Characterization and a role of *Pseudomonas aeruginosa* spermidine dehydrogenase in polyamine catabolism

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Pseudomonas aeruginosa PAO1 has two possible catabolic pathways of spermidine and spermine; one includes the *spuA* and *spuB* products with unknown functions and the other involves spermidine dehydrogenase (SpdH; EC 1.5.99.6) encoded by an unknown gene. The properties of SpdH in *P. aeruginosa* PAO1 were characterized and the corresponding *spdH* gene in this strain identified. The deduced SpdH (620 residues, calculated M_r of 68 861) had a signal sequence of 28 amino acids at the amino terminal and a potential transmembrane segment between residues 76 and 92, in accordance with membrane location of the enzyme. Purified SpdH oxidatively cleaved spermidine into 1,3-diaminopropane and 4-aminobutyraldehyde with a specific activity of 37 units (mg protein)^{−1} and a K_m value of 36 μ M. The enzyme also hydrolysed spermine into spermidine and 3-aminopropanaldehyde with a specific activity of 25 units (mg protein)^{−1} and a K_m of 18 μ M. Knockout of *spdH* had no apparent effect on the utilization of both polyamines, suggesting that this gene is minimally involved in polyamine catabolism. However, when *spdH* was fused to the polyamine-inducible promoter of *spuA*, it fully restored the ability of a *spuA* mutant to utilize spermidine. It is concluded that SpdH can perform a catabolic role *in vivo*, but *P. aeruginosa* PAO1 does not produce sufficient amounts of the enzyme to execute this function.

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INTRODUCTION

Pseudomonas species, including *Pseudomonas aeruginosa*, can utilize polyamines (putrescine, spermidine and spermine) as the sole source of carbon and nitrogen (Lu *et al.*, 2002; Nishijyo *et al.*, 2001; Stanier *et al.*, 1966). *P. aeruginosa* PAO1 initially converts putrescine into 4-aminobutyraldehyde via putrescine:pyruvate aminotransferase, encoded by *spuC* (Lu *et al.*, 2002). This intermediate aldehyde is then oxidized to 4-aminobutyrate by a dehydrogenase specified by *kauB* before being channelled into the TCA cycle (Jann *et al.*, 1988; Fig. 1). The *spuC* gene is part of the *spuABCDEF* operon, in which *spuA* and *spuB* encode putative enzymes with homology to glutamine amidotransferase and glutamine synthetase, respectively, and

the *spuDEFGH* cluster specifies a transport system of spermidine (Lu *et al.*, 2002). Expression of the *spu* operon is activated in the presence of a polyamine through the CbrA-CbrB two-component regulatory proteins (Lu *et al.*, 2002; Nishijyo *et al.*, 2001).

The functions of the SpuA and SpuB enzymes are obscure. However, the impaired growth of *spuA* and *spuB* mutants on spermidine and spermine suggests that the SpuA and SpuB enzymes play major roles in the catabolism of these polyamines. In addition, *P. aeruginosa* expresses spermidine dehydrogenase (SpdH; EC 1.5.99.6), which has also been identified in *Citrobacter freundii* and *Serratia marcescens* (Hisano *et al.*, 1990, 1992a; Tabor & Kellogg, 1970). The SpdH of *P. aeruginosa* IFO3080 is constitutively expressed and has been purified as a 63 kDa monomer, but it remains to be characterized in detail (Hisano *et al.*, 1990). In contrast, *C. freundii* and *S. marcescens* SpdHs are haem proteins that are inducible by spermidine and they both cleave spermidine to yield 1,3-diaminopropane and 4-aminobutyraldehyde (Fig. 1) and prefer potassium ferricyanide as an electron acceptor (Hisano *et al.*, 1992a, b; Tabor & Kellogg, 1970). The *S. marcescens* SpdH oxidizes spermine at an unknown scissile site, but to only about 30 % of its specific

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Two supplementary figures are available with the online version of this paper.

Fig. 1. Reactions catalysed by SpdH and possible catabolic pathways of reaction products in *P. aeruginosa* PAO1. Reactions catalysed *in vitro* by SpdH are indicated by dashed arrows and enzyme genes are assigned to each reaction according to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>): *spdH* (PA3713), spermidine dehydrogenase; *spuC*, putrescine aminotransferase; *kauB*, 3-aminopropanaldehyde/4-aminobutyraldehyde/4-guanidinobutyraldehyde dehydrogenase; *gabT*, 4-aminobutyrate aminotransferase; *gabD*, succinic semialdehyde dehydrogenase; *gltA*, citrate synthase. PA0132 and PA0130 might encode β -alanine aminotransferase and malonic semialdehyde dehydrogenase, respectively. The enzyme catalysing 1,3-diaminopropane deamination to 3-aminopropanaldehyde (dotted arrow) is unknown.

activity for spermidine (Tabor & Kellogg, 1970). The *spdH* gene has not been identified in any bacterium.

