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S-adenosylmethionine decarboxylase of *Bacillus subtilis* is closely related to archaebacterial counterparts

Agnieszka Sekowska,[1,2] Jean-Yves Coppée,[1] Jean-Pierre Le Caer,[3] Isabelle Martin-Verstraete[1] and Antoine Danchin[1,2]*

[1]*Régulation de l'Expression Génétique, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris, France.*

[2]*Hong Kong University Pasteur Research Center, Dexter HC Man Building, 8, Sassoon Road, Pokfulam, Hong Kong.*

[3]*Neurobiologie et Diversité Cellulaire, CNRS UMR 7637, Ecole Supérieure de Physique et de Chimie Industrielles de la ville de Paris, 10 rue Vauquelin, 75005 Paris, France.*

Summary

Bacillus subtilis **synthesizes polyamines by decarboxylating arginine to agmatine, which is subsequently hydrolysed to putrescine. Spermidine is synthesized from putrescine and decarboxylated S-adenosylmethionine (dAdoMet). In Gram-negative bacteria and in eukaryotes, AdoMet is decarboxylated by an unusual 'pyruvoyl' AdoMet decarboxylase (SpeD), the catalytic pyruvoyl moiety of which is generated by serinolysis of an internal serine with self-cleavage of the protein at the upstream peptide bond. Neither the Gram-positive bacterial nor the archaeal counterpart of the *Escherichia coli* SpeD enzyme were known. We have identified the corresponding *B. subtilis speD* gene (formely *ytcF*). Heterologous expression of the cognate *Methanococcus jannaschii* protein, MJ0315, demonstrated that it displays the same activity as *B. subtilis* SpeD, indicating that spermidine biosynthesis in Gram-positive bacteria and in archaea follows a pathway very similar to that of Gram-negatives and eukarya. In *B. subtilis*, transcription of *speD* is modulated by spermidine and methionine. Its expression is high under usual growth conditions. In contrast, the SpeD protein self-cleaves slowly *in vitro*, a noticeable difference with its archaeal counterpart. Under certain growth conditions (minimal medium containing succinate and glutamate as a carbon source), *speD* is co-transcribed with *gapB*, the gene encoding glyceraldehyde-3-phosphate dehydrogenase, an enzyme required for gluconeogenesis. This observation may couple polyamine metabolism to sulphur and carbon metabolism by a so far unknown mechanism.**

Received 8 January, 2000; revised 14 March, 2000; accepted 17 March, 2000. *For correspondence. E-mail adanchin@pasteur.fr, adanchin@hkucc.hku.hk; Tel. (+33) 1 45 68 84 41; Fax (+33) 1 45 68 89 48.

Introduction

Polyamines, although dispensable under routinely used laboratory growth conditions, are extremely important for the cell. They are involved in macromolecular syntheses and in particular in modulating the accuracy of translation, at steps which may be essential for survival of the cell populations. Their importance is reflected by the fact that their biosynthesis is energy costly (Cohen, 1998). This is especially true for the larger molecules, such as spermidine, spermine and their analogues. In particular, spermidine synthesis requires S-adenosylmethionine (AdoMet) as a precursor. Surprisingly, AdoMet is not used as such in the reaction but is first decarboxylated to 3-aminopropyl-S-adenosine (dAdoMet). The aminopropyl-moiety of the substrate is subsequently transferred onto one of the amino-terminal ends of putrescine, to generate spermidine. A further transfer on spermidine yields spermine in some organisms (Cohen, 1998).

Transamination and decarboxylation are ubiquitous steps in intermediary metabolism. They are generally achieved by enzymes carrying pyridoxal phosphate as a coenzyme. However, a noteworthy feature of the known AdoMet decarboxylation reaction is that it is achieved by an enzyme carrying not a pyridoxal but a pyruvoyl group as the catalytic residue (Smith *et al.*, 1991). Pyruvoyl enzymes perform a limited number of varied decarboxylation reactions (van Poelje and Snell, 1990), comprising the decarboxylation of AdoMet (Cohn *et al.*, 1977) in eukarya and Gram-negative bacteria. Apart from a preliminary report describing the existence of a counterpart in *Sulfolobus solfataricus* (Cacciapuoti *et al.*, 1991), nothing was known in the case of Gram-positive bacteria or archaea.

We unravelled the main features of polyamine biosynthesis in *Bacillus subtilis* (Sekowska *et al.*, 1998).

Fig. 1. Alignment of AdoMet decarboxylases. The sequences have been aligned using the software ClustalW (Higgins *et al.*, 1996) and the alignment has been further refined manually. a '=' sign indicates identical residues and a '+' sign indiactes conserved residues (AGPST; ILMV; FWY; DENQ; HKR; C). Data was extracted from 'complete genomes' and 'unfinished genomes' at the NCBI: http://www.ncbi.nlm.nih.gov/Entrez/Genome/org.html. Note the cluster of methionine residues at the beginning of the *B. subtilis* sequence. Asterisks indicate the complete genomes, the other ones are in progress. SPED_BACSU: **B. subtilis*; UNKN_CLODI: *Clostridium difficile*; UNKN_THEMA: **Thermotoga maritima*; UNKN_PYRHO: **Pyrococcus horikoshii* OT3; UNKN_PYRFU: *Pyrococcus furiosus*; UNKN_PSEAE1: *Pseudomonas aeruginosa*; SPED_METJA: **M. jannaschii*; UNKN_ARCFU: **Archaeoglobus fulgidus*; UNKN_THIFE: *Thiobacillus ferrooxidans*; UNKN_AERPY1: **Aeropyrum pernix* K1; UNKN_AERPY2: **Aeropyrum pernix* K1; UNKN_AQUAE: **Aquifex aeolicus*; UNKN_SULSO1: *S. solfataricus*; UNKN_SULSO2: *S. solfataricus*; UNKN_CLOAC: *Clostridium acetobutilicum*; UNKN_XANCA: *Xanthomonas campestris*; UNKN_PSEAE2: *P. aeruginosa*; SPED_SALTY: *Salmonella typhimurium*; SPED_YERPE: *Yersinia pestis*; SPED_ESCCO: **E. coli*.

