

Bacterial single-stranded DNA-binding proteins are phosphorylated on tyrosine

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ABSTRACT

Single-stranded DNA-binding proteins (SSBs) are required for repair, recombination and replication in all organisms. Eukaryotic SSBs are regulated by phosphorylation on serine and threonine residues. To our knowledge, phosphorylation of SSBs in bacteria has not been reported. A systematic search for phosphotyrosine-containing proteins in *Streptomyces griseus* by immunoaffinity chromatography identified bacterial SSBs as a novel target of bacterial tyrosine kinases. Since genes encoding protein-tyrosine kinases (PTKs) have not been recognized in streptomycetes, and SSBs from *Streptomyces coelicolor* (ScSSB) and *Bacillus subtilis* (BsSSB) share 38.7% identity, we used a *B.subtilis* protein-tyrosine kinase YwqD to phosphorylate two cognate SSBs (BsSSB and YwpH) *in vitro*. We demonstrate that *in vivo* phosphorylation of *B.subtilis* SSB occurs on tyrosine residue 82, and this reaction is affected antagonistically by kinase YwqD and phosphatase YwqE. Phosphorylation of *B.subtilis* SSB increased binding almost 200-fold to single-stranded DNA *in vitro*. Tyrosine phosphorylation of *B.subtilis*, *S.coelicolor* and *Escherichia coli* SSBs occurred while they were expressed in *E.coli*, indicating that tyrosine phosphorylation of SSBs is a conserved process of post-translational modification in taxonomically distant bacteria.

INTRODUCTION

Protein phosphorylation plays an important role in the regulation of cellular processes in all organisms. The

demonstration of protein-tyrosine phosphorylation in bacteria (1) challenged the notion that this type of modification is restricted to the eukaryotes. Bacterial protein-tyrosine kinases (PTKs) possess a Walker A motif as their active site (2), while their eukaryotic counterparts contain Hanks motifs (3). Most bacterial PTKs reported are involved in the regulation of exopolysaccharide production (4–6) and were initially believed to be exclusively autophosphorylating enzymes, phosphorylating tyrosine(s) at their C-termini (7,8).

Recently, two endogenous substrates of bacterial PTKs have been identified: UDP-glucose dehydrogenases in *B.subtilis* (9) and *E.coli* (10), and an RNA polymerase sigma factor in *E.coli* (11). Growth-related variation in protein tyrosine phosphorylation has been reported in streptomycetes (12) and *Myxococcus xanthus* (13). However, no direct evidence for tyrosine phosphorylation of a protein involved in bacterial DNA metabolism is available. We describe the isolation and identification of tyrosine phosphorylated single-stranded DNA-binding proteins (SSBs), which are ubiquitous proteins that bind DNA in a sequence independent manner to maintain genome integrity in various stages of DNA metabolism: replication (14,15), recombination (16,17) and repair (18). Besides stabilizing single-stranded DNA (ssDNA), SSBs interact with enzymes such as DNA polymerase (19), RNA polymerase (20) or DNA helicase (21) and modulate their activity. Although accomplishing similar functions, bacterial and eukaryotic SSBs differ considerably in their structure.

Until this study, phosphorylation of SSBs was detected only in eukaryotes (22); such SSBs (23) are hetero-trimers, whereas bacterial SSBs are homo-tetramers. Phosphorylation of eukaryotic SSBs takes place on serine and threonine residues on the central RPA2 subunit, and is cell cycle-dependent (24) or induced by DNA damage (25). The physiological role of SSB phosphorylation is not clear, since in some cases it increases ssDNA binding (26), but in others had no effect (27). Phosphorylation of SSBs did not interfere with replication or

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nucleotide excision repair (28), although a more recent study suggests that it prevents association of SSB with replication centers (29). Induced DNA damage or apoptosis favored SSB phosphorylation, and it has been suggested that hyperphosphorylated SSB participates in DNA repair (30,31).

E. coli SSB has been well characterized and it serves as a model for eubacterial SSBs (32,33). Bacterial SSBs have two distinct domains (34): a conserved N-terminal domain responsible for tetramerization and DNA-binding (35), and a less conserved C-terminal domain important for the interaction of SSBs with various proteins (36). Many bacteria encode two SSBs that differ in size. In *B. subtilis*, it was shown that the larger SSB is an essential protein and participates in DNA replication, while the short SSB, lacking most of the C-terminal domain, is non-essential but plays a role in natural transformation (37).

We describe the *in vivo* and *in vitro* tyrosine phosphorylation of bacterial SSBs from taxonomically distant bacterial species. Phosphorylations *in vitro* were performed on homologous and heterologous substrates with the *B. subtilis* PTK YwqD.

MATERIALS AND METHODS

DNA manipulations, *E. coli* and *B. subtilis* strains and growth conditions

Bacterial SSB-encoding genes were PCR-amplified from the respective genomic DNAs: *ssb* (NCBI, GeneID: 937911) and *ywpH* (NCBI, GeneID: 936910) from *B. subtilis*, *ssb* (NCBI, GeneID: 948570) from *E. coli* and *ssb* (NCBI, GeneID: 1099343) from *S. coelicolor*. The point-mutation BsSSB Y82F was obtained using two partially overlapping mutagenic primers. All PCR products were inserted between the BamHI and PstI sites of the vector pQE-30 (Qiagen) and introduced in *E. coli* NM522. Cloned PCR products that were used for gene expression were verified by sequencing. For the homologous constitutive expression of *B. subtilis* *ssb*, a different set of primers was used: the 5'-primer contained

