Isolation and characterization of the gene encoding single-stranded-DNA-binding protein (SSB) from four marine *Shewanella* strains that differ in their temperature and pressure optima for growth

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The *ssb* gene, coding for single-stranded-DNA-binding protein (SSB), was cloned from four marine *Shewanella* strains that differed in their temperature and pressure optima and ranges of growth. All four *Shewanella ssb* genes complemented *Escherichia coli ssb* point and deletion mutants, with efficiencies that varied with temperature and *ssb* gene source. The *Shewanella* SSBs are the largest bacterial SSBs identified to date (24.9–26.3 kDa) and may be divided into conserved amino- and carboxy-terminal regions and a highly variable central region. Greater amino acid sequence homology was observed between the *Shewanella* SSBs as a group (72–87%) than with other bacterial SSBs (52–69%). Analysis of the amino acid composition of the *Shewanella* SSBs revealed several features that could correlate with pressure or temperature adaptation. SSBs from the three low-temperature-adapted *Shewanella* strains were an order of magnitude more hydrophilic than that from the mesophilic strain, and differences in the distribution of eight amino acids were identified which could contribute to either the temperature or pressure adaptation of the proteins. The SSBs from all four *Shewanella* strains were overproduced and partially purified based upon their ability to bind single-stranded DNA. The differences found among the *Shewanella* SSBs suggest that these proteins will provide a useful system for exploring the adaptation of protein–protein and protein–DNA interactions at low temperature and high pressure.

Keywords: marine shewanellas, single-stranded-DNA-binding protein (SSB), temperature adaptation, pressure adaptation

INTRODUCTION

Life in most deep-sea environments is characterized by a temperature of approximately 2 °C, and by hydrostatic pressures extending up to 1100 atm (1 atm = 101 kPa = 1.01 bar). These selective constraints have exerted strong effects on protein evolution. Numerous examples exist of proteins from deep-sea fishes and invertebrates which possess enzymes and structural proteins whose activities and stabilities are optimized for low-temperature and high-pressure conditions (Somero,

1992a, b). A number of enzymes from low-temperature-adapted (psychrophilic) micro-organisms have been distinguished from those of their mesophilic or thermophilic counterparts by an increase in activity accompanied by a decrease in stability when such measurements are compared at a common temperature (Rentier-Delrue *et al.*, 1993; Hoffman & Jendrisak, 1990). It therefore seems reasonable to expect that proteins from deep-sea psychrophilic, barophilic ('pressure loving', also referred to as piezophilic; Yayanos, 1995) bacteria will possess proteins modified for function and turnover at extremes of low temperature and high pressure.

Studies with proteins from mesophiles indicate that proteins which form tetramers or aggregates of higher order may be particularly affected by low temperature

Abbreviation: SSB, single-stranded-DNA-binding protein.

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or high pressure. Under both conditions it is believed that subunit dissociation is followed by conformational changes which lead to loss of activity (Weber, 1992; Silva & Weber, 1993). At low temperatures, the conformationally drifted subunits or aggregates may predominate because of the high activation energy required for conversion back to the native state. At high pressures, it follows from Le Chatelier's principle that a decrease in subunit affinity will result if the volume change for dissociation is negative. The decreased volumes associated with many dissociated protein subunits arise from void volumes associated with subunit-subunit contacts and solvent-protein interactions.

In addition, protein-DNA association may also be sensitive to low temperature or high pressure. Low-temperature stress leads to the induction of a number of DNA-binding proteins in *Escherichia coli*, including DNA gyrase A subunit, H-NS, RecA, and CS7.4 (CspA) (Jones *et al.*, 1992). Elevated levels of certain DNA-binding proteins may be necessary at low temperature to compensate for decreased DNA binding, and the resulting effects on DNA structure and function. Although *E. coli* is not known to adapt to changes in hydrostatic pressure, high-pressure treatment of *E. coli* leads to the induction of some cold-shock proteins, including RecA and H-NS (Welch *et al.*, 1993). This suggests that bacterial cells may perceive a common signal under both low-temperature and high-pressure stresses. *In vitro* studies also indicate that both low temperature and high pressure can perturb protein-DNA association (Foguel & Silva, 1994; Macgregor, 1992; Royer *et al.*, 1990).

In order to begin to characterize the biochemical adaptation of protein-protein and protein-DNA interactions to low-temperature and high-pressure environments, we have elected to examine the deduced amino acid sequence of the single-stranded-DNA-binding protein (SSB) from closely related bacteria of the genus *Shewanella*. Shallow-water and deep-sea *Shewanella* isolates are available (DeLong & Yayanos, 1986; Jannasch & Wirsén, 1984; Jensen *et al.*, 1980; Yayanos *et al.*, 1982) which differ with regard to their temperature and pressure optima and ranges for growth (Table 2). SSB offers several advantages for such comparative analyses, including the fact that it is a small, conserved, homotetrameric DNA-binding protein which performs essential roles in DNA replication, recombination and repair, and for which fluorescence spectroscopic methods have been developed for examining its quaternary structure and DNA-binding characteristics (reviewed by Meyer & Laine, 1990). Here we present the cloning and sequencing of the *ssb* genes from four *Shewanella* strains, and the overproduction and partial purification of the SSB proteins.

METHODS

Strains and media. Strains and plasmids used in this study are listed in Table 1. More details of the *Shewanella* strains are given in Table 2. All *E. coli* strains were cultured at 37 °C with the exception of strain PAM5779, which was cultured at

30 °C. *E. coli* strains were grown in L broth (Davis *et al.*, 1980) and plated on L agar (L broth containing 1.5% agar, Difco). Medium containing ampicillin (100 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹) or mitomycin C (2.0 µg ml⁻¹) was used for the selection of resistant clones. *Shewanella* strains SC2A, F1A and *S. hanedai* were routinely cultured in 2216 Marine Medium (28 g l⁻¹, Difco), at their temperature optima (Table 2) and at 1 atm hydrostatic pressure. *Shewanella* strain PT99 was cultured at 9 °C and 680 atm hydrostatic pressure in filtered Marine Medium A (per litre: 28 g 2216, 5 g yeast extract, 10 g Casamino acids, 0.22 g trimethylamine-*N*-oxide dihydrate, 2 g sodium succinate, 2.38 g HEPES). Growth temperature optimum for *S. hanedai* was determined for cultures grown aerobically in 2216 Marine Medium at 1 atm; the growth pressure optimum was determined for cultures grown microaerobically in Marine Medium A.

