Structure–function relationships of two paralogous single-stranded DNA-binding proteins from *Streptomyces coelicolor*: implication of SsbB in chromosome segregation during sporulation

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ABSTRACT

The linear chromosome of *Streptomyces coelicolor* contains two paralogous *ssb* genes, *ssbA* and *ssbB*. Following mutational analysis, we concluded that *ssbA* is essential, whereas *ssbB* plays a key role in chromosome segregation during sporulation. In the *ssbB* mutant, ~30% of spores lacked DNA. The two *ssb* genes were expressed differently; in minimal medium, gene expression was prolonged for both genes and significantly upregulated for *ssbB*. The *ssbA* gene is transcribed as part of a polycistronic mRNA from two initiation sites, 163bp and 75bp upstream of the *rpsF* translational start codon. The *ssbB* gene is transcribed as a monocistronic mRNA, from an unusual promoter region, 73bp upstream of the AUG codon. Distinctive DNA-binding affinities of single-stranded DNA-binding proteins monitored by tryptophan fluorescent quenching and electrophoretic mobility shift were observed. The crystal structure of SsbB at 1.7 Å resolution revealed a common OB-fold, lack of the clamp-like structure conserved in SsbA and previously unpublished S-S bridges between the A/B and C/D subunits. This is the first report of the determination of paralogous single-stranded DNA-binding protein structures from the same organism. Phylogenetic analysis revealed frequent duplication of *ssb* genes in Actinobacteria, whereas their strong retention suggests that they are involved in important cellular functions.

INTRODUCTION

*Streptomyces* species are multicellular bacteria that exhibit a complex developmental programme and undergo morphological differentiation. Development of streptomyces is initiated by spore germination, hyphal growth and branching that leads to the formation of vegetative mycelium. From this mycelium, new branches grow into the air forming a lawn of aerial hyphae, which eventually generate long chains of unigenomic spores. Much current knowledge of streptomyces is based on genetic and genomic studies of *Streptomyces coelicolor M145* (1). The large, linear chromosome of this bacterium encodes 7825 ORFs with a large number of genes (12.3%) predicted to encode a regulatory proteins. In addition, genome analysis has revealed the distribution of different types of genes. Nearly all genes expected to be essential, such as those for cell division, replication, transcription, and the biosynthesis of important primary metabolites, are located in the core region. Exceptions to this rule are often duplicated genes (2). Amongst the latter group are two paralogous genes encoding single-stranded DNA-binding (SSBs) proteins.

SSB proteins, essential for cell survival, are found in all domains of life and in viruses (3). *Escherichia coli* SSB has been used extensively for several decades for studying protein structure–function relationships (4). SSB binds to single-stranded DNA (ssDNA) with high affinity in a sequence-independent manner, thus protecting ssDNA intermediates and disrupting unproductive secondary structures. During DNA replication, recombination and repair (5), SSBs interact with an array of chromosome

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maintenance proteins, mobilize them to the site of activity and often stimulate their activities (6,7).

The primary structures of SSB proteins display two distinct domains: an N-terminal domain containing a conserved oligonucleotide-oligosaccharide fold (OB-fold) responsible for ssDNA binding (8) and a C-terminal domain enriched in glycin and proline residues with acidic amino acids in a hexapeptide motif (D-D-D-I/L-P-F), which is important for protein interactions (9). Most bacterial SSBs function as homotetramers in which four OB-folds act together to bind to ssDNA. The crystal structure of SSB from E. coli was solved first (10), after which several crystal structures from other bacteria were described, among them an SSB from S. coelicolor (SsbA) (11). Two major binding modes of the SSB tetramer have been proposed. In the (SSB)_{35} binding mode, two subunits of the E. coli tetramer bind to 35 nucleotides, whereas in the (SSB)_{65} mode, all four subunits participate in binding to 65 nucleotides. It has been suggested that different binding modes may be used during different processes in DNA metabolism (12).

The biological role of paralogous SSB proteins in other bacteria is poorly studied. Transcriptional profiles of two ssb genes have been reported for Bacillus subtilis; in this naturally transformable bacterium, one SSB is essential, whereas the other participates in competence-related recombination (13). A division of labour between SsbA and SsbB during genetic recombination in B. subtilis was recently reported (14). In addition, differential binding properties of SsbA and SsbB from another naturally transformable bacterium, Streptococcus pneumoniae, were reported (15).

The biological roles of paralogous SSBs in streptomyces have not been reported until now. In this study, we have found a new and unexpected role of paralogous SSB protein in chromosomal segregation during morphological differentiation. In branching vegetative hyphae, chromosome copies are not segregated, and septation occurs unevenly (16). Nutrient depletion triggers the development of aerial mycelium and the start of the reproductive phase. In the aerial hyphae, intensive chromosome replication occurs producing ≥50 non-segregated linear chromosomes (17). Transformation of sporogenic aerial hyphae into chains of mature unigenomic spores requires two synchronous processes: formation of sporulation septa and chromosome segregation (18,19). Although progress has been made, there are still substantial gaps in understanding how dozens of linear chromosomes that are dispersed along aerial hyphae condense and accurately segregate into the nascent pre-spore compartments. Several proteins, such as FtsKSC, SMC, ParAB and ParJ, have been identified as contributing to chromosome organization and distribution during Streptomyces sporulation. Various ftsKSC mutants frequently have irregular DNA content owing to large terminal deletions of the chromosome (20). The sme/ftsK-truncation alleles, produced 7–15% of spores lacking DNA, whereas a segregation defect of 24% was observed for a sme/parB mutant (21,22). Mutants lacking parA, parB or parJ produce 19, 24 and 8% of spores lacking DNA, respectively (23–25). In summary, various combinations of mutant genes still produced viable spores, albeit at reduced frequency, suggesting that additional genes are responsible for proper chromosome segregation.

