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BIOTECHNOLOGY

Biological Containment of Genetically Modified *Bacillus subtilis*

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ABSTRACT Genetic manipulation of bacterial spores of the genus *Bacillus* has shown potential for vaccination and for delivery of drugs or enzymes. Remarkably, proteins displayed on the spore surface retain activity and generally are not degraded. The heat stability of spores, coupled with their desiccation resistance, makes them suitable for delivery to humans or to animals by the oral route. Despite these attributes, one regulatory obstacle has remained regarding the fate of recombinant spores shed into the environment as viable spores. We have addressed the biological containment of GMO spores by utilizing the concept of a thymineless death, a phenomenon first reported 6 decades ago. Using *Bacillus subtilis*, we have inserted chimeric genes in the two thymidylate synthase genes, *thyA* and *thyB*, using a two-step process. Insertion is made first at *thyA* and then at *thyB* whereby resistance to trimethoprim enables selection of recombinants. Importantly, this method requires introduction of no new antibiotic resistance genes. Recombinant spores have a strict dependence on thymine (or thymidine), and in its absence cells lyse and die. Insertions are stable with no evidence for suppression or reversion. Using this system, we have successfully created a number of spore vaccines as well as spores displaying active enzymes.

IMPORTANCE Genetic manipulation of bacterial spores offers a number of exciting possibilities for public and animal health, including their use as heat-stable vehicles for delivering vaccines or enzymes. Despite this, one remaining problem is the fate of recombinant spores released into the environment where they could survive in a dormant form indefinitely. We describe a solution whereby, following genetic manipulation, the bacterium is rendered dependent on thymine. As a consequence, spores if released would produce bacteria unable to survive, and they would exhibit a thymineless death due to rapid cessation of metabolism. The method we describe has been validated using a number of exemplars and solves a critical problem for containing spores of GMOs in the environment.

KEYWORDS thymineless death, thymidylate synthase, *Bacillus subtilis*, spores, GMO

Bacillus endospores, or spores, are dormant entities with well-known resistance properties, including heat stability and tolerance of exposure to noxious chemicals and desiccation (1). As such, spores offer a number of applications. First and foremost has been their use as mucosal vaccines wherein antigens can be displayed on the spore surface by fusion to a coat protein anchor (2). Immunization of recombinant spores by a mucosal route (oral, sublingual, or nasal) has shown promising results and in some cases levels of protection that could be efficacious in humans or animals (3–5). In the case of the latter, the ability to incorporate a vaccine in feed is particularly attractive and, for some animals, arguably the only way to vaccinate. For example, vaccinating farmed shrimp against viral pathogens such as white spot syndrome virus (WSSV) where recombinant spores expressing the WSSV VP26 and VP28 envelope proteins have been shown to confer protection in shrimps (6–9). A number of *Bacillus* species are considered food grade; that is, they are safe for human consumption, with a qualified

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presumption of safety (QPS) designation, as defined by the European Food Safety Authority (EFSA) (10). In the United States some *Bacillus* strains are considered "generally recognized as safe" (GRAS), as defined by the Food and Drug Administration. QPS and GRAS designations have supported the use of a number of *Bacillus* strains, including those of *Bacillus subtilis*, as probiotics in both human and animal feeds (11). The concept of recombinant probiotics (12), then, is a logical next step forward in exploiting the beneficial properties of *Bacillus* (13). In addition to use in vaccines, spores have been shown to facilitate expression of enzymes on the spore surface, for example, with the animal feed enzyme phytase (14). The ability to use probiotic bacteria that deliver enzymes negates the need to purify enzymes and could offer significant advantages to industry. Finally, streptavidin (SA) has been expressed on *B. subtilis* spores, enabling monoclonal antibodies to be conjugated to the spore surface and the targeting of spores loaded with anticancer drugs to cancer cells (15).

Despite the potential utility of *Bacillus* spores for industrial applications, there remain a number of obstacles regarding the deliberate release of genetically modified *Bacillus*. First is the use of antibiotic resistance (Ab^R) genes used in the engineering of stable recombinant strains. Regarding Ab^R, the majority of procedures requiring insertion of heterologous DNA into *B. subtilis* require ectopic insertion using an Ab^R gene for positive selection (16). Plasmid vectors carrying chloramphenicol or erythromycin resistance genes are typically used for ectopic insertion at a genetic locus that is redundant for cell growth, typically the alpha-amylase (*amyE*) or threonine C (*thrC*) gene (17). The potential risk of Ab^R gene transfer following release is recognized, and at least two systems in *B. subtilis* have now been described that enable insertion of heterologous genes without introduction of an Ab^R gene (18, 19).

The second, and most challenging, hurdle is the ultimate fate of recombinant spores once released since spores have been shown to be able to survive indefinitely in the environment (1). The soil is normally enriched with dormant spores (20), and their intrinsic robustness makes it difficult to argue that they would not persist after deliberate release. One approach might be to construct germination-deficient spores, but, at best, the germination rate can be reduced to 0.0015% (21), which is unlikely to satisfy regulatory authorities. One approach that could be considered is thymine starvation resulting in a thymineless death, a concept that has been well documented (22–24). Prokaryotes carrying a mutated thymidylate synthase gene are unable to grow in low concentrations of thymidine or thymine, resulting in cell death. Thymine starvation, then, is different from the biostatic effects commonly found with deprivation of other nutrients. Thymineless death has been documented for *B. subtilis* (25), and the bacterium carries two thymidylate synthase enzymes encoded by the *thyA* and *thyB* genes (26).

We have adopted the principles of a thymineless death to exploit *Bacillus* for the introduction of heterologous genes without the introduction of antibiotic resistance genes. These recombinant spores, should they germinate, will fail to survive in the environment and will die immediately. The approach we have developed is particularly novel since it is a two-step gene integration process enabling positive selection on successively higher levels of trimethoprim but without the introduction of any antibiotic resistance genes.

RESULTS AND DISCUSSION

Rationale. An absolute requirement for thymine in *B. subtilis* necessitates two thymidylate synthases (TSase) encoded by the unlinked *thyA* (TSaseA) and *thyB* (TSaseB) genes (26). These genes encode part of the folate biosynthetic pathway, which provides pyrimidines for cell growth (see Fig. S2 in the supplemental material). TSaseB is thermosensitive and retains only ~5 to 8% activity at a restrictive temperature of 46°C. Thus, inactivation of the *thyA* locus requires supplementation with thymine (or thymidine) for growth at 46°C. TSaseA is not thermosensitive, and so inactivation of *thyB* allows cells to grow at an elevated temperature. Inactivation of both *thyA* and *thyB*, however, produces an absolute requirement of thymine for growth at both 37°C and

