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The *metNPQ* operon of *Bacillus subtilis* encodes an ABC permease transporting methionine sulfoxide, D- and L-methionine

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Abstract

The *Bacillus subtilis yusCBA* operon, which encodes an ABC-type transporter, contains an S-box motif in its promoter region. We showed that the expression of these genes is repressed via the S-box system when methionine is available. The YusCB proteins are involved in the transport of both D- and L-methionine but also methionine sulfoxide. A *yusCB* mutant is unable to grow in the presence of 5 μM L-methionine or 100 μM methionine sulfoxide, while it grows similarly to the wild type with 100 μM L-methionine and 1 mM methionine sulfoxide. Other uptake systems are therefore present for these two compounds. In contrast, the Yus ABC transporter corresponds to the sole D-methionine uptake system. We propose to rename *yusC*, *yusB* and *yusA* as *metN*, *metP* and *metQ*, respectively.

Keywords: Sulfur metabolism; *Bacillus subtilis*; Methionine; S-box; ABC transporter

1. Introduction

Amino acid transport has been extensively investigated in Gram-negative bacteria and in *Saccharomyces cerevisiae* [6,17]. Two kinetically identifiable L-methionine transport systems are present in *Escherichia coli*: one with a very high affinity ($K_m = 0.1$ μM) and a second with lower affinity ($K_m = 40$ μM) [9]. The locus for the high affinity transporter system was called *metD*, since the *metD* mutants are unable to transport D-methionine and to utilize this compound as a methionine source [10,11]. The *metD* locus corresponds to the *abc*(*metN*), *yaeE*(*metI*) and *yaeC*(*metQ*) genes, which encode an ABC transporter necessary for L-methionine and D-methionine uptake [5,14,23]. However, *metI*, *metN* and *metQ* mutants can still grow in the presence of L-methionine [14,23]. This is probably due to the presence of the low affinity MetP system, which remains to be identified. The *metI*, *metN* and *metQ* genes are downregulated when methionine is available and this effect depends on MetJ, the common repressor of the methionine regulon [5,6,14,23]. In contrast, little is known about methionine transport in Gram-positive bacteria. In *Brevibacterium linens*, an Na^+-stimulated transport of L-methionine ($K_m = 55$ μM) has been described [4].

A number of *Bacillus subtilis* genes that are thought to be involved in methionine metabolism contain a highly conserved motif upstream from their coding sequence [7]. The S-box motif, which includes an intrinsic transcriptional terminator, is involved in the control of gene expression at the level of premature termination of transcription [2,3,7,13]. Using mutational analysis of the leader region of the methionine-regulated *yitJ* gene, Grundy and Henkin supported a model in which the 5′ portion of the leader forms an anti-antiterminator structure [7,13]. This anti-antiterminator sequesters sequences required for the formation of an antiterminator which, in turn, sequesters sequences required for the formation of the terminator. The direct binding of S-adenosyl-methionine (AdoMet) to RNA stabilizes the anti-antiterminator modulating the expression of the regulated genes [3,13]. S-box-controlled genes are involved in methionine biosynthesis (*metIC*, *metE*, *metK*, *yitJ*) and in methionine recycling via methylthioribose (*mtnKS*,

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mtnWXYZ) [1,2,16,19]. In contrast, the function of the *yxjG*, *yxjH*, *yoaDCB* and *yusCBA* genes remains to be characterized. Interestingly, the *B. subtilis* YusC, YusB and YusA proteins showed significant similarities to the MetN (48% identity), MetI (32% identity) and MetQ (32% identity) proteins of *E. coli*. These proteins are therefore good candidates for a methionine uptake system.