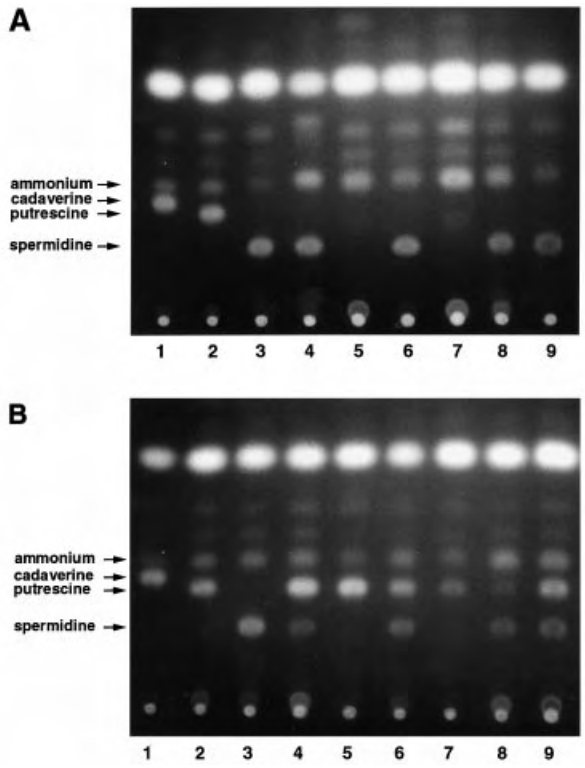

Fig. 2. Identification of dansyl polyamine derivatives of perchloric acid extracts of bacterial cultures by TLC in ethylacetate/cyclohexane (2:3, v/v).
A. Lane 1, cadaverine standard; lane 2, putrescine standard; lane 3, spermidine standard; lane 4, *B. subtilis* wild type; lane 5, BSIP7004 *B. subtilis speD* mutant; lane 6, BSIP7013–*speD* mutant complemented with the *B. subtilis speD* gene; lane 7, BSIP7014–*speD* mutant complemented with the *B. subtilis speD*S63A gene; lane 8, BSIP7015–*speD* mutant complemented with the *E. coli speD* gene; lane 9, BSIP7016–*speD* mutant complemented with the *M. jannaschii speD* gene.
B. Lane 1, cadaverine standard; lane 2, putrescine standard; lane 3, spermidine standard; lane 4, *E. coli* wild type; lane 5, HT375 *E. coli speD* mutant; lane 6, HT375 strain complemented with the *B. subtilis speD* gene; lane 7, HT375 strain complemented with the *B. subtilis speD*S63A gene; lane 8, HT375 strain complemented with the *E. coli speD* gene; lane 9, HT375 strain complemented with the *M. jannaschii speD* gene.

Spermidine was readily detectable in cell extracts, indicating that polyamine synthesis in *B. subtilis* is similar to that found in other organisms. Combining gene disruption experiments and biochemical identification of polyamines, we showed that the predominant pathway in *B. subtilis* proceeds from arginine via agmatine. We also observed that, in contrast to *Escherichia coli*, *B. subtilis* does not maintain a significant intracellular pool of putrescine under conditions where the level of spermidine is similar to that found in *E. coli*. It was therefore of great interest to identify the pathway leading to the addition of an *N*-propylamine group to putrescine, creating spermidine. In addition, this reaction yields the sulphur-rich molecule, methylthioadenosine (MTA) as a by-product. We have identified the nucleosidase encoded by the *mtn* (*yrrU*) gene as the first enzyme implicated in its recycling (Sekowska and Danchin, 1999). Identification of AdoMet decarboxylase was therefore the initial target of the present work.

Two classes of AdoMet decarboxylases, both pyruvoyl enzymes, are known: the AdoMet decarboxylase from bacteria and that from eukarya (van Poelje and Snell, 1990). Although they undergo the same zymogen-to-enzyme self-maturation process known as serinolysis (formation of the pyruvoyl group from a serine residue, splitting the precursor protein into two parts), most of these proteins do not share common structural features, at least at the level of their polypeptide sequence. Searches for similarities with sequences from either group (*E. coli* or *Saccharomyces cerevisiae*) revealed only a very weak similarity between the *E. coli speD* gene and a *B. subtilis* gene, *ytcF*. However, the similarity breaks down at the exact position of the putative catalytic site of the enzyme. By gene disruption, *in vitro* mutagenesis, cell-free protein synthesis and biochemical analysis of polyamines, we explored whether *ytcF* codes for the decarboxylase. Analysis of the phylogenetic relationships among bacterial enzymes demonstrated that the *B. subtilis* enzyme is very similar to several predicted proteins of unknown function from archaea. Therefore, the MJ0315 gene, which presumably encodes an AdoMet decarboxylase of *Methanococcus jannaschii*, was used to complement *B. subtilis ytcF and E. coli speD* mutants and was expressed in a cell-free system. Finally, analysis of the promoter region of the *B. subtilis speD* gene allowed identification of its transcription start point(s) as well as its major regulatory mechanisms.

Results

Identification of the S-adenosylmethionine decarboxylase gene in B. subtilis

Supposing that AdoMet decarboxylase could be a pyruvoyl containing enzyme (Cohn *et al.*, 1977), we scanned SubtiList (Moszer, 1998) with sequences upstream and downstream of the *E. coli* AdoMet decarboxylase pyruvoyl catalytic site. One coding sequence (CDS), *ytcF*, gave a putatively significant hit. The region upstream of the catalytic serine residue, however, had only little similarity to the *E. coli* SpeD protein (Fig. 1). Of particular interest was that the alignment predicted the presence of an intervening region immediately upstream of the active serine in the Gram-negative enzymes. This unusual finding prompted us to disrupt *ytcF* with a spectinomycin resistance cassette (strain BSIP7004).

Thin-layer chromatography (TLC) analyses, performed on cells grown in Luria–Bertani (LB) medium or in ED

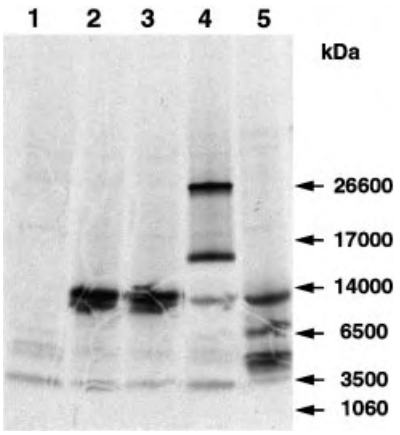

Fig. 3. In *vitro* transcription/translation and processing of AdoMet decarboxylases proenzymes. The proenzyme proteins were synthesized for 1 h as described under '*Experimental procedures*'. The samples were analysed on 16% Tris-Tricine SDS-polyacrylamide gels. Lane 1, pKK223-3 control plasmid; lane 2, pDIA5604 plasmid carrying the *speD* gene from *B. subtilis*; lane 3, pDIA5605 plasmid carrying the *speD*S63A gene from *B. subtilis*; lane 4, pDIA5606 plasmid carrying the *speD* gene from *E. coli*; lane 5, pDIA5607 plasmid carrying the *speD* gene from *M. jannashii*. Migration of protein standards of the M_r indicated is shown on the right.

minimal medium (MM) with fructose as the carbon source when they reached the end of their exponential growth phase (the OD_{600nm} of bacterial cultures was about three), demonstrated that the intracellular polyamine content in the *ytcF* disruptant is totally lacking spermidine (Fig. 2A). Thus, the strain behaved like a *speE* deficient mutant (Sekowska *et al*., 1998).

