Molecular Microbiology (1998) **29**(3), 851–858

Characterization of polyamine synthesis pathway in *Bacillus subtilis* 168

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Summary

The ubiquitous polyamines fulfil a variety of functions in all three kingdoms of life. However, little is known about the biosynthesis of these compounds in Gram-positive bacteria. We show that, in *Bacillus subtilis*, there is a single pathway to polyamines, starting from arginine, with agmatine as an intermediate. We first identified the structural gene of arginine decarboxylase, *speA* (formerly *cad*), and then described the *speE speB* operon, directing synthesis of spermidine synthase and agmatinase. This operon is transcribed into two messenger RNAs, a major one for the *speE* gene and a minor one for both *speE* and *speB*. The promoter of the operon was identified upstream from the *speE* gene by primer extension analysis. Transcription of this operon indicated that the level of agmatinase synthesis is very low, thus allowing a stringent control on the synthesis of putrescine and, therefore, of all polyamines. This is consistent with the level of polyamines measured in the cell.

Introduction

Polyamines fulfil a variety of functions in all three kingdoms of life, Eubacteria, Archaea and Eukarya. Although unable to synthesize polyamines, *Mycoplasma genitalium*, with a genome of only 580 kb, possesses a polyamine-specific transport system (Pollack *et al*., 1997). Bacterial pathogens require a polyamine supply from their host for growth (see references in Cohen, 1998). Indeed, these molecules, although dispensable under laboratory conditions, are extremely important for the cell. Beside proteins such as HU, H-NS, IHF or FIS that control both chromosome structure and gene expression (Yasuzawa *et al*., 1992), polyamines also play a significant role in the structural and functional organization of the chromoïd in *Escherichia coli* by compacting DNA and neutralizing negative charges (see Cohen, 1998). Furthermore, polyamines are implicated in RNA and protein biosynthesis. They stabilize functional ribosome structures and control or modulate the fidelity of translation. Finally, they are extremely important in membrane stability and in cell proliferation and differentiation in the higher organisms (Cohen, 1998). This prompted us to unravel polyamine biosynthesis in *B. subtilis*, the paradigm for Gram-positive bacteria. Combining biochemical analysis of the polyamine content of *B. subtilis* with the knowledge of its whole genome sequence (Kunst *et al*., 1997), we attempted to predict the biosynthetic pathways of these compounds. Spermidine was readily identified from cellular extracts, indicating that polyamine synthesis in *B. subtilis* starts from the urea cycle, as in most organisms. In contrast, protein prediction from the genome sequence show no clear-cut similarities to either arginine or ornithine decarboxylase. However, inactivation of the *cad* gene, previously identified as a lysine decarboxylase, showed that this gene codes for the missing arginine decarboxylase. This suggests that polyamine synthesis starts with agmatine. We further identified a bicistronic operon as directing the synthesis of spermidine synthase and agmatinase. Combining gene disruption experiments and biochemical identification of polyamines, we showed that, instead of two pathways as in *E. coli*, *B. subtilis* maintains only the agmatine pathway.

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Results

Identification of the biosynthetic arginine decarboxylase gene in B. subtilis

In *E. coli*, two pathways to putrescine exist. They proceed through decarboxylation of an amino acid, ornithine or arginine. *In silico* analysis of the *B. subtilis* genome did not reveal a counterpart for either ornithine decarboxylase or biosynthetic arginine decarboxylase. Two genes only, *yaaO* and *cad*, were similar to decarboxylases, namely *E. coli* degradative lysine decarboxylases. The *cad* gene product had been annotated in an earlier sequencing study (GenBank accession no. X58433) as producing cadaverine, hence its name. We therefore investigated whether *yaaO* or *cad* could code for the first step of polyamine biosynthesis. Thin-layer chromatography (TLC) analyses indicated that the intracellular polyamine content in a *yaaO* disruptant (a kind gift from Dr Asai Kei) grown in minimum