***Shewanella* SC2A cosmid library preparation.** The SC2A genomic library was prepared in the cosmid vector Supercos I as directed in the kit manual (Stratagene), using SC2A genomic DNA partially digested with *Sau*3A (30 kb average DNA fragment size; Sambrook *et al.*, 1989). The cosmid library was screened for the SC2A *ssb* gene by the method of de Vries & Wackernagel (1994), by complementation of the *E. coli* *ssb* point mutant PAM5779 (*ssb*-113).

DNA manipulations and hybridization. Chromosomal DNA was isolated by the method of Silhavy *et al.* (1984). DNA fragments containing the entire *ssb* gene from each of the *Shewanella* strains were identified by Southern blot analysis (Southern, 1975) using either the first 504 bp of the *Shewanella* SC2A *ssb* gene (see below) or the *E. coli* *ssb* gene (the 0.7 kb *Bam*HI-*Eco*RI fragment of pJA40; Table 1) as a probe. All probes were labelled with [α -³²P]dATP by random priming (Boehringer-Mannheim). Size fractionation of DNA, ligation into M13mp18, screening of the M13 library and subcloning into the plasmid vector pUC8 or pUC19, were performed as described by Sambrook *et al.* (1989). Plasmid ligations were introduced into either *E. coli* DH5 α or *E. coli* XL-1 Blue, by transformation of competent cells. Plasmids were prepared either by the alkaline lysis method (Sambrook *et al.*, 1989) or using QIAprep columns (QIAGEN). Gel-purified DNA fragments were obtained using the QIAquick gel purification kit (QIAGEN).

DNA sequencing and analysis. Double-stranded and single-stranded DNA sequencing was performed by the *Taq* DyeDeoxy thermal-cycle sequencing method (Applied Biosystems). Plasmids pLC10, pLC20, pLC30 and pLC40 were the source of the *Shewanella* SC2A, *S. hanedai*, *Shewanella* F1A, and *Shewanella* PT99 *ssb* genes, respectively. Additional information on the PT99 *ssb* sequence and proximal ORFs was obtained from an M13mp18 DNA clone. Primers used for sequencing included the M13 universal forward and reverse primers and several custom-synthesized primers specific to each *Shewanella* *ssb* sequence. Completed sequencing reactions were purified through Centri-Sep columns (Princeton Separations) and analysed on an Applied Biosystems model 373A automated sequencer.

Homology searches were conducted using the BLAST Network Service at the National Center for Biotechnology Information, National Institutes of Health (Altschul *et al.*, 1990). Multiple SSB sequence alignment was performed using the *NEWT* 87 programs *SCORE* and *PREALIGN* (Feng & Doolittle, 1990). The percentage identity/similarity values of the nucleotide and deduced protein sequences were calculated using the University of Wisconsin Genetics Computer Group (GCG)

Table 1. Strains and plasmids used in this study

Relevant genotype		Source or reference
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1</i>	Hanahan (1983)
F'DH5 α	F' derivative of DH5 α	Hanahan (1983)
XL1 Blue	<i>recA1 lac</i> {F' <i>proAB lacI</i> ^a Δ M15, Tn10}	Stratagene
PAM5779	<i>ssb</i> -113	Johnson (1977)
RDP268	Δ (<i>ssb-uvr A</i>)	Porter <i>et al.</i> (1990)
<i>Shewanella</i>		
SC2A	<i>ssb</i> ⁺	Yayanos <i>et al.</i> (1982)
<i>S. hanedai</i>	<i>ssb</i> ⁺	Jensen <i>et al.</i> (1980)
F1A	<i>ssb</i> ⁺	Jannasch & Wirsen (1984)
PT99	<i>ssb</i> ⁺	DeLong & Yayanos (1986)
Plasmids		
pJA40	<i>E. coli ssb</i> ⁺ gene in pUC9	Brandsma <i>et al.</i> (1985)
pSBH5	<i>E. coli ssb</i> ⁺ gene in pBluescript II KS	de Vries & Wackernagel (1994)
pACYC <i>ssb</i>	<i>E. coli ssb</i> ⁺ gene in pACYC184, Cm ^r	Porter <i>et al.</i> (1990)
pUC8	High copy number vector, Ap ^r	Norrandar <i>et al.</i> (1983)
pUC19	High copy number vector, Ap ^r	Norrandar <i>et al.</i> (1983)
pET23a	High copy number expression vector, Ap ^r	Novagen
pLC10	<i>S. SC2A ssb</i> ⁺ gene in pUC8	This work
pLC20	<i>S. hanedai ssb</i> ⁺ gene in pUC19	This work
pLC30	<i>S. F1A ssb</i> ⁺ gene in pUC19	This work
pLC40	<i>S. PT99 ssb</i> ⁺ gene in pUC19	This work
pLC101	<i>S. SC2A ssb</i> ⁺ gene in pET23a	This work
pLC201	<i>S. hanedai ssb</i> ⁺ gene in pET23a	This work
pLC301	<i>S. F1A ssb</i> ⁺ gene in pET23a	This work
pLC401	<i>S. PT99 ssb</i> ⁺ gene in pET23a	This work
Cosmids		
SuperCos I	Broad host range cosmid vector	Stratagene
Phage		
M13mp18	Phage cloning vector	Norrandar <i>et al.</i> (1983)
mGP1-2	M13 derivative containing phage T7 RNA polymerase on a <i>Bam</i> HI- <i>Sal</i> I fragment derived from the plasmid pGP1-1	Hostomski <i>et al.</i> (1989), Tabor & Richardson (1985), S. Tabor & C. C. Richardson, unpublished

Table 2. Description of *Shewanella* strains used in this study

Strain	T_{opt} [*] (°C)	Isolation depth (m)	P_{opt} [†] (atm)	Reference for T_{opt} and P_{opt}
SC2A	20	2000	136	Tobal (1993)
<i>S. hanedai</i>	12–18	0	1	This study
F1A	8	4900	300	Jannasch & Wirsen (1984)
PT99	9‡	8600	680‡	–

* Optimum growth temperature.

