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YsxC, a Putative GTP-Binding Protein Essential for Growth of *Bacillus subtilis* 168

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YsxC is a member of a family of GTP-binding proteins carried by a diverse range of organisms from bacteria to yeasts, plants, and humans. To resolve the issue of whether *ysxC* of *Bacillus subtilis* is essential for growth, we attempted to construct mutants in which *ysxC* was either inactivated or placed under the control of an inducible promoter. Viable mutants were obtained only in the latter case, and these were inducer dependent, demonstrating unambiguously that *ysxC* is an essential gene.

GTP-binding proteins are frequently involved in regulatory pathways as ubiquitous molecular switches (6), operating at various stages of growth and life cycles. The majority of these proteins are guanine nucleotide-binding proteins, while others use GTP as a substrate for phosphorylation and/or guanylation (10). In *Bacillus subtilis*, GTP-binding proteins are involved in translation initiation and elongation; cell division; protein secretion via the signal recognition particle pathway; biosynthesis of flagella; the synthesis of adenylosuccinate, pyrimidine, folic acid, and riboflavin; and the oxidation of thiophene (7). The function of several small putative GTP-binding proteins (e.g., Obg, Bex, EngA, YloQ, YsxC, and YyaF) is, as yet, unknown. YloQ is essential for the growth of *B. subtilis* (2), while Bex has been shown to complement the *Escherichia coli* essential gene *era* (EMBL accession no. U18532, available at http://www.embl-heidelberg.de/srs5/). Obg is essential for both growth and sporulation of *B. subtilis* (18, 21) and is required for the activation of σ^B by stress (17).

The putative GTP-binding protein YsxC (also called OrfX [13]) is encoded by a bicistronic operon that includes *lonA* (Fig. 1A), which encodes a cytosolic ATP-dependent serine endopeptidase (22). *ysxC* is likely to be transcribed together with *lonA*, since its start codon overlaps the *lonA* coding sequence and no *ysxC*-specific promoter or transcriptional initiation site has been detected. Furthermore, *lonA* and *ysxC* showed similar transcription patterns, including induction by heat and other stresses (13).

The insertional inactivation of *ysxC* was previously reported to have no effect on growth, nor did it result in any demonstrable phenotype (16). However, in a more recent genome-based approach designed to identify essential genes in *E. coli*, *B. subtilis*, and *Saccharomyces cerevisiae*, *ysxC* of *B. subtilis* was reported to be essential (2). In that study, which lacked experimental data, of six genes of unknown function that were essential for the growth of *E. coli*, five orthologs were essential for *B. subtilis* and one was essential for *S. cerevisiae*. Three of the five essential *B. subtilis* genes, namely, *obg*, *yloQ*, and *ysxC*, encode putative GTP-binding proteins, while *yrrA* (now called *trmU*, SWISS-PROT accession no. O35020) encodes a putative tRNA, (5-methylaminomethyl-2-thiouridylate)-methyltransferase, and *ydiE* encodes a putative metalloprotease (probably *o*-sialoglycoprotein endopeptidase). These conserved essential bacterial genes with nonessential orthologs in yeast represent potential targets for novel broad-spectrum antimicrobial agents (6).

Construction of integrational mutations in *ysxC*. To determine whether *ysxC* is essential for growth of *B. subtilis* 168, integrational mutants were constructed in which *ysxC* was either inactivated (knockout mutant) or placed under the control of a tightly regulated P_{spac} promoter (23) (fusion mutant). In the case of the knockout mutant, a 303-bp internal fragment of *ysxC* (bp 2879356 to 2879054 [7]) was cloned into the integrational plasmid pMUTIN4 (20), resulting in plasmid pYSXCK (Table 1). For the construction of the fusion mutant, a 164-bp fragment from the 5′ end of *ysxC*, incorporating the ribosome binding site (RBS) and start codon of *ysxC*, was also cloned into pMUTIN4, resulting in plasmid pYSXCF (Table 1).