Table 1. Bacterial strains and plasmids used or created in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
Escherichia coli		
MC1061	K12 F$^-$ *hsdR* Δ*lac*(*IPOZY*) *galE*115 *galK*16 *rpsL aro leu mcrA mcrB*	Dr V. Vagner
XL1-Blue	K12 *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F′[*pro*AB$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(tet^R)]	Laboratory collection
FB8	F$^-$	Bruni *et al*. (1977)
EWH331	K12 *thr*-1 *ara*-14 Δ*speD*98 Δ(*gpt-proA*)62 *lacY*1 *glnV*44(AS) *galK*2(Oc) λ$^-$ Δ(*speB-speA*)97 Δ(*speC-glcB*)63 *rpsE*2128(SpcR) *rpsL*25(strR) *xyl*A5 *mtl*-1 *thi*-1	Hafner *et al*. (1979)
EWH331/pHT*speB*	EWH331 + pHT1618*speB*$^+$	This work
Bacillus subtilis		
168	*trpC2*	Spizizen (1958)
BSIP7007	*trpC2 speE*$^+$*lacZ*	This work
BSIP7008	*trpC2 speE speB*	This work
BSIP7009	*trpC2 speB*	This work
BSIP7010	*trpC2 speA*	This work
BSIP7011	*trpC2 speB* pHT*speB*$^+$	This work
BSIP7012	*rpC2 speE speB*pHT*speB*$^+$	This work
Plasmids		
pMutin1 or 4mcs	Cloning vector, Erm^R	Dr V. Vagner
pDIA5392	pMutin4 *speE*$^+$	This work
pDIA5393	pMutin1::*speE*	This work
pDIA5394	pMutin4::*speB*	This work
pHT1618	Cloning vector, Tet^R	Lereclus and Arantes (1992)
pDIA5395	pHT1618 *speB*$^+$	This work
pUC19	Cloning vector, Amp^R	Yanisch-Perron *et al*. (1985)
pDIA5396	pUC19Δ*speA*Spec	This work

medium (see *Experimental procedures*) was identical to that of the wild type (data not shown). In contrast, when the *cad* gene was inactivated with a spectinomycin (Sp) resistance cassette (strain BSIP7010, Table 1), we no longer detected spermidine or putrescine in the cells. Finally, the addition of agmatine to the growth medium restored spermidine synthesis (Fig. 1). This shows that (i) in minimal growth medium, there is a single source of spermidine, i.e. decarboxylation of arginine to agmatine; and (ii) arginine decarboxylase is encoded by the *cad* gene. We therefore renamed the *cad* gene *speA* in conformity with the nomenclature in *E. coli*. If agmatine is the starting point for polyamine synthesis, there must exist an agmatinase in *B. subtilis*. Because the *ywhG* gene codes for a product similar to agmatinase, its structure and function were further investigated.

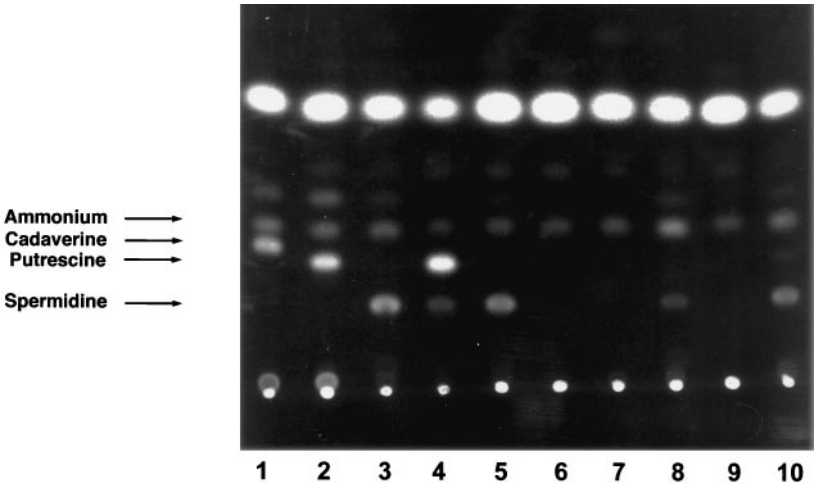


Fig. 1. Thin-layer chromatography identification of polyamines. Thin-layer chromatogram of dansyl polyamine derivatives of bacterial extracts chromatographed in ethylacetate/cyclohexane (2:3, v/v). Lane 1, cadaverine standard; lane 2, putrescine standard; lane 3, spermidine standard; lane 4, *E. coli* wild type; lane 5, *B. subtilis* wild type; lane 6, BSIP7008 – *speE* mutant; lane 7, BSIP7009 – *speB* mutant; lane 8, BSIP7011 – *speB* mutant complemented with *speB*; lane 9, BSIP7010 – *speA* mutant; lane 10, BSIP7010 – *speA* mutant plus 1 mM agmatine.