We purified and characterized the SpdH of *P. aeruginosa* PAO1 in the present study and then identified the corresponding *spdH* gene. Growth experiments with an *spdH* knockout mutant and a *spuA* mutant harbouring the *spdH* gene fused to the polyamine-inducible promoter for the *spu* operon showed that SpdH can catabolize spermidine *in vivo*. However, SpdH minimally participates in polyamine utilization by strain PAO1, because expression levels of the enzyme are too low to switch on the catabolic function.

METHODS

Strains, plasmids and media. We used the described *Escherichia coli* strains, *Pseudomonas aeruginosa* PAO1 (wild-type), PAO6100 (*kauB*::Tn5-751) and PAO5006 (*spuA*::Tc) and plasmids (Hoang *et al.*, 1998; Jann *et al.*, 1988; Nakada & Itoh, 2002; Lu *et al.*, 2002; Stover *et al.*, 2000). The *P. aeruginosa* strains TH2768, TH2774, TH2834, TH3990, TH3992, TH4239 and TH4240 were gifts from N. Gotoh, Department of Microbiology, Kyoto Pharmaceutical University, Kyoto, Japan. *P. aeruginosa* PAO4548 (*spdH*::Gm) was derived from *P. aeruginosa* PAO1 as described below. The *E. coli* and

P. aeruginosa strains were cultivated at 37 °C in Luria–Bertani (LB) and in nutrient yeast broth (NYB), respectively, supplemented with antibiotics when necessary (Haas *et al.*, 1997; Sambrook *et al.*, 1989). Minimal medium P (MMP; Haas *et al.*, 1997) was supplemented with the indicated carbon and nitrogen sources to examine spermidine and spermine utilization and to prepare *P. aeruginosa* cells for assay and for SpdH purification.

DNA manipulations. DNA purification, restriction enzyme analysis and other manipulations proceeded as described previously (Itoh, 1997; Nishijyo *et al.*, 2001). KOD DNA polymerase (Toyobo Biochemicals) was applied to the PCR under the reaction conditions recommended by the supplier and DNA was sequenced using an ABI310 DNA sequencer and Big-Dye Terminator sequencing kits (Applied Biosystems).

Construction of strains and plasmids. To construct an expression plasmid of SpdH and an *spdH* knockout mutant, we amplified the *spdH* coding region by PCR using PAO1 chromosomal DNA as the template and the oligonucleotide primers, 5'-TCATATGACCA-TCTCCCGCCGCGACTTCC-3' [corresponding to nucleotides +1 to +25 relative to the translation initiation codon ATG (bold type) with an *NdeI* site (underlined) at the 5' end] and 5'-GTCGCCGT-CCTTCAACCCAGTTCGCGGAC-3' (complementary to the 3'-non-coding region between nucleotides +1817 to +1846 from the ATG codon of *spdH*). The amplified DNA fragment was cloned into plasmid pUC118 (Vieira & Messing, 1987) at the *HincII* site to verify the nucleotides by sequencing. The 1.8 kb *spdH* region in the resultant plasmid, pYI431, was cleaved at the *NdeI* and *HindIII* (on the vector plasmid) sites and inserted into pET-22b+ (Novagen) between the corresponding restriction sites to produce pYI435. This plasmid was transformed into *E. coli* BL21(DE3) (Novagen). To construct an *spdH* knockout mutant, the 1.8 kb *spdH* segment was excised and cloned as a *BamHI*–*SphI* fragment from pYI431 into the mobilizable suicide plasmid pEX18Ap (Hoang *et al.*, 1998) between the corresponding sites, resulting in plasmid pYI432. A gentamicin (Gm)-resistance cassette (Hoang *et al.*, 1998) was inserted as a *SmaI* fragment into the *DraIII* site of *spdH* on this plasmid to create pYI436. This plasmid was then introduced into strain PAO1 by conjugation via helper *E. coli* S17-1 (Simon *et al.*, 1983). The *spdH*::Gm mutant strain PAO4548 was selected on MMP agar containing 100 μ g Gm ml⁻¹ and 20 mM glutamate as the sources of carbon and nitrogen, and then on LB agar containing 5% sucrose, which allows the growth of clones lost during the second cross-over of the plasmid sequence (*sacB*⁺) integrated into the chromosome (Hoang *et al.*, 1998). To create a *spuA*::*spdH* fusion gene, we amplified a 278 bp intergenic region between *spuA* and *spuI* (Lu *et al.*, 2002) by PCR using oligonucleotide primers designed to add the *NdeI* sites (underlined) at the translation initiation codons of these genes; 5'-GGGTACCGACATATGGCAACACCTT-3' (nucleotide -13 to +12 of *spuI*) and 5'-CAGGCGAGACATATGACACCCG-TTG-3 (complementary to nucleotide -13 to +12 of *spuA*). After cloning into plasmid pUC118 and verifying the nucleotides as described above, the amplified DNA fragment was isolated as an *NdeI* fragment and joined to *spdH* on plasmid pYI431 at the *NdeI* site. Because the DNA fragment inserted in the *spuA*::*spdH* and *spuI*::*spdH* orientations creates *Bpu1102I* fragments of 709 and 895 bp, respectively, we analysed the recombinant plasmids using *Bpu1102I* restriction endonuclease and identified a plasmid (pYI449) having *spuA*::*spdH*. We then digested plasmid pYI449 at the *BamHI* and *HindIII* sites (in the multiple cloning sites of pUC118) that flanked the fusion gene and cloned the resultant *BamHI*–*HindIII* fragment of *spuA*::*spdH* between the *BglII* and *HindIII* sites of plasmid pNIC6011 (Nakada & Itoh, 2002), resulting in plasmid pYI451.