2. Materials and methods

2.1. Bacterial culture conditions

E. coli cells were grown in LB broth [18], while *B. subtilis* cells were grown in SP medium or in minimal medium (6 mM K_2HPO_4, 4.4 mM KH_2PO_4, 0.3 mM trisodium citrate, 5 mM $MgCl_2$, 50 µM $CaCl_2$, 5 µM $MnCl_2$, 0.5% glucose, 50 mg l^{-1} L-tryptophan, 22 mg l^{-1} ferric ammonium citrate, 0.1% L-glutamine, 200 mM xylose) supplemented with L-methionine, D-methionine, L-methionine sulfoxide, sulfate, L-cystine or D,L-homocysteine. The concentration of the sulfur source is indicated in the text. In this minimal medium, a residual growth in the absence of any added sulfur source was observed. To avoid this problem, an exhausted minimal medium (EMM) was obtained by growing *B. subtilis* 168 in a sulfur-free minimal medium followed by centrifugation and filtration of the supernatant. In this EMM, a complete absence of growth was observed for the *B. subtilis* wild-type strain in the absence of any sulfur source. Antibiotics were added to the following concentrations when required: ampicillin, 100 µg ml^{-1} and chloramphenicol, 5 µg ml^{-1}. Solid media were prepared by addition of 20 g l^{-1} agar noble (Difco). Standard procedures were used to transform *E. coli* [18] and *B. subtilis* [12]. All experiments were performed in accordance with the European regulation requirements concerning the use of genetically modified organisms (level 1 containment, agreement n° 2735).

The resistance of *B. subtilis* strains to the toxic analogues of methionine, D,L-ethionine and seleno-L-methionine, was tested as follows: overnight cultures were grown in minimal medium containing 0.1% ammonium chloride instead of glutamine. The cells were diluted to an OD_{600} of 1 and spread on plates of the corresponding agar medium. A paper disk was laid on the agar and soaked with 10 µl of a 100 mM D,L-ethionine or seleno-L-methionine solution. The area of growth inhibition was measured.

The loss of amylase activity was detected as previously described [21]. β-Galactosidase specific activity was measured as described by Miller [15] with cell extracts obtained by lysozyme treatment. Protein concentration was determined by the method of Bradford. One unit of β-galactosidase is defined as the amount of enzyme that produces 1 nmol min^{-1} of *o*-nitrophenol at 28 °C. The mean values of at least three independent experiments are presented. Standard deviations were less than 15%.

2.2. DNA manipulations

Plasmids from *E. coli* and chromosomal DNA from *B. subtilis* were prepared according to standard procedures [18]. Restriction enzymes, Taq DNA polymerase and phage T4 DNA ligase were used as recommended by the manufacturers (New England Biolabs and Roche). DNA fragments were purified from agarose gels with the Qiaquick kit (Qiagen).

2.3. Plasmids and strains construction

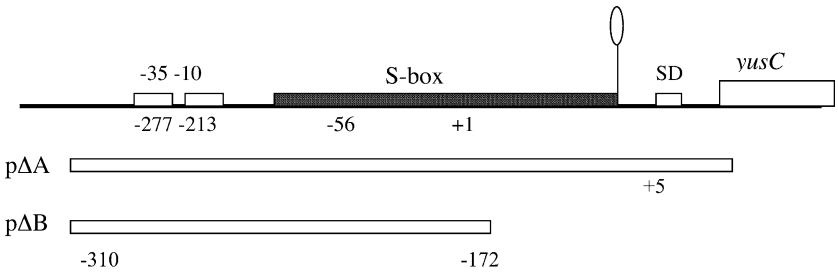
All the strains constructed during this study are derivatives from the 168 *B. subtilis* strain, whose genome has been sequenced [12].

Plasmid pAC6 [21] allowed the construction of transcriptional fusions between different *yusC* promoter regions and the promoterless *lacZ* gene. The ΔA (nucleotides −310 to +5 relative to the translational start site) and ΔB (nucleotides −310 to −172 relative to the translational start site) regions were amplified by PCR with the creation of *Eco*RI and *BamH*I sites. The PCR products were inserted into pAC6 to give pDIA5674 (pΔA) and pDIA5675 (pΔB), respectively. These plasmids were linearized with *Sca*I and used to transform the *B. subtilis* wild-type strain. This allowed the insertion of the transcriptional *yusC′–lacZ* fusions as a single copy at the *amyE* locus, giving strain BSIP1540 (pΔA) and BSIP1541 (pΔB) (see Fig. 1).

A Δ*yusCB* mutant was constructed by replacing the sequence of these genes by a chloramphenicol resistance cassette (*cat*) through a four-primer PCR procedure [22]. The *cat* gene was first amplified. The regions upstream from the *yusC* gene (nucleotides −679 to +296 relative to the translational start site of *yusC*) and downstream from the *yusB* gene (nucleotides −88 to +832 relative to the translational start site of *yusA*) were amplified by PCR so that 21-bp fragments corresponding to the *cat* gene were introduced at one of their ends. The *yusC* upstream region and the *yusB* downstream region overlapping at one of the ends the *cat* gene then served as long primers in a second PCR reaction using *cat* as a template, together with two external added primers. The final product, corresponding to the two regions flanking the *yusCB* genes with the inserted *cat* cassette in-between, was used to transform *B. subtilis* 168, giving BSIP1388.