a synthetic promoter of moderate strength (38) (H. H. Saxild, personal communication), and the 3'-primer encoded a 6xHis tag. The PCR product was inserted in the vector pDG268neo (39) and *B. subtilis* was transformed (40). This additional copy of the *ssb* gene integrated in the *amyE* locus is transcribed from the Synthetic Promoter providing SSB 6xHis-Tagged protein for the strain labeled *B. subtilis* SPSSBHT. For inactivation of the *ywqD* and *ywqE* genes in *B. subtilis* SPSSBHT, PCR products containing their respective central regions were inserted between the EcoRI and BamHI sites of pMUTIN-2 (41) and used to transform *B. subtilis* SPSSBHT. All PCR primers are listed in Table 1. Both *E. coli* and *B. subtilis* were grown in Luria-Bertani (LB) medium at 37°C. Ampicillin (100 µg/ml), kanamycin (25 µg/ml), erythromycin (1 µg/ml) and neomycin (5 µg/ml) were added as appropriate. DNA damage in *B. subtilis* was induced by adding mitomycin C (60 ng/ml), at the onset of exponential growth (OD₆₀₀ ~ 0.2).

Culture conditions and preparation of cell-free extracts from *S. griseus*

S-medium (42) was used to culture *Streptomyces*. Cells were grown at 30°C until late exponential phase and mycelium (wet weight 20–25 g/500 ml) was collected by centrifugation (7000 g) at 4°C, washed in 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 % glycerol, and resuspended in the same buffer supplemented with protease and phosphotyrosine protein phosphatase (PTP) inhibitors [0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml pepstatin, 2 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM Na₃VO₄]. The biomass was passed twice through a French pressure cell at 68.9 MPa. Cell debris was removed by centrifugation at 20000 g and the proteins in the supernatant fractionated with ammonium sulfate at 0–45%, 45–75% and 75–100% saturation. The precipitates were collected by centrifugation at 20000 g for 40 min, dissolved and dialyzed against the supplemented buffer. Protein concentration was determined by OD₂₈₀, and tyrosine phosphorylated (PY) proteins were detected using anti-PY western blots.

Table 1. PCR primers used for amplification and mutagenesis with underlined restriction sites and bold typed mutated codons

Amplification primers		
<i>B. subtilis</i> <i>ssb</i> forward	CGGGATCCATGCTTAACCGAGTTGTATTAGTCGG	BamHI
<i>B. subtilis</i> <i>ssb</i> reverse	AAA <u>ACTGCAGTT</u> AGAAATGGAAGATCATCATCCGAG	PstI
<i>B. subtilis</i> <i>ywpH</i> forward	CGGGATCCATGTTCAATCAGGTCATGCTTGTCCGACG	BamHI
<i>B. subtilis</i> <i>ywpH</i> reverse	AAA <u>ACTGCAGTT</u> AATCAGCAGCTTTTCCCGGGGTTAGGG	PstI
<i>B. subtilis</i> <i>ssb</i> SP forward	GCGAATTCCTCCGAAGTTTATTCTTGACATGGGATCAACTTCGCGTATAATAG GTAATTGTGAGTAATAGAAATTATTGCTCCTTGCCATTATGGGCCGCTTAGTCC	EcoRI
<i>B. subtilis</i> <i>ssb</i> HT reverse	AAAAGGAGGTGCAAACAGATGCTTAACCGAGTTGTATTAGTCGGAAGAC CGGGATCCCTTATCCGTGATGGTGATGGTATGGAATGGAAGATCATCATCCGA GATGTCATCG	BamHI
<i>E. coli</i> <i>ssb</i> forward	CGGGATCCATGGCCAGCAGAGGCGTAAACAAGG	BamHI
<i>E. coli</i> <i>ssb</i> reverse	TTTCTGCAGTCAGAACGGAATGTCATCATCAAAGTC	PstI
<i>S. coelicolor</i> <i>ssbL</i> forward	CGGGATCCATGGCAGGCGAGACCGTCATCACGGTC	BamHI
<i>S. coelicolor</i> <i>ssbL</i> reverse	TTTCTGCAGTCAGAAGGGGGCTCGTCCGAGTAGCCGCC	PstI
Mutagenic primers		
<i>B. subtilis</i> <i>ssb</i> Y82F forward	CAAACAAGAACTTTGAAAACCAGCAAGGACAGCGTGTCTTCGTG	NA
<i>B. subtilis</i> <i>ssb</i> Y82F reverse	GCTGGTTTTCAAAGTTTCTTGTGTTGTAACCGCCATCTACGCC	NA
Primers for gene inactivation with pMUTIN-2		
<i>B. subtilis</i> <i>ywqD</i> forward	CGGGATCCGGCAAATGAAATCAGTCATGATTACATCGGC	BamHI
<i>B. subtilis</i> <i>ywqD</i> reverse	ATAAGAATGCGGCCGCTAACCCAGAACAGAACCGTCTGCCACGTTGCC	NotI

Production, purification and coupling of the anti-PY antibody 4G10 to Affi-gel 10

Monoclonal antibody 4G10 was produced by hybridoma cells (43). The cell supernatant was supplemented with 0.02% sodium azide, adjusted to pH 8 with 1 M Tris-HCl and passed through a column containing 2.5 ml of protein A Sepharose beads, previously washed with borate/EDTA buffer (0.1 M borate, 0.5 M NaCl and 2.5 mM EDTA, pH 8). The bound antibody was eluted with 3.5 M MgCl₂, monitoring protein concentration by OD₂₈₀. The pooled protein fractions were dialyzed and concentrated with Centrprep-10 columns at 4°C. Hybridoma cells produced 7–10 mg of antibody per liter.

Purified 4G10 was covalently coupled to Affi-gel 10 (BioRad) as recommended by the manufacturer, except that the antibody solution was first saturated with 10 mM para-nitrophenyl phosphate (PNPP), to prevent coupling via the antigen binding site (M. R. Gold, personal communication). An aliquot of 33 mg of antibody was coupled per 2 ml of wet gel volume. The efficiency of binding of 4G10 to beads was monitored by reading the OD₂₈₀ in the supernatant. The beads were washed with phosphate-buffered saline (PBS) buffer, then with PBS and 1 M NaCl and finally stored in PBS with 10 mM PNPP and 0.02% sodium azide.