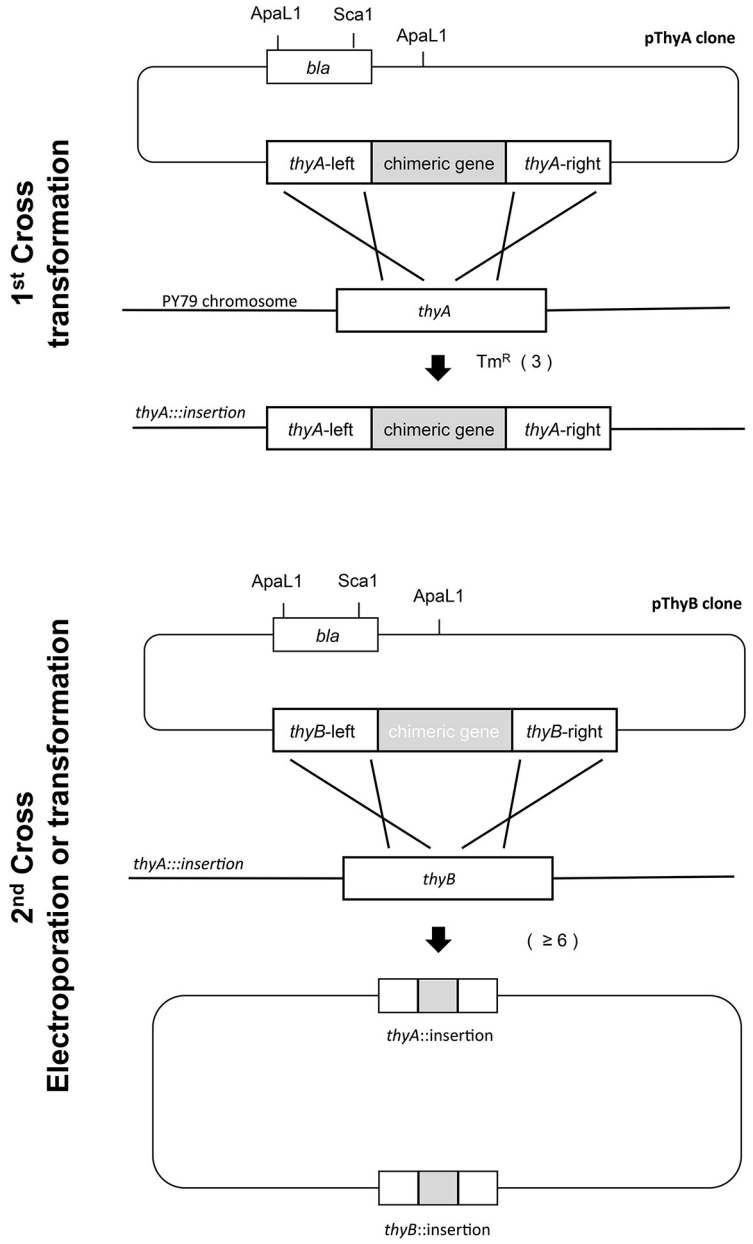

FIG 1 Strain constructions. To construct ectopic insertions at the *thyA* and *thyB* loci of *B. subtilis*, two steps are required. In the first step, a pThyA plasmid carrying a chimeric gene is linearized (ApaLI digestion) and introduced into cells of a wild-type *B. subtilis* strain (in this case strain PY79) by DNA-mediated transformation. Trimethoprim-resistant (Tm^r) transformants are selected on SMM agar containing trimethoprim (3 μg/ml) and thymine (50 μg/ml) and carry an insertion of homologous *thyA* DNA together with the chimeric gene, by marker replacement, as shown. In the second step, linearized plasmid DNA of a pThyB vector carrying the same or a different chimeric gene is introduced into cells of the *thyA* insertion strain created in the first step. Selection for Tm^r is made on SMM-CAA agar containing trimethoprim (>6 μg/ml) and thymine (50 μg/ml).

46°C. As shown by Neuhard et al. (26), inactivation of the *thy* genes produces resistance to the antifolate drug trimethoprim (or aminopterin) since the need for dihydrofolate reductase (DHFR), the target for trimethoprim, is dispensed with. However, the levels of resistance differ, with insertion at *thyA* producing a lower level of resistance than that found in a *thyA thyB* mutant (26). This attribute of differential resistance to trimethoprim enables a novel, two-step, ectopic cloning system to be designed (Fig. 1). In the first step, a gene is introduced at the *thyA* locus, and this is followed in the second

step by insertion at *thyB* using resistance to increasing concentrations of trimethoprim for positive selection.

To demonstrate proof of concept for ectopic cloning at the *thy* loci, we chose a number of heterologous genes whose products had previously been expressed on the spore surface. In each case expression had been achieved by fusion to a *B. subtilis* gene encoding a surface-expressed spore coat protein (either CotB or CotC). The proteins chosen included the following: (i) VP26 and VP28, both envelope proteins of the shrimp virus WSSV that when displayed on recombinant *B. subtilis* spores and incorporated in feed have been shown to confer protection to shrimp challenged with WSSV (7–9); (ii) a protein consisting of a C-terminal domain of *Clostridium difficile* toxin A ($TcdA_{26-39}$) that, when expressed on the spore surface, has been shown to confer protection from *C. difficile* infection (CDI) in hamsters dosed orally with these recombinant spores (4, 27); (iii) streptavidin (SA) that, when expressed on spores, can be conjugated to the monoclonal antibody cetuximab, enabling targeting to colon cancer cells (15); (iv) two enzymes, subtilisin E (AprE), an alkaline protease, and alpha-amylase (AmyE), which are both enzymes of industrial importance and commonly incorporated in animal feed (28, 29).

A two-step method for ectopic gene insertion. Two plasmids, pThyA and pThyB, were synthesized that carry the complete *thyA* or *thyB* gene interrupted at midpoint with a multiple cloning site (MCS) for insertion of heterologous DNA (Fig. 1 and Table S1). To ensure sufficient DNA homology to enable a double-crossover recombination event, the left and right arms carried the relevant *thy* segments as well as flanking upstream or downstream DNA (~900 bp at the proximal and distal ends). In-frame fusions of the *cotB* and *cotC* genes (encoding the spore coat anchors required for display of heterologous proteins) to the open reading frame (ORF) of *vp26*, *vp28*, $tcdA_{26-39}$, the SA gene, *aprE*, or *amyE* (Fig. S1A to E) were then cloned into the appropriate pThyA or pThyB vector. The pThyA plasmids were next linearized and used to transform competent cells of the wild-type strain PY79 with selection for trimethoprim resistance on plates supplemented with trimethoprim (3 μg/ml) and thymine at 37°C. Transformants carrying *thyA* insertions could be recognized by their failure to grow at 46°C without thymine. In the second step, for introduction of insertions at the *thyB* locus, classical DNA-mediated transformation of competent cells with the pThyB plasmid proved inefficient, with low integration frequencies. Instead, we developed an electroporation method that reliably and reproducibly enabled introduction of pThyB plasmids at the *thyB* locus in strains lacking *thyA* (see Materials and Methods) using selection with a higher concentration of trimethoprim (6 μg/ml). Strains carrying insertions at *thyA* and *thyB* were verified by their failure to grow at both 37°C and 46°C in the absence of thymine. Using this two-step process, we successfully constructed a number of strains carrying insertions (*vp26*, *vp28*, $tcdA_{26-39}$, and the SA gene) at the *thyA* and *thyB* loci (Table 1). In addition, we also made strains carrying single insertions of the *aprE* and *amyE* genes at the *thyA* locus. To create an absolute thymine dependence, we simply linearized an empty pThyB plasmid and introduced this DNA into the *thyA* mutant selecting for trimethoprim at 6 μg/ml. Strains we successfully constructed included monovalent (VP28, $TcdA_{26-39}$, streptavidin, subtilisin E, and amylase) as well as divalent (expression of VP26 and VP28 on one spore) expression by fusion to one or two different spore coat anchors (CotB and CotC). For each strain constructed and listed in Table 1, we confirmed by nucleotide sequence analysis the integrity of the *thyA* or *thyB* insertion.