If *ysxC* is essential for the growth of *B. subtilis* 168, it should not be possible to isolate an integrational mutant with pYSXCK, while integration of pYSXCF should lead to isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent growth. Repeated but unsuccessful attempts were made to generate a pYSXCK-based knockout mutant, irrespective of the presence of IPTG. In the case of the pYSXCF-based fusion mutant, about 100 erythromycin-resistant (Em^r) and lincomycin-resistant (Lm^r) transformants per μg of pYSXCF DNA were isolated in the presence of IPTG, while none were isolated in its absence. These data indicate that *ysxC* is essential for growth. The authenticity of the integration event in this mutant (BFA2414) (Fig. 1A) was confirmed by PCR (Fig. 1B). To ensure tight regulation of the P_{spac} promoter, BFA2414 was transformed with plasmid p65, which provides multiple copies of the *E. coli lacI* gene.

Expression of *ysxC* is essential for growth. The IPTG dependence of BFA2414(p65) was confirmed by growing the organism to exponential phase (optical density at 600 nm [OD_{600}] = ~0.3) in Luria-Bertani (LB) medium (15) containing 1 mM IPTG and 0.3 μg of erythromycin and 10 μg of kanamycin per ml. The cells were washed twice with prewarmed LB medium and diluted 10^{-5}-fold into prewarmed LB media containing erythromycin, kanamycin, and a range of concentrations of IPTG from 0 to 1 mM. As shown in Fig. 2, growth was eventually observed in each of the cultures. However, the time at which growth was first observed was increasingly delayed with decreasing IPTG concentrations. In the case of the culture with 0.1 mM IPTG, growth was delayed by

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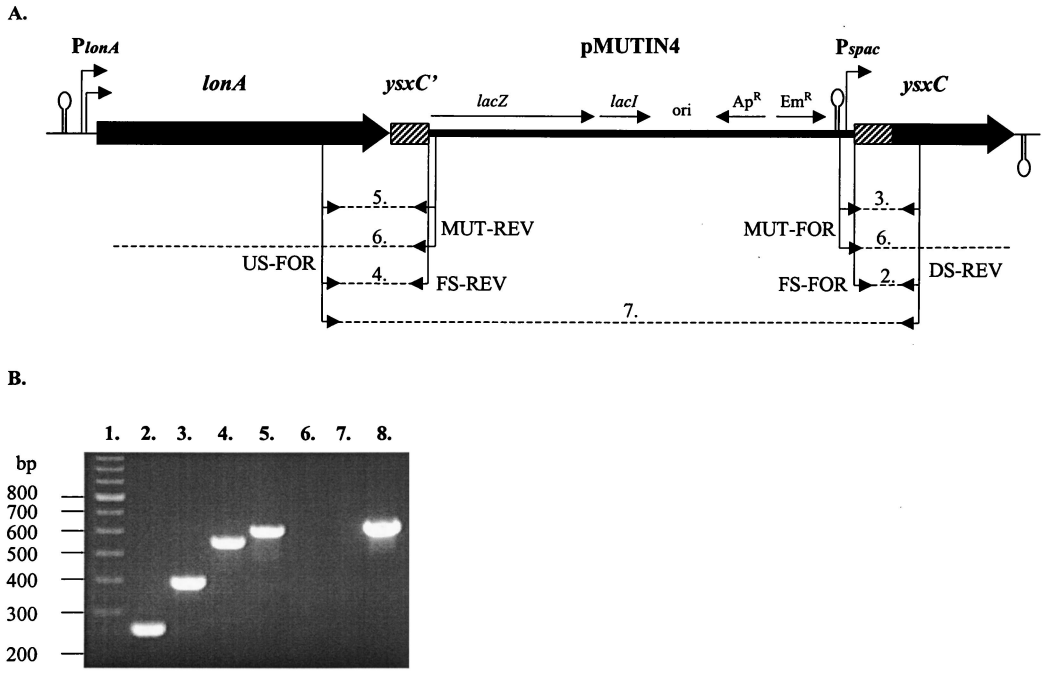