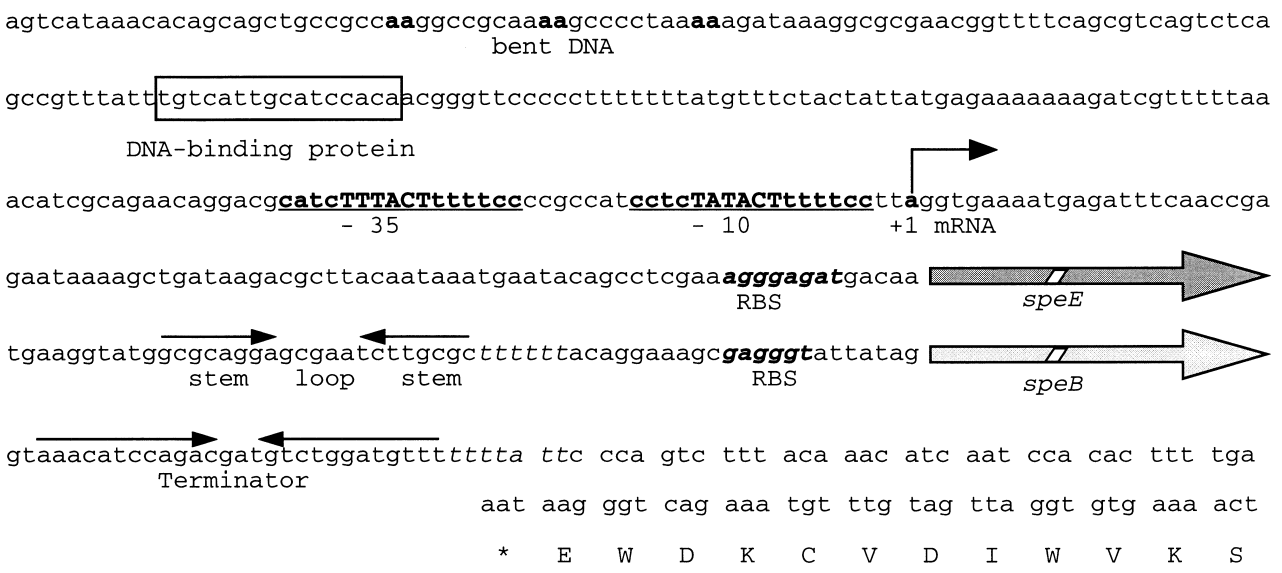


Fig. 2. The *speEB* operon (*speE*: STL|BG12460; *speB*: SP|P16936; STL, SUBTILIST; SP, SWISSPROT). Relevant features of the operon are as follows: a region of bent DNA (three aa dinucleotides separated by one helix turn are shown in bold letters) is predicted upstream from a putative DNA binding protein site (box), promoter consensus sites are in capital letters (−35: TTTACT; −10: TATACT), and the transcription start site is indicated by a broken arrow (+1). A tandem-repeated oligonucleotide, corresponding to the −35 and −10 regions of the promoter, is indicated (bold, underlined). Ribosome binding sites upstream of *speE* and *speB* are shown in bold italic letters. *speE* and *speB* CDSs are represented by full arrows. Between *speE* and *speB*, a putative stem and loop region and the hairpin terminator of the operon are indicated by thin arrows; polyT run is indicated in italic. It should be noted that the DNA coding for the Rho-independent terminator of the operon is symmetrical. It could operate equally well to terminate transcription of the convergent *ywhH* gene (its 3′ part is displayed upstream of the terminator).

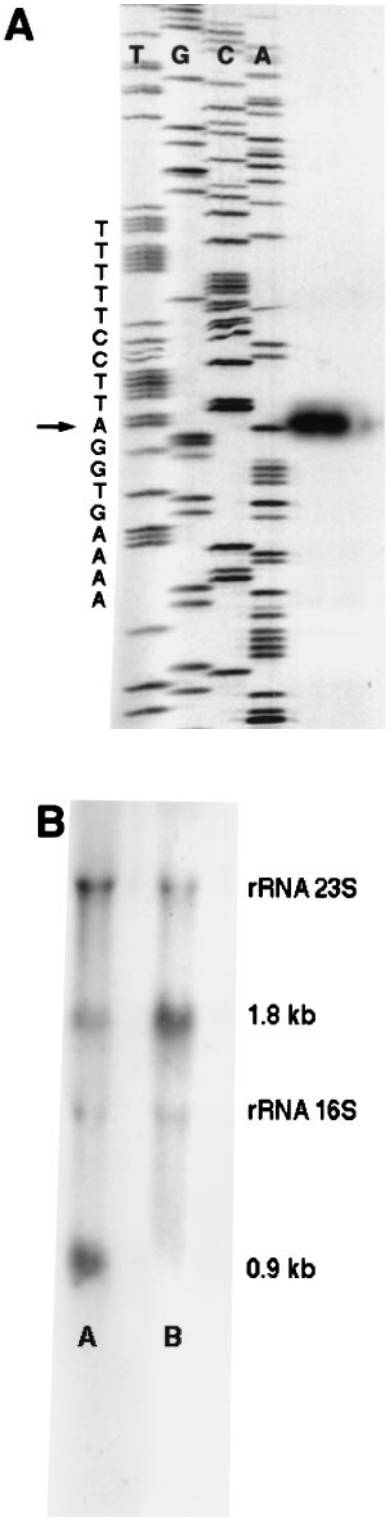
Identification of the speE *and* speB *gene functions*