To ascertain that this gene is encoding an AdoMet decarboxylase, we tried to complement the *speD* deficient *E. coli* strain HT375 with plasmids carrying the *ytcF* gene and, as a control, the *E. coli speD* gene. Spermidine synthesis was restored in both cases (Fig. 2B), substantiating SpeD identity. Finally, expression of the *B. subtilis ytcF* gene and the *speD* gene from *E. coli* in the *ytcF* disruptant, mediated by either the integration at the *amyE* locus or multicopy plasmids, restored spermidine synthesis (Fig. 2A and data not shown). The *ytcF* gene was therefore renamed *speD*.

speD *from* M. jannaschii *and the active serine residue*

Figure 1 displays an alignment of proteins found in Entrez libraries as well as in unfinished genome sequences that are significantly similar to *B. subtilis* SpeD. As mentioned in the preceding paragraph, the similarity between the proteins from Gram-negative bacteria and SpeD of *B. subtilis* is weak, with identities or conserved replacements limited to three short regions: a region of 14 residues (51–64 in *B. subtilis* SpeD), upstream of the putative active serine, a region of 12 residues with the active serine (65–76) and a region of 20 residues (77–97). In contrast, proteins from archaea display significant similarities to *B. subtilis* SpeD, both in the number of conserved residues and in length. Noticeably, the [TS]CG triplet (84–86) downstream from the putative serine reactive centre is conserved: the cysteine residue has been shown to participate in the reaction in the mammalian enzyme (Xiong *et al*., 1999). It is worth noting that two archaea, *S. solfataricus* and *Aeropyrum pernix*, have two *speD*-like genes. All predicted SpeD proteins from archaea were short proteins (in particular both proteins from *S. solfataricus* were 123 and 134 residues long respectively). They were predicted to be split by serinolysis into two very short subunits made up of 60–80 residues. Incidentally, *Pseudomonas aeruginosa* too has two *speD*-like genes, one coding for a protein similar to the Gram-positive bacteria/archaea enzyme and the other similar to the enzyme in Gram-negative bacteria. To determine whether the *M. jannaschii* gene MJ0315 encodes an AdoMet decarboxylase, we subcloned it in the *E. coli* and *B. subtilis speD* mutants. The gene MJ0315 restored normal spermidine synthesis in both organisms (Fig. 2 and data not presented), strongly suggesting that it does indeed encode an AdoMet decarboxylase. On the basis of the similarity between the aligned proteins, together with the complementation experiments, we propose that these genes code for AdoMet decarboxylases.

The signature for these enzymes can be associated to two types of strongly related sequences:
type 1:
G[AIV][ST][GV][AITV][AIV][ILV][ILV]XES#H[ILV]
[ACST][ILV]H[ST][FYW][NP]EX2[FLY][AV]X[AFILV][DE]
[FILV][FY][ST]CG
or
type 2:
G[AIV][ST][GV][AITV][AIV][ILV][ILV]XEX22–27DKS#H
[ILV][ACST][ILV]H[ST][FYW][NP]EX10R[AV]D[AFILV]
[DE][FILV][AS][ST]CG
(where # indicates the conserved serine residue responsible for serinolysis).

Starting from the [ST]CG conserved amino acid triplet, and counting upstream either 19 residues (type 1 signature) or 27 residues (type 2 signature), we find the counterpart of the *E. coli* SpeD active serine in different sequence contexts. In type 1 enzymes the immediately upstream residue is acidic, whereas in the longer type 2 enzyme it is a lysine residue. To ascertain the importance of the conserved serine, we mutated it to alanine [known to exist in the inactive protein obtained by the reduction of its pyruvoyl moiety by sodium borohydride ($NaCNBH_3$) (Xiong *et al*., 1997)] in the *B. subtilis* protein. Expression of the modified gene encoding SpeDS65A under conditions similar to those used for the wild type failed to restore the synthesis of dAdoMet in either *E. coli* or *B. subtilis* (Fig. 2).

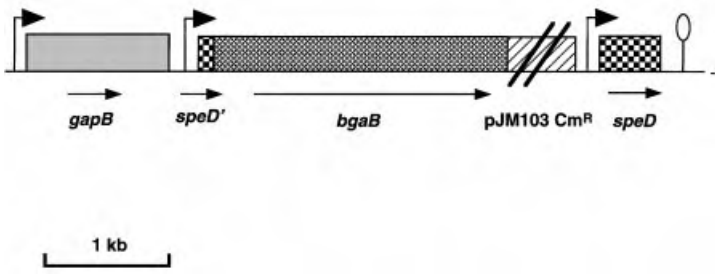

Fig. 4. Construction of the transcriptional fusion carried by strain BSIP7005. pJM103 is a non-replicative plasmid in *B. subtilis*, it carries an ampicillin resistance marker, the ColE1 origin of replication and a chloramphenicol resistance marker for selection in *B. subtilis* (Perego, 1993). The arrows above the boxed genes indicate the position of the transcription start sites. The loop indicates the position of a putative transcription termination site. The region crossed with two slash bars is not to scale (it represents the bulk of the plasmid insert).

Self-cleavage of the SpeD proteins

Pyruvoyl SpeD enzymes undergo self-cleavage at a reactive serine residue of the polypeptide chain, generating a heterodimeric protein (van Poelje and Snell, 1990). The various SpeD proteins could synthesise dAdoMet despite the fact that they were expressed in *E. coli*. This suggested that maturation of the preprotein could be accomplished in this bacterium. We therefore expressed the pKK223-3 derivatives carrying the *speD* gene of *E. coli*, *B. subtilis* and *M. jannaschii* in an *E. coli* cell-free transcription–translation coupled system in the presence of radioactive methionine. As shown in Fig. 3, the *E. coli speD* gene gave rise to three labelled bands, one of which corresponds to the preprotein, while the two smaller ones have molecular masses corresponding to those expected after serinolysis at the reactive centre serine residue (see Fig. 1). In the case of the *M. jannaschii speD* gene, a single band appears after serinolysis because the two parts of the protein have the same size. The smallest band is possibly due to degradation. In contrast, cleavage of the *B. subtilis* preprotein, at least at a high rate, was not observed either with the wild type or with the mutated *speD* gene. This could be due either to very slow self-cleavage or to the requirement for some activator molecule missing from the cell-free system. For this reason, we repeated the experiment in the presence of putrescine or AdoMet, which are known to activate maturation of the protein in some organisms (Xiong *et al*., 1997). No significant differences (i.e. a very low rate of cleavage) was observed under these conditions (data not shown). However, inspection of the gel revealed a second band with a molecular mass slightly lower than that of SpeD. This may be due to the existence of an alternative translation initiation site at the beginning of the *speD* gene (three methionine residues are clustered at the predicted amino-terminal end, see Fig. 1), or to changes in N-terminal processing (Rajagopalan and Pei, 1998).

Table 1. Expression of *speD*::*bgaB* transcriptional fusion (strain BSIP7005).

	β-Galactosidase activity (U mg^{-1} protein)[a]					
	BSIP7005		BSIP7005+spd[b]		BSIP7005+met[c]	
Carbon source	Exp.[d]	Stat.	Exp.	Stat.	Exp.	Stat.
Glucose	1600	140	1360	40	1470	950
Sucrose	180	190	130	80	150	900
Sorbitol	250	3000	200	1100	160	5500

a. For the β-galactosidase activity assay the bacteria were grown in the ED MM with different carbon sources.
b. Spermidine was added to the concentration of 1 mM.
c. Methionine was added to the concentration of 1 mM.
d. Exp., exponential-growth phase; Stat., stationary-growth phase.