FIG. 1. Construction of a fusion *B. subtilis* mutant of *ysxC*. (A) Schematic representation of the *ysxC* region of BFA2414 after integration of pYSXCF, which is a pMUTIN4-based integration plasmid. Filled thick arrows indicate structural genes, and putative ρ-independent terminators are shown as stem-loop structures. Two promoters upstream of *lonA* (P_{lonA}) are marked with fine broken arrows. Striped boxes show the tandem duplication of the RBS and 5′ end of *ysxC*. *ysxC′* is the 5′ end of *ysxC*. Plasmid pMUTIN4 is shown as a thick line. The *lacZ* reporter gene, *lacI*, and ampicillin resistance (Ap^r) and Em^r genes are marked with fine arrows, and promoter P_{spac} is marked with a fine broken arrow above that of pMUTIN4. The region in pMUTIN4 used for replication in *E. coli* is labeled "ori," and three terminators ($t_1t_2t_0$) upstream of P_{spac} are indicated as a stem-loop structure. The arrows below the genes indicate the location and orientation of the primers, while the dashed lines indicate their expected PCR products. The positions of the primers specific for *B. subtilis* 168 in respect to the entire genome (7, 11) are as follows: DS-REV, 2879355 to 2879372; FS-REV, 2879433 to 2879450; FS-FOR, 2879596 to 2879580; and US-FOR, 2879970 to 2879954. The 5′ ends of the forward (FOR) primers included a 10-bp linker with a *Hin*dIII restriction site, while the reverse (REV) primers included a 9-bp linker with a *Bam*HI site. Positions of the primers specific for pMUTIN4 were as follows: MUT-FOR, 147 to 165, and MUT-REV, 361 to 379. The numbers above the dashed lines correspond to the lanes of the agarose electrophoresis gel shown in panel B. (B) Diagnostic PCR confirming the correct integration of pYSXCF into *ysxC* in mutant BFA2414. Lane 1, 100-bp ladder (Amersham Pharmacia Biotech Inc., Little Chalfont, United Kingdom). PCR was performed with BFA2414 (lanes 2 through 7) or *B. subtilis* 168 (lane 8) chromosomal DNA. The primers (and expected product lengths) were as follows: lane 2, FS-FOR and DS-REV (261 bp); lane 3, MUT-FOR and DS-REV (399 bp); lane 4, US-FOR and FS-REV (557 bp); lane 5, US-FOR and MUT-REV (611 bp); lane 6, MUT-FOR and MUT-REV (no PCR product expected since these primers are oriented away from each other); lane 7, US-FOR and DS-REV (no PCR product expected since the polymerization reaction time of 50 s is too short for the synthesis of the 9,409-bp fragment); and lane 8, US-FOR and DS-REV (635 bp).

approximately 2 h with respect to the culture containing 1 mM IPTG, while growth of the culture with no added IPTG was delayed by more than 20 h. During exponential phase, the mean generation times of cultures with smaller amounts (≤0.1 mM) or no IPTG was increased to ~50 min, compared to ~30 min for the culture with 1 mM IPTG.

The transcription of *ysxC* was monitored (9) by fusing the *spoVG-lacZ* reporter gene of pMUTIN4 to its native promoter (Fig. 1A). Irrespective of the IPTG concentration, β-galactosidase production increased during exponential phase, reaching a peak of ca. 22 nmol of ONP/min/OD_{600} unit at or about the transition between exponential and stationary growth phase; thereafter, the values declined.

To determine whether the delayed growth and decreased growth rate of BFA2414(p65) at the lower (i.e., ≤0.1 mM) IPTG concentrations (Fig. 2) were due to overgrowth by suppressor mutants, stationary-phase samples were plated to determine the ratio of the IPTG-independent colonies (putative

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
B. subtilis		
168	*trpC2*	1
BFA2414	*trpC2 ysxC*::pYSXCF Em^r	This study (Fig. 1)
BFA2414(p65)	*trpC2 ysxC*::pYSXCF p65 Em^r Km^r	This study
BFA2414SUP(p65)	*trpC2 ysxC*::pYSXCF p65 Em^r Km^r, constitutive P_{spac} promoter	This study
Plasmids		
pMUTIN4	Ap^r Em^r *spoVG-lacZ* P_{spac} (8.61 kb)	20
pYSXCK	pMUTIN4 containing a 303-bp internal fragment of *ysxC* (8.913 kb)	This study
pYSXCF	pMUTIN4 containing a 164-bp fragment with RBS and 5′ end of *ysxC* (8.774 kb)	This study
p65	pUB110-based, Km^r P_{penP}::*lacI* (4.962 kb)	S. D. Ehrlich

[a] Km^r, kanamycin resistance.