ywhG is a component of a putative operon, *ywhFG*, lying in the 333° region of the *B. subtilis* chromosome (Kunst *et al*., 1997). Taking into account the complementarity between the region upstream of a putative start codon and the 3′ end of the 16S RNA, the *ywhG* CDS starts with an ATG codon, while the *ywhF* CDS starts with a TTG codon. The two CDSs are separated by a 60 nucleotide (nt) region (Fig. 2). Analysis of protein sequences repeatedly revealed similarity to agmatinase from organisms as diverse as *E. coli*, *Schizosaccharomyces pombe* and *Methanococcus jannaschii* for *ywhG*, and to spermidine synthase from organisms such as *E. coli*, *Saccharomyces cerevisiae* or *M. jannaschii* for *ywhF*. We renamed these genes *speB* and *speE* in conformity with the cognate nomenclature in *E. coli*.

The amino acid sequences deduced from *speB* and *speE* were compared with the sequences of known agmatinases and spermidine synthases. SpeB displayed remarkable similarity to both arginases and agmatinases from all three kingdoms of life, present in the SWISSPROT and TREMBL data libraries. In particular, the two manganese binding sites (Ouzounis and Kyrpides, 1994) are entirely conserved. In the same way, SpeE is very similar to known spermidine synthases. This family can be defined by the following consensuses in the central part of the protein: [ALP]-[EKR]-[EHKNS]-V-[AL]-[IV]-[ILV]-[DG]-G-X-X-X-X-[MLTV]-[AILV]-[HFR]-[EQ]-[ILMV]-X-[KR] and [FY]-D-[ALV]-[IV]-[FIM]-[CSTV]-D-[CLS]-[PRST]-[DE]-P-X-[GT]-[PV]-[AGSV]. Both recognize spermidine synthase sequences present in SWISSPROT (release 35) with 100% efficiency and no other sequences.

To identify the involvement of genes *speE* and *speB* in polyamine biosynthesis, we measured the putrescine and spermidine content in disrupted mutants compared with their wild-type counterpart. As shown in Fig. 1, wild-type *B. subtilis* did not show any significant level of intracellular putrescine. In contrast, the intracellular level of spermidine was similar to that found in *E. coli* (about 10 μM free molecules). Cadaverine was not detected in either organism. The spermidine content of a *speE* disruptant was undetectable. Similarly, a *speB* disruptant synthesized neither spermidine nor putrescine (Fig. 1). Spermidine synthesis was not restored even when putrescine was added to the medium (data not shown). To assess the role of *speB* in agmatinase synthesis, we expressed this gene in an *E. coli* strain lacking both ornithine decarboxylase and agmatinase activities. The complemented strain (EWH331/pHT*speB*) synthesized putrescine (data not shown). We also expressed the *speB* gene in the *B. subtilis speB* disruptant by constructing a replicative plasmid that contained the *speB* gene with its terminator but no *speE* gene (strain BSIP7011). Spermidine synthesis was restored in this

strain, confirming that *speE* had not been altered during the construction of the *speB* disruptant (Fig. 1). We also introduced the plasmid carrying the *speB* gene into the *B. subtilis speE speB* disruptant, giving rise to strain BSIP7012 (Table 1). No spermidine synthesis was observed (data not shown), in contrast to what we observed when *speE* was intact (spermidine synthesis was restored in the presence of a copy of the *speB* gene *in trans*). This provides evidence that *speE* codes for spermidine synthase.



Identification of the speE speB *promoter and transcripts*

To identify the promoter, we performed primer extension experiments (see *Experimental procedures*). As shown in Fig. 3A, bands were found 103 and 148 nt upstream from the two primer oligonucleotides respectively. The promoter lies 8 nt downstream from a −10 box (TATACT, Fig. 2). Upstream from this box is a −35 box (TTTACT), similar to the consensus sequence typical of sigma A-dependent promoters. The sequence displays some remarkable features: it is predicted to be strongly bent in its upstream 5′ end (3 amino acid runs spaced by one helix turn; Young *et al*., 1995); the bend is followed by a palindromic site strongly reminiscent of operators binding helix–turn–helix regulators (ATTTGTCATTGCATCCACAAC; Jansen *et al*., 1987; Gunasekera *et al*., 1992); finally, a repetition of 16 nt (CNTCTNTACTTTTTCC), which could constitute a binding site for a repressor molecule, overlaps the −35 and −10 boxes. Polyamines are nitrogen-containing molecules; however, no sigma 54 signature suggesting a direct nitrogen control could be found (Débarbouillé *et al*., 1991).