2.4. Methionine uptake

Cells were grown in minimal medium in the presence of sulfate as sole sulfur source to the middle of exponential growth phase. They were harvested by centrifugation for 10 min and washed twice with medium A (6 mM K_2HPO_4, 4.4 mM KH_2PO_4, 0.3 mM trisodium citrate, 200 mM xylose, 0.5% glucose and 60 µg/ml^{-1} chloramphenicol). To compare the L-methionine uptake in the wild-type and Δ*yusCB* strains, cells were mixed with the sub-



Sulfur source	β-Galactosidase activity (U mg^{-1} protein)	
	pΔA*yusC'-lacZ* (BSIP1540)	pΔB*yusC'-lacZ* (BSIP1541)
Methionine 1 mM	143	670
Methionine 50 μM	151	ND
Sulfate 1 mM	376	786
Methionine 1 mM + sulfate 1 mM	74	727
Cystine 1 mM	408	664
Homocysteine 1 mM	406	639
Methionine sulfoxide 1 mM	138	ND

Fig. 1. Structure of the *yusCBA* promoter region and expression of transcriptional *yusC′–lacZ* fusions in the presence of different sulfur sources. The promoter, the S-box motif and the transcriptional terminator are indicated. SD was indicated for Shine and Dalgarno sequence. Nucleotides are numbered relative to the translational start site of the *yusC* gene. Cells were grown in minimal medium containing 1 mM sulfate and/or 1 mM L-methionine, 1 mM cystine, 1 mM homocysteine or 1 mM methionine sulfoxide as the sulfur source. β-Galactosidase activities were determined in extracts prepared from exponentially growing cells. ND: Not determined.

strate L-(^{14}C)-methionine at 1 μM. This experiment was performed at 28 °C. Samples of 200 μl were withdrawn at intervals and filtered through 0.45 μm Durapore membranes (Millipore, HVLP02500). Filters were washed with 5 ml of medium A. After drying, radioactivity was measured by scintillation counting. To estimate the initial rate of methionine uptake, we measured three times the slope of the curve at 20 s in the presence of 2 μM L-(^{14}C)-methionine.

3. Results and discussion

3.1. Regulation of expression of the yusCBA *operon in response to methionine availability*

To study the regulation of expression of the putative *yusCBA* operon in response to sulfur availability and the role of the S-box sequence in this regulation, two different transcriptional *yusC′–lacZ* fusions were constructed. These fusions were integrated at the *amyE* locus of the *B. subtilis* 168 strain. Expression of the pΔA (−310, +5) *yusC′–lacZ* fusion, which contains the entire S-box motif, was 2.5- to 5-fold higher in the presence of sulfate than in the presence of L-methionine, methionine sulfoxide or sulfate and L-methionine (Fig. 1). Expression of this fusion was also high in the presence of cystine and homocysteine. In contrast, pΔB (−310, −172) *yusC′–lacZ* fusion caused a high β-galactosidase activity during growth with all the sulfur sources tested. This fusion only contains the promoter region and the 5′-end of the S-box (half of helix 1 and helix 2). The DNA fragment located between nucleotides −172 and +5 is therefore necessary for regulation in response to L-methionine availability. The regulation of the *yusCBA* operon requires the presence of the entire S-box sequence. This result is in agreement with the binding of S-adenosyl methionine (AdoMet) to the RNA leader region of the *yusCBA* operon and the AdoMet-dependent transcription termination observed in vitro [13]. The regulation of expression of the *yusCBA* operon clearly differs from MetJ-dependent regulation of the *metNIQ* operon of *E. coli* [5,23].