SpdH assay. Cells cultured to the exponential phase (OD₆₀₀ 0.6–0.8) in MMP containing 20 mM glutamate or 20 mM spermidine as

carbon and nitrogen sources were harvested, washed twice, and then resuspended in 50 mM potassium phosphate buffer (pH 7.2). Cell extracts were prepared by passage through a French pressure cell (SLM-AMINCO) at 20 000 p.s.i. (138 MPa), followed by centrifugation at 10 000 *g* for 30 min at 4 °C to remove intact cells and cell debris. Optionally, cytoplasm and cytoplasmic membrane/peptidoglycan fractions were separated by centrifugation at 100 000 *g* for 1 h at 4 °C. SpdH activity was determined in a reaction mixture (2 ml) containing 0.5 mM spermidine or spermine, 1.0 mM $K_3Fe(CN)_6$ and 200 mM Tris/HCl buffer (pH 7.2) at 25 °C. The reaction was initiated by adding a cell-free extract and the decrease in absorbance at 400 nm (ferricyanide reduction) was measured. One unit of SpdH activity was defined as described (Hisano *et al.*, 1990). Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad Protein Assay kit, with bovine serum albumin as the standard.

Purification of SpdH from strain PAO1. We prepared cell extracts as described above from *P. aeruginosa* PAO1 cells cultured at 37 °C in MMP (5 l) containing 20 mM glutamate as carbon and nitrogen sources. SpdH was precipitated with ammonium sulphate (40–60% saturation) dissolved in, and exhaustively dialysed against, 50 mM potassium phosphate buffer (pH 7.2). The dialysed enzyme was eluted through a Hiprep16/10 DEAE column (20 ml; Amersham Biosciences) using a linear gradient (0–1.0 M) of KCl in 50 mM potassium phosphate buffer (pH 7.2), with which the column had been equilibrated. The active fractions (5.0 ml each, eluted at 0.03 M KCl) were pooled, dialysed against the potassium phosphate buffer and applied to a MonoQ 5/50GL column (1.0 ml; Amersham Biosciences) equilibrated with 50 mM potassium phosphate buffer (pH 8.0). The enzyme was eluted with a KCl linear gradient (0–1.0 M) in the same buffer and the active fractions (0.6 ml each, eluted at 0.35 M KCl) were further purified by gel filtration through a column containing Superose12 HR 10/30 (Amersham Biosciences) using 50 mM potassium phosphate buffer (pH 7.2) containing 150 mM KCl as the elution buffer. The enzyme purity was examined by SDS-PAGE (Nakada & Itoh, 2002).