RNA synthesis in this region was analysed by Northern blotting in cells growing exponentially in minimal medium. As shown in Fig. 3B, two distinct bands (about 1800 nt and 900 nt) were observed using a probe in the *speE* gene. In contrast, only one band of about 1800 nt was detected with a probe in *speB*. This is compatible with transcription terminated through a stable stem and loop structure (-15 kcal mol^{-1}) typical of Rho-independent terminators (Fig. 2). The relative intensity of the short messenger band was 3.5 times stronger than that of the long band (measured by densitometry). Analysis of the *speE speB* intergenic region suggested that the transcript could fold into a stem and loop structure of moderate stability (-9.2 kcal mol^{-1}) (Fig. 2). This is consistent with polarity in transcription between *speE* and *speB*.

Fig. 3. Identification of the *speEB* promoter by primer extension, and of *speEB* transcripts by Northern blotting.
A. Identification of the transcription start site of the *speEB* operon. The size of the extended product is compared with a DNA sequencing ladder of the *speEB* promoter region. Primer extension and sequencing reactions were performed with the same primer. The +1 site is marked by an arrow.
B. Northern blot analysis of *B. subtilis* 168 *speEB* genes. A total of 15 μg of RNA was used. The probes were: lane A, specific *speE* fragment; lane B, specific *speB* fragment. rRNA 23S, rRNA 16S and estimated sizes of transcripts are indicated on the right.