Isolation of PY-proteins from *S.griseus*

4G10 affinity chromatography was used for enrichment of PY-proteins from the 0 to 45% ammonium sulfate fraction obtained from cell-free extracts of *S.griseus* using a modification of the published protocol (44). A typical purification cycle consists of the following steps. The most efficient binding of PY-proteins was obtained when 60 mg of fractionated extract in binding buffer (50 mM Tris-HCl, pH 7.7, 50 mM NaCl, 0.2% Triton X-100, 0.5 mM PMSF, 2 µg/ml pepstatin, 2 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM Na₃VO₄) was mixed with 2 ml of affinity matrix in a 10 ml column and placed on a rocking platform at 4°C for 4 h. The column was washed extensively with 50 mM Tris-HCl, pH 7.7, 50 mM NaCl, 0.2% Triton X-100 and 0.5 mM PMSF. Washing buffer supplemented with PNPP (2.6 mg/ml) was used for the elution of the PY-proteins. The eluates from 10 runs (~30 ml) were concentrated to one third of the initial volume on Centricon-10 columns (Amicon) and washed with 20 mM Tris-HCl, pH 7.7, 0.5 mM PMSF and 1 mM Na₃VO₄. Triton X-100 micelles did not pass through the concentrator membrane and were concentrated to 0.6%. A MonoQ ion exchange column (BioRad) was used for additional protein concentration and removal of Triton X-100 prior to separation of the isolated PY-proteins by preparative SDS-PAGE. Aliquots containing 10 ml of PY-protein fractions were loaded onto a 1 ml MonoQ column equilibrated with the same buffer at a flow rate of 0.3 ml/min. The column was washed with 20 mM Tris-HCl, pH 7.7, 2 mM EDTA, 0.5 mM PMSF, and then with the same buffer supplemented with 0.1 M NaCl. The proteins were eluted batchwise with 0.4 and 0.8 M NaCl, and the eluates analyzed by Western blotting. All fractions containing PY-proteins were pooled, desalted and concentrated 10-fold; an aliquot was run on 12% polyacrylamide-SDS gel and the gel was stained by standard silver staining procedure. The sample was submitted for peptide sequencing.

Synthesis and purification of tagged proteins

Synthesis of all 6xHis-tagged proteins was performed in *E.coli* NM522, except for YwqD and YwqD-123, whose reduced solubility required the use of a chaperon-overproducing strain (45). Protein purification and desalting were performed as described previously (9). When 6xHis-tagged SSB was purified from *B.subtilis* SPSSBHT, 10 mM sodium pyrophosphate was added to the cell lysate to inhibit the PTP YwqE (46); 8 M urea was added when the purified SSB was assayed by western blot.

Western blotting

PY-proteins purified by immunoaffinity chromatography were detected using 4G10 monoclonal anti-PY antibodies (Upstate Biotechnology) (12). Western blotting of purified 6xHis-tagged SSBs was performed as described previously (9). Approximately 50 ng of each protein (Bradford assay (Biorad)) were run in parallel on two identical pre-cast 10–20% gradient polyacrylamide-SDS gels (Cambrex) and each was electroblotted on to a PVDF membrane (Biorad). One membrane was treated with 10 U/ml of alkaline phosphatase in the blocking buffer. Both membranes were incubated for 1 h with the peroxidase-conjugate monoclonal anti-PY (Sigma) diluted 1:40 000. Peroxidase-conjugate anti-phosphoserine/anti-phosphothreonine (Upstate Biotechnology) diluted 1:5000 was used as negative control. The signals were visualized with a peroxidase AEC staining kit (Sigma).

In vitro phosphorylation assays

Protein phosphorylations were performed with 1 µM PTK, YwqD or YwqD-123 (the non-autophosphorylatable form of the kinase), and 1 µM YwqC-Nter, a truncated form of the transmembrane modulator of PTK activity (9). A typical 40 µl reaction contained 50 µM [γ -³²P]ATP (20 µCi/mmol), 1 mM MgCl₂, 100 mM Tris-HCl, pH 7.5 and 1–5 µM SSBs. Reactions were incubated at 37°C for 60 min. For the 'phosphate-sliding' assay, ~50 µg of YwqD were autophosphorylated in a scaled-up reaction described above, without SSB. [³²P]Tyr-YwqD and YwqC-Nter were then desalted (two successive PD-10 columns, Pharmacia) to remove the [γ -³²P]ATP, and to exchange initial buffer to 20 mM NH₄HCO₃ buffer. After lyophilization, the proteins were resuspended in reaction buffer without [γ -³²P]ATP, mixed with *B.subtilis* SSBs (BcSSB or YwpH) and incubated overnight at 37°C to permit the transfer of [³²P] from [³²P]Tyr-YwqD to the SSBs. All reactions were stopped by adding SDS-PAGE sample buffer and heating at 100°C for 5 min. Proteins were separated by electrophoresis on 15% SDS-polyacrylamide gels, then washed by boiling in 0.5 M HCl for 10 min to reduce the radioactive background and dried. Signals were visualized with the STORM PhosphoImager and quantified with ImageQuant (Amersham).

In vivo labeling of *B.subtilis* SSB

B.subtilis strains SPSSBHT, SPSSBHT- Δ ywqD and SPSSBHT- Δ ywqE were grown in 100 ml of LB medium supplemented with 300 µCi of [³³P]phosphate. Cells were harvested at the late exponential phase (OD₆₀₀ 0.8) and 6xHis-tagged SSB was purified from each strain as described. Purified

proteins were lyophilized in 20 mM NH_4HCO_3 , resuspended in 30 μl of 100 mM Tris-HCl, pH 7.5 and separated by electrophoresis on 15% SDS-polyacrylamide gels. Radioactive signals were visualized as described.