† Pressure for optimum growth at optimum temperature.

‡ P_{opt} and T_{opt} not determined; values indicate culture parameters.

program BESTFIT (Smith, 1988; Devereux *et al.*, 1984). The distribution of the amino acids in the entire SSB protein and the indicated portions was determined using the STADEN

program ANALYSEP (Staden, 1988) and the deduced hydrophilicity of the proteins was determined by the method of Hopp & Woods (1981).

Amplification by polymerase chain reaction and cloning of the *ssb* genes. All PCR reactions were performed using *Pfu* polymerase (Stratagene) at annealing temperatures ranging from 50 °C to 55 °C, in 100 µl buffer containing 20 mM Tris/HCl (pH 8.75), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 100 µg bovine serum albumin ml⁻¹, 250 µM of each dNTP, 1 µM each primer and about 250 ng genomic DNA (Saiki *et al.*, 1988). The following primers were employed for the amplification of the *Shewanella* *ssb* genes: *Shewanella* SC2A, 5' ATT CAT ATG GCT AGT CGT GGT GTG 3' (forward), 5' GCG AGC AAG CTT GAA ATA CGG CTG GCA 3' (reverse); *S. hanedai*, 5' AAT CAT ATG GCC AGT CGT GGT GTC 3' (forward), 5' GCT AAG CTT GCG TAG ATA TTG CTC CCT AGA ACG 3' (reverse); *Shewanella* F1A, 5' GCG AGC AAG CTT CTA GAA CGG GAT 3' (reverse); *Shewanella* PT99, 5' GCG ACG AAG CTT GCC GTA GCG AGG TTT GAG 3' (reverse). The *Shewanella* SC2A forward primer was used for amplifying the F1A and PT99 *ssb* genes. Each forward primer incorporated the *Nde*I restriction site (underlined), while the reverse primers incorporated a *Hind*III site (underlined) for ease of cloning into the expression vector pET23a (Novagen). Products of PCR reactions were purified using the QIAquick PCR purification kit, restricted with *Nde*I and *Hind*III, and ligated into correspondingly restricted pET23a. The ligations were introduced into *E. coli* F'DH5α by transformation of competent cells. The presence of insert was verified by restriction analyses, yielding plasmids pLC101 (*Shewanella* SC2A), pLC201 (*S. hanedai*), pLC301 (*Shewanella* F1A) and pLC401 (*Shewanella* PT99). The following primers were designed to amplify the first 504 bp of the SC2A *ssb* gene to use as a probe for *ssb* genes from the other *Shewanella* strains: 5' ATG GCT AGT CGT GGT GTG AA 3' (forward), 5' TTA CCC TGC TGC GGC AGG CGC AGT 3' (reverse). All PCR amplifications were performed using genomic DNA.

Complementation of *E. coli* PAM5779 (*ssb*-113) temperature sensitivity. *E. coli* PAM5779 was transformed with plasmids pLC10, pLC20, pLC30 and pLC40 and equal aliquots of the transformation were plated on L agar plates with ampicillin alone or with ampicillin and mitomycin C. Ampicillin-containing plates were incubated at 30 °C, and those containing both ampicillin and mitomycin C at 37 °C. Counts of Ap^r Mit^r colonies were normalized to counts of Ap^r colonies and expressed as a percentage. Colony counts on the ampicillin plates, for each transformation, were designated the 100% value. Transformations were performed in triplicate and the standard deviation of the normalized values was calculated.

Complementation of *E. coli* PAM5779 UV sensitivity. Aliquots (5 µl) of exponential-phase cultures (OD₅₉₀ 0.8) of *E. coli* PAM5779 transformed with pLC10, pLC20, pLC30 or pLC40 were gridded onto L agar plates. The gridded cultures were irradiated using a germicidal lamp (200–280 nm, Ultraviolet Products). UV emission was measured at 1.41 J m⁻² s⁻¹ (calibrated with an International Light IL-1500 research radiometer) with exposure to UV ranging from 0 to 127 J m⁻² along the grid. After irradiation, plates were covered and incubated at either 30 °C or 37 °C. Triplicate samples were irradiated and incubated at each temperature.

Complementation of the *E. coli* *ssb* deletion mutant RDP268. *E. coli* RDP268(pACYC*ssb*) (Porter & Black, 1991) was transformed with the *Shewanella* *ssb* plasmids pLC10, pLC20, pLC30 and pLC40. A single Ap^r Cm^r colony from each transformation was grown at 37 °C or 22 °C in L broth containing ampicillin and subcultured daily for 9 d. Aliquots were removed at 2 d intervals and dilutions replica-plated on L agar plates containing ampicillin. The relative proportion of

Ap^r Cm^r colonies was determined by replica gridding of Ap^r colonies on plates containing ampicillin, and ampicillin plus chloramphenicol.

Overproduction and partial purification of the *Shewanella* SSBs. Aliquots (100 µl) of overnight cultures of *E. coli* F'DH5α containing pLC101, pLC201, pLC301 or pLC401 were diluted into 6 ml L broth containing ampicillin and aerated at 37 °C until an OD₅₉₀ of 0.8 was obtained. Overproduction of the SSB protein was induced by delivery of T7 RNA polymerase through infection with the induction phage mGP1-2 at a multiplicity of infection of 10–100 (Hostomski *et al.*, 1989) and addition of IPTG (Sigma) to a final concentration of 0.2 mM. Aeration was continued for 90 min and the cells harvested by centrifugation at 14000 g for 3 min. SSB was partially purified from the induced cells by the method of de Vries & Wackernagel (1994) with the following modifications: affinity chromatography using ssDNA cellulose (Sigma; 1.5 ml ssDNA cellulose per ml lysate) was done by batch elution rather than on a column, with a 0.3 M KCl wash in buffer A (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, and 25%, v/v, glycerol) and elution with 2.0 M KCl in buffer A. The extent of protein overproduction and enrichment was visualized by electrophoresis of samples through a 15% SDS polyacrylamide gel (Laemmli, 1970).