Expression and purification of SpdH in *E. coli*. We expressed SpdH in *E. coli* BL21(DE3) harbouring plasmid pY1435 (see above). IPTG (1 mM) was added to the recombinant culture growing exponentially (OD₆₀₀ 0.3) in LB medium to induce SpdH synthesis. After 4 h incubation the cells were collected by centrifugation, suspended in 50 mM potassium phosphate buffer (pH 7.2) and ruptured by passage through an Amicon French press. Cell-free extract was obtained by centrifugation at 15 000 *g* for 30 min and recombinant SpdH was purified by chromatography using columns containing Hiprep16/10 DEAE, MonoQ 5/50GL and Superose12 HR 10/30 as described above.

HPLC analysis of polyamines and their reaction products. To determine the reaction products of spermidine and spermine, we incubated the polyamines with purified SpdH in the assay mixture (2 ml) as described above. Samples (0.25 ml) were removed at the indicated times and the reaction was terminated by adding 1 M HCl (20 µl ml⁻¹). Insoluble materials were removed by passage through a cellulose acetate membrane (pore size 0.45 µm; Advantec). Thereafter, portions (20 µl) were eluted through a Shodex Asahipak ODP-50 4E column (4.6 × 250 mm; Asahi Chemical Industry) using 200 mM sodium phosphate buffer (pH 7.7) containing 2.3 mM sodium 1-octane sulphonate as the mobile phase to resolve spermidine and its products, or through a Cadenza CD-C18 column (4.6 × 250 mm; Imtakt) to separate spermine and its products using 30% (v/v) acetonitrile as the mobile phase. The elution rate was 0.5 ml min⁻¹. The substrates and their products eluted from the HPLC columns were coupled with *o*-phthalaldehyde and detected by measuring emission at 455 nm (excitation at 345 nm) using a RF-10AXL fluorescence detector (Shimadzu) and an amino acid

detection system OPA (Shimadzu). We used the same HPLC columns under conditions identical to those applied to spermidine and spermine determinations in cultures. Wild-type PAO1 and *spdH* mutant cells (1×10^9 ml⁻¹) growing exponentially in MMP containing 20 mM spermidine (inducible substrate) or in 20 mM glutamate (non-inducible substrate) as the sole carbon and nitrogen sources were transferred to fresh MMP (10 ml) containing 20 mM spermidine or 20 mM spermine and shaken at 37 °C. Duplicate portions (0.2 ml) of the cultures were removed at the indicated time. The cells were sedimented by centrifugation and the supernatant was passed through cellulose acetate membranes. Thereafter, portions (20 µl) of the filtrate were eluted through the respective HPLC columns. Spermidine, spermine and 1,3-diaminopropane were purchased from Sigma and 4-aminobutyraldehyde and 3-aminopropanaldehyde were synthesized as described previously (Tanaka *et al.*, 2001; Yorifuji *et al.*, 1986).

Amino acid sequencing and MALDI-TOF mass spectrometry.

Purified SpdH was resolved by 10% SDS-PAGE and blotted onto a PVDF membrane (Amersham Biosciences). The amino-terminal sequence was determined using an HP10000 protein sequencer (Hewlett Packard). We measured the MALDI-TOF mass spectra of purified SpdH using a REFLEX II equipped with a SCOUT ion source and a pulsed ion extraction accessory (Bruker Daltonics). Protein samples were mixed with the same volume of a matrix solution (1% (w/v) of 3,5-dimethoxy-4-hydroxycinnamic acid (Bruker Daltonics) in acetonitrile/0.1% trifluoroacetic acid (1:2, v/v) and pulse-irradiated with a nitrogen laser at 337 nm for 3 ns each set in the linear mode at 20 kV of ion acceleration. Spectra were the means of at least 100 laser shots.

RESULTS

Occurrence and constitutive synthesis of SpdH among *P. aeruginosa* strains

Unlike *S. marcescens* ATCC 25179 and *C. freundii* IFO12681, which synthesize 6- and 20-fold more SpdH, respectively, in the presence of spermidine, *P. aeruginosa* IFO3080 constitutively produces this enzyme (Hisano *et al.*, 1990, 1992b; Tabor & Kellogg, 1970). To determine whether other *P. aeruginosa* strains also have SpdH and if so, whether they constitutively produce the enzyme, we cultured strain PAO1 and seven other independent *P. aeruginosa* strains in MMP containing 20 mM spermidine (inducing substrate) or 20 mM glutamate (non-inducing substrate) as the sole sources of carbon and nitrogen, and measured SpdH activities in the cell extracts. All tested strains formed similar amounts of the enzyme [about 0.02 units (mg protein)⁻¹] irrespective of the presence of the inducer, providing evidence that SpdH occurs universally and is constitutively synthesized among *P. aeruginosa* strains. Approximately half the enzymes were associated with the cell envelope (sediment after centrifugation at 100 000 *g* for 60 min) of all tested strains.