Gel filtration

Sephacryl™ S-300 (Pharmacia) gel filtration column (working buffer 100 mM Tris-HCl at pH 6.8) was calibrated with protein standards of following sizes: 14, 45, 57 and 98 kDa. Purified 6xHis-tagged *B.subtilis* proteins, SSB, SSB-Y82F, SSB-Y82E and YwpH, were loaded separately on the column, eluate was collected in 0.3 ml fractions, and the protein content monitored by the Bradford assay (Biorad).

Gel-shift assays

Random sequence oligonucleotides (50 bases) labeled with [^{33}P] were purchased from DNA Technology. Different ratios (indicated in the figure legend) of the oligonucleotide and BsSSB were mixed in 100 mM Tris-HCl, pH 7.5, incubated for 10 min at 37°C and loaded on a 12.5% native polyacrylamide gel (without SDS). After migration, the gel was dried, the radioactive signals visualized and quantified as described.

Determination of phosphorylation site by mass spectrometry

SSB purified from *B.subtilis* SPSSBHT (50 μg) was dissolved in 8 M urea, reduced with 1 μg of DTT, carboxyamido-methylated with 5 μg of iodoacetamide, diluted with 4 vol of 50 mM NH_4HCO_3 and digested O/N with 1 μg of trypsin. The digest was separated into 10 fractions on a Source™ 15RPC ST 4.6/100 column (Amersham Pharmacia Biotech). For phosphopeptide enrichment, each fraction was incubated with 50 μl of PHOS-Select™ IMAC beads (Sigma), according to the manufacturer's instructions. Eluted peptides were dried, resuspended in 1% trifluoroacetic acid, and LC-MS/MS analysis was performed on an 1100 nano-HPLC system (Agilent Technologies) coupled to a LTQ-FT mass spectrometer (Thermo Electron), as described previously (47), but without Single Ion Monitoring scans in the acquisition cycle. Spectra were acquired in the positive ion mode with acquisition cycle consisting of a full scan in the FT ICR cell, followed by MS/MS scans of the five most intense ions in the linear ion trap. Resulting mass spectra were searched against the NCBI database using the Mascot search engine (Matrix Science).

RESULTS AND DISCUSSION

Affinity purification of PY SSB from *S.griseus* and detection of PY SSBs in *B.subtilis* and *E.coli*

In order to apply affinity chromatography for purification of the PY-proteins from *Streptomyces griseus*, we produced a 4G10 monoclonal antibody specific for PY-proteins and coupled the antibody to Affi-gel 10 as described in Materials and Methods. We reported previously (12) that the pattern of PY-proteins varied during the growth phase; accordingly we harvested the cells at the logarithmic growth stage when

increased tyrosine phosphorylation was noted. The culture conditions, preparation of the cell-free extracts and ammonium sulphate fractionation are prepared as described in Materials and Methods. The 0–45% ammonium sulphate fraction contained the same number and size of proteins phosphorylated on tyrosine as detected in *S.griseus* previously (12). The maximal binding capacity of the affinity column was when 60 mg of 0–45% ammonium sulphate fraction was applied to 2 ml affinity matrix, no PY proteins were detected in the flow through fraction. The presence of the PY proteins in the eluates were detected by immunoblotting assay and the samples containing PY-proteins were pooled. According to published purification procedure (44) MonoQ ion-exchange chromatography was used for buffer exchange and protein concentration. The eluates were analysed by western blotting, the fractions with PY-proteins from 10 purification cycles were pooled, desalted and concentrated as described in Materials and Methods. Prior to submitting for sequencing the amount of the proteins was estimated by running an aliquot on the SDS-PAGE and silver staining (data not shown). Several protein bands were detected and estimated to be between 0.5 and 2 μg . The isolated PY-proteins were sequenced and the peptide (Q/K)AAENVAES(I/L)(Q/K)R identified as amino acids 62–73 of a putative *S.coelicolor* A3(2) SSB protein (NCBI protein databases, accession no. CAB42735).

No gene encoding a PTK has yet been identified in streptomycetes. To determine whether ScSSB could be phosphorylated *in vitro* with *S.coelicolor* cell extract the corresponding *S.coelicolor* gene (*ssb*) was cloned, and 6xHis-SSB purified from *E.coli*. *In vitro* phosphorylation of 6xHis-SSB was carried out with both cytoplasmic and membrane extracts from *S.coelicolor* as described previously (48), but no evidence of phosphorylation could be detected. A database search revealed a 38.7% identity in a 163 amino acid overlap between *S.coelicolor* SSB and *B.subtilis* SSB. Protein tyrosine kinase (YwqD) has been described in *B.subtilis* and shown to phosphorylate endogenous substrates (9). We observed that both genes are identically grouped on the chromosome with ribosomal protein genes (37). *S.coelicolor* and *B.subtilis* are both Gram-positive spore-forming genera (49,50) and we assumed that SSBs from both bacteria might be similarly phosphorylated on a tyrosine residue.