RESULTS

Cloning of the *ssb* gene from the marine *Shewanella* strains

The temperature- and UV-sensitive *E. coli* *ssb* mutant PAM5779 (*ssb*-113; Table 1) was transformed with a *Shewanella* SC2A genomic library prepared in the cosmid vector Supercos 1 (Stratagene). Among roughly 140000 transformants screened, 22 clones resistant to both ampicillin and mitomycin C were identified, all of which contained cosmids with an insert size of about 30 kb. Southern hybridization of three of the 22 clones with a probe consisting of the *E. coli* *ssb* gene identified a single strongly hybridizing band corresponding to a 1.6 kb *Eco*RI fragment. This fragment was subcloned onto the high-copy-number plasmid pUC8 (Norlander *et al.*, 1983), resulting in plasmid pLC10.

Chromosomal DNA fragments containing the entire *ssb* gene from each of the four *Shewanella* strains were identified by restriction enzyme analysis and Southern hybridization with the *Shewanella* SC2A *ssb* probe. This probe was generated by PCR amplification of the first 504 bp of the SC2A *ssb* gene, following nucleotide sequence determination (see below). After size fractionation of the restricted DNA, phage M13mp18 DNA libraries were constructed for *S. hanedai*, *Shewanella* F1A and *Shewanella* PT99, as described in Methods. The resulting plaques were screened for the *ssb* gene using the SC2A *ssb* probe. Strongly hybridizing plaques were plaque purified and insert sequences present in the phage vector were subcloned onto the plasmid vector pUC8 (*S. hanedai* *ssb*) or pUC19 (F1A *ssb* and PT99 *ssb*). This resulted in plasmids pLC20, pLC30 and pLC40, containing a 1.7 kb *Xho*I–*Sal*I *S. hanedai* DNA fragment, a 2.7 kb *Xba*I–*Hind*III *Shewanella* F1A DNA fragment, and a 3.7 kb *Eco*RI–*Hind*III *Shewanella* PT99 DNA fragment, respectively.

Fig. 1. Complementation of the temperature sensitivity of the *E. coli ssb-113* mutant PAM5779 by *Shewanella ssb* genes. Strain PAM5779 was transformed with plasmids pUC19 (vector control), pSBH5 (*E. coli ssb*), pLC10 (*Shewanella* SC2A *ssb*), pLC20 (*S. hanedai ssb*), pLC30 (*Shewanella* F1A *ssb*) and pLC40 (*Shewanella* PT99 *ssb*). The level of complementation of the mutant was determined as described in Methods.

Complementation of an *E. coli ssb* point mutant

The *E. coli ssb* point mutant PAM5779 (*ssb-113*) is sensitive to DNA-damaging agents such as UV irradiation and mitomycin C (Johnson, 1977). In the presence of mitomycin C, PAM5779 grows very poorly or not at all at 37 °C but is capable of normal growth at 30 °C. Transformation of the mutant with either pSBH5 (*E. coli ssb*; Table 1), or the *Shewanella ssb*-containing plasmids pLC10, pLC20, pLC30 or pLC40, restored growth ability at 37 °C in the presence of mitomycin C. The extent to which these plasmids complemented the temperature sensitivity of the mutant *E. coli* strain is shown in Fig. 1. *ssb* from the mesophilic *Shewanella* strain SC2A showed levels of complementation of the mutation comparable to that produced by the wild-type *E. coli ssb* gene. Complementation by *ssb* from the psychrotrophic *S. hanedai* and psychrophilic *Shewanella* strains F1A and PT99 was less efficient. Transformation of PAM5779 with the control plasmid, pUC19 alone, did not restore the ability to grow at 37 °C.

Resistance to UV irradiation was also evaluated for PAM5779 (*ssb-113*) in the presence or absence of high-copy-number plasmids bearing either the wild-type *E. coli ssb* or the marine *Shewanella ssb* genes. The effect of increasing UV irradiation on the *E. coli ssb-113* mutant when transformed with the plasmids pUC19, pSBH5, pLC10, pLC20, pLC30 and pLC40 is shown in Fig. 2. Under the conditions employed, the highly UV-sensitive *recA* *E. coli* strain DH5 α did not survive even 13 J m⁻² exposure, while the limit of UV exposure for PAM5779 survival was 51 J m⁻². As expected, the control plasmid pUC19 did not confer any UV resistance upon PAM5779. With the exception of pLC40, carrying the *Shewanella* PT99 *ssb*, all the *ssb*-containing plasmids increased the UV resistance of PAM5779. Wild-type *E. coli ssb* and *ssb* from the mesophilic *Shewanella*, SC2A, conferred UV resistance for an irradiation of up to 127 J m⁻². The *ssb* genes from the *S. hanedai* and *Shewanella* F1A did not complement the mutation as

Fig. 2. Complementation of the UV sensitivity of *E. coli* PAM5779 (*ssb-113*) by *Shewanella ssb* genes. Aliquots (5 μ l) of exponential-phase cultures (OD₅₉₀ 0.8) of *E. coli* strains DH5 α (*recA* mutant) and PAM5779, as well as strain PAM5779 transformed with the plasmids pUC19, pSBH5, pLC10, pLC20, pLC30 and pLC40 (indicated on the left of the figure) were gridded on L agar plates. The gridded cultures were incrementally exposed to UV irradiation from 0 to 127 J m⁻². The extent of subsequent growth at 37 °C after 18 h indicated the UV resistance of each strain.

efficiently, survival being restricted to irradiation of less than 102 J m⁻² and 76 J m⁻², respectively. The UV resistance of the *ssb-113* mutant strain bearing the *Shewanella* PT99 *ssb* plasmid, pLC40, was not significantly higher than that of the mutant alone. However, unlike the results obtained at either 30 °C or 37 °C, complementation of UV sensitivity by *Shewanella* PT99 *ssb* was evident at 22 °C (data not shown).