Regulation of speD *expression*

Despite this lack of self-cleavage in a cell-free extract, the *B. subtilis* enzyme efficiently complements a *speD* defect in *E. coli*. Does this reflect some unusual property of *speD* expression in *B. subtilis*? We constructed a transcriptional fusion by inserting the *Bacillus stearothermophilus bgaB* gene into the *speD* gene (strain BSIP7005) (Fig. 4). Unexpectedly, the level of expression of the *speD* gene (Table 1), as monitored by β-galactosidase assays, was amongst the highest values found for *B. subtilis* genes without known function (Vagner *et al*., 1998). Depending on the carbon source present in the medium, *speD* expression can fluctuate from 200 to 1600 U mg^{-1} of proteins in glucose or sorbitol/sucrose containing medium in the early exponential growth phase (the OD_{600nm} of bacterial cultures was about 0.2), and from 150 to 3000 U mg^{-1} of proteins in the medium containing glucose/succrose or sorbitol in the stationary growth phase (the OD_{600nm} of bacterial cultures was about 5) (see Table 1). The level of activity observed in the presence of glucose as a carbon source (1600 U mg^{-1} of proteins) corresponds to approximately 10 000 proteins per cubic micrometer of the cell volume, i.e. to a concentration comparable to that of ribosomes. Because the enzyme synthesises dAdoMet, a molecule expected to be present in limited amounts in the cell because it derives from AdoMet, a very expensive molecule, one would expect that the specific activity of the enzyme should be either very low or that the putative processing of SpeD should be tightly regulated. To ascertain whether

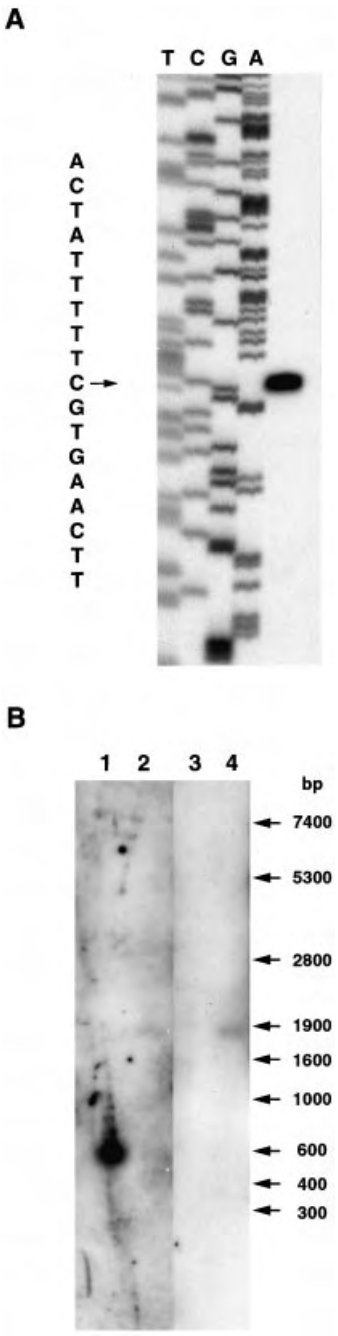

Fig. 5. Identification of the *speD* promoter by primer extension and of the *speD* transcripts by Northern blotting.
A. Identification of the transcription start site of the *speD* gene. The size of the extended product is compared with a DNA-sequencing ladder of the *speD* promoter region. Primer extension and sequencing reaction were performed with the same primer. The +1 site is marked by an arrow.
B. Northern blot analysis of the *B. subtilis* 168 *speD* gene. A total of 4 μg of RNA was used. The probes were: lanes 1 and 2, specific *speD* fragment and lanes 3 and 4, specific *gapB* fragment. RNA corresponding to lanes 1 and 3 was obtained from a culture grown in MM with glucose as a carbon source, and for lane 2 and 4 from a culture grown in MM with succinate and glutamate as a carbon source.

metabolites or analogues of metabolites involved in polyamine biosynthesis or in the neighbouring metabolic pathways may affect *speD* expression, we tested the following molecules: citrulline, ornithine, arginine, agmatine, putrescine, cadaverine, methylthioadenosine, norleucine, arginine hydroxamate, methionine hydroxamate, D-methionine, methionine sulfoximine, methyl-DL-methionine (assays were performed in ED glucose MM plates containing Xgal as an indicator of β-galactosidase activity by using paper discs impregnated with 10 μl of 100 mM solutions) and methionine, spermidine, methylthioribose and ethionine (assays were first performed on plates as above and subsequently on cultures grown in liquid ED minimal medium supplemented with either 1 mM methionine or spermidine, or 0.1 mM of either methylthioribose or ethionine). The addition of most of these molecules to the medium had a negligible effect on gene expression (data not shown). However, *speD* expression in the stationary growth phase (bacteria harvested at an OD_{600nm} between six and seven) was inhibited two- to fourfold by spermidine. In contrast, methionine alleviated the repressed stationary phase level by enhancing *speD* expression two- to sevenfold under similar conditions (Table 1). This pattern of expression is observed with different carbon sources such as glucose, sucrose or sorbitol (see Table 1).

The speD *promoter*

The putative promoter region of *speD* spans 230 nt downstream of the *gapB* gene. To identify the promoter of the *speD* gene, primer extension experiments were performed (see *Experimental procedures*). The RNA was prepared from bacteria grown in ED glucose MM during the exponential growth phase ($OD_{600nm} = 0.6$). As shown in Fig. 5A, bands, respectively, 138 nt and 78 nt (data not presented for the second primer), were found upstream from each of the two primers. The promoter start was found to lie 7 nt downstream from a putative −10 box identified in the sequence (TATACT). Upstream from this box is a −35 box (TTGCAA) with base sequence similar to the consensus sequence TTGACA that is typical of *B. subtilis* sigma A-dependent promoters (Moran, 1993). No clear-cut regulatory signal could be identified in the promoter region.

speD *is transcribed with* gapB *in minimal medium*

To examine transcription of *speD*, RNA synthesis was analysed by Northern blotting. RNA was extracted from exponentially growing cells in MM containing either glucose or succinate plus glutamate as carbon sources. As shown in Fig. 5B, when the glucose-containing ED medium was used, a band of about 600 nt, corresponding

Table 2. Variations observed in the proteome pattern of the *speD* deletion mutant (BSIP7004) in comparison with the wild-type strain (168).