In this study, we addressed the biological role of the paralogous ssb genes in S. coelicolor, ssbA and ssbB designated in UniProtKB as ssb2 and ssb1, respectively. We asked what is the driving force leading to the retention of alternative ssb genes. We found an unknown role of SsbB in chromosome segregation during reproductive growth of S. coelicolor. Consistent with the proposed cellular function of SSBs, transcriptional analysis showed that transcription of ssbB is upregulated in the later stage of growth and in a minimal medium that usually triggers spore formation. In addition, the ssbB promoter displayed unusual organizational features that might be important for its activity. Solved 3D structure of SsbB showed a unique interconnection by two S-S bridges between the A/B and C/D subunits. This is the first example of solved crystal structures of two paralogous SSB proteins from the same organism.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers

The bacterial strains, plasmids, cosmids and primers used during the course of this study are listed in the Supplementary Tables S1–S3.

Bacterial growth conditions

E. coli strains NM522, ET12567 and XL1-Blue were grown in Lauria Bertani (LB) medium at 250 r.p.m. and 37°C, whereas strains BW25113 (pIJ790) and BT340 at 30°C until induction of temperature-sensitive genes. The expression of recombinant proteins was induced with 1 mM isopropyl-b-D-thiogalactopyranoside. S. coelicolor M145 was grown at 250 r.p.m. and 30°C in liquid complete regeneration medium (CRM) (26), R5 or minimal medium (MM) and on solid media: Mannitol soya flour (MS), Tryptic soy broth (TSB) (Difco™) and MM (27). Appropriate antibiotics were added at the following concentration: 100 µg/ml of ampicillin, 50 µg/ml of apramycin, 25 µg/ml of chloramphenicol, 50 µg/ml of kanamycin, 25 µg/ml of nalidixic acid or 50 µg/ml of hygromycin, when necessary.

Gene cloning, heterologous over-expression and protein purification

The ssbA gene (previously ssb) from S. coelicolor (NCBI, Gene ID 1099343) was cloned as described previously (28). This construct was used to prepare all other recombinant ssbA genes used in this study. A DNA sequence containing ssbA and its upstream regulatory elements (promoter and SD sequence; pssbA) was reconstructed in the pGEM®-T vector and designated pGEM-pssbA, as shown (Supplementary Figure S1). This plasmid was digested with HindIII, treated with Klenow fragment to make blunt ends and again digested with SpeI. The DNA fragment carrying the pssbA sequence was further subcloned into the EcoRV and SpeI sites of pMS82b. This construct, pMS-pssbA as shown in
Supplementary Figure S1, was used for the complementation experiment. The \textit{ssb}B gene from \textit{S. coelicolor} (NCBI, Gene ID: 1098117) was cloned into pQE-30 and labelled pQE-ssB, using the same approach as described for \textit{ssb}A (28).

For expression of the His-tagged \textit{ssb}B gene in \textit{S. coelicolor}, this gene was subcloned into pGEM-T using S\textit{fl}ANTF and S\textit{fl}ANTR primers (Supplementary Table S3). This construct, pGEM-ssB, which contained the original \textit{ssb}B ribosomal binding site, was digested with EcoRI and HindIII and ligated into pANT849 generating pANT-ssB that was used for the transformation of \textit{S. coelicolor} protoplasts.

To obtain a construct with a DNA sequence covering the promoter and \textit{ssb}B, cloning was performed in the same way as for \textit{ssb}A; for details, see Supplementary Figure S2. The construct pMS-ssB was used for the complementation experiment. The \textit{ssb}BA\textit{C} gene, lacking the last 37 amino acids at the C-terminus was obtained by polymerase chain reaction (PCR) using SC-BF and SSBl19 R primers (Supplementary Table S3) with \textit{S. coelicolor} genomic DNA as a template. The amplified DNA fragment was cloned into the vector pQ\textit{E} as described for the \textit{ssb}A and \textit{ssb}B genes. In the next step, \textit{ssb}B from the pGEM-ssB construct was replaced by \textit{ssb}BA\textit{C} at the BamHI and HindIII restriction sites (Supplementary Figure S2). The construct pssB\textit{A}C was further subcloned into pMS82, giving pMS-ssB\textit{A}C. All constructs were verified by DNA sequencing.

Over-expression of the \textit{ssb} genes cloned into pQE expression system was achieved in \textit{E. coli} NM522 and in \textit{S. coelicolor} carrying pANT-ssB. \textit{S. coelicolor} was grown in MM (50 ml) for 48 h, mycelia was harvested by centrifugation, disrupted by sonication (4 \times 30 s) and the suspension was centrifuged at 10000g for 15 min to remove cell debris. Purification of His-tagged SsbB from cell-free extract was achieved by Ni-NTA resin (Qiagen), as reported previously (28). Fractions with purified protein were pooled, desalted on PD10 columns (GE Healthcare) and concentrated for microcrystallisation experiments to 17 mg/ml in 50 mM Tris–HCl (pH 7.0). Phosphate buffer (pH 6.9) was used for fluorimetric titration experiments. The proteins (50 ng/lane) were run on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and gel staining was performed with PhastGel® Blue R (Sigma). Western blot analysis as described previously (29) was performed with the anti-SsbB antibody obtained from Pineda, Anticorper-Service, Germany. Antibody binding was visualized with peroxidase-coupled anti-rabbit antibody and chemiluminescent detection (peroxidase ECL™ Primer Western Blotting Detection Reagent kit, GeHealthcare).

**Gene disruptions and complementation**

Gene disruption experiments were performed by allelic replacement with transposoned cosmids from a single gene transposon insertion cosmids library (30). Cosmids containing transposon Tn5062 inside \textit{ssb}B (6C07.1.c02, at 53rd aa) and \textit{ssb}A (h24-1.E01, at 124 aa) were introduced into the ET12567 non-methylating \textit{E. coli} strain containing the non-transmissible \textit{oriT} mobilizing plasmid, pUZ8002. Following a previously described method (31), conjugation to \textit{S. coelicolor} was performed. Exconjugants were selected for apramycin resistance and kanamycin sensitivity generating TSB01, and an allelic replacement by a double crossover recombination event was verified by PCR using primers complementary to the transposon cassette (EZR2 and EZL1) and \textit{ssb}B. The disrupted \textit{ssb}B gene was complemented by introducing an integrative vector, pMS82, carrying the pssB construct (pMS-ssB). This plasmid was introduced into \textit{E. coli} ET12567(pUZ8002) and transferred by conjugation into \textit{S. coelicolor}. Exconjugants were selected on hygromycin generating TSB01. PCR and specific primers (Supplementary Table S3) were used to confirm plasmid integration into the \textit{S. coelicolor} chromosome at the \textit{attB}_{\text{BAMHI}} site.