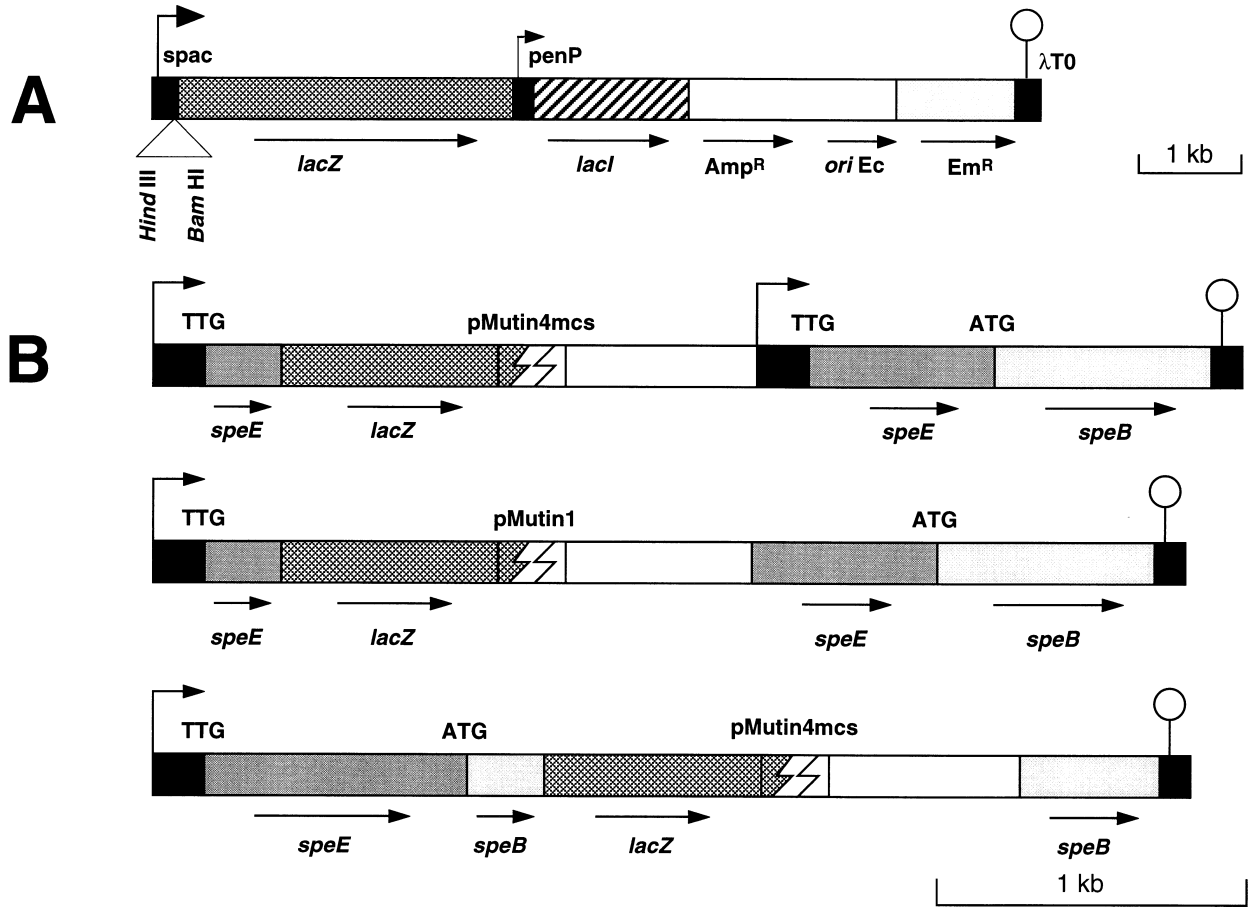


Fig. 4. pMutin1 vector map and construction of *spe*::*lacZ* fusions used in this study.
A. pMutin1 vector (a kind gift from Dr V. Vagner) was used to construct pDIA5393 plasmid, and pMutin4mcs was used to construct pDIA5392 and pDIA5394 plasmids (instead of a *Hin*dIII–*Bam*HI cloning site, this plasmid possesses a *Hin*dIII *Eco*RI *Sma*I *Not*I *Sac*II *Bam*HI multicloning site).
B. Constructions of three transcriptional fusions carried by strains BSIP7007, BSIP7008 and BSIP7009.

Regulation of the speE speB *operon expression*

To analyse the expression of the *speEB* operon, we constructed three transcriptional fusions by inserting the *E. coli lacZ* gene into the *speE* gene (strain BSIP7008) or into the *speB* gene (strain BSIP7009) and with the *speEB* promoter without gene inactivation (strain BSIP7007; Table 1, Fig. 4B). The β-galactosidase activity from the promoter-proximal fusion was about five times higher than that of the promoter-distal fusion (Table 2). Moreover, the expression of the *speE*::*lacZ* transcriptional fusion was always higher (1.4 times higher) when the *speE* gene was not interrupted.

The addition of metabolites involved in polyamine biosynthesis or in neighbouring metabolic pathways did not have any regulatory effects on *speEB* expression. However, when glutamine, but not ammonium, was used as

Table 2. Expression of *speEB*::*lacZ* transcriptional fusions.

	β-Galactosidase activity (U mg^{-1} protein) in strain[a]					
	BSIP7007		BSIP7008		BSIP7009	
Medium	Exp[b]	Stat	Exp	Stat	Exp	Stat
LB	668	186	550	138	142	31
MM + glutamine	415	195	301	156	48	21
MM + ammonium	130	117	116	108	26	17

a. BSIP7007, mutant strain carrying a *speEB*::*lacZ* transcriptional fusion without gene interruption; BSIP7008, *speE* mutant and BSIP7009, *speB* mutant.
b. Exp, exponential growth phase; stat, stationary growth phase.

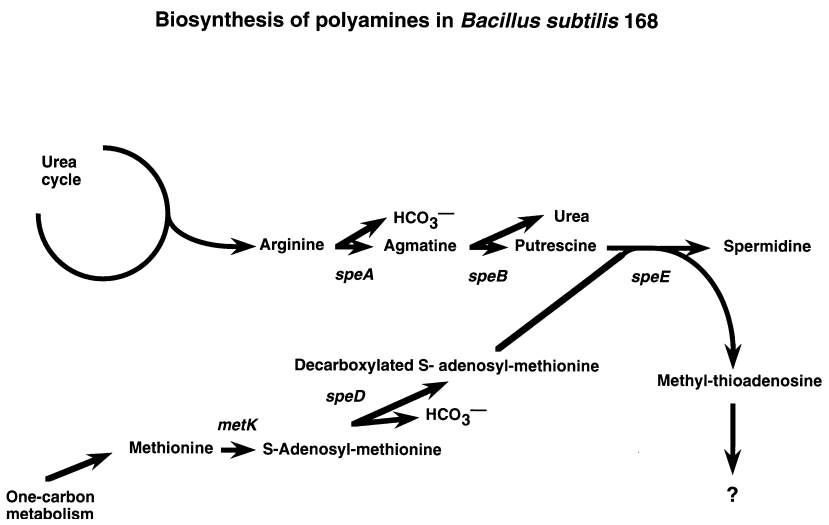


Fig. 5. Biosynthesis of polyamines in *Bacillus subtilis* 168. The main anabolic route (*speA*, *speB*, *speE*) has been identified in the present work.

a nitrogen source, the addition of spermidine reproducibly resulted in a significant repression of the *speE*::*lacZ* fusion expression (about 1.7 times) decreasing from about 400 U mg^{-1} to 240 U mg^{-1} protein. Under the same conditions, we observed an increased expression in the presence of carbamyl-phosphate (about 1.7 times) under stationary phase conditions (expression increased from about 170 U mg^{-1} to 290 U mg^{-1} protein). Finally, although agmatinase requires manganese as a cofactor, the addition of manganese or a manganese chelator such as 5-Br-PAPS had no significant effects on *speE*::*lacZ* expression.