Gene name	Function or similarity	Variation factor
murA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Absent
tasA	translocation-dependent antimicrobial spore component	Absent
yshD	similar to DNA mismatch repair protein	Absent
atpA	ATP synthase (α-subunit)	5 × repression
atpD	ATP synthase (β-subunit)	5 × repression
serA	phosphoglycerate dehydrogenase	4 × repression
ykwC	similar to 3-hydroxyisobutyrate dehydrogenase	4 × repression
ahpF	alkyl hydroperoxide reductase	4 × induction
hag	flagellin protein	10 × induction
metK	S-adenosylmethionine synthase	3.5 × induction

to the expected length of a transcript initiated at the *speD* promoter and terminating in a stem–loop structure, was observed for the *speD* gene probe (lane 1) and no band was detected with the *gapB* gene-specific probe (lane 3). In contrast, a single band of about 1800 nt, corresponding to the expected length of a *gapBspeD* transcript, was observed using a probe for either the *speD* gene or the *gapB* gene when bacteria were grown in the succinate/glutamate C medium under similar conditions (lane 2 and 4). These results indicate that *gapB* and *speD* form an operon under the latter conditions. *speD* transcription is therefore initiated at two regulatory regions: one which allows the transcription of the whole *gapBspeD* operon and a second, highly expressed in the presence of glucose, which is *speD* specific. RT-PCR experiments demonstrated that the operon structure of the *gapBspeD* transcript is independent of the growth phase, and occurs in the same way in the exponential and in the stationary growth phase (data not presented). It is remarkable that, when the *gapBspeD* transcript is formed, the short transcript for *speD* alone is no longer present, indicating occlusion of the *speD* promoter under these conditions. Apart from the signals which are characteristic of a sigma A-dependent promoter, we did not observe any signal that would be the landmark of transcription termination downstream of the *gapB* gene. This is consistent with the *gapBspeD* co-transcription we observed in glucogenic carbon sources. Promoter occlusion has been observed repeatedly since the discovery of the phenomenon (Adhya and Gottesman, 1982; Meng *et al*., 1997).

Proteome analysis of speD *mutant*

In an attempt to understand the underlying reasons for this apparent association between polyamines, carbon and sulphur metabolism, we compared the protein expression pattern (proteome) of a *speD* mutant with that of its wild-type counterpart. The cells were grown in ED glucose MM until they reached the middle of the exponential growth phase ($OD_{600nm} = 0.5$). The general pattern of the proteome was similar in the wild type and the mutant (data not presented). However, differences in several spots intensities were detected in the *speD* mutant (Table 2). Seven spots were either four to five times less intense or even entirely absent in the *speD* mutant, indicating that their expression is repressed (MurA, TasA, YshD, AtpA, AtpD, SerA and YkwC). Conversely, three spots, identified by mass spectrometry as MetK, Hag, AhpF proteins, were synthesized at a higher level in the *speD* mutant than in the parent strain. Overexpression of SAM synthase (MetK) was surprising, because one would have expected the opposite, i.e. that AdoMet would be less consumed in a *speD* mutant than in the wild-type strain. We therefore measured expression of a *metK–lacZ* fusion in a *speD* mutant and in its parent. Expression of *lacZ* was doubled in the *speD* mutant (increasing from about 150 U mg^{-1}–300 U mg^{-1}), in agreement with the proteome observation.

Discussion

Spermidine is the major polyamine of *B. subtilis*. As in other organisms, its synthesis presumably requires decarboxylated AdoMet as a precursor. The maintenance of a spermidine pool is therefore energy costly: synthesis of AdoMet requires individual hydrolysis of the three phosphates of ATP and its sulphur atom comes from the very energy-consuming reduction of sulphate. It was therefore of interest to investigate the nature of the gene coding for the enzyme that decarboxylates AdoMet. In the present paper we proved, using mutant strain constructions, complementation experiments and TLC polyamines analysis (Fig. 2), that *yctF* encodes AdoMet decarboxylase. Accordingly, the gene was renamed *speD*.

This study allowed us to complete the *B. subtilis* 168 polyamine biosynthesis pathway. Under our growth conditions, the latter consists of a single route, starting with arginine decarboxylase, encoded by the *speA* (*cad*) gene, and going through agmatinase and spermidine

Table 3. Bacterial strains and plasmids used or created in this study[a].

Strain or plasmid	Genotype or description	Source or reference
Strains		
Escherichia coli		
XL1-Blue	K12 *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F′[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(tet^R)]	Laboratory collection
TG1	K12 *supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′[*traD*36 *proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Laboratory collection
FB8	F$^-$	Bruni *et al*. (1977)
HT375	K12 Δ*speD*98 *lacZ*43 *rpsL*	Tabor *et al*. (1978)
HT375/*speD* (Bs)	HT375 pKK223-3*speD* $_{Bs}$	This work
HT375/*speD*S6A	HT375 pKK223-3*speD*S63A$_{Bs}$	This work
HT375/*speD* (Ec)	HT375 pKK223-3*speD* $_{Ec}$	This work
HT375/MJ0315	HT375 pKK223-3MJ0315 pACYC*lacI*	This work
Bacillus subtilis		
168	*trpC*2	Spizizen (1958)
BSIP1159	*trpC*2 *amyE*::(pro*metK-lacZ cat*)	This work
BSIP7004	*trpC*2 *speD*::*spc*	This work
BSIP7005	*trpC*2 *speD*$^+$-*bgaB*	This work
BSIP7013	*trpC*2 *speD*::*spc amyE*::(pxyl*speD*$_{Bs}$ *cat*)	This work
BSIP7014	*trpC*2 *speD*::*spc amyE*::(pxyl*speD*S63A$_{Bs}$ *cat*)	This work
BSIP7015	*trpC*2 *speD*::*spc amyE*::(pxyl*speD*$_{Ec}$ *cat*)	This work
BSIP7016	*trpC*2 *speD*::*spc amyE*::(pxylMJ0315 *cat*)	This work
BSIP7017	*trpC*2 *speD*::*spc amyE*::(pro*metK-lacZ cat*)	This work
BSIP7019	*trpC*2 *speD*::*spc* pHT1618	This work
BSIP7020	*trpC*2 *speD*::*spc* pHT1618 pxyl*speD*$_{Bs}$	This work
BSIP7021	*trpC*2 *speD*::*spc* pHT1618 pxyl*speD*S63A$_{Bs}$	This work
BSIP7022	*trpC*2 *speD*::*spc* pHT1618 pxyl*speD*$_{Ec}$	This work
BSIP7023	*trpC*2 *speD*::*spc* pHT1618 pxylMJ0315	This work
Plasmids		
pUC19	cloning vector, Amp^R	Yanisch-Perron *et al*. (1985)
pDIA5398	pUC19*speD*::*spc*	This work
pDL	cloning vector, Cm^R, Amp^R, *bgaB*	Yuan and Wong (1995)
pJM103	cloning vector, Cm^R, Amp^R	Perego (1993)
pDIA5399	pJM103 *speD–bgaB*	This work
pAC6	cloning vector, Cm^R, Amp^R	Stulke *et al*. (1997)
pDIA5538	pAC6 pro*metK*	This work
pKK223-3	cloning vector, Amp^R	Pharmacia Biotech
pDIA5604	pKK223-3 *speD* $_{Bs}$	This work
pDIA5605	pKK223-3 *speD*S63A$_{Bs}$	This work
pDIA5606	pKK223-3 *speD* $_{Ec}$	This work
pDIA5607	pKK223-3 MJ0315	This work
pDIA17	pACYC*lacI*	Glaser *et al*. (1991)
pX	cloning vector, Cm^R, Amp^R	Kim *et al*. (1996)
pDIA5608	pX *speD* $_{Bs}$	This work
pDIA5609	pX *speD*S63A$_{Bs}$	This work
pDIA5610	pX *speD* $_{Ec}$	This work
pDIA5611	pX MJ0315	This work
pHT1618	cloning vector, Tet^R, Amp^R	Lereclus and Arantes (1992)
pDIA5612	pHT1618 pxyl*speD*$_{Bs}$	This work
pDIA5613	pHT1618 pxyl*speD*S63A$_{Bs}$	This work
pDIA5614	pHT1618 pxyl*speD*$_{Ec}$	This work
pDIA5615	pHT1618 pxylMJ0315	This work

a. *cat* is the pC194 chloramphenicol acetyltransferase gene, *spc* is the spectinomycin resistance gene from *Staphylococcus aureus* and pxyl is the promoter of *xylA* gene. Bs, *B. subtilis*; Ec, *E. coli*.

synthase, both expressed from a single operon, *speEB* (Sekowska *et al*., 1998).