REDIRECT PCR-targeting technology (31) was used to replace the entire coding region of \textit{ssb}A by an apramycin (\textit{aac(3)IV}) resistance cassette. A mutagenic cassette (\textit{FRT-oriTaac(3)IV-FRT}) was PCR amplified using the primers SSBAko\textit{F} and SSBAko\textit{R} (Supplementary Table S3) and pIJ773 as a template. The PCR product was used to transform \textit{E. coli} BW25113(pIJ790) containing cosmid SCH24. Recombination was induced with L-arabinose, and colonies carrying the recombinant cosmid were selected as described previously (31). After PCR and restriction verification, a cosmid designated cSA01 (Supplementary Table S1) was introduced by transformation into \textit{E. coli} ET12567(pUZ8002) and transferred by conjugation into \textit{S. coelicolor}. This cosmid, cSA01, was used to generate an in-frame ‘scar’ mutation at the position of the resistance cassette. This was achieved by introducing cSA01 into \textit{E. coli} BT340 (harbouring pCP20 with the Flipase gene, FLP) in which temperature-induced recombination between both FRT mutagenesis cassette-flanking regions takes place. The resulting cosmid, cSA02, had an 81 bp ‘scar’ remaining in-frame with the adjacent ORF. This cosmid was used to transform \textit{S. coelicolor} protoplasts carrying an additional copy of \textit{ssb}A at the \textit{attB}_{\text{BAMHI}} site and the original \textit{ssb}A locus. KanR recombinants were selected and re-streaked without kanamycin. The resulting colonies were again cultured for one generation with and without antibiotic to identify loss of kanamycin resistance, i.e. double crossing over. Selected colonies were checked for the presence of the ‘scar’ sequence instead of the \textit{ssb}A gene by PCR using SSBAFRT and izaSSBA primers (Supplementary Table S3). In the original protocol, the unmarked ‘scar’ sequence was used for replacing the disruption cassette carrying the resistance gene (31). In our case, we applied the same approach to replace the undisrupted \textit{ssb}A, as the disruption cassette in place of \textit{ssb}A would not give any viable colonies.

**Transcription analysis**

Total RNA was isolated from \textit{S. coelicolor} M145 grown in 50 ml of CRM (26) or MM liquid media (27). Sampling (3 ml) was performed at 18, 24, 48 and 96 h of bacterial growth followed by immediate RNA isolation with the
FastRNA® Pro Blue Kit and FastPrep® instrument (Qbiogene, Inc, CA) for 40 s at a speed setting of 6.0. Dnase I was used to remove remaining DNA. The quality of RNA was checked spectrophotometrically and by agarose gel electrophoresis, whereas the absence of DNA was confirmed by PCR.

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random hexameric primers on 3 μg of total RNA isolated from each sample to examine expression of the ssb genes. Typically 2 μl of reaction volume with Complementary DNA (cDNA) and ssb primers, (Supplementary Table S3) were used for PCR. Amplified products were analysed by agarose gel electrophoresis.

Transcriptional start sites were determined by rapid amplification of cDNA ends (RACE) with the InvitrogenTM RACE kit according to the alternative protocol for FastRNA. Three micrograms of total RNA from the expo-

Without addition of trypsin, no crystals were obtained. Mass spectrometry analysis showed that SsbB in the crystal was 116 aa long, indicating that trypsin digested 40 aa of the flexible C-terminus, allowing the protein to crystallize. A complete data set to 1.7Å resolution was collected at BM14 beamline, ESRF (Grenoble, France) and processed using HKL2000 (34). The structure was solved by molecular replacement using the EPMR software (35) and SsbA (pdb code: 3EIV) as a starting model. Arp/Warp (36) was used for automatic model building and Coot for manual model building and real-space refinement (37). Restrained refinement was done using REFMAC5 (38). Crystallographic data collection and refinement statistics are shown in Supplementary Table S4. In the final model, there are 12 chains in the asymmetric unit. In chains A, D, E, F, I and K, all residues were built to fit the electron density (1–110). Chains B, C, G, H, J and L are missing some residues in the loop regions (B: 40–41, C: 40–43, 85–92, G: 39–44, 86–92, H: 42–43, J: 39–42, L: 40–42, 86–92).

**Electrophoretic mobility shift assay**

Formation of SsbA- or SsbB-ΔX174 DNA complexes were analysed by Electrophoretic mobility shift (EMSA). Various concentrations of SSB proteins were mixed with 7.7 nM of ssDNA in the reaction solutions containing 40 mM Tris acetate (pH 7.5), 10 mM MgCl2 and 1 mM ethylenediaminetetraacetic acid in a total volume of 20 μl (Figure 7A1 and 2). To test the inhibitory effect of dithiothreitol (DTT), increasing amounts (10–100 mM) were added to the binding buffer (Supplementary Figure S7C). Mixtures were incubated 15 min at 37°C and then analysed on a 0.5% agarose gel.

**Fluorescence measurements**

Fluorometric titrations of Ssb protein solutions were performed on a Varian Cary Eclipse spectrofluorimeter at room temperature using quartz cuvettes (1 cm), by excitation at a wavelength of 295 nm and emission monitored at 353 nm. Titrations were performed by titrating SSB proteins (c_{monomer} = 6.9 × 10^{-7} M) with small aliquots of dT35 stock solution (c = 1.9 × 10^{-5} M). After each addition, the solution was allowed to equilibrate for 5 min until no further change in fluorescence was observed. The fluorescence spectra were corrected for the buffer/NaCl baseline for each sample. The fluorometric titration data were analysed by non-linear fitting procedure to the Scatchard equation (39), to avoid large errors of linear processing of the fluorometric data (40).