This prompted us to examine the *in vivo* phosphorylation of SSB in *B.subtilis* SPSSBHT that was constructed to constitutively produce endogenous 6xHis-tagged SSB. Purified *B.subtilis* SSB reacted with the anti-PY antibody (Figure 1A, lane 1). In contrast to eukaryotic SSBs, no signal was obtained with anti-phosphoserine/anti-phosphothreonine antibody (data not shown). *B.subtilis* SSB was also phosphorylated when overproduced in *E.coli* (Figure 1A, lane 3), indicating that an *E.coli* PTK, possibly Wzc (8), might be involved. *E.coli* *ssb* was cloned, and 6xHis-SSB overproduced in *E.coli*. As expected, *E.coli* SSB strongly reacted with anti-PY antibodies (Figure 1A, lane 5). *S.coelicolor* SSB was also phosphorylated in the *E.coli* host (Figure 1A, lane 7). Differences in signal intensity are likely due to the structural variability of the SSB proteins as has been reported for *M.tuberculosis* and *E.coli* (51). To confirm the specificity of the signals, the samples were treated with the alkaline phosphatase and all signals were significantly reduced (Figure 1A, lanes 2, 4, 6 and 8). To exclude the possibility that the Western

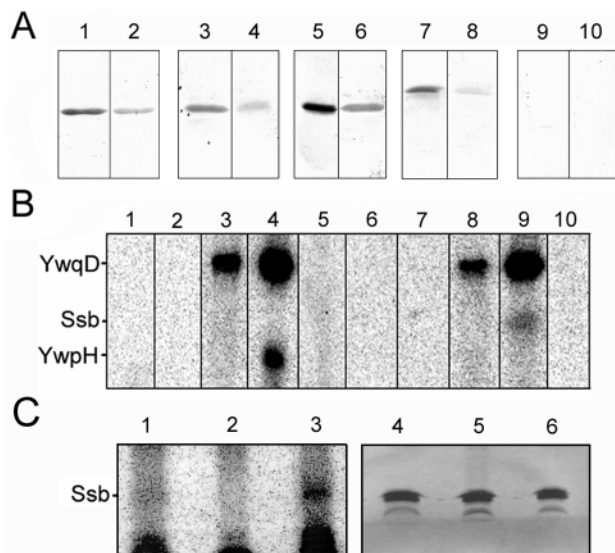


Figure 1. Tyrosine phosphorylation of different SSBs. (A) *In vivo* phosphorylation of bacterial SSBs. The odd-numbered lane contains purified 6xHis tagged protein (50 ng) separated on SDS-PAGE, electroblotted on PVDF membrane, and probed with anti-PY antibody, and the even-numbered lane contains the same sample treated with alkaline phosphatase. *B.subtilis* SSB purified from *B.subtilis* is in lanes 1 and 2, BsSSB purified from *E.coli* in lanes 3 and 4, EcSSB purified from *E.coli* in lanes 5 and 6, ScSSB purified from *E.coli* in lanes 7 and 8, and the eluate from the Ni-NTA column obtained from a crude extract of wild-type *B.subtilis* is in lanes 9 and 10. (B) *In vitro* phosphorylation of *B.subtilis* SSBs. Autoradiography of *in vitro* phosphorylation assays separated on SDS-PAGE. The lanes contained the products of reactions with the following proteins: YwpH (lane 1), YwpH and YwqC-NCTer (lane 2), YwpH and YwqD (lane 3), YwpH, YwqC-NCTer and YwqD (lane 4), BsSSB (lane 6), BsSSB and YwqC-NCTer (lane 7), BsSSB and YwqD (lane 8), BsSSB, YwqC-NCTer and YwqD (lane 9). Lanes 5 and 10 were the same as lanes 4 and 9, respectively, only YwqE and $MnCl_2$ were added after 60 min, and the reactions were left for an additional 120 min before loading on SDS-PAGE. (C) *In vivo* labeling of *B.subtilis* SSB. 6xHis SSB was purified from SPSSBHT, SPSSBHT- $\Delta ywqD$ and SPSSBHT- $\Delta ywqE$ grown in [^{33}P]phosphate. Purified BsSSB was separated by SDS-PAGE; the Coomassie-staining of the gel is shown in the right panel; and autoradiography signals in the left: SPSSBHT in lanes 1 and 4, SPSSBHT- $\Delta ywqD$ in lanes 2 and 5, and SPSSBHT- $\Delta ywqE$ in lanes 3 and 6.

signal came from a co-migrating protein, co-purifying with the BsSSB, we prepared an extract from the wild-type *B.subtilis* strain (no 6xHis-tagged SSB) that underwent the same purification procedure as that from SPSSBHT. Since this sample gave no signal (Figure 1A, lane 9), we concluded that the assay was specific for PY-SSB.

These findings provide the evidence that bacterial SSBs, until this study unidentified substrates of bacterial PTKs, are tyrosine phosphorylated and that this post-translational modification is conserved in Gram-positive and Gram-negative bacteria.

B.subtilis PTK YwqD phosphorylates cognate SSBs *in vitro*

We also tested the ability of YwqD to modify bacterial SSBs *in vitro*. YwqD interacts with the intracellular domain of a transmembrane modulator YwqC-NCTer during phosphorylation (9). The results of *in vitro* phosphorylation are presented in Figure 1B. Only when YwqD and YwqC-NCTer were

present in the phosphorylation mixture did YwpH and BsSSB phosphorylation occur (Figure 1B, lanes 4 and 9). YwqD-123, the non-autophosphorylatable form of YwqD (9), retained full capacity for SSB phosphorylation (data not shown). Although we have shown that *E.coli*, *B.subtilis* and *S.coelicolor* SSBs can be phosphorylated in *E.coli* (Figure 1A), the *E.coli* and *S.coelicolor* SSBs were not phosphorylated by *B.subtilis* YwqD *in vitro* (data not shown). This may reflect the low efficiency of the *in vitro* phosphorylation system with the artificial modulator (9).

The phosphorylation of *B.subtilis* SSBs by YwqD was compared with that of the UDP-glucose dehydrogenase YwqF (9). While YwpH was the most rapidly phosphorylated substrate in initial velocity measurements (9.8 nM/min), BsSSB phosphorylation (2.6 nM/min) was comparable with the phosphorylation of YwqF (3 nM/min) (9). As in the case of YwqF, phosphorylation of SSBs by YwqD was strictly ATP-dependent; [^{32}P]YwqD did not transfer phosphate to either BsSSB or YwpH in absence of ATP (data not shown). Optimal BsSSB phosphorylation occurred at a lower $MgCl_2$ concentration (1 mM) compared with YwqF (5 mM).