From the phylogenetic point of view, the *B. subtilis* SpeD protein exhibits higher similarities to the corresponding protein of *M. jannaschii* than to the *E. coli* one. Such a finding makes these genes interesting phylogenetic metabolic markers for the bacterial domain, witnessing either ancient origin or lateral gene transfer. A previous biochemical report for the existence of a SpeD protein in *S. solfataricus* gave a molecular mass of 32 kDa for the protein (Cacciapuoti *et al*., 1991), both much larger than that of *M. jannaschii* and not in agreement with the *S. solfataricus* genome data. We have no explanation for this discrepancy. We found that in a cell-free transcription–translation coupled system, the SpeD protein of *M. jannaschii* is self-cleaved, as expected for a pyruvoyl enzyme. This was also observed for the *E. coli* enzyme. However, the *B. subtilis* enzyme did not

show signs of self-cleavage (except perhaps a very faint band) under a variety of conditions (Fig. 3 and data not shown). It is not clear whether this could be the cause of some regulation of the catalytic properties of the enzyme which, when expressed at a high level, could lead to AdoMet depletion, a potentially toxic phenomenon. However, the observation that *B. subtilis* SpeD can complement an *E. coli speD* deficient strain would suggest that it is matured *in vivo*. Several hypotheses could account for this discrepancy between *in vivo* and *in vitro* conditions. First, among the factors missing in the cell-free *E. coli* transcription–translation system is an active deformylase, a protein extremely labile in the presence of molecular oxygen (Rajagopalan and Pei, 1998). Therefore, all proteins synthesized *in vitro* would have their formylmethionine capping residue, which in some cases can prevent the formation of catalytically active enzymes (Solbiati *et al*., 1999). Second, the protein might be intrinsically slow to cleave. Indeed, it has been shown that the rate of self-cleavage varies enormously according to the enzyme considered (Xiong *et al*., 1997). This leaves open the possibility that the pool of uncleaved SpeD may have some function in the cell.

We observed that in a glutamate–succinate medium *speD* is co-transcribed with *gapB*, a gene apparently functionally unrelated to *speD*. Comparison of the proteome of a *speD* deficient mutant to that of its parent strain revealed quantitative changes in proteins not obviously related to AdoMet decarboxylation. Overall, the relevant spots seem to be related to two classes of processes: those involved in proton synthesis and transfer (ATP synthase, NADH-dependent dehydrogenases) and those linked to genes expressed when cells enter into the stationary phase of growth [this is particularly the case of TasA, which has recently been shown to be a transition-phase gene (Stover and Driks, 1999)], a situation leading to strong repression of *speD*, except in the presence of methionine. A way to see both processes as going together is to involve proton metabolism at a specific step of the bacterial growth cycle linked to the entry into the stationary phase of growth. *speD* mutants behave as if the level of protons was lower than in normal exponential growth conditions (Table 1). Consistent with this hypothesis, it is known that entry into the stationary phase of growth is associated with pH changes and, in many cases, with a shift from glycolysis to gluconeogenesis. This might account for the link with *gapB*, which has been proposed to be the gluconeogenic glyceraldehyde dehydrogenase (Fillinger *et al*., 1999). Whether this was the case, we would expect some involvement of SpeD in this phenomenon, perhaps through the formation of multiprotein complexes (Danchin *et al*. 2000), thus associating polyamine biosynthesis, sulphur metabolism and carbon metabolism into a specific process linked to the stationary growth phase transition. Finally, the puzzling observation that *metK* expression is enhanced in a *speD* defective background supports the hypothesis that SpeD itself, decarboxylated AdoMet or spermidine regulate AdoMet availability. Further work is needed to unravel this interesting phenomenon.

Experimental procedures

Bacterial strains, plasmids and growth media

E. coli and *B. subtilis* strains as well as plasmids used in this work are listed in Table 3. *E. coli* TG1 and XL1-Blue were used for cloning experiments (TG1 for single cross-over recombination and XL1-Blue for double cross-over recombination). *E. coli* FB8 was used as a wild-type strain for polyamine synthesis and HT375 (a kind gift from Dr M. Berlyn from the Genetic Stock Center, http://cgsc.biology.yale.edu) was used for complementation experiments. The plasmid carrying the *M. jannaschii* MJ0315 gene (AMJAZ62) was obtained from TIGR/ATCC.

E. coli and *B. subtilis* were grown in LB medium (Bertani, 1951), in ED MM medium: K_2HPO_4, 8 mM; KH_2PO_4, 4.4 mM; Na_3-citrate, 3 mM; L-glutamine, 15 mM; L-tryptophan, 0.244 mM; ferric citrate, 33.5 μM; $MgSO_4$, 2 mM; $MgCl_2$, 0.61 mM; $CaCl_2$, 49.5 μM; $FeCl_3$, 49.9 μM; $MnCl_2$, 5.05 μM; $ZnCl_2$, 12.4 μM; $CuCl_2$, 2.52 μM; $CoCl_2$, 2.5 μM; Na_2MoO_4, 2.48 μM, with glucose, fructose, sucrose or sorbitol (0.5%) as a carbon source (Anagnostopoulos and Spizizen, 1961) and in C MM: $(NH_4)_2SO_4$, 25 mM; K_2HPO_4, 70 mM; KH_2PO_4, 30 mM; ferric citrate, 33.5 μM; $MgSO_4$, 0.5 mM; $MnSO_4$ 5 μM; L-tryptophan, 0.244 mM, with glutamate and succinate (0.5%) as a carbon source (Msadek *et al*., 1990). The MM (ED or C) were supplemented with 0.1% casamino acids, 1 mg l^{-1} thiamine and 0.5% xylose when necessary. LB and ED plates were prepared by the addition of 17 g l^{-1} Bacto agar or Agar Noble (Difco), respectively, to the medium. When included, antibiotics were added to the following concentrations: ampicillin, 100 mg L^{-1}; spectinomycin, 100 mg L^{-1}; tetracycline, 10 mg L^{-1}; chloramphenicol 5 mg l^{-1} (*B. subtilis*) or 20 mg l^{-1} (*E. coli*). Bacteria were grown at 37°C. The optical density (OD) of bacterial cultures was measured at 600 nm.

Transformation

Standard procedures were used to transform *E. coli* (Sambrook *et al*., 1989) and transformants were selected on LB plates containing ampicillin, ampicillin plus spectinomycin or ampicillin plus chloramphenicol. *B. subtilis* cells were transformed with plasmid DNA according to the two-step protocol described previously (Kunst and Rapoport, 1995). Transformants were selected on LB plates containing spectinomycin, chloramphenicol, spectinomycin plus chloramphenicol or spectinomycin plus tetracycline.