**Spore counting**

Spore produced by M145 and TSB01 strains were counted using a Neubauer-improved haemocytometer (depth 0.02 mm). The spores were scraped with a microbiological loop from 3 spots of the same MS plate for each strain (~1/8 or 7 cm² of the plate). Spores were re-suspended in 1 ml of 0.1% Tween 80 (Sigma) and agitated vigorously with vortex mixer to disperse the clumps. The original spore suspension and 10⁻¹ dilution of each sample were counted in the haemocytometer.
Spore resistance to heat and lysozyme

Heat resistance was assayed by incubating spores \((10^6/\text{mL})\) of the wild-type and \(ssbB\) mutant strain spores at \(30^\circ\text{C}\) and \(60^\circ\text{C}\) simultaneously. Five microlitres of the sample was taken every 10 min and spotted onto LB agar. To examine resistance to lysozyme, \(10^4\) spores of the wild-type and \(ssbB\) mutant strains were streaked in patches and \(5\mu\text{g}/10\mu\text{L}\) of lysozyme was applied to LB agar plates. Plates were incubated for 3 days at \(30^\circ\text{C}\) before being photographed (41).

Phylogenetic analysis

Sequence similarity searches were performed with BLASTP, whereas comparison, sorting and multiple sequence alignments were obtained with Promals (42). Substitution modelling was completed using ProtTest (43). Construction of the phylogenetic tree was made by the maximum likelihood method using PhyML (44). An aLRT (45) was used to test branch support. Seaview (46) was used for statistical report and CorelDRAW® for graphic presentation of the results. Protein similarity searches with \(S.\ coelicolor\) SsbA against complete genome sequences from the phylum Actinobacteria were performed. Representative paralogous genes from each suborder of Actinobacteria were used to construct the phylogenetic tree.

RESULTS

Analysis of \(ssb\) genes

Both \(ssbA\) and \(ssbB\) are positioned in the central region of the chromosome (Figure 1) that is not exposed to frequent deletion events and where many essential genes are located (2). SCO3907 (\(ssbA\)) encodes a protein of 199 aa, whereas SCO2683 (\(ssbB\)) encodes a protein of 156 aa. The two SSB proteins share an overall 35% sequence identity, mostly conserved in the first 110 aa that contains the OB-fold sequence, at the very end of this protein, is essential for interacting with proteins (6) or with the OB-fold (47). In SsbB, the C-terminus is reduced to 50 aa, and it lacks the acidic tail, which is highly conserved in all SSB sequences (6).

Distinctive functions of paralogous SSBs

Retention of duplicate genes depends on their adoption of novel functions in the cell (48). To elucidate the biological role of SSBs in \(S.\ coelicolor\), gene disruption experiments were performed as described in the ‘Materials and Methods’ section.

To disrupt \(ssbA\) by semi-targeted \textit{in vitro} transposon mutagenesis, we introduced h24.EO1 (apramycin resistance—Apr\(^R\), kanamycin resistance—Kan\(^R\)) by intergeneric conjugation and selected for Apr\(^R\) exconjugants. After screening 500 colonies for the Apr\(^R\) Kan\(^R\) phenotype indicative of integration of h24.EO1 by a double crossover event, all colonies displayed the Apr\(^R\) Kan\(^R\), phenotype indicative of a single recombination event. The failure to obtain Apr\(^R\) Kan\(^S\) colonies suggests that either allelic exchange could not occur easily or that \(ssbA\) is an essential gene. As \(ssbA\) is clustered with genes encoding ribosomal proteins (\(rpsF\) and \(rpsR\)), as in \(B. subtilis\) in which co-regulation of \(ssbA\) and ribosomal genes was reported (13), we created an in-frame deletion of \(ssbA\) to allow transcription of downstream gene(s). \(S.\ coelicolor\) transformed with the cSA02 where \(ssbA\) was replaced by the ‘scar mutation’, and transformants were re-streaked without kanamycin. Single colonies were tested for the Kan\(^R\) phenotype indicative of a double recombination event, but none contained \(ssbA\) replaced by the ‘scar’ fragment. Deletion of \(ssbA\) is indicative of a double recombination event, but none contained \(ssbA\) replaced by the ‘scar’ mutation. Based on this result, we concluded that \(ssbA\) is essential for \(S.\ coelicolor\) survival.

To examine the biological role of \(ssbB\), this gene was disrupted with Tn5062. Interestingly, after 4 days of growth, the mutant strain (TSB01) displayed white aerial mycelium on MS medium (Figure 2A). Formation of the white aerial hyphae [\textit{whi} phenotype (49)] is characteristic of \textit{Streptomyces} mutants that fail to develop grey pigment associated with mature spores. Further, we observed that after prolonged growth (10 days), the surface of our mutant turned light grey (Figure 2B), indicating that a small proportion of mutant hyphae could complete the spore maturation process. To exclude the possibility of an unpredicted polar mutation, the strain TSB01 lacking \(ssbB\) was complemented with the native gene integrated at the \(attB_{BT1}\) site under the control of its own promoter. Strain TSB02, carrying this complementation vector, fully restored the original phenotype (Figure 2). Occasionally, TSB02 displayed darker grey pigmentation indicating higher production of spores. We speculate that this could be ascribed to \(ssbB\) chromosome relocation that consequently changed gene expression. To check