To confirm the identity of the modified residues in *B.subtilis* SSBs, the phosphorylation mixtures containing [^{32}P]YwpH and [^{32}P]BsSSB were treated with a specific PTP, YwqE (46) to remove the radioactive label from both phosphoproteins (Figure 1B, lanes 5 and 10), confirming the presence of labeled PY in the SSBs.

B.subtilis PTK YwqD and PTP YwqE control the phosphorylation state of BsSSB *in vivo*

Since *in vitro* phosphorylation assays show that YwqD and YwqE act on *B.subtilis* SSB, we examined if the same enzymes control the phosphorylation state of the SSB *in vivo*. *B.subtilis* strains SPSSBHT, SPSSBHT- $\Delta ywqD$ and SPSSBHT- $\Delta ywqE$ were grown in the presence of [^{33}P]inorganic phosphate, and the purified BsSSB assayed by autoradiography (Figure 1C). A weak radioactive signal comigrated with BsSSB purified from SPSSBHT (Figure 1C, lane 1), indicating *in vivo* phosphorylation. This signal was no longer detectable in the kinase deficient strain (Figure 1C, lane 2), and the signal was strongly enhanced in the phosphatase deficient strain (Figure 1C, lane 3). These results confirm that the kinase YwqD is indeed responsible for phosphorylating *B.subtilis* SSB, and the phosphatase YwqE carries out its dephosphorylation *in vivo*.

Phosphorylation of *B.subtilis* SSB increases ssDNA binding *in vitro*

Effective binding to ssDNA requires tetramers of bacterial SSBs (32). The oligomerization state of purified BsSSB and YwpH was checked by gel filtration, indicating that both proteins are tetramers in solution (data not shown). The efficiency of ssDNA binding was assayed by gel-shift analysis using a 50 base ssDNA fragment as substrate (Figure 2A, lanes 1–4). The ssDNA fragment binds one or two SSB tetramers, confirming observation that the bacterial SSB tetramer could wrap 25–30 bases of ssDNA (52). To test whether *B.subtilis* SSB phosphorylation affects ssDNA binding, 2 pmol of BsSSB were pre-phosphorylated with YwqD and YwqC-NCTer. As

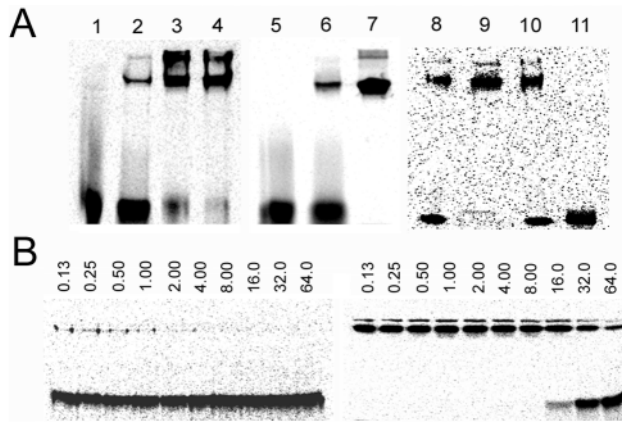


Figure 2. The influence of *B. subtilis* SSB phosphorylation on ssDNA binding. (A) Gel shift assay with 0.2 pmol of [33 P]labeled ssDNA (present in all lanes) with different quantities of *B. subtilis* SSB. Lane 1 contained only the ssDNA; lanes 2–4 contained 1, 10 and 100 pmol of SSB, respectively; lane 5 contained YwqD, YwqC-NCTer, 1 mM MgCl₂ and 5 mM ATP (negative control); lane 6 contained 2 pmol of SSB; lane 7 contained 2 pmol of SSB pretreated with YwqD, YwqC-NCTer, 1 mM MgCl₂ and 5 mM ATP for 1 h before the gel-shift. Lane 8 contained 5 pmol of SSB; in lane 9 the same quantity of SSB was *in vitro* phosphorylated (same treatment as in lane 7), and the sample from lane 9 was dephosphorylated by YwqE in the presence of 1 mM MnCl₂ for 20 min (lane 10) and 60 min (lane 11). (B) Gel shift assay with SSB purified from SPSSBHT- Δ ywqD. All reactions contained 125 nM SSB, and the total ssDNA concentration in nM (same amount of radiolabeled and increasing amount of unlabelled probe) is indicated above each lane. Left panel: unphosphorylated SSB, right panel: SSB phosphorylated *in vitro* before the gel shift study.

shown in Figure 2A (lane 6), this amount of BsSSB shifts a fraction of the probe, but when the same amount of pre-phosphorylated BsSSB was used, the probe was shifted completely (Figure 2A, lane 7). Since the same amount of YwqD and YwqC-NCTer did not lead to ssDNA shift (negative control, Figure 2A, lane 5), we assumed that the SSB phosphorylation might increase ssDNA-binding. To verify this, the amount of BsSSB was increased to 5 pmol (shifting ~50% of the probe) (Figure 2A, lane 8); the binding could still be stimulated by phosphorylation (Figure 2A, lane 9), but when the *in vitro* phosphorylated sample (lane 9) was treated with the PTP YwqE binding was again reduced (Figure 2A, lanes 10–11). A 60 min treatment with YwqE abolished all binding, suggesting that YwqE removed phosphates attached to BsSSB *in vivo*. The SSB purified from the kinase deficient strain SPSSBHT- Δ ywqD exhibited very weak binding in the gel shift assay using a wide range of ssDNA concentrations (Figure 2B, left panel). Phosphorylation of this sample *in vitro* increased its affinity for ssDNA almost 200-fold, supporting the conclusion that this modification facilitates the ssDNA binding. The mechanism of this activation is presently not known. Phosphotyrosine would repel the negatively charged DNA polymer in direct contact, so it is more likely that the phosphorylation maintains the SSB tetramer in a state of higher affinity for ssDNA.