3.2. Growth of a ΔyusCB *mutant in the presence of various sulfur sources*

To test the involvement of the *yusCBA* operon in methionine transport, a mutant was constructed by replacing the sequence of the *yusCB* genes by a chloramphenicol resistance cassette (*cat*) (see Section 2). As the methionine toxic analogues seleno-L-methionine and D,L-ethionine inhibit the uptake of D-methionine by *E. coli* [8], resistance to seleno-L-methionine or D,L-ethionine was tested for both strains 168 and BSIP1388 (Δ*yusCB*). In both cases, the area of growth inhibition was 10% smaller for the Δ*yusCB* mutant than for the wild-type strain, indicating a possible role of the *yusCB* genes in methionine uptake (data not shown).

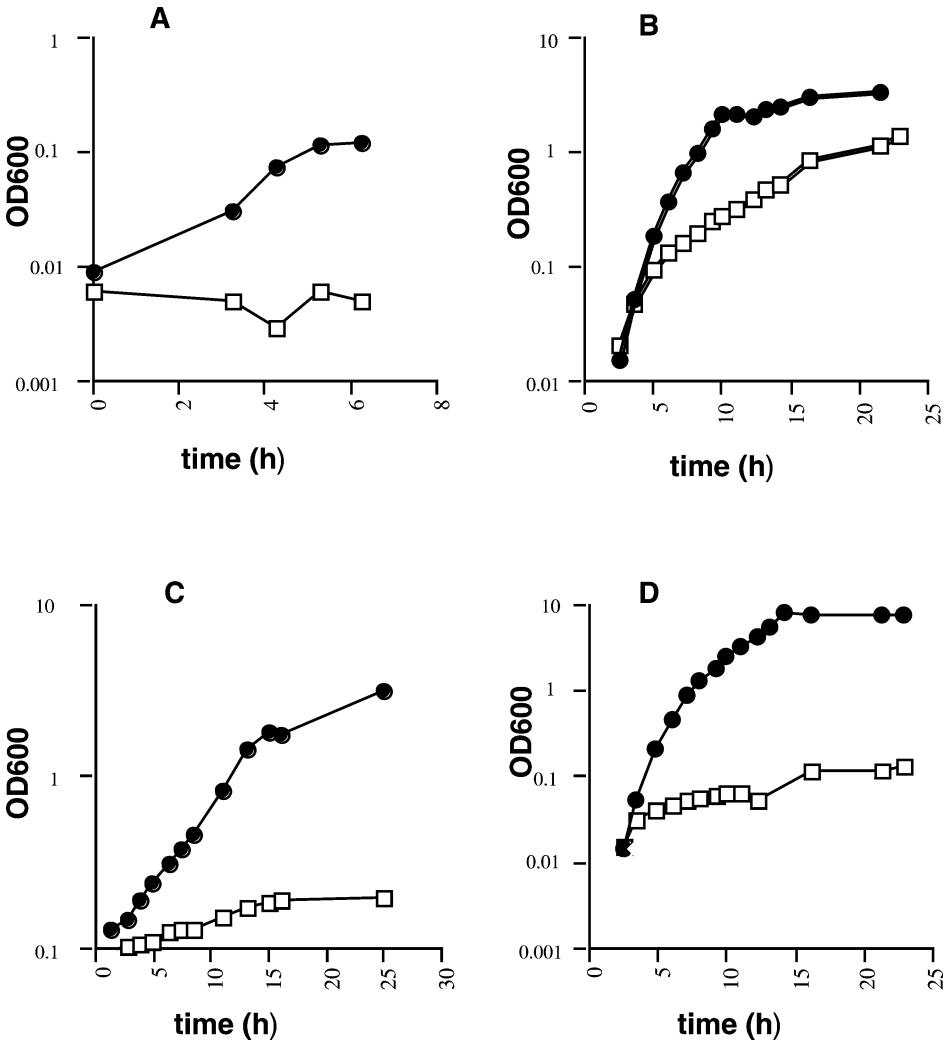


Fig. 2. Growth of the wild type (circles) and Δ*yusCB* mutant (squares) in exhausted minimal medium (EMM) with different sulfur sources: (A) 5 μM L-methionine; (B) 50 μM L-methionine; (C) 1 mM D-methionine; (D) 100 μM methionine sulfoxide.

