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Purification, molecular cloning and expression in *Escherichia coli* of homospermidine synthase from *Rhodopseudomonas viridis*

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Homospermidine synthase (HSS) catalyzes the synthesis of the polyamine homospermidine from 2 mol putrescine in an NAD^+-dependent reaction. In this study, the enzyme was purified from anaerobically grown cultures of the photosynthetic bacterium *Rhodopseudomonas viridis* to electrophoretic homogeneity using a three-step procedure. The enzyme was shown to be a homodimer of 52-kDa subunits. Six endopeptidase LysC fragments were sequenced from the purified protein. With the aid of degenerate primers designed against these peptides, specific PCR products from *R. viridis* DNA were obtained that were used as hybridization probes to isolate the *hss* gene from a library constructed in λEMBL4. The *hss* gene and flanking regions were sequenced and were shown to exist as a single copy in the *R. viridis* genome. HSS is translated from a monocistronic mRNA and possesses no detectable similarity to previously sequenced gene products. *Escherichia coli*, which lacks HSS activity, was transformed with an expression plasmid containing the *hss* coding region under the control of a bacteriophage T_7 promoter. Upon induction, transformed *E. coli* cells accumulate enzymatically active and highly stable *R. viridis* HSS at levels corresponding to 40–50% of the soluble protein in crude extracts.

Keywords: *Rhodopseudomonas viridis*; homospermidine; homospermidine synthase; molecular cloning; nucleotide sequence.

Homospermidine is a linear aliphatic polyamine found in some bacteria (Busse and Auling, 1988; Hamana and Matsuzaki, 1992), plants (Smith, 1985), and even animals (Matsuzaki et al., 1982). Due to its limited distribution, it is considered to be one of the so-called uncommon polyamines. It is the characteristic polyamine of the genera of the α-2 subclass of the proteobacteria (Stackebrandt et al., 1988), which includes the purple photosynthetic bacteria such as *Rhodopseudomonas* and their non-photosynthetic relatives such as *Nitrobacter*, *Agrobacterium*, and *Rhizobium* (Busse and Auling, 1988; Hamana and Matsuzaki, 1992). Within the cyanobacteria, species belonging to the order Nostocales contain homospermidine ubiquitously as the major polyamine (Busse and Auling, 1988; Hamana and Matsuzaki, 1992).

In eukaryotes, the common polyamines putrescine, spermidine, and spermine occur in various amounts in all species. They are occasionally accompanied but never substituted by homospermidine. In animals, homospermidine has been detected in amphibian and reptile tissues and hamster epididymis (Matsuzaki et al., 1982). In plants, isolated occurrences are known in eukaryotic algae (Kneifel, 1977; Hamana and Matsuzaki, 1982) and higher plants, e.g. leaves of *Santalum album* (Kuttan et al., 1971), roots of *Eichhornia crassipes* (Yamamoto et al., 1983) as well as root-nodules (Hamana et al., 1992) and seeds (Fujihara et al., 1994) of several legumes.

The function of homospermidine is still uncertain. In prokaryotes, the frequent occurrence of homospermidine in N_2-fixing species of archaebacteria (*Methanosarcina*; Scherer and Kneifel, 1983), eubacteria, and cyanobacteria led to the suggestion that homospermidine might be functionally related to N_2 fixation. In certain higher plants, homospermidine is a precursor of simple polyamine alkaloids (Guggisberg and Hesse, 1983) and the specific intermediate in the biosynthesis of the complex pyrrolizidine alkaloids (Rana and Robins, 1983; Khan and Robins, 1985). The latter compounds represent a typical class of secondary compounds that play an important role in plant defense against herbivores (Hartmann and Witte, 1995). Homospermidine is the first pathway-specific intermediate in the biosynthesis of pyrrolizidine alkaloids (Böttcher et al., 1993).

The enzymatic formation of homospermidine by homospermidine synthase (HSS) was first described by Tait (1979) using partially purified enzyme preparations from the bacterium *Rhodopseudomonas viridis*. HSS activity was also detected in seedlings of the legume *Lathyrus sativus* (Srivenugopal and Adiga, 1980). More recently, HSS has been purified from the bacterium *Acinetobacter tartarogenes* (Yamamoto et al., 1993) and from the plants *Eupatorium cannabinum* and *Senecio vulgaris* (Asteraceae) (Böttcher et al., 1993).

In the latter case, HSS is part of the specific biosynthetic pathway leading to pyrrolizidine alkaloids, and our primary interest in the enzyme concerns its pivotal role in alkaloid-mediated plant defense. The plant enzyme is extremely unstable (Böttcher et al., 1993), which renders its purification to homo-

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Abbreviations. HSS, homospermidine synthase; *hss*, homospermidine synthase gene.

Note. The novel nucleotide sequence data reported in this paper have been deposited with GenBank and are available under the accession number L77975.