We examined growth of the insertions in Spizizen's minimal medium (SMM) at 37°C; Fig. 2 shows examples of a strain (SH14) carrying *cotB-*$tcdA_{26-39}$ and *cotC-*$tcdA_{26-39}$ insertions at the *thyA* and *thyB* loci. As expected, the strain with the *thyA* insertion grew normally with or without thymine (Fig. 2B) and was indistinguishable from wild-type PY79 (Fig. 2A). In contrast, the *thyA thyB* insertion strain was thymine dependent but in the presence of thymine had reduced fitness, as shown from the lower maximal optical density (OD) (Fig. 2C).

TABLE 1 Phenotypes of *B. subtilis* recombinant strains

Strain	Genotype	Growth at 37°C[a]		Growth at 46°C[a]		Trimethoprim MIC (mg/ml)[b]	Sporulation (CFU/ml [% of total])[c]	
		− Thy	+ Thy	− Thy	+ Thy		Total no. of spores	No. of heat-resistant spores
PY79	*thyA*$^+$ *thyB*$^+$	+	+	+	+	0.25	2.8×10^8	2.4×10^8 (85.7)
SH11	*thyA*::*cotC-vp26*	+	+	−	+	16	1.9×10^8	1.6×10^8 (82)
SH12	*thyA*::*cotC-vp26 thyB*::*cotB-vp28*	−	+	−	+	>64	2×10^8	1.5×10^8 (75)
AC01	*thyA*::*cotB-vp28*	+	+	−	+	16	2.6×10^8	2.2×10^8 (85)
AC02	*thyA*::*cotB-vp28 thyB*::*cotB-vp28*	−	+	−	+	>64	1.9×10^8	1.5×10^8 (78.6)
SH13	*thyA*::*cotB-tcdA*$_{26-39}$	+	+	−	+	16	2.2×10^8	1.8×10^8 (85)
SH14	*thyA*::*cotB-tcdA*$_{26-39}$ *thyB*::*cotC-tcdA*$_{26-39}$	−	+	−	+	>64	2.5×10^8	1.9×10^8 (77.2)
SH15	*thyA*::*cotB*-SA gene	+	+	−	+	16	2.8×10^8	2.3×10^8 (82)
SH16	*thyA*::*cotB*-SA gene *thyB*::*cotB*-SA gene	−	+	−	+	>64	3.1×10^8	2.4×10^8 (77.4)
SH17	*thyA*::*cotB-amyE*	+	+	−	+	16	7.4×10^8	6.1×10^8 (82.5)
SH18	*thyA*::*cotB-amyE thyB*::MCS	−	+	−	+	>64	1.28×10^8	9.8×10^7 (76.5)
SH19	*thyA*::*cotB-aprE*	+	+	−	+	16	4.1×10^8	3.7×10^8 (90)
SH20	*thyA*::*cotB-aprE thyB*::MCS	−	+	−	+	>64	8.1×10^7	6.8×10^7 (83.9)

[a]Growth (+) or no growth (−) on SMM agar with or without thymine (50 μg/ml).
[b]The MIC was determined using a microdilution method (51).
[c]Spores were from cultures grown overnight in DSM (with thymine) at 37°C.

In addition, using whole-genome sequencing, we confirmed that strain SH12 (*thyA*::*cotC-vp26 thyB*::*cotB-vp28*) and SH14 (*thyA*::*cotB-tcdA*$_{26-39}$ *thyA*::*cotC-tcdA*$_{26-39}$) carried the specific insertions at the *thy* loci without any chromosomal aberrations (data not shown). Expression of chimeric proteins was confirmed by Western blotting of proteins extracted from purified spores. Figure 3 shows blots for detection of the VP26, VP28, TcdA$_{26-39}$, and SA chimeras in SH12, SH14, and SH16, which demonstrated bands of the expected molecular masses. A second method was detection of whole spores by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies to the corresponding heterologous antigen. Figure 4 shows surface detection of the TcdA$_{26-39}$ (Fig. 4A) and VP28 (Fig. 4B) antigens by whole-spore ELISA. As expected, levels of the TcdA$_{26-39}$ protein carried at both the *thyA* and *thyB* loci (Fig. 4A, SH14) were greater than the expression level at one locus (i.e., *thyA*, SH13). In parallel, we also measured the abundance of TcdA$_{26-39}$ in spores of PP108 that carried the same antigen fused to CotB and CotC but inserted at the *thrC* and *amyE* loci, respectively (4). Expression levels were somewhat lower, but it should be noted that when an anti-spore polyclonal antibody (PAb) was used to measure levels of spore coat proteins, expression levels were correspondingly reduced.

Both thymine and thymidine could be used for growth of strains carrying insertions in the *thyA* and *thyB* loci. Using SH14, we showed that, for optimal growth, 15 μg/ml

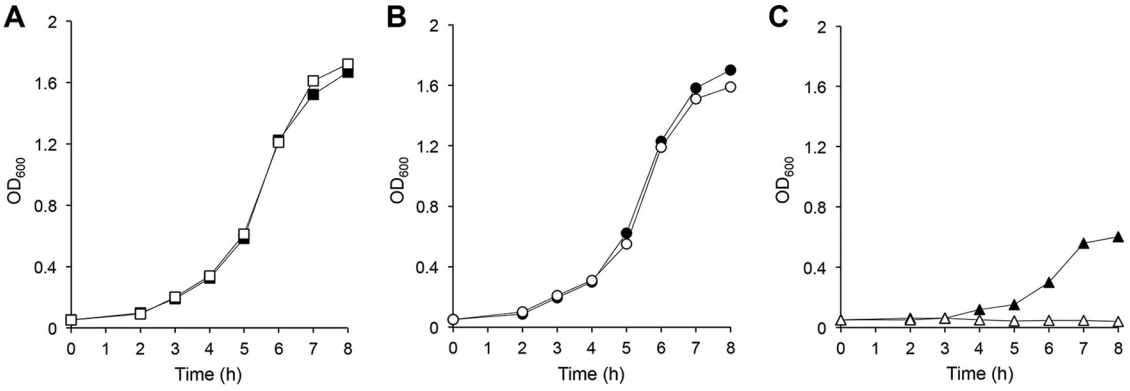

FIG 2 Growth of strains in minimal medium. Wild-type PY79 (A), SH13 *thyA*::*cotB-tcdA*$_{26-39}$ (B), and SH14 *thyA*::*cotB-tcdA*$_{26-39}$ *thyB*::*cotC-tcdA*$_{26-39}$ (C) were grown in SMM at 37°C with (filled symbols) or without (open symbols) thymine supplementation (50 μg/ml).