![Figure 1. Physical map of \(S.\ coelicolor\) chromosomal region with two paralogous \(ssb\) genes. The gene SCO3907 (\(ssbA\)) encoding a protein of 19.9 kDa is located 40 kb on the right side of the origin of replication (\(oriC\)), whereas the gene SCO2683 (\(ssbB\)) encoding protein of 16.8 kDa is located 1.36 Mb on the left from the \(oriC\). The both genes are positioned in a region that is not exposed to frequent deletion events and contains many essential genes.](image-url)
whether C-terminus of SsbB is important for DNA segregation during sporulation, we constructed TSB03 strain, i.e. mutant carrying ssbB^{DC} (see ‘Materials and Methods’ section); synthesis of SsbB/C1C in TSB03 was confirmed by western blot (not shown). This strain also exhibited white phenotype as shown for TSB01 (Supplementary Figure S3A). Thus, this result confirmed that SsbB truncated at the C-terminus and with intact OB-fold could not restore spore maturation process. Both TSB01 and TSB03 displayed a similar phenotype on R5 medium. We observed a delay in formation of aerial mycelium and after prolonged incubation mutant colonies displayed a more pronounced pink colour (Supplementary Figure S3B) (50). Microscopy analysis also showed it was more difficult to find regions with abundant spore chains in TSB01. We performed simultaneous counting of the spores collected from the wild-type and TSB01, as described in ‘Materials and Methods’ section. The mean value of three independent counts for each strain showed that mutant strain produced 32.8% less spores than the wild-type strain. As shown in Figure 3A1–3, closer inspection showed that spores in the mutant strain frequently lacked DNA (30%; n = 2200) or contained excessive amounts of DNA (23%, n = 900), suggesting a defect in chromosome segregation. In contrast, the complemented strain (TSB02) displayed restoration of the wild-type phenotype (Figure 3C1 and 2), with just 1% of spores lacking DNA in TSB02 compared with the wild-type 0.8–1.1% of spores (21,25). To exclude the possibility that observed defect is an artifact because of the difficulty in stain penetration through the spore wall, we performed in vivo staining of the S. coelicolor wild-type, TSB01, TSB02 and TSB03 (Supplementary Figure S4). Staining with DAPI showed similar results as observed previously, in TSB01 25%, TSB03 26% and wild-type 1.5% of spores were deficient in DNA (n = 800). We further examined sensitivity of the spores for the wild-type strain and TSB01, by exposing them to moderate heat and lysozyme. Although both strains displayed the same sensitivity towards heat, TSB01 showed slightly higher sensitivity towards lysozyme (Supplementary Figure S5A and B). Statistical analysis showed that TSB01 had a slightly increased spore length and number of spores in spore chains (Table 1). The length of the spores in TSB01 was less uniform than in the wild-type, as can be seen from the standard deviation values. An F-test was also applied and showed that TSB01 spores were significantly longer than the wild-type strain (P < 0.001). The aberrant distribution of DNA in the spores and irregularly sized spores suggest that SsbB has a distinctive function for chromosome segregation (18). To the best of our knowledge, the role of an SSB protein in DNA partitioning was not previously reported. We also observed that the strain deficient in SsbB occasionally produced longer and coiled aerial hyphae, (Suplementary Figure S6) as previously reported for the whiA mutant (51).

Expression profiles of ssb genes

The genetic evidence suggested that the ssb paralogs perform different biological roles: ssbA is essential for cell survival, whereas ssbB is important for production of viable spores. As the latter function is more pronounced at the end of the cell cycle, which correlates with nutrient depletion, we studied the effect of time and growth conditions on gene expression. S. coelicolor was grown in MM and CRM media for 4 days, and total RNA was isolated at different time intervals that coincided with metabolic
changes, as monitored by accumulation of actinorhodin, a red/blue acid/base indicator pigment with weak antibiotic properties (Figure 4C) (27). Reverse transcriptase (RT)-PCR analysis of ssa indicated that this gene is expressed as a long mRNA with rpsF and most likely rpsR from a promoter positioned upstream of rpsF. Expression was more pronounced in the exponential phase of growth in rich medium (Figure 4A1), whereas in minimal medium (Figure 4A2), expression was prolonged into stationary phase. Under the same conditions, the ssbB transcript was hardly detectable in MM, whereas in rich medium, it was undetectable. Therefore, RT-PCR was extended to 35 cycles; even under modified condition, ssbB mRNA was barely detectable in rich medium. On the contrary, in minimal medium, the gene is expressed more or less equally throughout 96 h of growth (Figure 4B).

Identification of the promoter regions of ssa and ssb
As no obvious conserved promoter regions are present in the upstream regions of the two ssa genes, we analysed mRNA transcripts using 5′-RACE. As shown in Figure 4A, RT-PCR revealed that ssa was transcribed as a long mRNA with ribosomal gene(s). Two primers, GSP1 located at the beginning of ssa and RPR located at the end of rpsF (Figure 4) were used to obtain cDNA in a RACE experiment. Two transcriptional start points (TS1 and TS2) of rpsF were identified. TS1 was determined to be a G or C 75 nucleotides upstream of the start codon of ssa, whereas TS2 was located 163 nucleotides upstream of the translational start codon of rpsF. By inspecting upstream regions of TS1 and TS2, we could not find obvious promoter consensus sequences. However, detailed analysis of the upstream region showed a TTTACT sequence, 6 nucleotides apart from TS1 and a GAC motif 16 bp upstream of this sequence (Figure 5). These two putative promoter elements most closely resembled previously reported subclass G of Streptomyces promoters, GAC (N18/19) T(N4)T (52). Comparative analysis of these regions from other Streptomyces genomes displayed genetic variation upstream of the TS1 owing to short insertion and deletion events. Therefore, despite the synteny, the proposed promoter elements of the proximal promoter are located in the poorly conserved DNA region. The sequence homology blocks were found upstream of the TS2 (not shown). The transcriptional start of ssbB was determined to be a C, 73 nucleotides upstream of the AUG codon. Analysis

Table 1. Effect of the absence of Ssb in S. coelicolor

| Variable | Mean ± SD | 95% CI | P-value |
|----------|-----------|--------|---------|
| M145     | 27.20 ± 5.99 | 25.50–28.90 | 0.001 |
| TSB01    | 40.42 ± 13.6 | 36.54–44.30 |         |
| Number of spores in chain (n = 100) | 13.22 | 9.04–17.40 | <0.001 |
| Spore length (n = 200) | 1.24 ± 0.19 | 1.21–1.28 | 0.12 | <0.001 |
|          | 1.36 ± 0.35 | 1.30–1.43 |         |

The mutant strain, TSB01, has an increased number of spores in spore chains and slightly increased spore length.