β-Galactosidase assay

B. subtilis cells containing *lacZ* or *bgaB* fusions were assayed

for β-galactosidase activity as described previously (Msadek *et al.*, 1998; 1990). In contrast to β-galactosidase from *E. coli* (LacZ), which displays only limited stability in *B. subtilis* cells or cell extracts, β-galactosidase from *B. stearothermophilus* (BgaB) is very stable. Specific activity was expressed in Units per mg protein. The Unit used is equivalent to 0.28 nmols min^{-1} at 28°C. Protein concentration was determined by Bradford's method using a protein assay Kit (Bio-Rad Laboratories). At least two independent cultures were monitored.

Molecular genetics procedures

Plasmid DNA was prepared from *E. coli* by standard procedures (Sambrook *et al.*, 1989). *B. subtilis* chromosomal DNA was purified as described by Saunders (Saunders *et al.*, 1984). Restriction enzymes and T4 DNA ligase were used as specified by the manufacturers. Southern blot analysis was performed according to Southern (1975) using a non-radioactive DNA labelling and detection kit 'Dig-UTP labelling' from Roche.

DNA fragments used for cloning experiments were prepared by PCR using *Pfu* Turbo DNA polymerase (Stratagene). Amplified fragments were purified using the QIAquick PCR Purification Kit (Qiagen).

DNA fragments were purified from a gel using Spin-X columns from Corning Costar by subsequent centrifugation and precipitation.

All experiments were performed under the rules established for GMO manipulation by the Ministry of Education Research and Technology (level 1 containment, agreement no. 2735).

Plasmid constructions

To construct the *speD* deletion strain, a *Sma*I restricted spectinomycin resistance cassette (Murphy, 1985) was used. Two DNA fragments, one upstream from the *speD* gene (nucleotides −465 to −9 relative to the translational start point of *speD*) and the second one downstream from the *speD* gene (nucleotides −25 to +398 relative to the *speD* stop codon) were amplified by PCR using primers introducing, for the first one, an *Eco*RI cloning site at the 5′ end and a *Stu*I cloning site at the 3′ end of the fragment, and for the second one, a *Stu*I cloning site at the 5′ end and a *Bam*HI site at the 3′ end of the fragment. PCR products and the spectinomycin cassette were ligated and inserted into the *Eco*RI and *Bam*HI sites of pUC19 (Roche) producing plasmid pDIA5398. Prior to transformation, this plasmid was linearized at its unique *Bsa*I site. Complete deletion of the gene was obtained by a double cross-over event, giving strain BSIP7004.

For transcriptional fusion of *speD* with the *bgaB* gene from *B. stearothermophilus*, a DNA segment upstream from the *speD* gene (nucleotides −180 to +157 relative to the translation start point) was amplified by PCR using primers introducing an *Eco*RI cloning site at the 5′ end and a *Bam*HI cloning site at the 3′ end of the fragment, then inserted into the *Eco*RI and *Bam*HI sites of plasmid pDL carrying the *bgaB* gene. A DNA fragment corresponding to the inserted *speD* upstream fragment fused with the *bgaB* gene was cut out from plasmid pDL, using *Eco*RI and *Sac*I. After gel purification, this fragment was inserted into plasmid pJM103 and digested with the *Eco*RI and *Sac*I restriction enzymes, producing plasmid pDIA5399. This plasmid was introduced into the chromosome by a single cross-over event, giving strain BSIP7005. This strain carries an intact copy of *speD* and a *speD–bgaB* transcriptional fusion. The construction was checked by Southern blot.

For transcriptional fusion of *metK* with the *lacZ* gene, a DNA segment upstream from the *metK* gene (nucleotides −390 to +56 relative to the translation start point) was amplified by PCR using primers introducing an *Eco*RI cloning site at the 5′ end and a *Bam*HI cloning site at the 3′ end of the fragment. This fragment was inserted into the *Eco*RI and *Bam*HI sites of plasmid pAC6, producing plasmid pDIA5538. Prior to transformation, this plasmid was linearized at its unique *Sca*I site. Integration of the gene was obtained by a double cross-over event at the *amyE* locus giving strain BSIP1159. To obtain a stain carrying both a *metK* fusion and a *speD* deletion, strain BSIP1159 was transformed with chromosomal DNA obtained from strain BSIP7004, giving rise to strain BSIP7017.

For site-directed mutagenesis and complementation of *E. coli* by the *speD* mutant (HT375), plasmid pKK223-3 with an IPTG inducible promoter was used. The complete *speD* genes with its ribosome binding site (nucleotides −25 relative to the translational start point and ending 22 bp after the stop codon of *speD*), *E. coli* (nucleotides −25 relative to the translational start point and ending 5 bp after the stop codon of *speD*) and *M. jannaschii* (nucleotides −20 relative to the translational start point and ending 1 bp after the stop codon of *speD*) were amplified by PCR using primers introducing an *Eco*RI restriction site at their 5′ end and a *Pst*I restriction site at their 3′ end. The amplified fragments were inserted into the *Eco*RI and *Pst*I sites of pKK223-3, producing plasmids pDIA5604, pDIA5606 and pDIA5607 respectively. Plasmid pDIA5605, deriving from pDIA5604 and carrying the *B. subtilis speD* gene substituted for serine 63 with alanine, was obtained after site-directed mutagenesis (see below). All four plasmids were used to complement the HT375 strain. To obtain a mutant carrying the pDIA5607 plasmid (with the *M. jannaschii speD* gene), we co-transformed the cells with a compatible plasmid (pDIA17) carrying additional copies of the *lacI* gene. Failure of transformation with pDIA5607 alone was probably due to leakage of the *tac* promoter leading to overexpression and toxicity of *M. jannaschii* DNA.

Plasmid pX with its xylose-inducible promoter was used to complement *B. subtilis* with different *speD* genes. The DNAs corresponding to complete CDS with their RBS were amplified from *B. subtilis* (nucleotides −25 relative to the translational start point and ending 26 bp after the stop codon of *speD*), *E. coli* (nucleotides −25 relative to the translational start point and ending 5 bp after the stop codon of *speD*) and *M. jannaschii* (nucleotides −20 relative to the translational start point and ending 1 bp after the stop codon of *speD*) and were amplified by PCR using primers introducing a *Spe*I restriction site at their 5′ end and a *Bam*HI restriction site at their 3′ end. The amplified fragments were inserted into the *Spe*I and *Bam*HI sites of pX, producing plasmids pDIA5608, pDIA5610 and pDIA5611 respectively. To obtain a pX

plasmid carrying a *B. subtilis speD* gene substituted for serine 65 with alanine, plasmid pDIA5605 was used as a template, and the *speD*S63A gene was amplified by PCR and introduced into plasmid pX as described above, producing plasmid pDIA5609. Prior to transformation, these plasmids were linearized at their unique *Hpa*I site (pDIA5608 and pDIA5609) or *Sca*I site (pDIA5610 and pDIA5611). Complete integration of the genes was obtained by a double cross-over event at the *amyE* locus, giving strains BSIP7013, BSIP7014, BSIP7015 and BSIP7016.