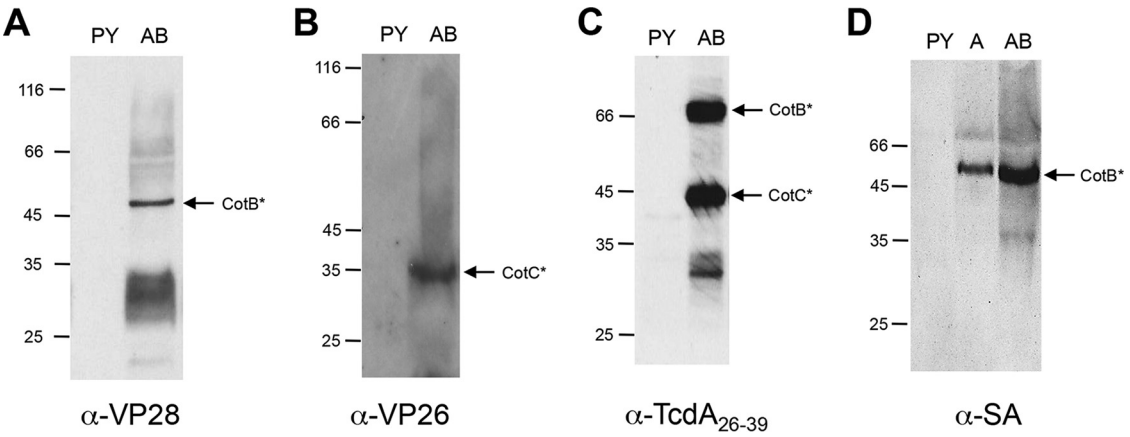

FIG 3 Spore coat expression. *B. subtilis* strains carrying insertions at the *thyA* and *thyB* loci were examined by Western blotting of spore coat proteins extracted from preparations of pure spores. Panels show bands obtained in extracts of wild-type spores (PY), spores carrying *thyA* and *thyB* insertions (AB), or spores carrying only a *thyA* insertion (A). Panels A and B show analysis of SH12 *thyA*::*cotB-vp28 thyB*::*cotC-vp26* with anti-VP28 and anti-VP26 antibodies. Panel C shows analysis of SH14 *thyA*::*cotB-tcdA*$_{26-39}$ *thyB*::*cotC-tcdA*$_{26-39}$ with TcdA$_{26-39}$-specific antibodies. Panel D shows analysis of SH16 *thyA*::*cotB*-SA gene *thyB*::*cotB*-SA gene with SA-specific antibodies. Bands corresponding to the chimeric proteins are indicated (*) with the relevant coat protein anchor (CotB or CotC). The protein loaded per well corresponded to an extraction from 2 × 10^8 spores (see Materials and Methods).

of thymine or 20 μg/ml of thymidine was sufficient (Fig. S3). This was significantly less than the 50 μg/ml reported before (26) and most probably reflects differences in the backgrounds of the strains used. Cells carrying insertions at the *thyA* and *thyB* loci, although able to grow in medium supplemented with thymine, underwent a massive loss in cell viability (~5 logs in 5 h) when thymine was removed, a hallmark of a thymineless death (Fig. S4).

Finally, using this cloning system, we were able to introduce insertions that were of the same or opposite transcriptional polarity to the respective *thy* gene (Fig. S1A to E), indicating no evidence of readthrough from the proximal *thy* coding sequences.

Experimental considerations for use of ectopic insertion at the *thy* loci. (i) Growth in rich medium. In rich medium such as LB medium and Difco sporulation medium (DSM), the single *thyA* insertion strain was able to grow at 37°C without thymine supplementation while the *thyA thyB* strain with the double insertion showed no growth (Fig. 5B and D). Intriguingly, supplementing LB medium or DSM with

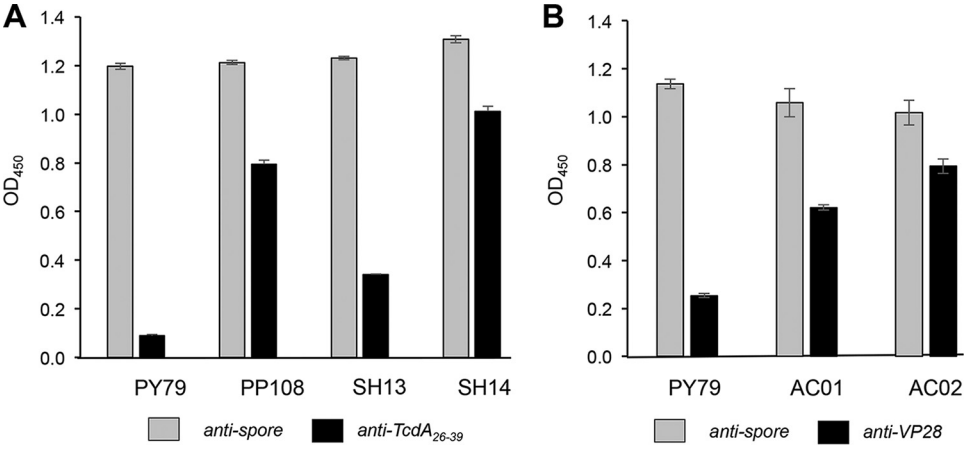

FIG 4 Surface expression determined by whole-spore ELISA. (A) Microtiter plates were coated with spores (2 × 10^8 spores/well) of PY79 (*spo*$^+$), PP108 (*amyE*::*cotC-tcdA*$_{26-39}$ *thrC*::*cotB-tcdA*$_{26-39}$), SH13 (*thyA*::*cotB-tcdA*$_{26-39}$), and SH14 (*thyA*::*cotB-tcdA*$_{26-39}$ *thyB*::*cotC-tcdA*$_{26-39}$) and then probed with either anti-spore (1:1,000) or anti-TcdA$_{26-39}$ (1:500) rabbit PAb. Secondary PAbs were diluted 1:5,000, and naive serum was used for comparison, with basal levels subtracted. (B) The experiment as described for panel A but using spores of PY79, AC01 (*thyA*::*cotB-vp28*) and AC02 (*thyA*::*cotB-vp28 thyB*::*cotB-vp28*) probed with either anti-spore (1:1,000) or anti-VP28 (1:300) rabbit PAb.

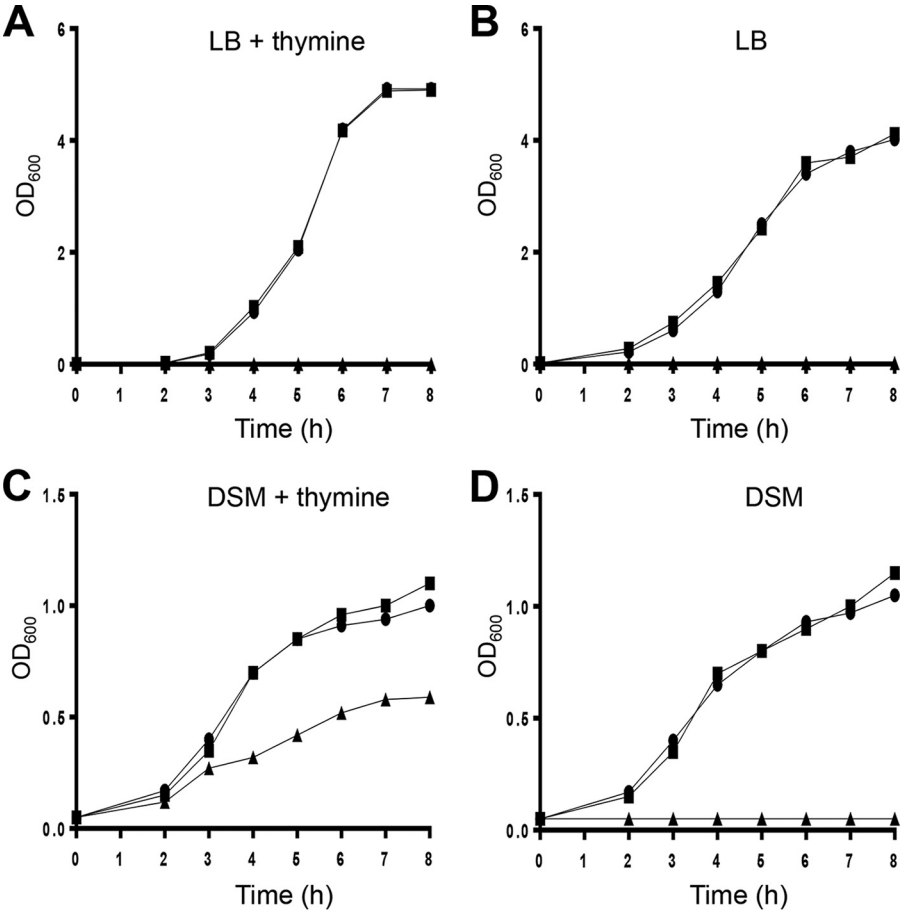

FIG 5 Growth of strains in rich medium. Growth at 37°C under the indicated conditions is shown for PY79 (■), SH13 (●), and SH14 (▲). Thymine was used at 50 μg/ml.