Identification of *spdH*

As described in Methods, we purified SpdH 2100-fold from PAO1 cells with a yield of 2.6% (2 µg protein from 1 l culture). Separation by SDS-PAGE showed that the purified enzyme contained 69 and 47 kDa proteins (see

Supplementary Fig. S1, available with the online version of this paper). Because the molecular mass of SpdH determined by gel-filtration chromatography (see Methods) was 70 kDa, we sequenced the first 15 amino-terminal residues (APGGRYYPALTGLR) of the 69 kDa protein. A BLAST P search using this amino-terminal sequence against the protein sequences in the *Pseudomonas* genome database (<http://www.pseudomonas.com>) identified the PA3713 gene, which encodes a protein with the same sequence between residues 29 and 43. The calculated M_r (65 907) of mature PA3713 protein (29–620 residues) is in agreement within experimental error with the determined M_r of SpdH, and the PA3713 protein expressed in *E. coli* displayed SpdH activity (see below), substantiating the notion that PA3713 is the gene encoding SpdH. We hereinafter refer to PA3713 as *spdH*. This gene is also present in *P. aeruginosa* strains 2192 (accession no. ZP_00973472), C3719 (ZP_00967906) and UCBPP-PA14 (ZP_00137108), the genome sequences of which have been determined. However, the gene is absent in closely related *Pseudomonas* species such as *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas syringae*. Homologues of SpdH ($\geq 43\%$ identity and $\geq 56\%$ similarity) occur in a few beta- and epsilonproteobacterial species, including *Chromobacterium violaceum* (Vasconcelos *et al.*, 2003), *Campylobacter lari* (Fouts *et al.*, 2005), *Campylobacter jejuni* (accession no. ZP_01099292), *Ralstonia metallidurans* (AAAI03000006.1), *Ralstonia eutropha* (AAZ60824) and culicine *Anopheles gambiae* (AAAB01002848.1).

Purification and characterization of SpdH expressed in *E. coli*

To confirm that *spdH* encodes SpdH and to characterize the enzyme, we cloned the coding DNA region into the plasmid pET-22b(+) between the *Nde*I and *Hind*III sites, so that the cloned gene was transcribed from the T7 promoter on the expression plasmid. The resultant plasmid pYI435 was introduced into *E. coli* BL21(DE3) that carries the IPTG-inducible T7 RNA polymerase gene. Cultivation of the recombinant *E. coli* in the presence of 1 mM IPTG induced sufficient SpdH synthesis to produce 7.0 units (mg protein)⁻¹ of the enzyme, which was about 300-fold more than the amount produced by strain PAO1. Three column chromatography separations (see Methods) yielded 4 mg apparently homogeneous SpdH per litre of culture, as judged by SDS-PAGE (see Fig. S1, lane 2). The first 16 amino-terminal residues of the purified enzyme were TISRDFLNGVALTI. This sequence precisely matched the deduced amino-terminal sequence except for the first methionine, which appeared to be post-translationally eliminated. The signal peptide was not cleaved in the *E. coli* host. Presumably a signal peptidase of the heterologous host cannot recognize this sequence, or the abundant synthesized *spdH* mRNA is translated in the cytoplasm by free ribosomes.

Absorption peaks of purified SpdH appeared at 280 and 415 nm in the oxidized state. In the reduced state (in the

presence of substrate), enzyme absorption peaked at 340, 530 and 560 nm. These spectrometric profiles are typical of haem proteins and are very similar to those observed with *S. marcescens* and *C. freundii* SpdHs (Hisano *et al.*, 1992b; Tabor & Kellogg, 1970). The molecular mass of the recombinant SpdH determined by MALDI-TOF-mass spectrometry was 69 850. This value is larger than the calculated M_r (68 730) by 1120 Da. We consider possible prosthetic groups of SpdH in the Discussion. The purified SpdH preferentially used ferricyanide as an electron acceptor. Phenazine methosulphate and dichloroindophenol also served as electron acceptors but at about 10% of the efficiency of ferricyanide. Cytochrome *c* was a poor electron acceptor for this enzyme (2% ferricyanide). Measurements of initial reaction velocities with different substrate concentrations determined the specific activity and the K_m value of the enzyme for spermidine as 37 ± 2 units (mg protein)⁻¹ and 36 ± 2 μ M, respectively. The enzyme also used spermine as a substrate. The specific activity towards spermine was 25 ± 2 units (mg protein)⁻¹ and the K_m value for this polyamine was 18 ± 1 μ M.