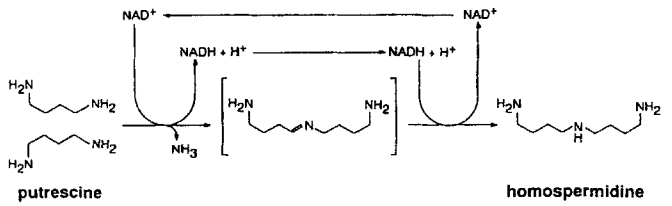

Fig. 1. Predicted reaction catalyzed by homospermidine synthase (HSS).

geneity intractable with current methods. However, HSS from *R. viridis* and its counterpart from the pyrrolizidine alkaloid pathway in *E. cannabinum* are very similar in their physical and kinetic properties (Böttcher et al., 1994). The enzyme from both sources catalyzes the NAD^+-dependent conversion of 2 mol putrescine into homospermidine (Fig. 1), whereby spermidine can substitute for the first putrescine moiety, in which case diaminopropane instead of ammonia is released. HSS is unique in that NAD^+ functions as a prosthetic group in the reaction, i.e. enzyme-bound NAD^+ is a hydride acceptor in the first step of catalysis and a hydride donor in the second (Böttcher et al., 1994).

Our main objective is to establish the function of HSS in plant pyrrolizidine alkaloid biosynthesis, e.g. its tissue-specific expression in the roots and its role as a control element of a pathway leading to final products lacking any significant turnover (Hartmann and Witte, 1995; Hartmann, 1995). Furthermore, the sporadic occurrence of homospermidine in nature provokes the question of the phylogenetic relations between HSS from different sources as well as the relations of the pathways of pyrrolizidine alkaloids in unrelated plant taxa. To address these objectives, molecular tools are needed, and thus as the first step of a molecular approach towards isolating plant HSS, we have investigated the more stable and accessible enzyme from *R. viridis*. Here, we report the purification, cloning and expression in *E. coli* of bacterial HSS.

MATERIALS AND METHODS

Bacteria. *R. viridis* (DSM 134) was obtained from the Deutsche Sammlung für Mikroorganismen (DSM Braunschweig). The strain was cultured anaerobically in the medium according to Malik (1983) in 500-ml screw-capped or 2-l aluminium capped flasks under low-intensity (40 W) lamp light at 20–27 °C. For DNA and protein isolation, cells were harvested at the beginning of the stationary phase (approximately 7 days after inoculation) by centrifugation at 8000×*g* at 4 °C.

Enzyme purification. Acetone dry-powder was prepared in a Waring blender by adding approximately 100 ml acetone (+4 °C) to the wet cells harvested from a 10-l culture, brief blending (about 10 s), followed by addition of 500 ml acetone (−8 °C), and homogenization at full speed for 30 s. The procedure was repeated with 100 ml acetone (−18 °C) and the precipitate was recovered on a Büchner funnel, washed with 100 ml acetone (−18 °C), air-dried, and ground in a mortar. The acetone dry-powder could be stored at −18 °C for months without significant loss of enzyme activity.

Crude enzyme extracts were prepared by adding 10 g acetone dry-powder to 100 ml buffer A, which consists of 50 mM KH_2PO_4 and 2 mM dithioerythritol, pH adjusted to 8.5 with 5 M NaOH. The mixture was stirred for 60 min at 4 °C, and centrifuged at 20000×*g*. The supernatant was subjected to a protamine sulfate precipitation (0.35 mg · ml^{-1}), stirred for 30 min, and centrifuged at 27000×*g* for 15 min. The supernatant was loaded onto a DEAE-Fractogel (EMD DEAE-650 M; Merck) column (gel bed volume 45 ml) equilibrated with buffer A. The column was washed with 70 ml buffer A. Elution of the enzyme was achieved at a flow rate of 1.0 ml · min^{-1} with 80 ml of a linear KCl gradient (0 M to 0.6 M KCl). HSS activity eluted at 0.2 M to 0.3 M KCl. The pooled fractions (approximately 30 ml) were desalted by ultrafiltration (Filtron Pro Vario-3) and concentrated via a second DEAE-Fractogel column (gel bed volume 20 ml) at a flow rate of 1.0 ml · min^{-1} in a 0 M to 0.6 M KCl gradient. The fractions containing HSS activity (about 12 ml) were pooled, concentrated to a maximum volume of 4 ml in Filtron-Microsep concentrators (30-kDa cut-off membrane) and 2-ml portions were loaded onto a Superdex 200 HiLoad (16/60) column in FPLC mode. The column had been previously equilibrated with buffer A. Elution was carried out with buffer A at a flow rate of 0.5 ml · min^{-1}; 2-ml fractions were collected. Fractions with HSS activity (fractions 36 ml to 45 ml) were pooled. Further purification was achieved by affinity chromatography using a putrescine–Sepharose column (gel bed volume 15 ml), equilibrated with buffer A. Putrescine was coupled to CH Sepharose 4B (Pharmacia) according to the manufacturer's protocol. The Superdex 200 eluate (about 25 ml) was applied to the affinity column (superloop; FPLC mode) at a flow rate of 0.2 ml · min^{-1} to ensure complete binding of HSS. The column was washed with 100 ml buffer A containing 100 mM KCl (flow rate 0.4 ml · min^{-1}) followed by elution of HSS with buffer A containing 100 mM putrescine (flow rate 0.2 ml · min^{-1}). Fractions of 1 ml were collected, HSS eluted in fractions 10–30. The active fractions were pooled, concentrated to 100–300 µl with Filtron-Microsep devices and 100-µl portions were applied to a Superdex 200 HR (10/30) column at a flow rate of 0.2 ml · min^{-1}. Fractionation was performed with buffer A at a flow rate of 0.4 ml · min^{-1}; 1-ml fractions were collected; HSS activity eluted in fractions 11 and 12.