Discussion

Despite the intrinsic importance of polyamines in all living organisms, the starting point of polyamine synthesis appeared to be missing in *B. subtilis* metabolism reconstruction (Selkov *et al*., 1998). Indeed, one could not readily identify biosynthetic ornithine or arginine decarboxylases from the genome sequence. In the present work, we proved by mutant strain construction and TLC polyamine analysis (Fig. 1) that the *cad* gene, previously annotated to code for a lysine decarboxylase, in fact encodes an arginine decarboxylase. We renamed this gene *speA* accordingly. We demonstrated further that genes *ywhF* and *ywhG* encoded spermidine synthase and agmatinase, respectively, thus establishing the pathway of polyamine biosynthesis in *B. subtilis* 168 as summarized in Fig. 5.

The biosynthetic pathway to spermidine consists of a single route (at least in minimum medium supplemented with ammonium or glutamine as a nitrogen source), starting with arginine decarboxylase, coded by the *speA* gene and going through agmatinase and spermidine synthase, expressed from a single operon, *speEB*. Both enzymes of this operon are homologous to their counterparts in the three kingdoms constituting life, thus witnessing their likely origin in an organism before kingdom separation (i.e. very early in the history of life). As already proposed by Ouzounis and Kyrpides (1994), this makes these genes excellent candidates as metabolism phylogenetic markers, which should be used as a complement to rRNA genes in the study of the early evolution of life. Moreover, the very high similarities among these enzymes suggest that the structure and function of corresponding genes are subjected to strong and constant selection pressure.

Because arginine must be used for anabolism (protein and polyamine synthesis) as well as degraded as a nitrogen source, the polyamine biosynthesis must be subjected to a complex regulatory network. The *speEB* promoter region may indeed comprise several regulatory sites (Fig. 2). In addition, as in many biosynthetic operons, the distal gene is responsible for the first step of biosynthesis and expressed at a fairly low level compared with that of the proximal gene. An interesting feature of the operon structure is a putative transcription termination signal (moderately stable stem and loop followed by a polyU track) situated between *speE* and *speB*, suggesting an attenuation process limiting agmatinase concentration in the cell. This is supported by the relative concentration of the proximal mRNA and the full-length operon mRNA (Fig. 4B). As a case in point, synthesis of the enzymes needed for putrescine and spermidine synthesis seems to be subjected to some regulation by the pool of available spermidine. However, no nitrogen-related feature was found in the promoter of the *speEB* operon.

The most perplexing observation of the polyamine content in *B. subtilis* cells was that no hint of free putrescine could be found in the cells grown under a variety of conditions. This was in sharp contrast to the situation in *E. coli*, in which putrescine could be readily identified (Fig. 1) (Cohen, 1998). This amazing feature deserves further investigation.

Experimental procedures

Bacterial strains, cultures, transformation and enzyme assay

Escherichia coli, *B. subtilis* strains and plasmids are listed in Table 1. *Escherichia coli* MC1061 and XL1-Blue were used for cloning experiments. *Escherichia coli* FB8 was used as a wild-type strain for polyamine synthesis, and EWH331 (a kind gift from the *E. coli* Genetic Stock Center, http://cgsc.biology.yale.edu) was used for complementation experiments.

Escherichia coli and *B. subtilis* were grown in Luria–Bertani (LB) medium and/or minimal salt medium (Anagnostopoulos and Spizizen, 1961) with 27.7 mM glucose and 15 mM L-glutamine or 15 mM ammonium. The medium was supplemented with 0.244 mM L-tryptophan or 3 μM thiamine when necessary. When included, antibiotics were added to the following concentrations: ampicillin, 100 mg l^{-1}; tetracycline, 10 mg l^{-1}; spectinomycin, 100 mg l^{-1}; erythromycin plus lincomycin, 1 mg l^{-1} and 25 mg l^{-1}. Bacteria were grown at 37°C.

Escherichia coli and *B. subtilis* were transformed as described by Kunst and Rapoport (1995) and Sambrook *et al*. (1989), and transformants were selected on appropriate LB plates.