Cleavage modes on spermidine and spermine

We next investigated where SpdH cleaves spermidine and spermine using HPLC analysis of the reaction products. This enzyme yielded mostly 1,3-diaminopropane and 4-aminobutyraldehyde from spermidine and trace amounts of 3-aminopropanaldehyde (Fig. 2A). Thus, like *S. marcescens* and *C. freundii* SpdHs (Hisano *et al.*, 1992b; Tabor & Kellogg, 1970), *P. aeruginosa* SpdH selectively cleaves the N-4-aminobutylimino bond of spermidine. This enzyme generated 3-aminopropanaldehyde and spermidine from spermine during the initial stage of the reaction, and the produced spermidine was decomposed into 1,3-diaminopropane and 4-aminobutyraldehyde at a later stage of the reaction (Fig. 2B), showing that *P. aeruginosa* SpdH cleaves spermine at the N-3-aminopropylimino bonds.

Growth phenotypes of *spdH* and *kauB* mutants

To determine whether *P. aeruginosa* PAO1 SpdH contributes to spermidine and spermine utilization in any way, we inactivated *spdH* by inserting a Gm cassette (Hoang *et al.*, 1998) into the *Dra*III site at nucleotide 1117 of *spdH* (1863 bp). The resultant *spdH* mutant, strain PAO4548 (*spdH*::Gm), had no SpdH activity either in the membranes or in the cytoplasm, indicating that both enzymes are the *spdH* product. Possibly SpdH dissociates from the membranes during cell disruption. The *spdH*::Gm strain proliferated as normally as wild-type PAO1 in MMP supplemented with 20 mM spermidine or spermine as the sole source of both carbon and nitrogen (data not shown). The *spdH* mutant also grew normally in MMP containing glutamate as the sole source of carbon and nitrogen, or succinate and ammonia. To examine whether SpdH participates at any level in polyamine catabolism *in vivo*, we cultivated strains PAO1 (wild-type) and PAO4548 (*spdH*::Gm) cells in MMP containing 20 mM glutamate

Fig. 2. HPLC analysis of products of reactions between spermidine (A) and spermine (B) with SpdH. Reaction mixtures were sampled at the indicated times and analysed by HPLC. Retention times of substrates and products were determined using commercial or synthesized compounds: a, spermidine; b, 1,3-diaminopropane; c, 4-aminobutyraldehyde; d, spermine; e, 3-aminopropanaldehyde. Fluorescence emission intensity differs depending on the compound and hence does not reflect chemical amounts.

or 20 mM spermidine to the exponential growth phase and transferred the induced and non-induced cells into fresh MMP containing 20 mM spermidine or 20 mM spermine. The polyamine amounts in the cultures determined by HPLC showed that the non-induced cells of both strains utilized the polyamines very slowly and at comparable rates (see Supplementary Fig. S2, available with the online version of this paper). The induced cells, regardless of the *spdH* mutation, actively and again similarly consumed both the polyamines. Spermidine was utilized three times faster than spermine (see Fig. S2) and hence is a more efficient source of both carbon and nitrogen than spermine (see below). Thus, SpdH contributed little to polyamine catabolism by *P. aeruginosa* PAO1 under our growth conditions.

The *kauB* gene encodes an aldehyde dehydrogenase, which catalyses the oxidative conversion of 4-guanidinobutyraldehyde to 4-guanidinobutyrate in the arginine dehydrogenase pathway and of 4-aminobutyraldehyde to 4-aminobutyrate in the putrescine catabolic pathway. The mutation of *kauB* therefore simultaneously abolishes the utilization of 2-ketoarginine and putrescine (Jann *et al.*, 1988). We found that the *kauB* mutant strain PAO6100 (*kauB*::Tn5-751) (Jann *et al.*, 1988) also could not grow on any of spermidine, spermine, or 1,3-diaminopropane.