For the preparation of pure HSS for protein sequencing, the last step was slightly modified: the column was loaded with 200-µl and 0.5-ml fractions were collected, the HSS peak fraction (0.5 ml) was recovered, diluted 250-fold with distilled water and concentrated to 0.25 ml with Centricon-30 (Amicon) devices.

Enzyme assay. HSS assay was performed at 37 °C in a total volume of 62.5 µl or 125 µl buffer A containing 1.0 mM [^{14}C]putrescine (0.025 µCi/assay or 0.05 µCi/assay) and 0.2 mM NAD^+. A K^+ concentration of 50 mM is essential for maximal activity; significant decreases in activity are observed at K^+ concentrations less than 25 mM and greater than 75 mM (see also Tait, 1979; Yamamoto et al., 1993). Assays were incubated for 10–20 min and stopped by applying a 10-µl aliquot directly onto a TLC plate (silica gel 60 F_{254}; Merck). Putrescine (R_f 0.45) and homospermidine (R_f 0.11) were separated in the solvent system 4:3:2 (by vol.) acetone/methanol/25% (by vol.) ammonium hydroxide. The amount of product was calculated from the ratio of labelled putrescine/homospermidine obtained by radioscanning using a TLC multichannel analyzer (RITA, Raytest) (Böttcher et al., 1993). Probes from crude extracts and protamine sulfate supernatants and pooled fractions from DEAE-Fractogel and putrescine–Sepharose affinity purification steps were assayed after desalting via PD10 columns (Pharmacia) equilibrated with buffer A.

Molecular mass determination. The molecular mass of the purified protein was determined by gel filtration on a Superdex 200 HR (10/30) column (Pharmacia) calibrated with standard proteins (Serva) supplemented with ferritin (440 kDa). Samples were applied in a volume of 100 µl and eluted with buffer A at a rate of 0.25 ml · min^{-1}. Elution volumes of proteins and blue dextran were calculated from the peak retention time and flow

rate. The subunit molecular mass was determined by SDS/PAGE in comparison with low-molecular-mass standard proteins (Pharmacia).

Protein determination and SDS/PAGE. Protein concentrations were determined according to Bradford (1976) modified as described by Read and Northcote (1981) with BSA as a standard. For SDS/PAGE, the respective samples were desalted with buffer A on PD10 columns (Pharmacia) and concentrated on Centricon-30 concentrators (Amicon) if necessary. SDS/PAGE was performed according to Laemmli (1970).

Protein sequencing. Peptides from purified HSS were obtained by digestion with endoproteinase LysC as described by Eckerskorn and Lottspeich (1989). Peptides were separated on a 2 mm×125 mm Supersher 60 reverse-phase select B column (Merck) at a flow rate of 300 µl · min^{-1} in a gradient (1% · min^{-1}) of 0.1% (by vol.) trifluoroacetic acid in water to 0.1% (by vol.) trifluoroacetic acid in acetonitrile. Sequencing of the isolated peptides was carried out by automated Edman degradation (Edman and Begg, 1967) in a Porton 3600 sequencer (Beckman) and amino acids were identified in a Mikrobore HPLC System Gold (Beckman).

Isolation of hybridization probes for DNA library screening. Six peptide sequences were obtained after digestion of purified HSS with endopeptidase LysC and sequencing of the fragments: 1, LVVIDPSDEARK; 2, GIHIAERDTQRASFPK; 3, PGGTXAVSXRGANPGMVSXFVK; 4, TDWPVYHRI; 5, FALVNLA; 6, RQSDWRILDETEIVDGIDELGVLLYGH. Peptide 4 represents the N-terminus of the enzyme. We synthesized coding (+) and non-coding (−) strand degenerate oligonucleotides of 17−20 bases in length against peptides 1−3, respectively, and against the (+) strand of the N-terminal peptide. The least degenerate regions of each peptide were used (underlined). From peptide 6, one (+) and one (−) oligonucleotide was chosen against motifs at both ends of the peptide (underlined). Oligonucleotides were chemically synthesized with the Pharmacia oligosynthesizer. For PCR amplification with *R. viridis* DNA, we employed all possible ± combinations of all primers under various temperature profiles. The successful PCR reaction (25 µl) with oligonucleotides 5′-GTRTCNCGYTCNGCDATRTG-3′ (BM27) and 5′-GGNGCNAAYCCNGGNATGGT-3′ (BM28) constructed against the peptide motifs HIAERDT and GANPGMV, respectively, contained 50 mM KCl, 10 mM Tris/HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 µM of each dNTP, 2 µM of each primer, 100 ng isolated *R. viridis* DNA and 0.75 U Taq Polymerase (Boehringer). Amplification was performed in a Perkin Elmer thermocycler using 35 cycles of 93°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The 0.16-kb PCR amplification product was reamplified under the same conditions, isolated from a 1.5% agarose gel, and subcloned blunt ended into *Sma*I-cut pBluescript KS^+ (Stratagene). The identity of the amplification product was confirmed by dideoxynucleotide sequencing (Sanger et al., 1977) of the resulting plasmid pPCRHs3.2 and comparison with peptide sequences of the HSS protein. The *Xba*I−*Eco*RI 0.16-kb fragment of pPCRHs3.2 was isolated by electroelution and DE-52 ion-exchange chromatography and used as a hybridization probe for screening the *R. viridis* DNA library.