SSB needs to be removed from and re-attached to ssDNA during bacterial growth in concert with various stages of DNA metabolism, such as DNA replication or lesion repair (32,53). The results of this study suggest that SSB phosphorylation could participate in this process by changing its affinity to bind to ssDNA.

SSB phosphorylation in *B. subtilis* decreases during DNA damage response

Investigating all potential implications of SSB phosphorylation is beyond the scope of the present study. To initiate this research, we examined the relation of this post-translational modification to the DNA lesion repair since a consensus exists regarding the link between SSB phosphorylation and DNA lesion repair in *Eukarya* (23). Concomitant to DNA damage, the ssDNA-dependent protein kinase is activated to phosphorylate the SSB, but it is not known how hyper-phosphorylated SSB enhances lesion repair.

When the lesions are induced, the fraction of ssDNA is likely to increase in the cells. As presented, the ssDNA exerted an inhibitory effect on *in vitro* phosphorylation of YwqH (Figure 3A) and SSB (similar results, data not shown). An equivalent amount of double-stranded DNA had a less pronounced inhibitory effect; ssDNA also inhibited YwqD autophosphorylation, irrespective of the presence of SSBs (data not shown), suggesting that ssDNA exerts its effect directly on the kinase. Furthermore, we examined SSB phosphorylation *in vivo* while DNA damage was induced by adding mitomycin C at the onset of exponentially growing *B. subtilis* SPSSBHT cultures. The phosphorylation state of 6xHis-SSB was checked at different time intervals using anti-PY antibodies (Figure 3B). BsSSB was phosphorylated to a significantly lower extent in the mitomycin-treated cells, suggesting that dephosphorylated BsSSB after induction of DNA damage might play a role in DNA repair. According to this *Eukarya* and *Bacteria* might respond differently to DNA damaging conditions.

Therefore, we compared the growth of *B. subtilis* SPSSBHT- Δ ywqD cells with *B. subtilis* SPSSBHT in the presence of mitomycin C. Cultures of *B. subtilis* SPSSBHT- Δ ywqD reached a higher OD₆₀₀ than *B. subtilis* SPSSBHT before growth finally stopped (Figure 3C). Since DNA damaging agents often cause cell filamentation, thus rendering OD measurements non-reliable for estimating cell proliferation (54), we also compared the colony-forming ability of the two strains after induction of DNA damage (Figure 3D). The CFU of both strains fell dramatically after the addition of mitomycin, but *B. subtilis* SPSSBHT- Δ ywqD retained a 2-fold higher colony forming ability throughout the experiment. This could indicate that kinase activity might affect DNA repair. However, our data could also be ascribed to a pleiotropic effect of ywqD deletion. Nevertheless, it can not be neglected, as depicted in Figure 3B, that BsSSB phosphorylation is inhibited in the mitomycin-treated cells with wild type kinase activity. The reduction in the level of BsSSB phosphorylation that decreases its affinity for ssDNA is in agreement with published data. SSB pre-bound to ssDNA inhibits the nucleation stage of RecA and subsequent RecA-dependent repair. On the other hand, SSB is crucial for maximal activity of RecA-mediated reactions, suggesting that the interplay of affinity for DNA binding between SSB and RecA must be precisely tuned in concert with cell requirements (53). If BsSSB phosphorylation participates in this tuning, deletion of the PTP-encoding gene ywqE should also affect cell growth and survival. However, SPSSBHT- Δ ywqE exhibited no significant phenotype (data not shown). It might be that other *B. subtilis* PTPs, such as YwqE and YfkJ (46), were also capable of dephosphorylating PY-SSB.