In vivo catabolic function of SpdH

Although *P. aeruginosa* PAO1 SpdH actively degrades spermidine and spermine *in vitro*, it seemed not to be involved significantly in their catabolism and utilization. Perhaps the constitutive levels of SpdH, being below 5 % of the induced levels in *C. freundii* (Hisano *et al.*, 1990), are insufficient to do so. To demonstrate a catabolic function of *P. aeruginosa* SpdH *in vivo*, we fused in-frame the *spdH* structural gene to the polyamine-inducible promoter of *spuA* at the translation initiation codons of these genes and cloned the resultant *spuA*::*spdH* chimeric gene into plasmid pNIC6011 (Nishijyo *et al.*, 2001), to create pYI451 (see Methods). When this plasmid was introduced into strain PAO5006 (*spuA*::Tc), which grows poorly on either spermidine (Lu *et al.*, 2002; Fig. 3) or spermine (data not shown), the *spuA* mutant initiated growth in MMP + 20 mM spermidine much earlier than wild-type PAO1 and proliferated at a growth rate (doubling time of about 110 min) similar to that of the wild-type strain (Fig. 3). PAO1, PAO5006 and PAO5006/pNIC6011 cells growing in MMP + 20 mM glutamate or 20 mM spermidine produced approximately 0.02 units SpdH (mg protein)⁻¹. PAO5006/pYI451 cells contained 0.07 units SpdH (mg protein)⁻¹ during growth in MMP + 20 mM glutamate. However,

Fig. 3. Catabolic function of SpdH. Strains were incubated in MMP containing 20 mM spermidine at 37 °C and growth was followed by measuring optical density at 660 nm. ○, *P. aeruginosa* PAO1; ●, *P. aeruginosa* PAO5006 (*spuA*::Tc); □, *P. aeruginosa* PAO5006/pNIC6011; ▲, *P. aeruginosa* PAO5006/pYI451 (*spuA*::*spdH*).

when incubated in MMP containing 20 mM spermidine the recombinant cells induced SpdH synthesis to form 0.24 units (mg protein)⁻¹, amounts that were fairly similar to those generated (0.2–0.6 units (mg protein)⁻¹) in the induced *C. freundii* and *S. marcescens* (Hisano *et al.*, 1990; Tabor & Kellogg, 1970). Plasmid pYI451 did not support growth of the *spuA* mutant on spermine. Furthermore, *P. aeruginosa* PAO1 grew much more slowly (doubling time of 150 min) in MMP containing 20 mM spermine than in that containing spermidine. Spermine might not effectively induce the *spuA* promoter.

DISCUSSION

P. aeruginosa PAO1 SpdH shares similar properties with its *S. marcescens* and *C. freundii* counterparts (Hisano *et al.*, 1990; Tabor & Kellogg, 1970). These are monomers of haem proteins associated with cytoplasmic membranes that cleave spermidine into 1,3-diaminopropane and 4-aminobutyraldehyde and preferentially use ferricyanide as an electron acceptor *in vitro*. Like *S. marcescens* SpdH (Okada *et al.*, 1979; Tabor & Kellogg, 1970), the *P. aeruginosa* enzyme can also cleave spermine, but at a different scissile site from the *S. marcescens* enzyme. Spermidine and 3-aminopropanaldehyde are the sole products of spermine during the initial stage of the reaction (Fig. 2B); 1,3-diaminopropane becomes detectable only after spermidine has accumulated and the amount of the diamine increases in parallel with an increase in 4-aminobutyraldehyde (Fig. 2B). These profiles of the reaction products show that the diamine is derived from the product spermidine and not the initial substrate, spermine. Thus *P. aeruginosa* SpdH specifically acts on the propylimino bonds of spermine, whereas *S. marcescens* SpdH preferentially cleaves spermine at the butylimino

bonds, yielding 1,3-diaminopropane and *N*-3-aminopropionyl-4-aminobutyraldehyde (Okada *et al.*, 1979).