Cloning of *R. viridis* HSS. DNA from one-week-old *R. viridis* cells was isolated using a modified version of the method of Marmur (1961) and Tandeau de Marsac et al. (1982). Cells (2−3 g) from a 500-ml culture were frozen in liquid nitrogen and resuspended in 100−150 ml 10 mM Tris/HCl, pH 8.0, containing 120 mM NaCl, 50 mM EDTA. After incubation with 1 mg · ml^{-1} lysozyme for 15 min at 37°C and 1% SDS for 10 min at 60°C, the solution was extracted by mixing with 0.5 vol. 80% phenol followed by 0.5 vol. 24:1 (by vol.) chloroform/isoamyl alcohol. Nucleic acids were precipitated from the aqueous phase by adding 0.1 vol. 3 M NaOAc and 2 vol. ethanol. After dissolving in 20 ml Tris/EDTA (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and incubation with 0.1 mg · ml^{-1} ribonuclease (Serva) followed by 0.1 mg · ml^{-1} proteinase K (Merck), a second phenol/chloroform extraction was performed. Reprecipitated DNA was dissolved in 4−5 ml Tris/EDTA buffer. 15 µg isolated DNA was partially digested with *Sau*3AI. Fragments of 15−20 kb length were isolated by gel electrophoresis with DE 81 paper (Whatman) (Dretzen et al., 1981); 400 ng of these fragments were ligated into 300 ng *Bam*HI digested λEMBL4 (Frischauf et al., 1983) and packaged with extracts prepared as described (Sambrook et al., 1989). The *R. viridis* library (20000 original recombinants) was amplified to 1×10^6 plaque-forming units · μl^{-1}. Recombinant clones, plated on *Escherichia coli* K803, were screened by plaque hybridization (Sambrook et al., 1989) at 60°C in 3×NaCl/EDTA/P_i (1×NaCl/EDTA/P_i is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.5) containing 0.1% SDS, 0.02% polyvinylpyrrolidone, 0.02% Ficoll-400 with 10 ng · ml^{-1} hybridization probe. The hybridization probe, random labelled with [α-^{32}P]dCTP (Feinberg and Vogelstein, 1984), was the 0.16-kb *Xba*I−*Eco*RI fragment of pPCR Hs3.2. Filters were washed twice for 15 min at 60°C in 2×NaCl/EDTA/P_i, 0.1% SDS, and autoradiographed overnight on XAR films (Kodak) at −70°C. From 13 positively hybridizing and purified clones, λRv30.1 was selected for further analysis on the basis of restriction mapping and Southern hybridization. Hybridizing fragments were subcloned into pBluescript KS^+ plasmids (Stratagene). DNA sequences were determined by the dideoxynucleotide chain-termination method with modified T_7 DNA polymerase (Tabor and Richardson, 1987) using radioactive (Sanger et al., 1977) or fluorescence (Ansorge et al., 1986) detection methods. Sequencing with dITPs was carried out using the USB Sequenase kit. Sequencing templates were double-stranded alkaline-denatured plasmids (Chen and Seeburg, 1985) or in most cases single-stranded plasmid DNA prepared with M13 helper phages following the manufacturer's instructions (Stratagene). Sequencing was performed on both strands with synthetic primers or overlapping deletion clones in pBluescript KS^+ or SK^+ generated with exonuclease III according to Henikoff (1984) using mung bean nuclease (Pharmacia) instead of S1 nuclease. Other molecular techniques were performed as described (Sambrook et al., 1989). Sequence analysis was performed with the GCG package (Devereux et al., 1984).

Southern blot analysis. DNA (1 µg) of recombinant λ clones and 10 µg *R. viridis* DNA were cut with appropriate restriction enzymes. Restriction products were separated by electrophoresis on an 0.7% agarose gel, transferred to a Hybond-N (Amersham) nylon membrane according to the manufacturer's protocol. Prehybridization and hybridization of the filters were carried out at 65°C in 5×NaCl/Cit (1×NaCl/Cit is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.1% *N*-lauroylsarcosine 0.02% SDS, 1% blocking reagent using the nonradioactive digoxigenin DNA labelling and detection kit (Boehringer). Hybridization was performed overnight with 5−10 ng · ml^{-1} 0.16-kb *Xba*I−*Eco*RI pPCRHs3.2 fragment, random labelled with digoxigenin-11-dUTP. Filter washes and colorimetric detection were performed as described by the manufacturer.

Northern blot analysis. Total RNA was isolated from a 300-ml culture of *R. viridis*, grown to mid-logarithmic phase (A_{540} 0.5), according to the method of Aiba et al. (1981). Samples of 10 µg and 20 µg total RNA were electrophoresed on a 1.2% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham) by capillary transfer as described by Ausubel et al. (1989). Hybridization was carried out at 72°C in 3×NaCl/EDTA/P_i containing 0.1% SDS, 0.02% polyvinyl-

pyrrolidone, 0.02% Ficoll-400 with 10 ng · ml^{-1} *Nde*I–*Sfi*I fragment of pHsRvT$_7$2 random labelled (Feinberg and Vogelstein, 1984) with [α-^{32}P]dCTP. Filters were washed twice for 15 min with 2×NaCl/EDTA/P$_i$, 0.1% SDS, at 72°C and autoradiographed at −70°C for 96 h.