The transcriptional start of ssbB was determined to be a C, 73 nucleotides upstream of the AUG codon. Analysis

Figure 4. Expression profiles of ssa genes. Samples were taken at different stages of growth characterized by visible actinorhodin accumulation. Gene expression was analysed by RT-PCR as described in ‘Materials and Methods’ section. Total cDNA was obtained using random hexanucleotide primers, and PCR was performed with gene-specific primers positioned as indicated in (A) and (B). (A1) Expression of ssa in rich media (RM), (A2) expression of ssa in minimal media (MM). (B) expression of ssb gene in RM and MM. (C) Control reactions and S. coelicolor culture flasks at given times, 16s—upper panel shows expression of 16S rRNA in RM, whereas 16s—lower panel in MM, -RT shows control reactions without reverse transcriptase.
of the ssbB promoter region did not show typical Streptomyces-E. coli like, promoter sequence [SEP-like sequences; TTGACA-18bp-TCTTAT (54) and TTGACPu-18-TAgPuPuT (53)], although we observed its activity in E. coli (unpublished data). Detailed inspection showed a complex DNA region with a palindromic sequence, DnaA box and two long imperfect direct repeats as indicated in Figure 5. Moreover, part of this long repeat (GGCTGCCACCCGTGC) was found at three additional positions in this promoter region.

**Determination of SsbB crystal structure and comparison with SsbA**

We determined the crystal structure of a tryptic SsbB fragment (1–116 aa) lacking the structurally disordered C-terminus at 1.7 Å resolution. There are 12 copies of the SsbB monomer in the asymmetric unit, forming three homotetramers. The r.m.s. deviation of Cα atoms between SsbB tetramers is 0.6 Å [calculated using the SUPERPOSE programme from the CCP4 suite (55)]. Each SsbB monomer possesses a single OB-fold comprising five β strands forming a β barrel capped by an α helix and one additional β strand. One feature unique to SsbB is the presence of two intermolecular disulphide bonds between the Cys7 residues from the AB and CD subunits (Figure 6). The presence of S-S bridges was detected by sodium dodecyl sulphate–polyacrylamide gel electrophoresis or western blot (Supplementary Figure S7A and B) using SsbB purified from S. coelicolor and E. coli cells. The specificity of protein band at the expected position of SsbB dimer was confirmed by western blotting. The dimer structure of SsbB was disrupted on addition of DTT (100 mM). In addition, we showed that 10 mM DTT almost completely abolished SsbB binding to ssDNA, whereas affinity binding of SsbA was not changed even in the presence of 100 mM DTT (Supplementary Figure S7C). According to PISA server calculations (56), the free energy of the tetramer dissociation (ΔG^\text{diss}) for the entire SsbB tetramer is 19.5 kcal mol^{-1} (average value for the three tetramers), whereas for SsbA, this value is 11.7 kcal mol^{-1}. Superposition of the quaternary structures of SsbA and SsbB from S. coelicolor is shown in Figure 6. The r.m.s. deviation of Cα atoms between SsbB and SsbA is 1.4 Å for entire tetramers and 1.0 Å for the superimposed monomers. The rotation of the BD subunit with respect to the AC subunit remains the same in both SsbA and SsbB (Figure 6).

**Binding properties of paralogous SSB proteins**

Cooperative binding mode of SSB proteins is important for DNA replication (6). To reinforce our finding that SsbA plays an important role during DNA replication, we evaluated the binding efficiency of SsbA and SsbB to the long ssDNA fragment by EMSA. Binding was tested using the circular ssDNA of 1748 nt. When increasing concentrations of SSB proteins were incubated with a fixed concentration of 1748 DNA, as described in ‘Materials and Methods’ section, a progressive decrease in the mobility of circular ssDNA could be seen for both proteins (Figure 7A1). However, a moderate concentration of salt (100 mM NaCl) significantly reduced cooperative binding of SsbB (Figure 7A2).
on addition of dT35 showed that the binding affinities for both SSB proteins dropped significantly with the addition of NaCl (Figure 7B1 and 2). Furthermore, without salt, the binding constant (K35) of SsbA is 7.9 × 10^6 M^{-1}, whereas (K35) of SsbB is 6.3 × 10^7 M^{-1}, showing that SsbB binds to ssDNA dT35 at almost tenfold higher affinity.

**Phylogenetic analysis of SSB proteins from Actinobacteria**

Our results clearly show that two paralogous genes have adopted different cellular functions through evolution. We determined whether this duplication is common to other members of the same phylum. To gain a better insight into the phylogenetic relationships of SSB proteins, we collected and analysed 129 available SSB sequences from 60 genera of Actinobacteria. We found that ~90% of the analysed species possess at least two ssb genes. Representative paralogous genes from each suborder belonging to five orders of Actinobacteria, and two paralogous SSBs from *B. subtilis* as an outgroup, were used to construct a maximum likelihood tree. Our phylogenetic analysis (Figure 8) demonstrated that SsbB and SsbA are clustered separately within the orders Actinomycetales and Bifidobacteriales. Branch lengths indicate that cluster with *S. coelicolor* SsbB protein are more heterogeneous than cluster with SsbB, perhaps indicative of the essentiality of that cluster of proteins. Two species with three SSBs (Stackebrandtia nausensis and Corynebacterium glutamicum) were also included in the analysis. As shown (Figure 8), their SsbC proteins were clustered differently; C. glutamicum SsbC was clustered with the SSB-A group, whereas SsbC from *S. nausensis* was positioned separately. Orders Coriobacteriales, Rubrobacterales and Salinibrobacterales are branched together with high confidence (see aLRT values at nodes); however, their SsbB proteins, when present, do not form a separate cluster. As an outgroup, the two SSBs from *B. subtilis* (14) were grouped together.