The SpdH of *S. marcescens* is a flavoprotein and that of *C. freundii* is a quinoprotein (Hisano *et al.*, 1992a; Tabor & Kellogg, 1970). The *P. aeruginosa* SpdH does not appear to be a quinoprotein, because it has no sequence similarity to known quinoproteins that have a conserved pyrroloquinoline quinone (PQQ)-binding domain and a homologous haem C-binding sequence in the carboxyl-terminal region (Oubrie, 2003; Yamada *et al.*, 2003). The SpdH of *P. aeruginosa* has a pfam_fs:FAD_binding_2-FAD binding domain (N-score, 14, 35; E-value, 9.4e⁻⁰⁸) and the pfam_fs:DAO motif (N-score, 15, 33; E-value, 1e⁻⁰⁸) of FAD-dependent oxidoreductase (<http://kr.expasy.org/prosite>) between residues 75 and 114. The carboxyl-terminal region (520–620 residues) contains the pfam_fs:Amino_oxidase motif (N-score, 12, 39; E-value, 8.6e⁻⁰⁶) of an amine oxidoreductase that contains flavin. These motifs are conserved in oxidoreductases containing flavin such as glycine/D-amino acid oxidase (Mattevi *et al.*, 1997; Mortl *et al.*, 2004) and FixC dehydrogenase (Edgren & Nordlund, 2004). The predicted molecular mass of the prosthetic groups (*M_r* 1120) is close to the combined molecular mass of haem (*M_r* ~620) and FMN (*M_r* 456). The structure of the prosthetic flavin should be confirmed by chemical analysis. The SpdH purified from PAO1 cells is truncated at the amino terminal by 28 residues. This region has features typical of a signal peptide (peak value, 2.10; an amino-terminal basic region with a net charge of 1, a central hydrophobic region of 16 residues and a discriminant score of 2.03) according to the PSORT program (<http://psort.ims.u-tokyo.ac.jp>). The TMPred program (<http://www.ch.embnet.org>) predicted a short transmembrane segment (score of 1661) between residues 76 and 92. These features of the SpdH sequence are consistent with the membrane location of the enzyme and account for weak interaction with the cytoplasmic membranes, since sonication, low ionic concentration buffers and detergents can dissociate SpdH from membranes.

SpdH appears to occur universally and be constitutively expressed at low levels in *P. aeruginosa* strains. Knockout of *spdH* did not apparently affect growth on spermidine of *P. aeruginosa* PAO1, because this strain has the *spuAB* genes that encode inducible enzymes for spermidine catabolism (Lu *et al.*, 2002). However, a knockout mutant of *spuA* can still grow, though slowly, on spermidine (Lu *et al.*, 2002; Fig. 3), suggesting that *spdH* contributes to this slow growth of the *spuA* mutant. When SpdH is expressed *in vivo* in appropriate amounts, this enzyme can fully support growth on spermidine of a *spuA* mutant (Fig. 3), demonstrating the *in vivo* catabolic function of this enzyme. 4-Aminobutyraldehyde, one of the degradation products of spermidine by SpdH, would be converted by KauB aldehyde dehydrogenase to 4-aminobutyrate, which is then led to succinate via 4-aminobutyrate aminotransferase (GabT or GoaD) and succinic semialdehyde dehydrogenase (GabD) (<http://www.pseudomonas.com>) as in *E. coli*

(Kurihara *et al.*, 2005; Schneider *et al.*, 2002) (Fig. 1). The other product, 1,3-diaminopropane, can serve as a source of both carbon and nitrogen for *P. aeruginosa* PAO1. In *Arthrobacter* spp. an aminotransferase converts 1,3-diaminopropane along with pyruvate into 3-aminopropanaldehyde and alanine (Yorifuji *et al.*, 1997). The catabolic enzymes of this diamine have not been studied in *P. aeruginosa*. However, the *kauB* mutation abolished growth on 1,3-diaminopropane, suggesting that *P. aeruginosa* PAO1 also catabolizes the diamine via 3-aminopropanaldehyde, which is then oxidized to β -alanine by *KauB* dehydrogenase (Fig. 1). According to the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>), *PA0132* and *PA0130* are good candidates for β -alanine aminotransferase and malonic semialdehyde dehydrogenase, respectively (Fig. 1). *P. aeruginosa* PAO1 thus appears to have all the enzymes required to catabolize the compounds yielded from spermidine and spermine by SpdH.

Since the inactivation of *spuA* and *spuB* results in defective spermidine (Lu *et al.*, 2002) and spermine (data not shown) utilization, the products of these genes should play key roles in polyamine catabolism. Kurihara *et al.* (2005) recently reported that *E. coli* converts putrescine by YcjK to *N*- γ -glutamylputrescine, which is subsequently oxidatively deaminated to γ -glutamylaminobutyraldehyde that is in turn oxidized to γ -glutamylaminobutyrate. YcjL hydrolyses γ -glutamylaminobutyrate into glutamate and 4-aminobutyrate. *SpuA* and *SpuB* share similar amino acid sequences with YcjL (60 % similarity) and YcjK (50 % similarity), respectively. Such similarity and the impaired growth of a *kauB* mutant on spermidine and spermine imply that the catabolism of these polyamines in *P. aeruginosa* proceeds via γ -glutamyl intermediates and that *KauB* aldehyde dehydrogenase is involved in the oxidation of intermediate aldehydes. We are presently analysing the catabolic intermediates that accumulate in *spuAB* and *kauB* mutants to establish the catabolic routes of spermidine and spermine in *P. aeruginosa* PAO1.

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