Complementation of an *E. coli ssb* deletion mutant

SSB is an essential protein and *E. coli ssb* deletion mutants have been shown to be non-viable (Porter & Black, 1991). The *E. coli ssb* deletion mutant RDP268 (Table 1) carries a helper plasmid pACYC*ssb* which provides the essential *ssb* gene along with a chloramphenicol-resistance marker. Plasmid bumping experiments were carried out to replace the *E. coli ssb*-bearing plasmid in the deletion mutant with the *Shewanella ssb*-bearing plasmids pLC10, pLC20, pLC30 and pLC40, as outlined in Methods. It has been previously demonstrated that the *ssb* genes from the enteric bacteria *Serratia marcescens* and *Proteus mirabilis* can complement the *E. coli* deletion mutant and replace pACYC*ssb* (de Vries & Wackernagel, 1994; de Vries *et al.*, 1994). RDP268 transformants containing both the *E. coli ssb* plasmid (Cm^r) and the *Shewanella ssb* plasmids (Ap^r) were grown at 22 °C or 37 °C and selection was maintained only for the ampicillin-resistance marker on the *Shewanella ssb* plasmids. Loss of the original pACYC*ssb* plasmid resulted in a Ap^r Cm^s strain. The *Shewanella ssb* plasmids pLC10, pLC20 and

Fig. 3. Schematic view of the size, position and orientation of the cloned *Shewanella* *ssb* genes and additional proximal ORFs present in plasmids pLC10, pLC20, pLC30 and pLC40. Solid box, *lac* promoter; stippled box, *lacZ* gene; boxes with overlying arrows, identified ORFs; arrows, ORF polarity; H, HindIII site; R, EcoRI site; S, SalI site; X, XhoI site; Xb, XbaI site; a, portion of an ORF with homology to the *csgF* gene of *E. coli*.

pLC30 replaced the *E. coli* *ssb* plasmid at both 22 °C and 37 °C. Within the duration of the experiment, the *Shewanella* PT99 *ssb* plasmid pLC40 replaced pACYC*ssb* at 22 °C but not at 37 °C.

Sequence analyses of the four *Shewanella* *ssb* genes

The nucleotide sequences of the *ssb* genes on plasmids pLC10, pLC20, pLC30 and pLC40 were determined using a combination of vector-specific and *ssb* internal sequencing primers. Fig. 3 presents the size, position and orientation of the four *ssb* genes. In addition to the *ssb* genes, a number of additional ORFs with similarity to other gene products of known function were identified using the BLAST network service (Altschul *et al.*, 1990) to search the National Center for Biotechnology Information nucleic acid databases. For example, a portion of an ORF with strong homology to the UvrA protein of *Haemophilus influenzae* (*P* value 2.5×10^{-66}), *E. coli* (*P* value 2.7×10^{-63}) and *Salmonella typhimurium* (*P* value 2.8×10^{-63}), among others, was found about 1800 bp upstream on the *Shewanella* PT99 *ssb* gene. Between this ORF and the *ssb* gene is a third ORF oriented in the direction of the *ssb* gene and having strong homology to a variety of antiporter proteins isolated from *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis*, among others. This arrangement of the *ssb* and *uvrA* gene differs from that seen in the enteric bacterial *ssb* genes examined to date (de Vries & Wackernagel, 1994; de Vries *et al.*, 1994; Jarosik & Hansen, 1994). Although the *ssb* and *uvrA* genes are divergently transcribed in *Shewanella* PT99, as is the case with many enteric bacteria, the PT99 genes are interrupted by a third gene whose product has no obvious significance to DNA repair. In enteric bacteria, *uvrA* and *ssb* gene expression is regulated by the DNA-damage-inducible LexA protein (Walker, 1984). No sequences similar to the canonical LexA-binding sequence of *E. coli* (Walker, 1984; also referred to as the SOS box) are evident in the apparent intergenic region between the UvrA-like and antiporter-like ORFs. However, a sequence with similarity to the consensus sequence of the *E. coli* SOS box is located 82–98 bp upstream of the *Shewanella* PT99 *ssb* gene.

Approximately 860 bp upstream of the *S. hanedai* *ssb* is part of an ORF with significant homology (*P* value 0.036) to the *csgF* gene of *E. coli* K-12. The *csgF* gene is believed to be necessary for the transport and assembly of curli polymers, thin coiled surface structures that mediate binding to extracellular matrix and serum proteins by *E. coli* K-12 (Hammar *et al.*, 1995).

The *Shewanella* *ssb* genes were 75–93 % identical to one another, while the homologies to other bacterial *ssb* genes ranged from 63 to 70 %. At the protein level, the extent of homology shared between the deduced *Shewanella* SSB amino acid sequences ranged from 72 to 87 %, whereas the identity shared between any of the *Shewanella* SSBs and any of the bacterial SSBs ranged from 52 to 69 %. The *Shewanella* SSBs were also distinguished in size from one another and as a group from other bacterial SSBs. They range from 226 aa residues (*S. hanedai* SSB) to 243 aa residues (*Shewanella* SC2A SSB) and are considerably larger than the *E. coli* SSB, which has 177 residues. In contrast, all of the remaining bacterial SSBs characterized are of similar size to *E. coli* SSB. These data suggest that the *Shewanella* SSBs constitute a distinct subfamily of SSB proteins. Indeed, construction of an SSB protein phylogenetic tree using the maximum parsimony PAUP program (Swofford, 1991) indicated branching at the 100 % confidence level between the *Shewanella* SSBs and all other bacterial SSBs (data not shown).