**DISCUSSION**

Until now, two limited studies were performed on SsbA, reporting close structural similarities to mycobacterial SSBs (11,57) as well as tyrosine phosphorylation (28). *S. coelicolor* possesses two ssb genes; both genes are located in the core region of the linear chromosome of *S. coelicolor* (Figure 1) defined by the presence of essential genes (2). We hypothesized that the retention of a duplicate ssb gene might be associated with an important cellular function (48).

*S. coelicolor* survives without SsbB but displays defect in chromosome segregation during sporulation

Mutational analysis allowed new insight into the biological role of SsbA and SsbB. As described in ‘Results’ section, we failed to disrupt ssbA with Tn5062. This gene lies between rpsF and rpsR, encoding ribosomal proteins. Conserved synteny of these genes in distantly related bacteria and coupled transcription were reported previously (13). Therefore, a polar effect caused by insertional gene replacement was anticipated, and a ‘scar’ mutation at the ssbA locus was constructed. Despite this strategy, deletion of the ssbA locus was obtained only in a strain with an additional copy of the wild-type gene, indicating that ssbA is essential for *S. coelicolor* survival, in line with other bacteria (4,6,13). In contrast, the flanking regions of ssbB with neighbouring genes are >200 bp, and a polar effect was not anticipated. The ssbB gene was disrupted by replacing the original chromosomal sequence with an *S. coelicolor* mutagenized cosmid. Numerous recombinant colonies were obtained, indicating that this gene is not essential.
However, the ssbB mutant (TSB01) displayed a white phenotype (Figure 2, Supplementary Figure S3A) and, although TSB01 differentiated on solid agar plates to form aerial hyphae, did not exhibit the grey colour characteristic of mature spores. As we could restore the parental phenotype by introducing an additional copy of ssbB (Figure 2, strain TSB02), we concluded that the white phenotype was a consequence of the mutation in ssbB. This was unexpected and intriguing, as a role of SSB in morphological development had not been reported previously. A white phenotype is often associated with pleiotropic developmental regulators (18,58). Several mutants displaying this phenotype have been studied for decades. Genes blocked at early stages (whiG, −A−B−H and −I) are defective in sporulation septation, whereas whiD and whiE mutants are blocked in spore maturation (59). In addition, two mutants that belong to the SsgA-like proteins (SALP family), ssgA and ssgB, also display a non-sporulating white phenotype, whereas ssgG showed, after 6 days of growth, a light grey appearance similar to

Figure 8. Phylogenetic tree of SSB proteins based on multiple alignment of full-length protein sequences from phylum Actinobacteria. The representatives of different orders are coloured as shown in legend, and aLRT values are shown for main branches.
the ssbB mutant (58). However, in this mutant, the unusually large spores, three to four times normal size, with proportionally segregated chromosomes were detected (58). As shown in Figure 3 and Supplementary Figure S4, the ssbB mutant produced spore chains with a severe nucleoid segregation defect. Unequal distribution of DNA in spore chains showed that the ssbB mutant was impaired in the segregation process rather than in replication. The percentage of spores lacking DNA in analysed spore chains showed that the ssbB mutant was impaired in the segregation process rather than in replication. The percentage of spores lacking DNA in analysed spore chains was ~30%. This is higher than for mutants in other genes associated with chromosome segregation (22,23,25). At present, it is not easy to conclude by what mechanism SsbB participates in chromosomal partitioning. Complementation with a truncated ssbB gene encoding protein with preserved tetramer structure, demonstrated that C-terminus of SsbB is important for its activity during chromosomal segregation. Thus, we speculate that SsbB interacts and supports the function of other DNA-binding proteins known to be important for chromosomal segregation. For example, ParB binds to numerous parS sites near oriC, whereas ParA provides support for proper distribution of these nucleoprotein complexes (49). FtsK controls the partitioning of chromosome before septal closure (20), whereas SMC binds to DNA and promotes its condensation (22). Single, double and triple mutations of these genes (21) did not completely block nucleoid segregation during sporulation; moreover, the percentage of spores lacking DNA in analysed spore chains was <25%. This indicates that SsbB makes an important contribution to chromosome segregation during sporulation.

Our analysis (Table 1) showed that the ssbB mutant had an increased number of spores in spore chains and slightly increased spore length. Although sensitivity test towards heat and lysozyme did not show similar differences between wild-type strain and mutant (Supplementary Figure S5), major changes in spore wall morphology were anticipated, and it is next task to examine this in detail. It was previously reported that strains with a DNA segregation defect caused by smc, ftsK or parB mutations produced spores that were similar in shape and size to the wild-type strain (21). Contrary to the smc, ftsK and parB mutants, the ssbB mutant also displayed a white phenotype, and we hypothesize that our observation are due to more severe chromosome partitioning defect than those reported previously or that the ssbB mutation causes a distinct defect at a final stage of spore maturation. In any case, it will be a key challenge to clarify the role of the SsbB protein in the complex mechanism of cell division in Streptomyces.

Transcriptional profile of ssb genes correlates with their proposed biological functions

Differences in the transcriptional profile of ssb genes were in agreement with their proposed biological functions. The ssbA is expressed throughout bacterial growth, being significantly upregulated in rich medium during the logarithmic phase, whereas under starvation conditions, its expression is prolonged (Figure 4A2). This result corresponds to the cellular level of SsbA from B. subtilis, which is higher during intensive DNA replication in fast-growing vegetative hyphae (13) and indicates that S. coelicolor SsbA might be a crucial component of DNA metabolism. Prolonged transcription of ssbA in minimal medium could be correlated to nutrient-limiting conditions that trigger aerial mycelium formation. This consequently leads to intensive replication in the apical regions of hyphae (17). Transcriptional analysis clearly showed that ssbA is expressed at a much higher level than ssbB as reported for B. subtilis (13). Even when PCR was extended to 35 cycles, ssbB transcripts were barely detectable in rich medium, whereas in minimal medium, the gene is expressed more or less equally throughout 96 h of growth (Figure 4B). This is consistent with the proposed role of SsbB during reproductive stage of Streptomyces growth.