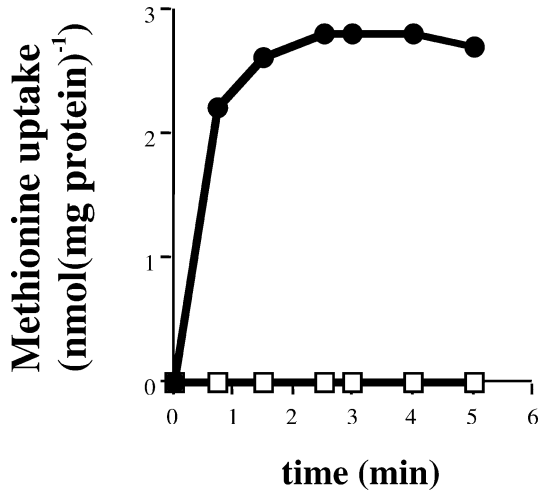


Fig. 3. L-methionine uptake by the wild-type strain (circles) and by the Δ*yusCB* mutant (squares). Methionine uptake was performed in minimal medium in the presence of 1 μM (^{14}C)L-methionine as indicated in Section 2.

We further examined the effect of *yusCB* gene disruption on growth of *B. subtilis* in a sulfur-exhausted minimal medium (EMM) in the presence of L-methionine. The Δ*yusCB* mutant did not grow in EMM supplemented with 5 μM L-methionine while the wild-type strain was able to grow (Fig. 2A). In the presence of 50 μM L-methionine, strain BSIP1388 displayed significant growth retardation as compared to *B. subtilis* 168 (Fig. 2B), while both strains grew similarly in the presence of 100 μM L-methionine (data not shown). In contrast with *E. coli metNI* or *metI* mutants [14,23], the Δ*yusCB* mutant displays a clear absence of growth in the presence of low L-methionine concentration. The YusC protein and/or the YusB protein are therefore necessary for growth with 5 μM L-methionine. Although direct experimental evidence for the participation of YusA in the methionine transport is lacking, it seems very likely that YusCBA forms an L-methionine transporter as observed for MetNIQ from *E. coli*. The growth of the Δ*yusCB* mutant with higher L-methionine