Alignment of the deduced amino acid sequence of the *Shewanella* strains with that of *E. coli* SSB revealed several interesting features (Fig. 4). All five SSBs show a high degree of similarity up to position 114 of the *Shewanella* SSBs (position 126 of the *E. coli* SSB). Within this region, nearly all the residues shown to be involved either in DNA binding (W40, W54, F60, W88) or subunit interaction (H55) in *E. coli* (reviewed by Meyer & Laine, 1990) are found in identical positions in the *Shewanella* SSBs. Of the residues listed above, only F60 is substituted with a tyrosine residue in all four *Shewanella* SSBs. In this portion of the protein, there are only three unconserved substitutions within the shewanellas, in positions 48, 93 and 106, positions known to be highly

<i>E. coli</i>	1	MASRGVNKVLVGNLQDPEVRYPNGGAVANITLATSESWRDKATGEMKEQTEWHRVVLFGKLAEVASE	70
<i>S. SC2A</i>	1		69
<i>S. hanedai</i>	1		69
<i>S. F1A</i>	1		69
<i>S. PT99</i>	1		69
		+	+
Amino-terminal/Variable			
<i>E. coli</i>	71	YLRKGSQVYIEGQLRTRKWTQ.SGQDRYTTEVVNVGGTMQLGGR.....QGGGA.PAGGNIGGGQPQ	132
<i>S. SC2A</i>	70	K KV L KLQ S Q TT K INAM...E DS NSGDNAG QYQGGQSSGQNAAG	136
<i>S. hanedai</i>	70	K KI I KLQ S Q AT K INAS...E DSGQG Q.QGGGDYGGQONQ	131
<i>S. F1A</i>	70	K KV I KLQ S Q AT K INAN...E DSAQG Q.QGGYSQQQDQQ	131
<i>S. PT99</i>	70	K KV I KLQ S Q AT K INAH...E DSGQS Q.QGDYGSQQAAQQ	131
		+	+
<i>E. coli</i>	133	SGWGQPQ.....QPQGNQFSGGAQSRPQQ.....	157
<i>S. SC2A</i>	137	QNMGQQ SRPAPAPQARPPQAQYNQTAPAAGNAAYQAQPNAG QGGGYQQQSAPAAQQAAPAYAPKPAAP	206
<i>S. hanedai</i>	132	YNAPQQ ...APQGGYAPKPSAPQQPAYAPKPSAPQPAQ QG.GYQSQQQ...PQAPAYAPKPAAP	195
<i>S. F1A</i>	132	QQAPQQ RQQAPQPQTSYQGGQTPQPSSQSGYGQQQAAPQ QAPAYAPKQQGAQQAP.QQAASKAAP	200
<i>S. PT99</i>	132	SSGGYQ .QALQQQGSQQ..QAAQQKSTQDS.GQQQAAPQ QAPAYAPKQQEAQKAP.QQAVSKAAP	196
		+	+
Variable/carboxy-terminal			
<i>E. coli</i>	158	SAPAAPSNEPPMDF.....DDDIPF	177
<i>S. SC2A</i>	207	QGMFQQQNAPAPQAPQPQNFPTDLDEGW	242
<i>S. hanedai</i>	195	QQRAPAPQQAAPQNFPTDLDDGW	226
<i>S. F1A</i>	201	QQRAPAPQQAAPQNFPTDLDDGW	231
<i>S. PT99</i>	198	QQRAPAPQQAAPQNFPTDLDDGW	227
		++	+++++++ ++

Fig. 4. Alignment of amino acid sequence of SSBs from *E. coli* and *Shewanella* SC2A, *S. hanedai*, *Shewanella* F1A and *Shewanella* PT99. Boxed regions indicate the amino-terminal, carboxy-terminal and central variable regions of the protein. Spaces indicate amino acid residues that are identical in all five SSB sequences; plus signs indicate amino acid residues which are identical between the *Shewanella* SSB sequences alone; dots indicate gaps in the sequence. The first and last amino acid in each line of sequence is numbered.

variable among the other bacterial SSBs. Most of the remainder of the *Shewanella* SSBs consists of a highly variable central portion, much of which is absent in other bacterial SSBs. A stretch of six identical amino acid residues, PAYAPK, is seen in this region of all four *Shewanella* SSBs but is absent in other SSBs. *S. hanedai* has an internal repeat of these residues. Finally, all of the *Shewanella* SSBs contain a well-conserved carboxy-terminal domain of 19 residues, the last six amino acids of which are also shared with other bacterial SSBs. Within these 19 residues, the *Shewanella* SC2A SSB has a single conserved substitution of a glutamic acid residue for an aspartic acid residue at position 234.

Overproduction and partial purification of the *Shewanella* SSBs

The *Shewanella* *ssb* genes were cloned into the expression vector pET23a (Stratagene) to facilitate the controlled overproduction of the cloned *ssb* gene products. Incorporation of an *Nde*I site into one of the primers employed for PCR amplification and cloning permitted the optimal placement of the *ssb* start codons downstream of the ribosome-binding site of the expression vector and under the control of the T7 promoter. Plasmids pLC101, pLC201, pLC301 and

pLC401 were constructed for the purification of SSB from *Shewanella* SC2A, *S. hanedai*, *Shewanella* F1A and *Shewanella* PT99, respectively. Upon induction, each expression plasmid directed the overproduction of a single polypeptide to approximately 10% of the total cell protein in 90 min and 30% of the total cell protein in 4 h. No proteins were induced in control experiments involving plasmid pET23a alone. For the purpose of partially purifying the recombinant SSBs, the 90 min induction period was utilized in order to reduce the amount of SSB localizing to inclusion bodies within the cells (our unpublished results). Fig. 5 shows the level of SSB production before and 90 min after T7 promoter activation. Based on their deduced amino acid sequences the molecular masses of the *Shewanella* SSBs are 24.9 kDa (*S. hanedai*), 26.3 kDa (SC2A), 25.6 kDa (F1A), and 25.2 kDa (PT99). As observed for other bacterial SSBs, all four *Shewanella* SSBs migrated somewhat more slowly in SDS-PAGE gels than would be expected based on their predicted molecular masses. SSBs from SC2A and *S. hanedai* were found at positions corresponding to a molecular mass of about 33 kDa, while the F1A SSB was found at about 35 kDa and the PT99 SSB at about 36 kDa.