thymine did not restore normal levels of growth. In LB medium the double-insertion strain failed to grow (Fig. 5A), while in DSM plus thymine, limited growth was observed (Fig. 5C). However, after 24 h of growth in DSM, sporulation was shown to have occurred, and the numbers of heat-resistant spores were essentially equivalent to those of PY79 (Table 1).

Since the *thyA thyB* insertion strain could grow, albeit with reduced fitness, in minimal medium supplemented with thymine (Fig. 2C), we reasoned that one or more components of the rich medium might inhibit growth potentially by interfering with the folate pathway. A prime candidate was yeast extract (YE) that is present in LB medium and DSM at 5 mg/ml and 2 mg/ml, respectively, and absent in SMM. YE has been shown to inhibit thymine mutants of *Escherichia coli* strains, and the active bactericidal ingredient has been identified as adenosine (30). A second possibility, though, was *p*-aminobenzoic acid (31), a component of YE and directly involved in the folate pathway (Fig. S1) but also found to be bactericidal to *E. coli* (32).

We grew strains (*thy*$^+$ and *thyA thyB*) in LB medium with thymine with various levels of YE and found that YE concentrations of $\geq$2 mg/ml inhibited growth of strains carrying two *thy* (*thyA* and *thyB*) insertions (Fig. S5). The inhibitory activity of YE could therefore explain why in LB medium the *thyA thyB* double-insertion strain failed to grow in the presence of thymine (Fig. 5A). Similarly, in DSM, where YE is present at a lower concentration, reduced growth was observed (Fig. 5C). Finally, the inhibitory action of YE necessitated the need for a modified growth medium (SOC2) lacking YE for preparation of cells for the electroporation step used in the strain constructions (see Materials and Methods). Although YE clearly inhibits growth, we have been unable to attribute this definitively to either adenosine or *p*-aminobenzoic acid (data not shown).

(ii) Gene transfer. As mentioned earlier, DNA-mediated transformation of competent cells could be used to introduce plasmid DNA into *B. subtilis* cells. However, for introducing DNA into *thyA* cells in the second step, electroporation using a modified medium lacking YE yielded higher frequencies of integration.

We found that following the first genetic cross, two colony types, in equal proportions, were apparent on SMM agar supplemented with thymine and trimethoprim (3 μg/ml) (Fig. S6). Type 1 colonies were large and opaque (2 to 3 mm), and type 2 colonies were translucent, smaller (1 mm), and grew slowly. All colonies could grow at 46°C in the presence of thymine, indicating *prima facie* a *thyA* insertion. However, only about one-third of type 1 colonies were found, using PCR, to carry stable *thyA* insertions while no type 2 colonies carried insertions. We assume that these colonies able to grow on trimethoprim plates must carry some form of compensatory, yet unstable, mutation(s) allowing growth in the presence of the antibiotic.

A second important finding was that for the second genetic transfer, recombinants could be selected only on SMM supplemented with thymine, trimethoprim (6 μg/ml), and Casamino Acids (CAA). If plated directly onto agar lacking CAA, small ($<$1 mm), slow-growing colonies would result, but after reculturing these were found to have lost the *thyB* insertion, as determined by colony PCR. Even in the presence of CAA, all colonies were small, and only about 20% of those growing on trimethoprim (6 μg/ml) carried a stable *thyB* insertion. Work in *E. coli* as well as in *B. subtilis* has shown that disruption of the folate pathway can lead to depletion of key amino acids as well as of purines and pyrimidines (33, 34). Trimethoprim-mediated inactivation of dihydrofolic acid reductase (DHFR) would deplete intracellular levels of tetrahydrofolic acid (THF), methylenetetrahydrofolic acid (MTHF), and dihydrofolate (DHF). In turn, this would affect the reversible interconversion of serine and glycine with THF, a vital reaction in the synthesis of purines and catalyzed by a serine hydroxymethyltransferase ([SHMT] or GlyA) (35, 36). MTHF is also utilized in the final step of the biosynthetic pathways of cysteine and methionine (37), and disruption of the pathway by the *thyA thyB* alleles could introduce a requirement for methionine.

We measured the growth of the *thyA thyB* insertion strain SH14 in SMM supplemented with CAA, adenine, and thymine (Fig. S7). In the presence of trimethoprim (6 μg/ml) growth was optimal, reaching a maximal OD at 600 nm (OD_{600}) of $\sim$2 only in SMM containing CAA, while in medium containing no CAA or carrying only adenine and/or thymine, growth was markedly reduced. In the absence of trimethoprim, growth of SH14 remained weak compared to that of the wild-type strain PY79. Growth, however, was superior to that in the presence of the antibiotic and was restored to normal fitness only in the presence of CAA. Trimethoprim therefore disrupts the folate pathway, significantly reducing strain fitness, and this could not be restored by supplementation with purines or pyrimidines but only with CAA.

Therefore, for the second genetic transfer step (i.e., for construction of the double, *thyA thyB*, insertion), selective medium must provide all amino acids. However, once the strain is constructed, the use of trimethoprim is no longer required, and strains can be cultivated on any medium so long as three criteria are met: first, that the medium contains thymine or thymidine; second, that YE is either absent or at a concentration less than 2 mg/ml; and third, that amino acids are provided in the growth medium.

(iii) Choice of one coat protein anchor. For expression of heterologous proteins on the spore surface, the coat proteins CotB and CotC can be used for both mono- and divalent expression. As expected, when two different spore coat anchors were used, protein expression was higher than when only one anchor was used. So, as shown in Fig. 4A, $TcdA_{26-39}$ levels were higher in SH14 spores carrying *thyA*::*cotB-tcdA*$_{26-39}$ and *thyB*::*cotC-tcdA*$_{26-39}$ insertions than in SH13 spores carrying only a *thyA*::*cotB-tcdA*$_{26-39}$ insertion. Interestingly, using fusion of VP28 to CotB and insertion of this chimera at the *thyA* locus alone (strain AC01) or at both the *thyA* and *thyB* loci (strain AC02) led to higher levels of expression in the latter. This finding requires some consideration since it might be assumed that each spore would carry a defined number of CotB monomers

TABLE 2 Reversion

Culture round[a]	Growth in culture 1 (CFU/ml)		Growth in culture 2 (CFU/ml)	
	DSM + thymine	DSM	DSM + thymine	DSM
1st	2.8×10^8	0	2.1×10^8	0
2nd	1.8×10^8	0	3.4×10^8	0
3rd	1.85×10^8	0	1.73×10^8	0
4th	1.82×10^8	0	1.6×10^8	0
5th	1.54×10^8	0	2.4×10^8	0