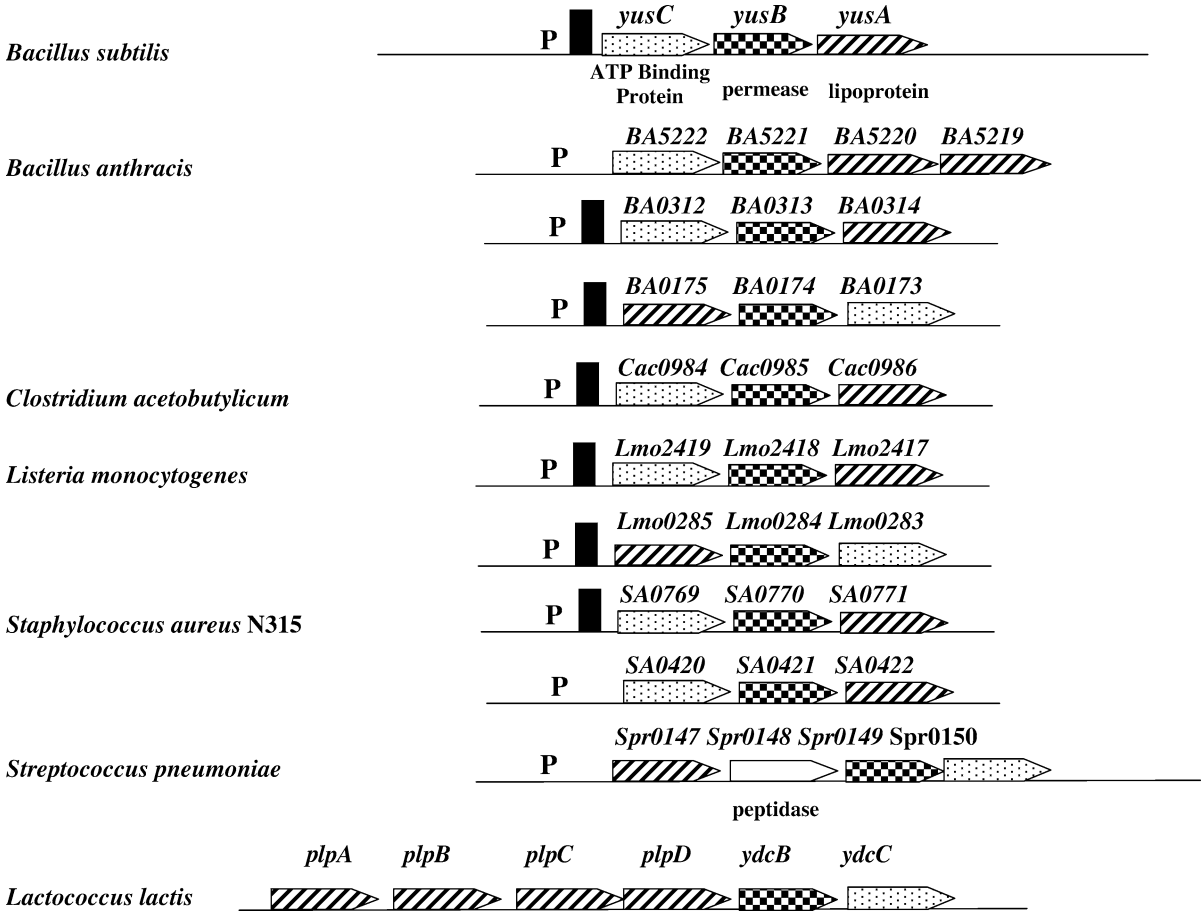


Fig. 4. Genetic organization of the *yusCBA* locus of *B. subtilis* and other sequenced Gram-positive bacteria with a low GC content. The presence of a highly conserved 5′-half helix 1 and helix 2 of the S-box was searched. The S-box motif is indicated as a black box. The dotted boxes correspond to the ATP binding cassettes, the checkered boxes to the membrane permeases and the crosshatched boxes to the solute binding proteins. A similar genetic organization was found in *B. subtilis* and *B. halodurans*, in *B. anthracis* and *B. cereus*, or in *L. monocytogenes* and *L. innocua*. An additional isolated copy of the *yusA* gene is present in *B. subtilis* (*yhcJ*) and *C. acetobutylicum* (CAC1400). These copies are not indicated in this figure.

concentrations indicates the existence of one or several other L-methionine transporter(s). Several genes encoding candidates for these transporter(s) have been tested. The *yoaB* gene is a member of the S-box regulon, which encodes a permease belonging to the major facilitator family. The YhcL protein shares similarities with sodium-glutamate symporters and the expression of the *yhcL* gene is modulated in response to methionine availability [1]. The *yoaB* and *yhcL* genes had been disrupted by fusion with the *lacZ* reporter gene within the framework of European Union and Japanese projects for the functional analysis of the *B. subtilis* genome (http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl and http://bacillus.genome.ad.jp). Δ*yusCB yhcL* or Δ*yusCB yoaB* double mutants were constructed and their ability to grow in the presence of 50 μM L-methionine was tested. These double mutants grew similarly to the Δ*yusCB* mutant, indicating that the YoaB and YhcL proteins are either not involved in L-methionine uptake or that at least three L-methionine transporters exist in *B. subtilis*. Further work is needed to identify the other L-methionine transporter(s).

Growth of the BSIP1388 strain was also tested in the presence of D-methionine. Disruption of the *yusCB* genes completely abolished growth of *B. subtilis* in the presence of 1 mM D-methionine (Fig. 2C). The YusCB proteins and potentially the YusA protein constitute the sole D-methionine transporter of *B. subtilis*. This is in agreement with the characteristics of the MetNIQ system from *E. coli* [5,8,14,23]. Interestingly, the growth of the *yusCB* mutant in the presence of 100 μM methionine-sulfoxide was completely abolished (Fig. 2D) while the growth of this mutant was restored in the presence of 1 mM methionine sulfoxide. In addition to the transport of D- and L-methionine previously observed for the MetNIQ system from *E. coli*, the YusCB proteins also participate in methionine sulfoxide transport. This system corresponds to the highest affinity transporter for this compound. The growth of the *yusCB* mutant in the presence of high methionine sulfoxide concentration indicates that at least two methionine sulfoxide transporters exist in *B. subtilis*. An usual biotope of *B. subtilis* is the phylloplane. If methionine is exuded from plant leaves, then this occurs in the presence of high levels of dioxygen, probably leading

to the conversion of methionine to methionine sulfoxide, an excellent sulfur source for *B. subtilis* [20].

