(T_{35}) that was bound to the SsbB protein increased with increasing protein concentration until all of the d(T_{35}) was incorporated into a SsbB–d(T_{35}) complex. A similar pattern of binding was observed with the previously purified

S. pneumoniae SsbA protein and with the *E. coli* SSB protein (Fig. 5). Although the mobilities of the complexes that were formed with the various proteins were different (presumably due to the difference in the sizes of the proteins), the dependence of complex formation on protein concentration was similar for the three SSB proteins.

It has been shown that the *E. coli* SSB protein forms a stable tetramer in solution and that it is the tetrameric form of the protein that binds to ssDNA [4]. Furthermore, previous determinations of the DNA binding stoichiometry of the *E. coli* SSB protein indicate that the d(T_{35}) oligomer used in our study is sufficient to bind only a single tetramer of *E. coli* SSB protein [4]. Thus, the close similarity of the DNA binding titrations shown in Fig. 5 indicates that the SsbB protein (and SsbA protein) also binds to d(T_{35}) as a tetramer and with an affinity that is similar to that of the *E. coli* SSB protein. Consistent with this interpretation, we have recently shown by gel-exclusion chromatography that the purified SsbB protein (and SsbA protein), like the *E. coli* SSB protein, forms a stable tetramer in solution [10].

Discussion

We have developed an efficient IPTG-inducible *E. coli* expression system and purification procedure which provides highly purified *S. pneumoniae* SsbB protein. We have also demonstrated that the purified SsbB protein binds to d(T_{35}) to form a SsbB–d(T_{35}) complex with a concentration dependence similar to that exhibited by the previously purified SsbA protein and to the extensively studied *E. coli* SSB protein. These results confirm the expectation (based on comparative sequence analysis) that the SsbB protein is a single-stranded DNA binding protein.

The requirements for the expression of the SsbB protein differed significantly from those for the SsbA protein. Although we had previously shown that the SsbA protein was expressed strongly in the *E. coli* strain BL21(DE3)pLysS, no expression of the SsbB protein was detected in this strain. However, the SsbB protein was expressed strongly in the Rosetta(DE3)pLysS strain. This indicated that the expression of the SsbB protein required the pRARE plasmid that is present in the Rosetta strain which presumably furnishes tRNAs for the codons CUA (Leu 53 and Leu 57) and AGA (Arg 56) that are present in the SsbB coding sequence. These codons are rarely used in *E. coli*. An analysis of the *E. coli* genome sequence shows that of the six codons for arginine, the AGA codon is used only 3.85% of the time. Similarly, of the six codons for leucine, the CUA codon is used only 3.68% of the time (TIGR codon usage tables; http://www.tigr.org). Interestingly, however, these codons are not as rarely used in *S. pneumoniae*. An analysis of the *S. pneumoniae* genome sequence shows the arginine codon AGA is used 17.54% of the time, and that the leucine codon CUA is used 11.22% of the time (TIGR codon usage tables; http://www.tigr.org). Nevertheless, to express the *S. pneumoniae* SsbB protein in *E. coli*, it was apparently necessary to supply the tRNAs for these codons through the pRARE plasmid. Since these codons are not present in the SsbA coding sequence, the SsbA protein was expressed with similar efficiencies in either the BL21(DE3)pLysS or the Rosetta(DE3)pLysS strains. Although the stated purpose of the Rosetta strains is to enhance the expression of eucaryotic proteins which may contain codons rarely used in *E. coli* (Novagen), this work demonstrates that these strains are also useful for the expression of proteins from other bacterial species that may have codon usage patterns differing from that of *E. coli*.

The results presented here represent the first purification and characterization of a transformation-specific SSB protein from any bacterial species. The expression and purification procedures that are reported here will facilitate further investigations into the biological role of the *S. pneumoniae* SsbB protein and related proteins from other bacterial species.

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