[a]Two 25-ml cultures of SH14 (*thyA*::*cotB-tcdA*$_{26-39}$ *thyA*::*cotC-tcdA*$_{26-39}$) were made in DSM with (+) thymine (50 μg/ml). For the first culture round, cells were grown for 24h at 37°C, and samples were analyzed for CFU counts on DSM agar with or without thymine (50 μg/ml). A sample was also used to subculture fresh medium (25 ml), and the process was repeated four times (2nd to 5th rounds).

that could assemble onto the spore surface and that simply increasing the number should not lead to higher levels of incorporation in the coat. These strains, however, would carry an intact *cotB* gene (residing at its normal chromosomal locus), so in cells carrying a *thyA*::*cotB-vp28* insertion (i.e., AC01), we would predict that 50% of displayed CotB proteins would present a wild-type CotB, and 50% would be CotB-VP28. In a double *thyA thyB* insertion strain (AC02), we would predict that ~66% of displayed CotB proteins would present VP28, and ~33% would express wild-type CotB. Although speculative, the stoichiometric ratio of CotB-VP28 and CotB might help explain the ELISA detection data of VP28 as shown in Fig. 4B.

Reversion. An insertion generated by a double-crossover recombination event should be inherently stable, yet there exists the possibility of acquisition of a compensatory suppressor or bypass mutation. To address this, we conducted a straightforward experiment to determine whether upon repeated culture in the absence of any selective pressure, the thymine dependence could be lost. As shown in Table 2 repeated culture and reculture of SH14 (*thyA*::*cotB-tcdA*$_{26-39}$ *thyA*::*cotC-tcdA*$_{26-39}$) yielded no loss of thymine dependency, showing that the insertions were stable and suggesting that the acquisition of compensatory mutations, if they were to occur, must be an extremely rare event.

***In vivo* fate of spores lacking *thyA* and *thyB* in the GI tract.** It has been demonstrated that for *E. coli* to colonize the murine gastrointestinal (GI) tract, synthesis of purines and pyrimidines is necessary (38). This implies that the low levels of purines and pyrimidines that might result from digested food or from spurious lysis of resident gut microbiota might not be sufficient to permit growth of a *B. subtilis thyA thyB* mutant. In humans, the intestinal concentration of thymidine is estimated as 0.075 μM, and in pigs it is ~1.0 μM (39). We gave mice a single oral dose of 2×10^9 spores of SH250 (*thyA*$^+$ *thyB*$^+$ Cm^r) or of SH14 (*thyA*::*cotB-tcdA*$_{26-39}$ *thyA*::*cotC-tcdA*$_{26-39}$) by gavage. Subsequent shedding of heat-resistant spores in freshly voided feces was determined. For SH250, the strain carried a silent insertion of the *cat* gene (chloramphenicol acetyltransferase), so after serial dilution, counts of heat-resistant CFU were determined on chloramphenicol plates. SH14 spores were shed in the feces and could be detected only on agar plates supplemented with thymine, but when spores were plated on agar lacking thymine, no CFU could be detected (Fig. 6). After 10 days the number of SH14 spores being shed in the feces was at levels ($<10^3$ spores/g of feces) at the threshold of detection. These *in vivo* data show that the kinetics of shedding of spores lacking both *thyA* and *thyB* is indistinguishable from that of spores of a wild-type strain. We have confirmed that SH14 spores germinate efficiently and as well as wild-type spores (data not shown). Although it is not possible to definitively determine whether SH14 spores could proliferate in the GI tract, it was possible to show that *thyA thyB* spores that had transited the GI tract were unable to survive in the absence of thymine. Also, there was no evidence of reversion or acquisition of markers that might enable germinated spores to survive.

Utility of spore display. We used three exemplars to demonstrate that spores carrying insertions at the *thyA* and *thyB* loci were suitable for applied purposes, as

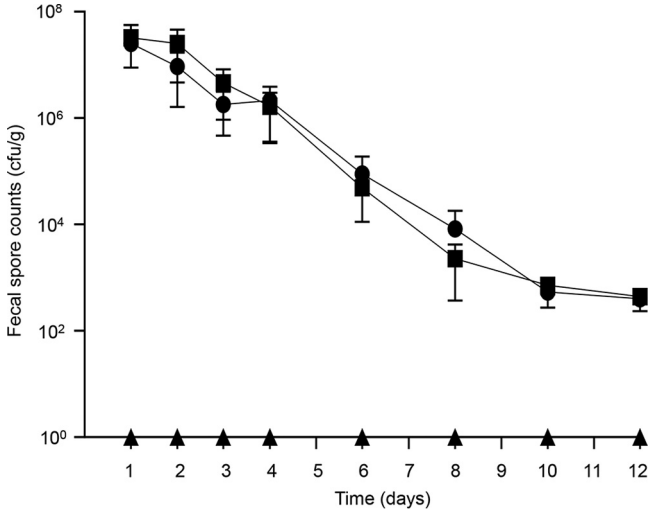

FIG 6 Survival in the murine GI tract. Mice ($n = 5$/group) were given a single oral dose of spores (2×10^9) of SH14 carrying insertions in the *thyA* and *thyB* loci or of SH250, an essentially wild-type strain tagged with a chloramphenicol resistance marker. The numbers of heat-resistant spores in feces was determined for 12 days postdosing. SH14 numbers were determined on agar supplemented with trimethoprim and thymine (■). In the absence of thymine, no CFU were detectable (◆). SH250 CFU counts were determined on agar supplemented with chloramphenicol (●).

vaccine delivery vehicles, for conjugation of proteins to the spore surface, and finally for display of active enzymes. As vaccines we used SH14 spores that express the $TcdA_{26-39}$ antigen of *C. difficile* fused to two spore coat protein anchors, CotB and CotC. SH14 is equivalent to strain PP108 that has previously been shown to confer protection against *C. difficile* infection (CDI) in murine and hamster models of infection (4, 27). We immunized mice with spores of SH14 and PP108 using oral administration, and after a total of four doses, we measured $TcdA_{26-39}$-specific IgG (Fig. 7A) and IgA (Fig. 7B) levels in the serum and feces, respectively. Compared to results with the control groups (naive animals and mice dosed with naked PY79 spores) that exhibited no responses, both PP108 and SH14 spores generated high titers of IgG and IgA, and based on previous work, we would predict that these levels of antibodies would be protective.