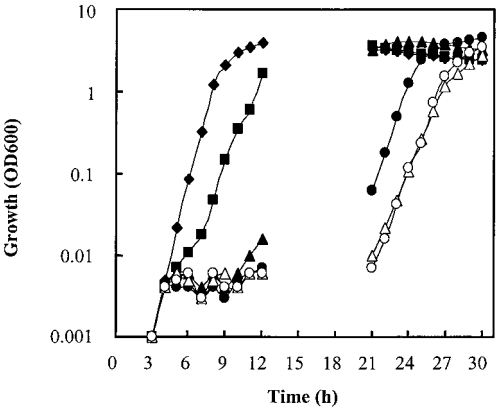

FIG. 2. OD_{600} of BFA2414(p65) in LB media with the following concentrations of IPTG: 1 mM (◆), 0.1 mM (■), 0.01 mM (▲), 0.001 mM (●), 0.0001 mM (△), and 0 mM (○).

suppressor mutants) to total CFU. There was a marked difference in the plating efficiencies of the various cultures. In the case of the culture with 1 mM IPTG, the plating efficiency was $(4.9 \pm 1.2) \times 10^{-7}$. The number of the IPTG-independent colonies increased as the IPTG concentration decreased, and cultures with 0.001 mM IPTG or less exhibited a plating efficiency close to 1. These data suggest that the growth in the latter cultures was due to the accumulation of derivatives with suppressor mutations.

To determine the location of putative suppressor mutation(s), chromosomal DNA from IPTG-independent colonies [BFA2414SUP(p65)] was used to transform *B. subtilis* 168. In each case, similar numbers (ca. $10^3/\mu g$ of DNA) of Em^r Lm^r transformants were observed, irrespective of the presence of IPTG. In contrast, when chromosomal DNA from the IPTG-dependent BFA2414 was used, transformants (ca. $9 \times 10^2/\mu g$ of DNA) were obtained only in the presence of IPTG. These results suggested that the observed suppression was linked to the integrated pYSXCF.

One possibility was that the suppressor mutation(s) occurred in the "oid" *lac* operator (14) of the P_{spac} promoter, leading to its constitutive expression. The oid, or ideal, *lac* operator has perfect symmetry and a 10-fold-higher affinity for the Lac repressor than the native *lac* operator. Consequently, the native *lac* operator associated with the P_{spac} promoter of integrational vector pMUTIN2 was replaced by the oid operator in pMUTIN4 to reduce the noninduced level of expression of this promoter (20). The P_{spac} promoter regions from several IPTG-independent BFA2414SUP(p65) mutants were PCR amplified using primers MUT-FOR and DS-REV (Fig. 1A). Sequencing of the PCR products revealed that all of the BFA2414SUP (p65) mutants contained a single C→T transition at nucleotide 10 of the oid *lac* operator. An identical base pair substitution at the same nucleotide of the native *lac* operator has been shown to decrease its affinity for the Lac repressor by 96% and to generate a constitutive phenotype (3). We therefore concluded that the observed IPTG-independent growth is due to the selection of clones with a mutation in the integrated pYSXCF that severely reduces the capacity of the *lac* operator upstream of the functional *ysxC* gene to bind the lactose repressor. Since a single spontaneous mutation in the *lac* operator of pMUTIN4 can result in the loss of the IPTG dependence of target gene expression, this may lead to an underestimation of the number of essential genes when a gene fusion rather than a gene knockout strategy is initially used to isolate such mutants. Our results indicated that appropriate care needs to be taken when selective pressure is applied to this controllable promoter system.

YsxC protein family. YsxC is a member of a family of small GTP-binding proteins that are carried by a diverse range of organisms from bacteria to yeast, plants, and humans. An analysis of 29 members of the YsxC protein family (Fig. 3) shows that they can be classified into four distinct phylogenetic groups. Group I includes YsxC orthologs from gram-negative bacteria and *Mycoplasma* species, group II includes orthologs from gram-positive bacteria (e.g., *Bacillus*, *Clostridium*, and *Staphylococcus*), group III includes orthologs from the *Archaea*, and group IV includes orthologs from *S. cerevisiae*, *Homo sapiens*, *Arabidopsis thaliana*, and, interestingly, two hyperthermophilic bacteria, *Aquifex aeolicus* and *Thermotoga maritima*.