Expression of HSS in *E. coli*. For expression with the T$_7$ polymerase system (Studier et al., 1990), the HSS coding region was amplified by PCR with pHsRv4.5 as template and the two primers 5′-ATATTTGATCACTTATGCCCTGATCGACTGAGG-3′ (D26) and 5′-ATATTCATATGACCGATTGGCCGGTTTATCAC-3′ (D28). PCR conditions were as described above with the following modifications: reactions contained 10 ng plasmid DNA and 0.1 µM of each primer. After 3 min initial denaturation at 97°C, 2 U Taq polymerase were added and 30 cycles were carried out for 1 min at 97°C, 1 min at 60°C, and 2 min at 72°C. For cloning, the amplification product was isolated from 100 µl preparative reactions by gel electrophoresis and the use of Microcon 30 devices (Amicon) according to the manufacturer's protocol. The purified product was cut with *Bcl*I and *Nde*I and ligated into *Bam*HI/*Nde*I cut pET-3a vector (Studier et al., 1990). After transformation of *E. coli* NM522 (Gough and Murray, 1983), one clone with the expected *Bcl*I-*Nde*I fragment was chosen and its plasmid (pHsRvT$_7$2) transformed in *E. coli* BL21(DH3) (Studier and Moffatt, 1986). Two ampicillin-resistant transformants (BL21/pHsRvT$_7$2.1 and T$_7$2.2) were grown overnight at 37°C in 200 ml LB medium containing 100 µg · ml^{-1} ampicillin to A_{600} 1.5. 50 ml fresh LB medium was added and the cells were induced with 0.4 mM isopropyl thio-β-D-galactoside after 1 h. At 30-min or 60-min intervals after induction, 1-ml aliquots of the culture were harvested, pelleted cells were washed with lysis buffer (50 mM Tris/HCl, pH 8.0, 2 mM EDTA), centrifuged, and resuspended in 400 µl lysis buffer containing 100 µg · ml^{-1} lysozyme. After incubation for 15 min on ice, cells were disrupted by sonication for two bursts of 30 s each and the cell debris centrifuged at 4°C. 5 µl supernatant was directly used for SDS/PAGE. 380 µl were loaded on a NAP 10-column (Pharmacia) and eluted with 1.5 ml ice-cold buffer A. 25 µl eluate was used for the activity assay. The BL21 wild-type control was handled in the same manner.

RESULTS

Purification of HSS from *R. viridis*. In contrast to HSS from higher plants (Böttcher et al., 1993), HSS from *R. viridis* is a relatively stable enzyme and we have developed a protocol that enabled us to recover electrophoretically homogeneous HSS from this bacterial source. Starting with crude acetone dry-powder extracts separated from nucleic acids by protamine sulfate precipitation, HSS was purified in three steps by a combination of ion-exchange chromatography, gel filtration, and putrescine–Sepharose affinity chromatography. The procedure led to a 224-fold purification with a total recovery of 18% of enzyme activity (Table 1). The putrescine affinity chromatography was the most efficient purification step, yielding an approximately 20-fold increase in specific activity. The final Superdex 200 HR gel-filtration step did not significantly enhance the purification procedure, but was necessary to separate the protein from excess putrescine that binds unspecifically to the protein and also to remove a protein band which in some preparations contaminates the pure HSS protein. SDS/PAGE of the final preparation shows a single protein band with molecular mass 52 kDa (Fig. 4B). An aliquot of the purified enzyme was subjected to native molecular mass determination via gel filtration on Superdex 200 HR against known standard proteins and shown to have a native molecular mass of 100 kDa (data not shown), which indicates

Table 1. Purification of HSS from *R. viridis*. HSS was prepared from 20 g acetone dry-powder corresponding to about 200 g wet cells from a 40-l culture.

Purification step	Protein	Total activity	Specific activity	Yield	
	mg	nkat	nkat · mg^{-1}	%	-fold
Acetone dry-powder	768	283	0.37	100	1
Protamine sulfate	776	299	0.39	105	1
DEAE-Fractogel	188	211	1.12	74	3
Superdex 200 Hiload	37	149	4.04	53	11
Putrescine-Sepharose	0.6	51	82.78	18	224

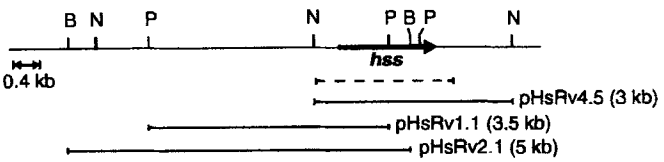

Fig. 2. Restriction map of pHsRv4.5, pHsRv1.1, and pHsRv2.1. The arrow indicates the location of the *hss* gene. The sequenced region is shown (– – –). B (*Bam*HI); N (*Not*I); P (*Pst*I).

that at this point of our investigation the active enzyme was either a homodimer or a heterodimer of subunits of nearly identical size.