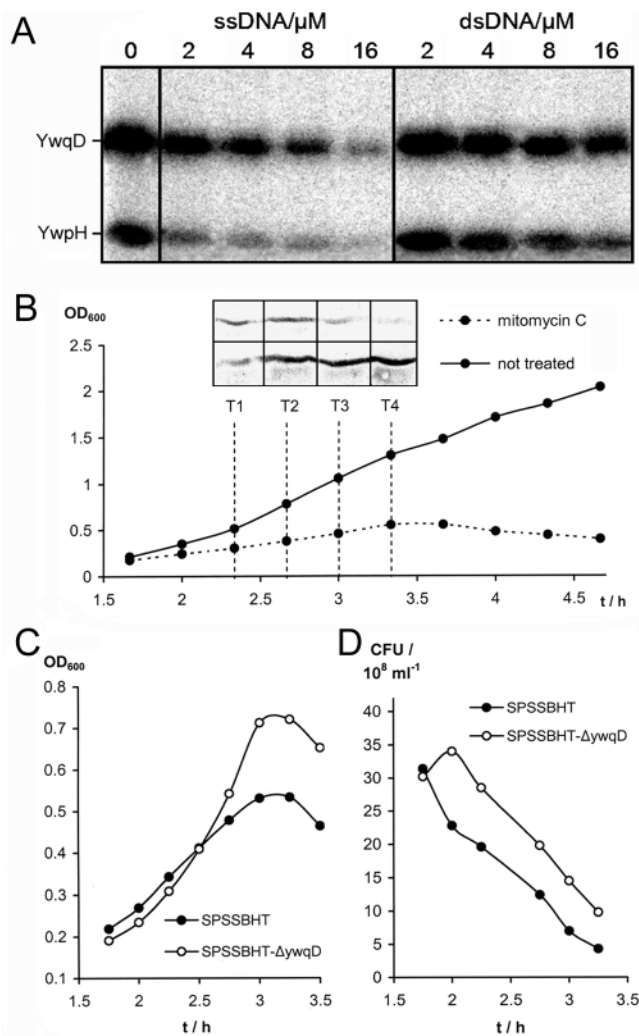


Figure 3. Effect of DNA damage on BsSSB phosphorylation. (A) Control kinase reaction is shown in lane 1. The same reaction was performed in presence of increasing amounts of ssDNA (lanes 2–5) and dsDNA (lanes 6–9). (B) BsSSB phosphorylation followed by anti-PY western blotting in *B. subtilis* strain SPSSBHT, with and without 60 ng/ml mitomycin C (added at 1.5 h). Samples from both cultures were taken at regular time intervals and anti-PY western blotting was performed with equal quantities (about 50 ng) of purified BsSSB. Growth of *B. subtilis* SPSSBHT- $\Delta ywwqD$ and *B. subtilis* SPSSBHT strains was compared in DNA damaging conditions. Mitomycin treatment was the same as above, the OD₆₀₀ (C) and the colony forming ability (D) of the growing cultures were monitored. One representative of four independent experiments is shown.

B. subtilis SSB is phosphorylated on tyrosine 82 *in vivo*

In order to identify the phosphorylated residue, purified SSB from *B. subtilis* SPSSBHT was analyzed by mass spectrometry (Figure 4A). LC-MS analysis detected a doubly charged peptide at m/z 608.748, the mass of which matched (within 12 p.p.m.) the theoretical mass of the *B. subtilis* SSB peptide NYENQQGQR containing one phosphate group. An MS/MS spectrum of the peptide showed pronounced y-ion series, confirming its sequence and pointing to Y82 as the phosphorylation site. Identification was furthermore confirmed by comparison with the fragmentation spectrum of the non-phosphorylated peptide (data not shown). As shown in

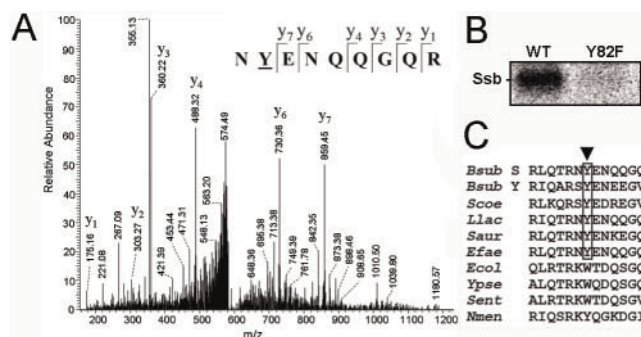


Figure 4. Identification of the tyrosine phosphorylated residue in *B. subtilis* SSB. (A) MS/MS spectrum of the precursor ion at m/z 608.748, corresponding to the *B. subtilis* SSB peptide NYENQQGQR with one phosphorylated residue. C-terminal y-ion series confirms the peptide sequence and indicates Y82 as the phosphorylation site. The low intensity of the spectrum reflects low occupancy of the phosphorylation site. (B) *In vitro* phosphorylation of mutant protein BsSSB Y82F compared with wild-type BsSSB. Reactions were performed with YwqD-123. (C) Sequence alignment of the region surrounding the phosphorylated residue Y82 in *B. subtilis* SSB. Aligned bacterial SSBs are from *B. subtilis* (*Bsub*), *S. coelicolor* (*Scoe*), *Lactococcus lactis* (*Llac*), *Staphylococcus aureus* (*Saur*) *Enterococcus faecalis* (*Efae*), *Escherichia coli* (*Ecol*), *Yersinia pseudotuberculosis* (*Ypse*) *Salmonella enterica* (*Sent*) and *Neisseria meningitidis* (*Nmen*). *B. subtilis* SSB and YwpH are denoted by 'S' and 'Y', respectively.

Figure 4B, when the residue Y82 in *B. subtilis* SSB was replaced by phenylalanine, *in vitro* phosphorylation of the mutant protein was severely impaired, with residual phosphorylation still detectable. The BsSSB Y82F migrated as tetramer on gel filtration column (data not shown), suggesting that its overall structure was not disrupted by this mutation. This residue is highly conserved in SSBs from Gram-positive bacteria (Figure 4C). *E. coli* SSB has no tyrosine at this position, but was also found to be tyrosine-phosphorylated. How can this be explained? *E. coli* possesses four tyrosine residues in the SSB protein. Among these, two tyrosine residues (78 and 98) are located close to tyrosine residue 82 in BsSSB identified as phosphorylation site. Three-dimensional modeling of *B. subtilis* and *S. coelicolor* SSBs strongly implies (A. Krisko and D. Vujaklija, unpublished data) that tyrosine residue 98 in EcSSB occupy nearly the same molecular space as tyrosine residue 82 in BsSSB and therefore is the most likely phosphorylation site in EcSSB. This is in agreement with the crystal structure data of the EcSSB tetramer which reveals that tyrosine residue 78 is placed inside the tetramer while tyrosine residue 98 is exposed on the surface.

CONCLUSION

We have shown that SSBs are the target of bacterial PTKs and that this specific post-translational modification occurs in *Streptomyces* spp., *B. subtilis* and *E. coli*. This suggests that tyrosine phosphorylation of SSBs may be a general process in Gram-positive and Gram-negative bacteria. Focusing on the *B. subtilis* system, we have identified the kinase (YwqD) and the phosphatase (YwqE) that affect the phosphorylation state of the SSB on its residue Y82. Phosphorylation of BsSSB increased almost 200-fold its binding affinity for ssDNA. During DNA damage response, tyrosine phosphorylation of

BsSSB was reduced and this could suggest one biological aspect of this process in the regulation of DNA metabolism.

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