In group II organisms, *ysxC* is located downstream of *lonA* or *clpX*, both of which code for class III ATP-dependent heat shock proteases. In the case of *Bacillus* and *Brevibacillus*, *ysxC* is located downstream of *lonA*, and its start codon overlaps the 3′ end of the *lonA* coding sequence by one nucleotide. In *Clostridium difficile* and *Clostridium acetobutylicum*, the coding sequence of *ysxC* overlaps the 3′ end of *lonA* by eight nucleotides. In *Streptococcus* spp., *Enterococcus faecalis*, *Staphylococcus aureus*, and *Lactococcus lactis*, *ysxC* is in a putative operon with *clpX*. In *L. lactis* the start codon of *ysxC* overlaps the 3′ end of the *clpX* coding sequence by one nucleotide. In the other three phylogenetic groups (I, III, and IV), *lonA* or *clpX* is located at a distal site on the chromosome with respect to *ysxC*. Linkage between an ATP-dependent protease and *ysxC* was also observed in *Pyrococcus horikoshii* from group III, in which the *ysxC* ortholog, PH0200, is located 59 bp downstream of a gene encoding a putative regulatory subunit of the ATP-dependent 26S protease. In addition to YsxC orthologs in eukaryotes, two bacterial homologs from *A. aeolicus* and *T. maritima* belong to group IV. *A. aeolicus* is one of the earliest diverging and most thermophilic bacteria known, and as a chemolithoautotroph, it can grow on hydrogen, oxygen, carbon dioxide, and mineral salts (5). *T. maritima* is one of the deepest and most slowly evolving lineages in the *Eubacteria*. Although the core of *T. maritima* may be eubacterial, almost one quarter of the genome is archaeal in nature (12). *A. thaliana* has at least two homologs of YsxC, one 219 and the other 318 amino acid residues in length, encoded on chromosomes II and V, respectively (8). The length of the smaller homolog is in the range of prokaryotic YsxC proteins (190 to 219 amino acids). The length of the longer YsxC homolog is similar to that of the YsxC orthologs from *S. cerevisiae* and *H. sapiens*. With respect to YsxC of *B. subtilis*, these proteins have a 100- to 110-amino-acid extension at their amino terminus. Their N termini, which are highly conserved in *S. cerevisiae* and *H. sapiens* but not in *A. thaliana*, showed no homology to any other bacterial and archaeal proteins. In the case of *A. thaliana*, the N terminus contained a putative transmembrane helix (19) between amino acid residues 11 and 29 and the protein is currently the only putative membrane-bound member of the YsxC family.

No homologs of *ysxC* were observed on the complete genome sequences of *Borrelia burgdorferi*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Deinococcus radiodurans*, *Mycobacterium tubercolosis*, and *Treponema pallidum* using databases at The Institute for Genomic Research (TIGR; http://www.tigr.org/tdb) and the Pasteur Institute (http://genolist.pasteur.fr).

An alignment of YsxC and 28 homologs (data not shown) revealed four regions of conservation: (i) (G/R)X(S/T)N(V/A)GKS(S/T), a putative GTP-binding motif located toward the amino terminus; (ii) PGXTXXX(N/I), located 15 to 23 residues downstream of the first region; (iii) DXPG(Y/F)G(Y/F), a second putative GTP-binding motif located 10 to 20 residues