Protein microsequencing and generation of a HSS-specific probe. The final preparation from protein purification was subjected to digestion with endopeptidase LysC, the resulting fragments were separated by HPLC, and sequenced by automatic Edman degradation. Six peptides were obtained (see Materials and Methods section). Peptide 4 could be identified as the N-terminus of the purified enzyme. With the primer combination BM27–5′-GTRTCNCGYTCNGCDATRTG-3′ (384-fold) and BM28–5′-GGNGCNAAYCCNGGNATGGT-3′ (512-fold) against the motifs HIAERDT and GANPGMV in peptides 2 and 3, respectively, we obtained an amplification product of 160 bp only when *R. viridis* DNA was present as a substrate and only when both primers were included in the PCR reaction. This PCR product was cloned in a blunt-ended form into *Sma*I-cut pBluescript KS$^+$ to yield plasmid pPCRHs3.2. pPCRHs3.2 was shown by sequencing to contain the expected terminal peptides in addition to peptide 5 which was present as an internal sequence, which unambiguously identifies the insert of pPCRHs3.2 as a HSS-specific gene probe.

Isolation of the *R. viridis hss* gene. From 20000 recombinant clones of the amplified *R. viridis* library screened with the radioactive-labelled *Xba*I–*Eco*RI insert of pPCRHs3.2, 26 positively hybridizing clones were detected, 13 of which were purified for further study. Restriction mapping of these clones revealed that they all correspond to the same locus and that λRv26 and λRv30.1 overlapped, containing identical hybridizing 5-kb *Bam*HI, 3.5-kb *Pst*I and 3-kb *Not*I fragments (Fig. 2). These restriction fragments were subcloned into pBluescript KS$^+$ vectors digested with the appropriate enzyme to yield pHsRv1.1 (*Pst*I fragment), pHsRv2.1 (*Bam*HI fragment), and pHsRv4.5 (*Not*I fragment). The sequence of the *hss* gene, including 0.18 kb and 0.25 kb of the 5′ and 3′ non-coding regions, respectively, was determined (Fig. 3). Due to the very high G+C content of the gene (93.5% at the third codon positions, 68% overall for the sequenced region), numerous sequencing runs and synthetic primers had to be employed before the identity of all bases from

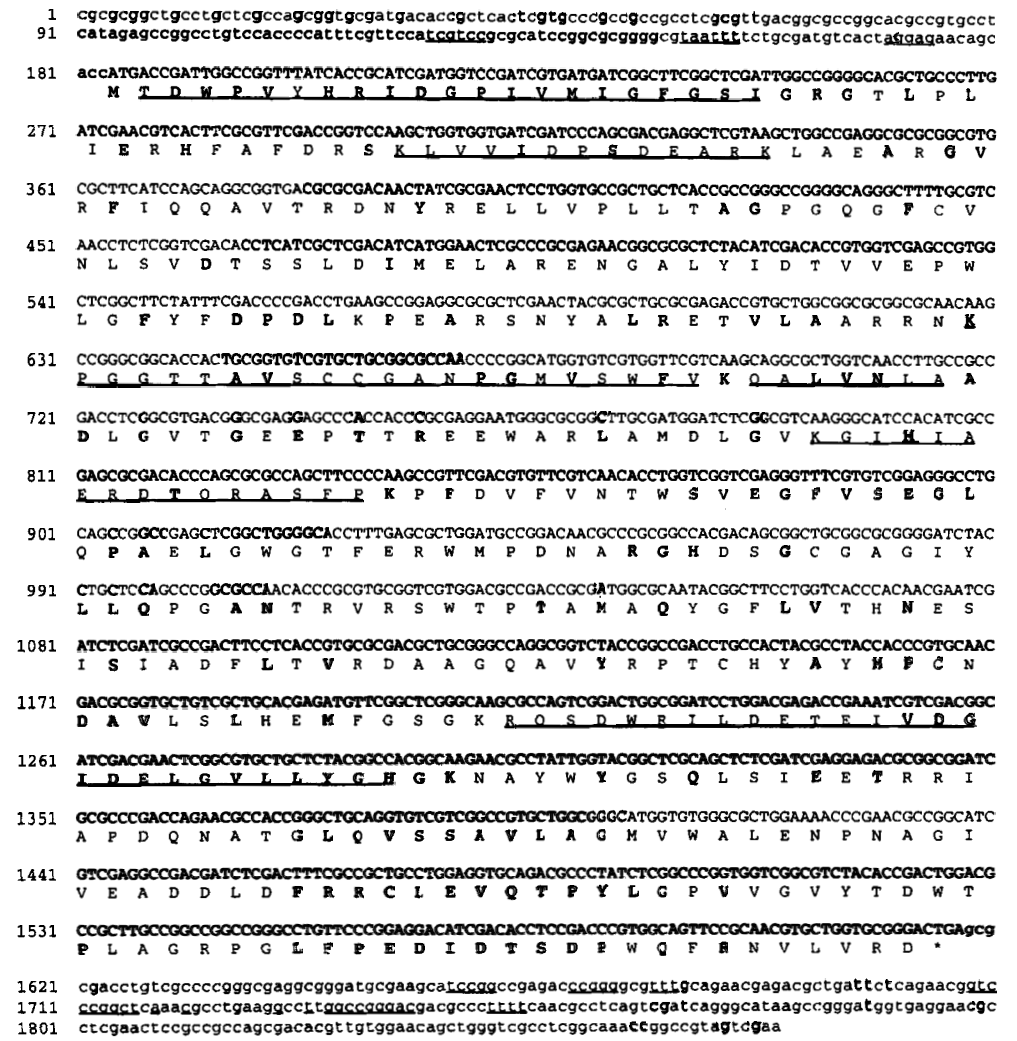

Fig. 3. Nucleotide and deduced amino acid sequences of HSS from *R. viridis*. The sequenced region according to Fig. 2 is shown. Regions sequenced from the isolated protein are underlined. Non-coding regions are given in lower-case letters. A putative ribosome-binding site, *E. coli*-like -10 and -35 promotor regions, and potential termination structures are underlined.