After induction the *Shewanella* SSBs in cell lysates were qualitatively assessed for ssDNA-binding ability fol-

Fig. 5. Overproduction and partial purification of SSB protein from *Shewanella* SC2A, *S. hanedai*, *Shewanella* F1A and *Shewanella* PT99. Overproduction of *Shewanella* SSB protein was induced in *E. coli* F' DH5 α transformed with pLC101, pLC201, pLC301 and pLC401. The figure shows an SDS-PAGE gel with lysate from each of the transformants, prior to induction (lanes 1) and after a 90 min induction (lanes 2). Lane 3 for each strain shows enrichment for the overproduced protein after equilibration with ssDNA cellulose and elution with 2 M KCl. Positions of molecular mass markers are indicated.

lowing the SSB purification protocol of de Vries & Wackernagel (1994). Cell lysates were incubated with ssDNA cellulose at 4 °C followed by removal of unbound and loosely bound proteins from the resins by several buffer washes. Proteins were eluted from stable protein–DNA complexes by washing resins in buffer containing 2 M KCl. As seen in Fig. 5, fractions obtained by a high-salt elution from the ssDNA cellulose were considerably enriched in SSB, verifying that overproduced SSB from each of the four marine *Shewanella* strains was capable of binding to ssDNA. SSBs from the barophilic shewanellas F1A and PT99 appeared to bind the ssDNA with lower affinity than SSBs from SC2A and *S. hanedai*. This was evident in the high amounts of F1A and PT99 SSB removed in the 0.3 M KCl wash and in the reduced SSB yield obtained during the 2 M KCl treatment.

DISCUSSION

In this study the gene encoding SSB was isolated from four related *Shewanella* strains which differ with regard to temperature and pressure adaptation. Using several different criteria the *ssb* genes were shown to comp-

lement *E. coli* *ssb* mutants. In addition, the nucleotide sequences of the *ssb* genes were obtained, and the *ssb* genes were overexpressed and SSBs shown to bind ssDNA.

The complementation of the *E. coli* *ssb* mutants by the *Shewanella* *ssb* genes is remarkable in that each of the SSBs so tested is functioning outside its normal osmolarity, temperature and pressure. Changes in any of these conditions could affect protein quaternary structure as well as ligand interaction (Jaenicke, 1991; Ludlow & Clark, 1991; Royer, 1995; Robinson & Sligar, 1995). *E. coli* SSB protein binds ssDNA as a tetramer rather than by the assembly of the tetramers on DNA after the binding of monomers or dimers (Bujalowski & Lohman, 1991). Thus, the quaternary structure of these recombinant proteins *in vivo* is relevant to their ability to complement *E. coli* *ssb* mutations. From the ability of the overproduced protein to bind ssDNA *in vitro* (Fig. 5), it may be assumed that at the high protein concentrations utilized in these experiments, the SSB protein is in the tetrameric form. *In vivo*, however, SSB protein concentration controlled by the high-copy-number plasmids pLC10, pLC20, pLC30 and pLC40 in *E. coli* is not known. In each of these plasmids expression of the *ssb* gene would appear to be controlled by its own promoter rather than by the *lac* promoter of the plasmid vector. pLC10 contains the *ssb* gene in the opposite orientation to the *lac* promoter, while the *ssb* genes on pLC30 and pLC40 are located more than a kilobase downstream of the *lac* promoter. While the *S. hanedai* *ssb* gene on pLC20 could be expressed from the *lac* promoter of the vector, complementation of the *E. coli* *ssb* mutant PAM5779 also occurred in an *S. hanedai* plasmid clone containing the *ssb* gene in the opposite orientation (data not shown). *E. coli* ribosome-binding and σ^{70} promoter-like sequences are found upstream of all of the cloned *ssb* genes.

pLC40, bearing the *Shewanella* PT99 *ssb* gene, and to a lesser extent plasmids pLC20 and pLC30, bearing the *S. hanedai* and *Shewanella* F1A *ssb* genes, exhibited weaker complementation abilities than the *E. coli* or *Shewanella* SC2A *ssb* plasmids. Complementation of either the *E. coli* *ssb* deletion mutant or the *ssb* point mutant by the PT99 *ssb* was higher at 22 °C than at 37 °C. It is tempting to speculate that the SSBs from the psychrotrophic or psychrophilic and barophilic shewanellas, *S. hanedai*, F1A and PT99, require conditions of low temperature or high pressure, or both, to function as efficiently as the SSBs from *E. coli* or from the mesophilic, moderate barophile *Shewanella* SC2A. However, we can not rule out the possibility that differences in the levels of the SSB proteins are also being reflected in the observed complementation patterns. An additional complicating factor is that the degree of *Shewanella* SSB–*E. coli* SSB heterotetramer formation is unknown.

The *Shewanella* SSB monomers range in size from 24.7 kDa (*S. hanedai*) to 26.3 kDa (*Shewanella* SC2A), considerably larger than the *E. coli* SSB (18.8 kDa). This makes the *Shewanella* SSBs the largest bacterial SSBs

Table 3. Percentage composition of eight amino acids as a function of total SSB protein, and conserved or variable protein sectionsA, *E. coli* SSB; B, *Shewanella* SC2A SSB; C, *S. hanedai* SSB; D, *Shewanella* F1A SSB; E, *Shewanella* PT99 SSB.

Amino acid residue	Total SSB protein					Conserved portions of SSB protein*					Central portion of SSB protein†				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Arg (R)	5.6	4.1	3.5	3.9	3.5	7.4	5.9	5.9	5.9	5.9	1.8	2.4	0.9	1.8	0.9
Asn (N)	4.5	7.0	4.4	3.9	3.5	4.1	5.9	5.9	6.7	5.9	5.4	8.1	2.8	0.9	0.9
Gln (Q)	10.2	16.1	18.6	22.9	22.0	5.7	7.6	7.6	8.4	8.5	19.6	27.5	30.8	45.0	41.3
Gly (G)	16.4	10.3	9.7	8.7	8.4	11.5	7.6	7.6	7.6	7.6	25.0	17.5	12.2	12.5	11.3
Lys (K)	3.4	4.1	5.3	4.8	5.7	4.9	7.6	7.6	7.6	7.6	0.0	0.0	2.8	1.3	5.0
Pro (P)	6.8	8.3	10.6	8.2	6.2	2.5	2.5	3.4	2.5	2.5	16.1	10.0	18.7	12.5	6.3
Ser (S)	5.6	4.5	4.4	5.2	6.2	4.9	5.0	5.9	5.0	5.1	8.9	6.3	2.8	6.3	10.0
Tyr (Y)	2.3	4.1	4.9	3.9	3.5	3.3	4.2	4.2	4.2	4.2	0.0	4.1	5.6	3.6	2.8

* Defined as residues 1–114 of the *Shewanella* SSBs (1–126 for the *E. coli* SSB) and the last six residues of the carboxy-terminal end of protein for all the SSBs.