We also identified transcriptional start sites for ssbA and ssbB (Figure 5), and these results were in concert with the transcriptional starts identified independently by RNAseq (Dr. K. McDowall, personal communication). Although ssbA is transcribed throughout growth, the proposed proximal promoter region is more active (Dr. K. McDowall, personal communication) and does not display a typical Streptomyces vegetative σ factor consensus sequence. In contrary, the proposed distal promoter elements most closely resemble SEP-like promoters (53,54). By inspecting promoter regions of ssbA, we found only short motifs that partially resembled previously described regulatory elements (54). Altogether this is not surprising, as streptomyces promoter regions are poorly understood and highly divergent (52–54) owing to their numerous σ factors encoded by these organisms (2). The promoter region of ssbB is complex and unusual. It is extremely GC rich (78.9%) in comparison with average GC content of Streptomyces promoter regions estimated to be 57–62% (53). It also possesses various repeats and palindrome sequences as shown in Figure 5. We speculate that this may indicate interactions of the promoter region with DNA-regulatory protein(s) required for the tight regulation of ssbB expression. A similar expression profile and possible promoter regulatory elements are found in other genes involved in chromosome segregation. For example, the gene-proximal promoter (P1), governing the expression of parAB throughout growth on minimal medium, displays a similar pattern of activity to ssbB, whereas the gene-distal promoter (P2) is upregulated during spore chain formation (23).

Unexpectedly, the ssbB promoter was active in E. coli causing filamentous growth (data not shown), as reported previously (4). At present, it is difficult to explain how an extremely GC-rich sequence and no obvious SEP-like promoter could act as a regulatory element in a distantly related bacterium.

Crystal structure of SsbB revealed unique variations

This is the first example of solved crystal structures of two SSBs from the same organism. Although the quaternary structure of SsbB reported here highly resembles the quaternary structure of S. coelicolor SsbA (12), distinctive variations were observed. The clamp mechanism found...
in SsbA, which includes a conserved ATAKVTK sequence (Figure 6) characteristic of high GC Gram-positive bacteria, is not preserved in SsbB. It has been proposed that this structure contributes to the stability of the SsbA homotetramer that is possibly important for the special requirement of high-GC content genomes (11). Therefore, we propose here that in the SsbB structure, this clamp is functionally substituted by two disulphide bridges that keep the quaternary structure highly stable and rigid. The presence of disulphide bridges in SSB structures has not been previously reported. As shown in Supplementary Figure S7A and B, disulphide bonds were detected in SsbB protein samples isolated from homologous and heterologous hosts. Although we could not exclude the possibility that S-S bond formation had occurred during protein purification, our results clearly indicated that binding affinity had decreased significantly in the presence of DTT (Supplementary Figure S7C). At present, we speculate that binding activity of the SsbB might be regulated during oxidative stress in *S. coelicolor*, and that formation of disulphide bridges increases its binding activity (60). In addition, calculations of the free energy of the tetramer dissociation (ΔGdiss) showed that SsbB is the most stable of all bacterial SSB proteins described so far (11).

**SsbA and SsbB bind to ssDNA with different affinity**

The EMSA experiment showed that SsbA and SsbB form complexes with circular ΦX1748 ssDNA. The results of this binding experiment indicated that SsbA binds to long ssDNA in a more cooperative way, as reported for SsbA from *S. pneumoniae* and SSB from *E. coli* (15,61). In addition, cooperative binding of *E. coli* SSB is favoured at low salt concentrations (61). The cooperative binding mode has been proposed to function in DNA replication (6). In agreement with this, our results support the predicted biological role of SsbA. Cooperative binding of SsbB was less pronounced and almost completely abolished under moderate salt concentrations (Figure 7A).

Different ssDNA binding affinities to dT35 were also observed for paralogous SSBs. Similarly to SSBs from *S. pneumoniae* (15), SsbB displayed a higher affinity for binding to shorter oligomers (dT35) than SsbA (Figure 7B). Interestingly, the opposite result was published recently for SsbA and SsbB from *B. subtilis* (14). This discrepancy can be explained by differences in the C-terminal domain. Previous studies showed that the size of C-terminal region as well as conserved acidic tail play an important role in binding affinity (47,62). At present, we speculate that different binding properties of *S. coelicolor* SSB proteins might be a consequence of their biological functions.

**Phylogenetic analysis indicates frequent duplication and retention of ssb genes in Actinobacteria**

Our results show that two paralogous genes have adopted different biological functions through evolution that possibly correlate with the complex life cycle of streptomycetes. To see whether this was confined to the genus *Streptomyces*, we determined the phylogeny of SSB proteins by analysing representative SSBs from related orders of the phylum Actinobacteria. Phylogenetic analyses (Figure 8) revealed that SsbA and SsbB from orders Actinomycetales and Bifidobacteriales are clustered, indicating that duplication occurred in a common ancestor. Retention of paralogs in most members of these orders indicates that these gene products have well-established roles (48). Although the SsbB proteins are branched together, they evolved much faster, as indicated by branch lengths. In contrast, among SsbA proteins, homology is conserved, probably reflecting their essential biological role. In the case of SSBs from *S. nausensis* and *C. glutamicum*, the third SSB, SsbC could be the result of new duplication or it could be a consequence of recent horizontal transfer. Members of other analysed orders belong to the deepest branches of Actinobacteria (63). Interestingly, their clustering might indicate that some of them underwent recent duplication, as their SSBs were grouped together. Some genomes did not retain duplicated genes or duplication did not occur.

**ACCESSION NUMBERS**

PDB ID: 4DAM.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1–4, Supplementary Figures 1–7 and Supplementary References [26,29,64–68].

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