both strands had been unambiguously determined. The deduced amino acid sequence of *R. viridis* HSS contains all six directly sequenced peptides and encodes a protein with a predicted molecular mass of 52.6 kDa, which is very close to the molecular mass determined for the purified protein (52 kDa). These data suggest that *R. viridis* HSS is a dimer of identical subunits. No additional open reading frames either upstream or downstream of the *hss* gene were detected. *E. coli* promotor-like sequences were identified 5′ of the gene (Fig. 3). Some G+C-rich stem loops were found 5′ of a run of T residues (at 1677 bp and 1751 bp, respectively) possibly indicating ϱ-independent termination structures. To show clearly that *hss* is not cotranscribed with other genes, we performed Northern hybridization with an *hss*-specific probe against total RNA isolated from exponentially growing *R. viridis* cells. A transcript of approximately 1.5 kb was detected (data not shown). Since the coding region of *hss* is 1433 bp long, this clearly indicates that HSS is translated from a monocistronic mRNA and is not cotranscribed as part of an operon. Southern blots of *R. viridis* total DNA probed with the *Xba*I−*Eco*RI fragment of pPCRHs3.2 revealed only one band each, which indicates that this bacterium contains only one copy of *hss* (data not shown).

Overexpression of HSS in *E. coli*. The identification of all six peptide sequences determined from the isolated protein in the *hss* gene strongly suggested that HSS is a homodimer, but did not exclude the possibility that a second subunit of considerably smaller (or conceivably equal) size may be essential for enzyme activity. To clarify this, we amplified the *hss* coding region (denaturing temperatures of 97 °C were necessary) and cloned it in frame into a *Nde*I/*Bam*HI-cut pET-3a vector (Studier et al., 1990) to yield the plasmid pHsRvT$_7$2 for expression in *E. coli*. Since *E. coli* BL21 does not possess HSS activity (Table 2), transformants of the strain could be selected on the basis of plasmid integrity and HSS enzyme activity. Strains BL21/pHsRvT$_7$ 2.1 and 2.2, respectively, contain *R. viridis* HSS under the control of the T$_7$ promotor. HSS activity in *E. coli* BL21/pHsRvT$_7$2.1 crude lysates prior to induction is 5.7 nkat · ml^{-1} (t_0) and increases after induction of T$_7$ RNA polymerase with isopropyl thio-β-D-galactoside up to 11.5 nkat · ml^{-1} (Table 2). SDS/PAGE of crude lysates (Fig. 4A) shows the expected HSS band of the 52-kDa subunit. HSS is expressed at high levels and is stable in *E. coli*. According to its activity, the amount of the enzyme protein increases twofold within 4.5 h after induction. Approximately 40−50% of soluble protein in the crude extracts of BL21/pHsRvT$_7$2.1 and 2.2 consists of *R. viridis* HSS. The yield of the overexpressed enzyme is about 160−200 mg · l^{-1} culture. HSS is expressed prior to addition of isopropyl thio-β-D-galactoside. This is due to a basal level of T$_7$ RNA polymerase

Table 2. HSS activity in crude extracts of BL21 strains transformed with pHsRvT$_7$2 and BL21 wild-type. Activity was measured per ml crude extract prepared from the cultures at different times after induction of the T$_7$ RNA polymerase with isopropyl thio-β-D-galactoside.

Time after induciton	HSS activity		
	BL21	BL21/ pHsRvT$_7$2.1	BL21/ pHsRvT$_7$2.2
	nkat · ml^{-1}		
0	–	5.74	8.28
0.5	–	8.05	7.59
1.0	–	8.42	8.27
1.5	–	9.53	9.18
2.0	–	10.34	10.29
3.0	–	11.05	10.93
4.5	–	11.70	11.63
5.0	–	11.49	11.14