For conjugation of proteins to streptavidin displayed on the spore surface, we first biotinylated a rabbit polyclonal $TcdA_{26-39}$-specific antibody and demonstrated that the antibody had conjugated to SH16 spores using immunofluorescence

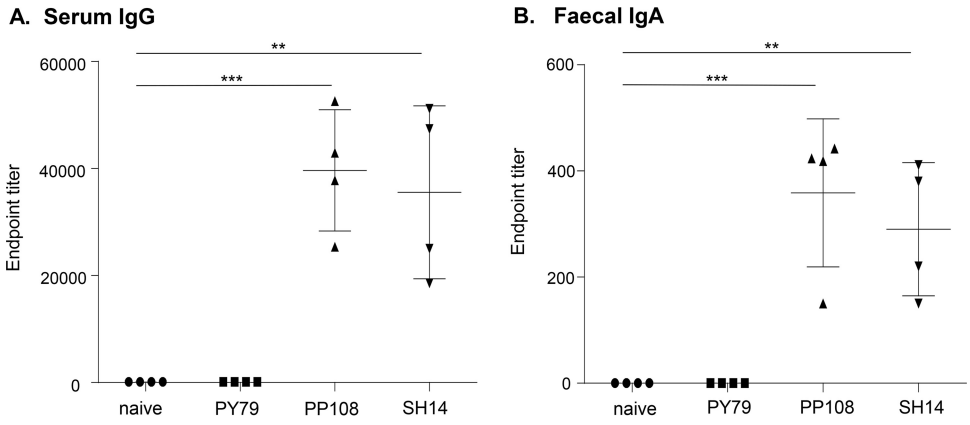

FIG 7 Immunogenicity of SH14 spores expressing the *C. difficile* $TcdA_{26-39}$ antigen. Spores (5×10^{10}) of SH14 (*thyA*::*cotB-tcdA*$_{26-39}$ *thyB*::*cotC-tcdA*$_{26-39}$), PP108 (*amyE*::*cotC-tcdA*$_{26-39}$ *thrC*::*cotB-tcdA*$_{26-39}$), and PY79 (*spo*$^+$) were administered to mice ($n = 4$) by oral gavage on days 1, 14, 35, and 57. Serum IgG and fecal IgA levels specific to $TcdA_{26-39}$ were determined by ELISA, and endpoint titers are shown. **, $P < 0.005$; ***, $P < 0.0002$.

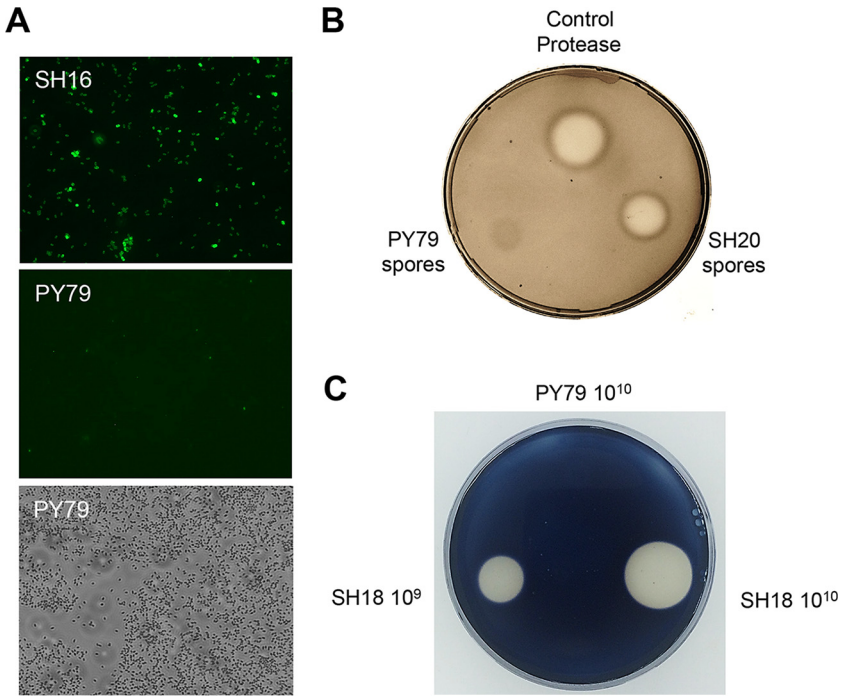

FIG 8 Conjugation of antibodies to spore-displayed streptavidin and enzyme display. (A) Visualization of conjugation of anti-$TcdA_{26-39}$ antibodies to SH16 spores (top panel) using immunofluorescence. As a control, PY79 spores lacking SA failed to conjugate (middle panel). The phase-contrast image confirms the presence of PY79 spores (bottom panel). (B) Activity of subtilisin E displayed on the spore surface. Casein agar was used to visualize protease activity. Plates were spotted with 20 μl of suspensions of SH20 (*thyA*::*aprE thyB*::MCS) or PY79 spores. In each case the inoculum carried 5×10^8 spores. As a positive control, 0.02 U of *Streptomyces griseus* protease (P5147; Sigma) was applied. After 24 h of incubation at 37°C, plates were stained with bromocresol green and incubated for 30 min at RT to reveal zones of degradation. (C) Starch plates stained with Lugol solution. Suspensions of pure spores of SH18 (10^9 and 10^{10} CFU) or PY79 (10^{10} CFU) were spotted on plates and incubated for 48 h. Plates carried three antibiotics to prevent bacterial growth. Spores displaying active amylase produced a zone of clearing.

detection (Fig. 8A), and also confirmed this by using whole-spore ELISA (data not shown). Using these conjugated spores, we asked whether SH16 spores displaying $TcdA_{26-39}$ IgG could remove *C. difficile* toxins from a crude cell-free lysate. As shown in Table 3, incubation of conjugated spores with toxin-containing lysates for just 5 min reduced toxicity by 90%. Interestingly, PY79 spores also had some ability to bind $TcdA_{26-39}$ antibodies and were able to provide a modest reduction (10 to 20%) in toxin activity. Used as an example, this experiment demonstrates that spores might have potential for therapeutic purposes, for example, in the oral administration of antibodies.

Enzymes are commonly included in animal feeds where they improve digestion and nutrition. Proteases and amylases are pertinent examples used here to show that it is possible to display on the spore surface an enzyme that retains activity. Using casein agar, we showed that SH20 spores expressing the alkaline protease, subtilisin E, carried enzymatic activity (Fig. 8B). In liquid suspensions we found that 10^{10} spores of SH20 had ~0.13 units of protease activity. Similarly, SH18 spores expressing amylase were

TABLE 3 Subtraction of *C. difficile* toxins[a]

Mixture	Cytotoxicity (%)
Medium only	0
Toxins only	100
PY79-treated spores + toxins	80–90
SH16-conjugated spores + toxins	10

[a]Cytotoxicity in HT29 cells was measured using crude toxins from *C. difficile* or from the same toxins pretreated with SH16 spores conjugated with a polyclonal antibody to $TcdA_{26-39}$ or with PY79-treated spores.

TABLE 4 PCR primer list

Primer name[a]	5′–3′[b]	Purpose
aprE-F	TATA**AAGCTT**GTGAGAGGCAAAAAGGTATG	Amplification of *aprE*
aprE-R	TATA**GAATTC**TTACTGAGCTGCCGCCTGTAC	
amyE-F	TATA**AAGCTT**GAAACTGCAAACAAATCGAA	Amplification of *amyE*
amyE-R	TATA**GAATTC**TTAATGCGGAAGATAACCGTTTAA	
cotB-F	TATA**GGATCC**ACGGATTAGGCCGTTTG	Amplification of *cotB* and fusion to *aprE* or *amyE*
cotB-R	TATA**AAGCTT**GGATGATTGATCATCTGAAGATTTTAG	
thyA-F	GTCTAAATGGAGAAAAAGTGGATC	Verification of the *thyA* gene with or without insertion
thyA-R	GTTAAGGCCATTGCGTCTAATTC	
thyB-F	GATATTAAAACAAATCCGAACTC	Verification of the *thyB* gene with or without insertion
thyB-R	GTCAGACACATAGAATTG	

[a]F, forward; R, reverse.
[b]Embedded restriction endonuclease sites (HindIII, EcoR1, and BamH1) are in boldface.

found to be able to express active amylase on their surfaces (Fig. 8C), and 10^{10} spores carried ~3 units of amylase activity.