† Defined as the central variable portion of the SSB proteins (as shown in Fig. 4).

identified to date (de Vries & Wackernagel, 1993, 1994; Jarosik & Hansen, 1994). An interesting pattern of migration is observed for the *Shewanella* SSBs on SDS-PAGE gels (Fig. 5). As with the *E. coli* and *Proteus mirabilis* SSBs (de Vries & Wackernagel, 1994; Lohman *et al.*, 1986), the *Shewanella* SSBs all migrate more slowly than would be indicated by their calculated molecular masses. Even within the *Shewanella* SSB proteins there is no correlation between size and migration distance in SDS-PAGE. Curiously, the SSBs from the more extreme barophiles, *Shewanella* F1A and PT99, migrate the most slowly.

The division of the SSBs into highly conserved amino-terminal and carboxy-terminal portions separated by a variable central portion is in accordance with the presumed functions of these regions. The amino-terminal region, up to residue 115, of the *E. coli* SSB is involved in both subunit interaction and the DNA-binding activity of the protein, and the conserved residues of the carboxy terminus are believed to interact with other proteins such as DNA polymerase II (Curth *et al.*, 1996; also reviewed by Meyer & Laine, 1990). It is not surprising therefore, that these regions are well conserved in the *Shewanella* SSBs. Interestingly, there are only three unconserved amino acid substitutions between the *Shewanella* SSBs in these regions. The replacement of polar residues with hydrophobic methionine residues at positions 48 and 106 in SSB from the mesophilic *Shewanella* SC2A could reflect adaptations to function at higher temperatures (Russell, 1990). Despite the variable nature of the central portion it is undoubtedly critical to SSB function. *In vitro* and *in vivo* analyses of *E. coli* SSBs bearing various deletions in the carboxy-terminal third of the protein indicate that this variable region of the protein is not required for DNA binding. However, a mutant *E. coli* SSB lacking the

central variable region of the protein displays decreased affinity for ssDNA, suggesting that this region influences the DNA-binding capacity of the protein (Curth *et al.*, 1996). Thus, the observed differences in the central portion of the *Shewanella* SSBs could be important to regulating DNA binding at different temperatures and pressures.

In all four *Shewanella* SSBs the variable central region is predominantly composed of alanine, glutamine, glycine and proline residues, but the distribution of these and other residues is not the same in all four *Shewanella* SSBs. For example, the central portion of the F1A and PT99 SSBs contains an extraordinarily high proportion of glutamine, 37% and 38% respectively, but the glutamine content of this portion of the SC2A SSB is only 24.4%. Table 3 gives a breakdown of the percentage amino acid composition of eight amino acids, showing the greatest differences among the *Shewanella* SSBs presented as a function of the total protein, and conserved or variable protein sections.

Some of the differences in amino acid composition among the *Shewanella* SSBs correlate with differences previously observed in comparisons of homologous proteins adapted to different temperature regimes. Measurements of the hydrophilic character of the SSBs by the method of Hopp & Woods (1981) indicate that the lower-temperature-adapted SSBs (*S. hanedai*, F1A and PT99) are dramatically more hydrophilic overall than their mesophilic counterparts (hydrophilicity values of 0.113 for SC2A SSB and 1.2–1.3 for *S. hanedai*, F1A and PT99 SSBs). Proteins from mesophiles often possess fewer hydrophobic residues than their thermophilic counterparts (Zwickl *et al.*, 1990; Zuber, 1988), where they are believed to play a major role in protein thermostability. It may also be noteworthy that the major cold-shock protein from *E. coli*, a protein which

must function at the lower temperature limits for growth and survival of the organism, is also a highly hydrophilic protein (Goldstein *et al.*, 1990). Comparison of the *Shewanella* SSBs also revealed decreased asparagine/glutamine and arginine/lysine ratios with increased adaptation of the source organism to lower temperatures. This follows the trend observed among lactate dehydrogenases (Zuber, 1988). Asparagine predominates over glutamine in thermophilic lactate dehydrogenases for steric reasons. The arginine/lysine results are also consistent with the analysis of a variety of thermophilic and mesophilic proteins (reviewed by Mozhaev & Martinek, 1984). Arginine is generally able to form one more intramolecular hydrogen bond than lysine (Mrabet *et al.*, 1992), and thus may also be important in protein thermostability.

Perhaps the most intriguing amino acid substitutions observed were those identified among the *Shewanella* SSBs which correlate with pressure adaptation of the source organism. This is because amino acid substitutions important for protein structure/function at biologically relevant elevated pressures have yet to be identified. The striking hydrophilicity of the deep-sea bacterial SSBs is significant in this context because hydrophobic interactions have been implicated in the high-pressure stabilization of proteins from thermophiles (Hei & Clark, 1994). Curiously, among the polar residues there is a barophilic bias towards serine in place of tyrosine. Another striking difference between the *Shewanella* SSBs is in the combined total of the helix-destabilizing glycine residues, and the helix-breaking proline residues. There is a progressive decrease in the number of these residues extending from *S. hanedai*, which was isolated from the shallowest waters, to *Shewanella* PT99, which was isolated from the deepest marine environment and is an obligately barophilic isolate. This trend is even more obvious when only the central portion of the SSBs is examined. A loss of prolines and glycines argues for a decrease in flexibility. Globular proteins with low flexibility also exhibit low compressibility (Gross & Jaenicke, 1994). Interestingly, proline to glycine substitutions in staphylococcal nuclease which decrease chain flexibility increase the stability of the protein at high pressure (Royer *et al.*, 1993). Therefore, increased structural homogeneity could be favoured by the high-pressure environments of the deep sea.

In order to determine SSB residues critical to psychro- and baro-adaptation it will be necessary to perform comparative studies of the effects of low temperature and high pressure on the quaternary structure and the DNA-binding capacity of purified *Shewanella* SSBs and mutant proteins obtained by site-directed mutagenesis. Some of these experiments are now in progress.

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