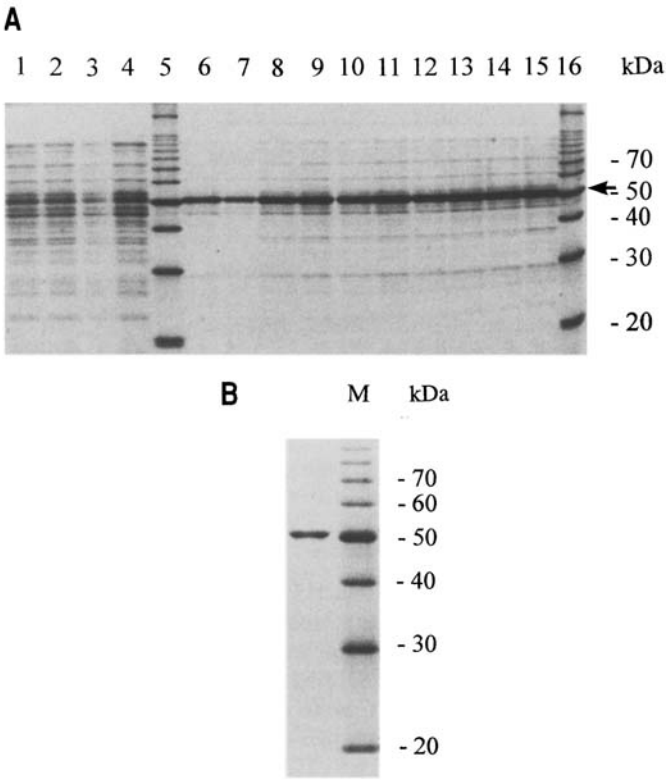

Fig. 4. Overproduction of HSS in BL21/pHsRvT$_7$2.1 and BL21/ pHsRvT$_7$2.2 with the T$_7$ expression system; SDS/PAGE of protein extracts (A) and purified HSS (B). (A) 1-ml aliquots were taken from cultures at different times after induction of T$_7$ polymerase. 5 µl crude extract was loaded per lane. The arrow indicates the 52-kDa subunit of induced HSS. Lanes 5 and 16, molecular markers; BL21 wild-type with lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; and lane 4, 3 h after induction. BL21/pHsRvT$_7$2.1 with lane 6, 0 h; lane 8, 0.5 h; lane 10, 1 h; lane 12, 2 h; and lane 14, 3 h after induction; BL21/pHsRvT$_7$2.2 with lane 7, 0 h; lane 9, 0.5 h; lane 11, 1 h; lane 13, 2 h; and lane 15, 3 h after induction. (B) M, molecular markers. In A and B, proteins were stained with Coomassie brilliant blue.

activity promoting some transcription of the target gene in the uninduced cell (Studier et al., 1990). The wild-type BL21 strain does not contain any HSS activity; its protein pattern does not change during induction.

DISCUSSION

The enzyme from *Acinetobacter tartarogenes* is the only HSS that has been purified to homogeneity to date (Yamamoto et al., 1993). Here, we describe the cloning and nucleotide sequence of the *hss* gene from *R. viridis*. The sequence of the *hss* gene, which includes 0.18 kb and 0.25 kb, respectively, of the 5′ and 3′ non-coding regions, encodes a protein of 477 amino acid residues. Two lines of evidence confirm the identity of gene. First, the deduced amino acid sequence contains all six peptides determined from the purified HSS protein. Secondly, catalytically active HSS was obtained from expression of the gene in *E. coli*, the wild type of which does not produce HSS. Database searching revealed no detectable similarity with known sequences on the nucleotide or amino acid level.

The molecular mass of native *R. viridis* HSS was determined to be 100 kDa. SDS/PAGE and overexpression of HSS in *E. coli* clearly show that the enzyme consists of two identical subunits of 52 kDa (i.e. 52.6 kDa as calculated from the amino acid residues). This is in contrast to previous data from Tait (1979) who found a molecular mass of 73 kDa for the native *R. viridis* enzyme. Studies of HSS purified from *A. tartarogenes* (Yamamoto et al., 1993) show a molecular mass of 102 kDa for the native enzyme and 52 kDa for the subunit and are in good agreement with our results. The N-terminal sequences of both bacterial enzymes are the same for 8 out of 15 amino acids (53%). The enzymes also share similarities regarding their substrate kinetics, enzyme stabilisation, and assay parameters (Böttcher et al., 1994; Yamamoto et al., 1993). This indicates that both enzymes are similar although they are encoded in taxonomically distinct species.

From our studies with plant and bacterial HSS, we suggested a reaction mechanism based on the existence of two binding sites at the catalytic centre for the two substrate molecules (Böttcher et al., 1994). In this model, one site is less substrate specific (i.e. it also accepts homologous diamines such as 1,3-diaminopropane, cadaverine, 1,6-diaminohexane and even the polyamine spermidine) and the other binds only putrescine (Ober, D., Tholl, D., Martin, W. and Hartmann, T., unpublished results). Expression of *R. viridis* HSS in *E. coli* led to *in vivo* formation of homospermidine and its by-product 4-aminobutylcadaverine in the presence of intracellular cadaverine. Both HSS products were absent from wild-type *E. coli* (Ober, D., Tholl, D., Martin, W. and Hartmann, T., unpublished results). The formation of 4-aminobutylcadaverine through a side reaction of HSS was also demonstrated for the *A. tartarogenes* enzyme (Yamamoto et al., 1993) and more recently by crude preparations of bacteroids from root nodules of adzuki beans (*Vigna angularis*) (Fujihara et al., 1995). In the latter case, it has been conclusively demonstrated that the aminobutanal moiety of putrescine is transferred to one of the primary amino groups of cadaverine. Since bacterial HSS is composed of two identical subunits, the question arises whether each subunit has its own catalytic centre with two binding sites or if two subunits form only one catalytic site. X-ray studies of crystallized HSS from the overexpressing *E. coli* strain will reveal more answers to this question. The molecular mass of native HSS from a representative pyrrolizidine-alkaloid-containing plant such as *Senecio vernalis* was determined to be 110 kDa. Polyclonal antisera against *R. viridis* HSS showed cross-reactivity to the plant enzyme and a protein band of about 55 kDa in SDS/PAGE of *Eupatorium cannabinum* protein extracts (data not shown).

The sequence of the *R. viridis hss* gene should provide a valuable tool for the characterization of *hss* genes from other eubacteria and cyanobacteria in order to obtain information on sequence homologies among the prokaryotic homologues.

Furthermore, on the basis of the biochemical similarities between bacterial and plant HSS, the bacterial genes should facilitate the isolation of cDNAs for plant HSS. Studies in this direction are currently in progress.

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