β-Galactosidase was assayed as described by Msadek *et al*. (1990).

Molecular genetic procedures

Standard procedures were used for extracting plasmids from *E. coli* and chromosomal DNA from *B. subtilis* (Saunders *et al*., 1984; Sambrook *et al*., 1989). 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 Boehringer Mannheim). DNA fragments used for cloning experiments were prepared by polymerase chain reaction (PCR) using *Pfu*$^+$ DNA polymerase (Stratagene). Amplified fragments were purified by QIAquick PCR purification kit (Qiagen).

The DNA upstream from the *speE* gene (nucleotides −144 to +232 relative to the translation start point) was amplified by PCR using primers introducing a *Hin*dIII cloning site at the 5′ end and a *Bam*HI cloning site at the 3′ end of the fragment, then inserted into the *Hin*dIII and *Bam*HI sites of plasmid pMutin4mcs (a kind gift from Dr V. Vagner, unpublished) producing plasmid pDIA5392. Plasmids disrupting the *speE* and *speB* genes were obtained by PCR amplification of downstream regions of *speE* (+10 to +232) and *speB* (+35 to +246) as described for pDIA5392. The PCR products were inserted into the *Hin*dIII and *Bam*HI sites of pMutin1 and pMutin4mcs, respectively, producing the plasmids pDIA5393 and pDIA5394. These plasmids were introduced into the chromosome by a single cross-over event. The constructions were checked by Southern blot.

To construct the *speA* deletion strain, a *Sma*I-restricted spectinomycin resistance cassette (Murphy, 1985) was used. Two DNA fragments, one upstream from the *speA* gene (nucleotides −528 to −6 relative to the translational start point of *speA*) and the second one downstream from the *speA* gene (nucleotides +43 to +528 relative to the *speA* stop codon) were amplified by PCR using primers introducing, for the first one, a *Bam*HI 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 *Sma*I cloning site at the 5′ end and an *Eco*RI 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 (Boehringer Mannheim) producing plasmid pDIA5396. Before transformation, this plasmid was linearized at its unique *Sca*I site. Complete deletion of the gene was obtained by a double cross-over event.

Plasmid pHT1618 (a kind gift from Dr D. Lereclus) was used to construct a complementation vector with the *speB* gene (Lereclus and Arantes, 1992), producing plasmid pDIA5395.

RNA isolation and analysis

Total RNA was obtained from cells by the modified extraction method of Hagen and Young (1978). Briefly, before the addition of extraction buffer, the bacterial pellet was resuspended in 200 μl of lysozyme solution (40 mg ml^{-1} in 12.5 mM Tris, 5 mM EDTA, 10% glucose). RNA was separated on 1% agarose gels and transferred to nylon membranes (Hybond-N; Amersham). Membranes were hybridized with *speE*- and *speB*-specific probes using a non-radioactive DNA labelling and detection kit ('Dig-UTP labelling' as recommended by Boehringer Mannheim). The relative band intensity was measured on a SPARC station 5 microcomputer (Sun Microsystems).

Primer extension analysis using reverse transcriptase was performed as described by Piekielny and Rosbash (1985) with two oligonucleotides from position +66 to +96 and +22 to +51 with respect to the translation start point of *speE*. DNA sequences were determined using Sander's dideoxy chain termination method as described by Moszer *et al*. (1991).

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 centrifuged. The supernatant was dansylated and extracted with toluene for polyamine determination by thin-layer chromatography (TLC) on silica gel G plates (Merck). The dansylated polyamines were separated by development in ethylacetate/cyclohexane (2:3, v/v; a standard separation system) or in butanol–90% acetic acid–water (75:15:10, v/v) to separate agmatine from putrescine, 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 UV light, and the areas corresponding to dansylated authentic polyamine standards were scraped off and extracted with 3 ml of redistilled ethanol by vigorous shaking, followed by 10 min centrifugation at 14 000 × *g*. The fluorescence of the supernatant solution was read in a Perkin-Elmer LS-5B luminescence spectrofluorimeter at 335 and 515 nm for activation and emission respectively.

Acknowledgements

We wish to thank Dr P. Glaser for his kind support at the

beginning of this work, Professor A. Hénaut for his continuous interest, Professor Chun-Kwun Wun for critical and attentive reading of this manuscript and E. Turlin for technical assistance. We thank Drs D. Lereclus and V. Vagner for the gift of plasmids, and Dr A. Kei for the gift of the *yaaO*::*lacZ* fusion strain. This work was supported by a grant (BIOTECH PL950278) from the European Union General Directorate XII, Groupement d'Etudes et de Recherches sur les Génomes (GREG), Université de Versailles-Saint-Quentin.

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