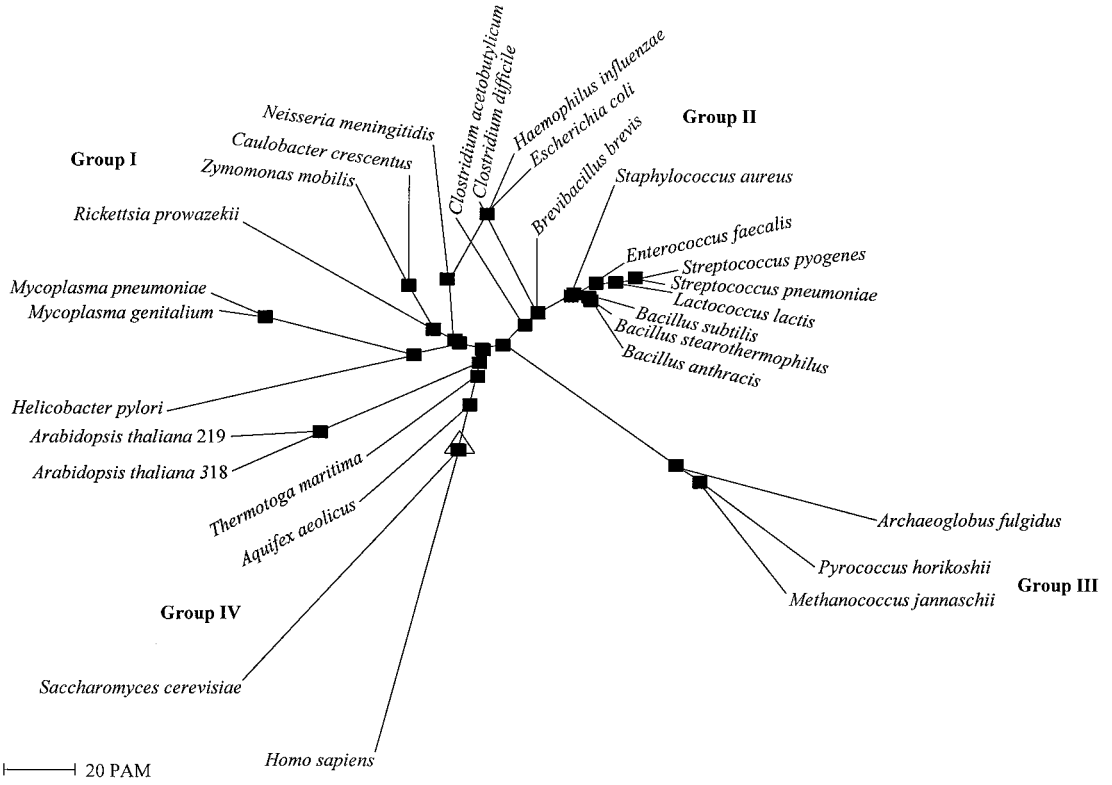

FIG. 3. Radial phylogenetic tree of the YsxC protein family. Multiple-sequence alignment and phylogenetic analysis were performed using the web site of the Institut National de la Recherche Agronomique (http://www.toulouse.inra.fr/multalin.html; see reference 4). The symbol comparison table was Blosum62, the gap weight was 12, and the gap length weight was 2. The root of the tree is marked with a triangle. Proteins with SWISS-PROT accession numbers (in parentheses) are as follows: *B. subtilis* YsxC (P38424), *E. coli* YihA (P24253), *Haemophilus influenzae* HI1118 (P46453), *Helicobacter pylori* HP1567 (O26087), *Mycoplasma genitalium* MG335 (P47577), *M. pneumoniae* MP359 (P75303), *Methanococcus jannaschii* MJ0320 (Q57768), *Rickettsia prowazekii* RP102 (Q9ZE46), *A. aeolicus* AQ_1815 (O67679), and *Caulobacter crescentus* CgpA (Q9ZG89). Proteins with GenBank accession number are as follows: *Archaeoglobus fulgidus* AF1326 (AAB89919), *P. horikoshii* PH0200 (BAA29269), *T. maritima* TM1466 (AAD36534), *L. lactis* (AAF63739), *Neisseria meningitidis* NMB1806 (AAF42143), *Zymomonas mobilis* CgpA (AAD56911), *S. cerevisiae* Ydr336wp (AAB64772), *H. sapiens* HSPC135 (AAF29099), *A. thaliana* 219 is At2g22870 (AAC32434) with a length of 219 amino acid residues, and *A. thaliana* 318 is F15N18_70 (CAB87708) with a length of 318 amino acid residues. YsxC orthologs of *B. anthracis*, *B. stearothermophilus*, *C. acetobutylicum*, *C. difficile*, *E. faecalis*, *S. aureus*, *S. pneumoniae*, and *S. pyogenes* were obtained from unfinished genome sequencing projects at TIGR, the Sanger Centre, Genome Therapeutics Corporation, and the University of Oklahoma. In *B. stearothermophilus*, the 130 amino acids at the N terminus of the YsxC homolog were deduced from sequence contig 552. In *B. brevis*, the 162 amino acids at the N terminus were deduced from the GenBank sequence (D00863) after inserting one nucleotide between positions 2745 and 2746 (16). PAM, percent accepted mutation.

downstream of the second region; and (iv) KXDK, located 56 to 74 residues downstream of the third motif. The second motif is shorter in *S. cerevisiae* (GXTXXXN), and only the threonine residue is conserved in the case of *H. sapiens*.

We were not able to generate a viable mutant of *ysxC* with pYSXCK, which, after integration into the *B. subtilis* chromosome, generates a strain carrying a YsxC protein that is truncated at its C terminus by just 23 amino acids. Since this protein includes the four conserved motifs described above, this indicates that the highly charged (5 K residues, 2 E residues, 1 D residue, 1 R residue, and a serine dyad) C terminus is essential for function.

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