Plasmid pHT1618 was used for complementation of *B. subtilis* with different *speD* genes expressed from high-copy number plasmids. All *speD* genes were amplified by PCR together with the xylose-inducible promoter from constructions obtained using the pX plasmid (pDIA5608, pDIA5609, pDIA5610 and pDIA5611 as a template for the *B. subtilis speD* gene, *B. subtilis speD*S63A gene, *E. coli speD* gene and *M. jannaschii speD* gene, respectively, see above). All these genes were amplified by PCR using primers introducing an *Eco*RI restriction site at their 5′ end and a *Bam*HI restriction site at their 3′ end. The amplified fragments were inserted into the *Eco*RI and *Bam*HI sites of pHT1618, producing plasmids pDIA5612, pDIA5613, pDIA5614 and pDIA5615 respectively. The strain BSIP7004 was transformed with plasmid pHT1618 as a control and with pDIA5612, pDIA5613, pDIA5614 and pDIA5615 giving strains BSIP7019, BSIP7020, BSIP7021, BSIP7022 and BSIP7023.

Site-directed mutagenesis

Plasmid pDIA5604, which contains a *speD* gene inserted into plasmid pKK223-3 was used for mutagenesis. The 'GeneEditor™ *in vitro* Site-Directed Mutagenesis System' (Promega) was utilized for creating a site-directed point mutation according to the manufacturer's instructions. The following oligodeoxynucleotide was synthesized for use as a mutagenic primer to produce the change of a serine residue to an alanine residue (Ser-65→Ala): 5′-TATTTCTGAAGCACACT-TAACG-3′ (the mismatch is underlined). The complete coding sequence of mutated clones was checked to confirm that the desired mutation was present and that no other mutations occurred.

In vitro *transcription and translation of* speD *gene*

The '*E. coli* S30 Extract System for Circular DNA' (Promega) was used for coupled transcription and translation from the control (pKK223-3) and the *speD* gene expressing plasmids (pDIA5604, pDIA5605, pDIA5606 and pDIA5607). Reactions were carried out according to the manufacturer's specifications with 1 μg of plasmid DNA and 13 pmol of [^{35}S] methionine (1500 Ci mmol^{-1}, Amersham). In some experiments, 0.2 mM of putrescine or 0.1 mM of S-adenosylmethionine was added to the reaction mixtures. The tubes were incubated at 37°C for 1 h, and translation was stoped by putting them in ice for 5 min and precipitated with acetone. The protein products were separated by SDS–PAGE using Tris-Tricine 16% gels. The gels were dried and the radioactivity was detected by autoradiography using X-OMAT AR Kodak films.

RNA isolation and analysis

Total RNA was obtained from cells using the 'High Pure RNA Isolation Kit' from Roche. RNA molecules were separated on 1% agarose gels and transferred to nylon membranes (Hybond-N, Amersham). Efficiency of transfer and location of rRNAs were monitored by analysis of ethidium bromide-stained material. Membranes were prehybridized at 50°C for 1 h in DIG Easy Hyb buffer from Roche. Hybridization was performed under the same conditions with *speD* or *gapB* specific probes using a non-radioactive DNA labelling and detection kit 'Dig-UTP labelling' from Roche.

Primer extension analysis using reverse transcriptase AMV (Roche) was performed as described by Piekielny and Rosbash (1985) with two oligonucleotides: 5′-ATAACGTGA-CGCCCCATTGTTTCCATAGTC-3′ and 5′-AGTGAAATTC-CAACAACTGGGTCGGCCATT-3′ (+26 to −4 and −33 to −62 with respect to +1 translation site respectively). The same primers were used for the generation of sequence ladders. Reaction products were separated on 7% denaturing polyacrylamide gel containing 8 M urea. DNA sequences were determined using Sanger's dideoxy chain-termination method with the 'Thermo Sequenase radiolabelled terminator cycle sequencing kit' from Amersham Pharmacia Biotech.

RT-PCR experiments were performed using the RT-PCR System (Promega) as specified by the manufacturer.

Determination of polyamine intracellular concentration

Polyamines were identified as described by Dion and Herbst (1970). Aliquots from bacterial cultures were pelleted, washed and resuspended in 0.2 M perchloric acid. They were subsequently disrupted by sonication and centrifugated. The supernatant was subjected to dansylation. Toluene extraction was used for polyamine determination by TLC on silica gel G plates (Merck). Separation of the dansylated polyamines was performed by development in ethylacetate/cyclohexane (2:3, v/v) followed immediately by spraying the developed TLC plate with triethanolamine/isopropanol (1:4, v/v) to enhance and stabilize fluorescence. After drying, spots were visualized under ultraviolet light and photographed.

Two-dimensional polyacrylamide gel analysis

Next, 50 ml of exponentially grown cells were harvested by centrifugation and washed twice in TE buffer (Tris 10 mM pH 7.5, EDTA 1 mM). The pellet was then resuspended in 1 ml of sonication buffer (Tris 10 mM pH 7.5, EDTA 1 mM, Urea 8 M, DTT 100 mM, Triton X-100 1%, CHAPS 4%, PMSF 2 mM) with 15 μl of DNAse (1 mg ml^{-1})/RNAse (0.5 mg ml^{-1}). Cells were disrupted by ultrasonication on ice (Branson Sonifier 250, 5 × 1 min) and the cells debris were removed by ultracentrifugation (60 min, 4°C, 35 000 r.p.m.). Approximatively 100 μg of proteins was resuspended in sample buffer (Pharmalyte 3–10 2%, urea 8 M, DTT 100 mM, Non-Idet P40 2%). For the isoelectric focusing, the Immobiline DryStrips (pH 4–7) and the IPGPhor Isoelectric

System from Pharmacia were used. Strips were rehydrated for 6 h at 20°C and then focused for 45 000Vh. The second dimension was performed with 11.5% polyacrylamide gels in the presence of SDS using the Protean II 2D Multi-Cell system from Bio-Rad. After running, the gels were fixed in 40% ethanol/10% acetic acid and silver stained. The gels were digitalized using a JX-330 scanner (Sharp). Spot detection and quantification were performed on a SPARC station 5 microcomputer (Sun Microsystem) using the PDQUEST software package (Bio-Rad). For each strain, proteins were extracted from two independent cultures and at least three gels were analysed for each of the protein preparations.

Protein spots were cut and in-gel digested with trypsin as described by Shevchenko *et al*. (1996). Digests were resuspended in 20 μl formic acid 1% and desalted using Zip Tips $_{C18}$ from Millipore and eluted with 50% acetonitrile. MALDI-TOF spectra of the peptides were obtained with a Voyager-Elite Biospectrometry Workstation mass spectrometer (Perseptive Biosystems). The matrix used is 2,5-dihydroxybenzoic acid. The sample was loaded on the target by the dried droplet method. Spectra obtained for the whole protein were calibrated externally using the $[M+H^+]$ ion from Des Arg Bradykin peptide (904.47) and ACTH peptide (2465.13). Internal calibration was performed using peptides from the autodigestion of trypsin. The analyses were performed in the reflectron mode, with an accelerating voltage of 25 000 V, an extraction delay of 200 ns and around 250 scans were averaged. Data mining was performed using the ProFound and MS-FIT softwares against non-redundant databases.

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