Lower growth yield was also observed with strain BSIP1388 as compared to *B. subtilis* 168 after 24 h in EMM with several other methionine derivatives at a concentration of 5 µM: F-Met (−50%) and peptides Met-Ala, (−60%), Met-Gly (−50%) and Met-Leu (−40%). This suggests that the *yusCB* genes could also be involved in the transport of these compounds.

3.3. L-*Methionine uptake in the wild-type strain and in the* ΔyusCB *mutant*

To confirm the growth phenotype, L-methionine uptake was tested in the Δ*yusCB* mutant and in *B. subtilis* 168. These strains were grown in minimal medium in the presence of sulfate (1 mM), leading to a high level of expression of the *yusCBA* operon (Fig. 1). (^{14}C) L-Methionine uptake assays were performed as described in Section 2. A significant methionine uptake was observed in the wild-type strain while no uptake was detected in the Δ*yusCB* mutant (Fig. 3). In the *B. subtilis* wild-type strain, we estimated the initial rate of methionine uptake to about 7.7 nmol min^{-1} mg^{-1} protein in the presence of 2 µM L-methionine (data not shown). To compare the uptake of L-methionine, methionine sulfoxide or D-methionine by the YusCBA system, we tested the inhibition of (^{14}C)L-methionine transport (2 µM) in the presence of 2 µM cold methionine sulfoxide or D-methionine. The addition of these analogues reduced the L-methionine uptake to about 50% and 40%, respectively (data not shown). These results confirmed that the YusCB system is involved in the uptake of D-methionine and methionine sulfoxide.

3.4. YusCBA-*like systems in several low GC content Gram-positive bacteria*

The presence of *yusCBA*-like operons has been searched in several low GC content Gram-positive bacteria whose genome is entirely sequenced (Fig. 4). Using the highly conserved 5′-half helix 1 and helix 2 of the S-box, we also determined the presence or absence of this motif in the leader region of these genes. A YusCBA-like system is present in the genome of the low GC content Gram-positive bacteria with the exception of *Clostridium perfringens*. However, the number of Yus-like systems, the genetic organization of the corresponding genes and the presence of the S-box motif vary from one organism to another (Fig. 4). A *yusCBA*-like operon with an S-box motif in its leader region is present in *B. subtilis*, *Bacillus halodurans*, *Bacillus anthracis*, *Bacillus cereus*, *Clostridium acetobutylicum*, *Listeria monocytogenes* and *Staphylococcus aureus*. One or two additional copies of this operon are present in *L. monocytogenes*, *S. aureus*, *B. cereus* and *B. anthracis*, but the order of the genes or the regulatory motif is usually not conserved (Fig. 4). Whether all these systems participate in the uptake of L-methionine remains to be established. However, it seems likely that the S-box-containing loci play a role in the transport of methionine or related compounds. Surprisingly, in *B. anthracis*, an S-box motif seems to be absent upstream from the BA5222 operon, which encodes the ABC transporter most similar to YusCBA, but is present upstream from the two less similar systems. The physiological role of these multiple Yus systems is intriguing.

We also observed the presence of several genes encoding solute binding proteins. Two to four copies are adjacent to the membrane permease in *B. anthracis* and *Lactococcus lactis* (Fig. 4) or a second copy is located outside the *yus* locus as found in *B. subtilis* and *C. acetobutylicum*. In *B. subtilis*, the YhcJ protein shows 28% identity with YusA. As the *yusCBA* system is probably involved in the uptake of several compounds including L-methionine, D-methionine and methionine sulfoxide, the YusA protein might contain several binding sites for all these compounds. Alternatively, the binding proteins may be separately encoded with a possible role of the *B. subtilis* YhcJ protein. In *E. coli*, MetQ is involved in the transport of both L- and D-methionine while the second copy of the solute binding protein, NlpA, may be a poor receptor for these two compounds [23]. The YusCBA system of *B. subtilis* clearly belongs to the MUT family (TC#3.A.1.23) described by Zhang et al. [23]. We therefore propose to rename *yusC*, *yusB* and *yusA* as *metN*, *metP* and *metQ*, respectively. Further investigations are needed to elucidate the involvement of YusA and/or YhcJ in the specific binding of L-methionine, D-methionine and methionine sulfoxide and to identify the other L-methionine transporter(s) present in *B. subtilis*.

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