Summary. We have described a straightforward method to contain genetically modified bacterial spores. Our approach is conceptually similar to methods described for *Lactobacillus acidophilus* (40) and *Lactococcus lactis* (39) that rely on the indigenous suicide resulting from a thymineless death. First described in 1954 (22), thymine dependence differs from other auxotrophies in that the absence of thymine is bactericidal, and so bacteria carrying defects in the thymidylate synthase genes cannot accumulate in the environment. *Bacillus* species carry two thymidylate synthase genes (*thyA* and *thyB*) and require inactivation of both loci to achieve complete dependence on thymine. We have demonstrated here a two-step cloning procedure requiring insertional inactivation of first the *thyA* locus and then the *thyB* locus that renders recipient cells thymine dependent. Our approach does not require the introduction of antibiotic resistance markers for selection but, rather, the development of increasing levels of resistance to trimethoprim that arise from successive disruption of the folate pathway. Coupled with the temperature-sensitive phenotype of *thyA thyB* recombinant strains, this method enables both selection and screening of insertions although technically there are a number of constraints that must be considered. We show that the absence of thymine is bactericidal, and we observe no evidence for reversion or suppression despite repeated passage of these strains. Of course, the purpose of our cloning system is to construct *Bacillus* strains able to express proteins for applied purposes, for example, for the expression of heterologous antigens or enzymes. We have used examples here showing that chimeric proteins comprised of a heterologous protein fused to a spore coat protein can be displayed on the spore surface. This has included the delivery of two enzymes (subtilisin E and alpha-amylase), putative vaccine protective antigens, as well as streptavidin, which was used here for conjugation to a polyclonal antibody. It is clear, though, that this system could equally be used for expression of proteins in, or secretion from, the vegetative cell.

MATERIALS AND METHODS

Strains, medium, and general methods. PY79 is a prototrophic strain of *B. subtilis* derived from the type strain 168 (41). PP108 (*amyE*::*cotC-tcdA*$_{26-39}$ *thrC*::*cotB-tcdA*$_{26-39}$) has been described elsewhere (4). SH250 is a prototrophic derivative of PY79 carrying the *cat* gene (encoding resistance to chloramphenicol) inserted at the *amyE* locus. General methods for work with *B. subtilis*, including the two-step transformation procedure, were performed as described previously (42). DSM is a standard medium for growth and sporulation of *B. subtilis* (43). For Western blotting, purified spores were prepared, and 2×10^8 spores were suspended in 40 μl of Bolt LDS buffer (Life Technologies) and incubated at 95°C for 10 min. The spore suspension was centrifuged (10 min at 18,000 $\times$ *g*), and 20 μl of supernatant was run on a 12% SDS-PAGE gel.

Primers. DNA primers used for PCR amplification are shown in Table 4.

pThy vectors. pThyA (4,274 bp) carried a 1,910-bp segment comprising the left (900 bp) and right (950 bp) arms of the *B. subtilis thyA* gene surrounding a multiple cloning site (MCS) cloned into pMA-RQ (2,556 bp; Genscript, USA). Both arms carried additional proximal and distal DNA sequences adjacent to *thyA*. Similarly, pThyB1 (4,973 bp) carried a 2,057-bp segment comprising the left (900 bp) and right (1.1 kb) arms of the *B. subtilis thyB* gene surrounding an MCS cloned into pBluescript SK(+) (2,958 bp).

Plasmids carried the *bla* gene, and the nucleotide sequences of the *thyA* and *thyB* segments are given in Table S1 in the supplemental material and shown schematically in Fig. 1. pThyA and pThyB plasmids were constructed which carried chimeric genes inserted at the MCSs of each vector. Chimeric genes (not optimized for codon usage) containing an in-frame fusion between the 5′ segment of *B. subtilis cotB* or *cotC* to the *vp26*, *vp28*, *tcdA*$_{26-39}$, or SA gene coding ORF were first synthesized with suitable 5′ and 3′ ends for subcloning in the MCSs of the pThyA and pThyB vectors. For two genes, *aprE* and *amyE*, we amplified fragments from chromosomal DNA templates and then fused them to *cotB*. The *aprE* gene (encoding subtilisin E) was PCR amplified from a *B. subtilis* strain (SG115) in our collection, and the amplified coding segment lacked the N-terminal regions involved in protein secretion (presequence) and activation (prosequence) (44). The *amyE* gene (encoding alpha-amylase) was amplified from a lab strain of *Bacillus amyloliquefaciens* and was cloned devoid of the secretory signal sequence. The gene fusions used for this work are shown in Fig. S1.

Construction of *B. subtilis* strains carrying *thyA* and *thyB* insertions. The procedure developed here consisted of two steps. In the first stage, cells of a wild-type recipient strain (in the work described here the prototrophic strain PY79 was used) were made competent using a two-step transformation procedure described by Dubnau and Davidoff-Abelson (45) and in common use in *Bacillus* labs (45, 46). pThyA plasmids carrying the chimeric gene were linearized with either ApaLI or ScaI digestion, and cells were plated on SMM (42) agar supplemented with thymine (50 μg/ml) and trimethoprim (3 μg/ml). After 72 to 96 h of growth, single colonies were colony purified and assessed for growth at 37°C and 46°C on SMM agar with or without thymine (50 μg/ml) and trimethoprim (3 μg/ml). Cells carrying an insertion at the *thyA* locus could grow at 37°C with or without thymine but were unable to grow at 46°C unless supplemented with thymine. A further verification was to amplify, by PCR, the presence of the chimeric gene from transformants using primers annealing to the *thyA* sequences. Typically, transformation frequencies were about 2×10^3 transformants/μg of competent cells, with about 15 to 20% of colonies carrying the correct insertion (see explanation below). In the second stage, a linearized (ApaL1 or Sca1) pThyB plasmid carrying a chimeric gene was introduced into cells of the *thyA* insertion strain by electroporation. Electroporated cells were plated on SMM containing 0.2% (wt/vol) CAA (SMM-CAA) containing thymine (50 μg/ml) and trimethoprim (6 μg/ml) and incubated at 37°C for 48 h. To confirm the presence of both a *thyA* and a *thyB* insertion, colonies were streaked on SMM-CAA agar with or without thymine (50 μg/ml) and grown at 37°C. Cells carrying two insertions were unable to grow at both 37°C and 46°C unless supplemented with thymine. A final verification was made using PCR primers that amplified the two insertions. Using electroporation, integration frequencies were about 1×10^3 insertions/μg of linear DNA, with ~20% of trimethoprim resistance colonies carrying two insertions (*thyA* and *thyB*).

Electroporation. The procedure used here was modified from established methods (47) for electroporation in *Bacillus* primarily with the use of SOC2 medium that contained no yeast extract. SOC2 medium contains tryptone (2%, wt/vol), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (5 mM), $MgSO_4 \cdot 7H_2O$ (5 mM), and glucose (20 mM). An overnight culture of the strain carrying a *thyA* insertion was subcultured in 25 ml of SOC2 medium (supplemented with 0.5 M sorbitol) to give a starting OD_{600} of 0.2. The culture was grown at 37°C to an OD_{600} of 1.4, cooled on ice for 10 min, and then harvested by centrifugation at 4°C (5,000 $\times$ *g*, 5 min.). Cells were washed four times in ice-cold electroporation solution (0.5 M sorbitol, 0.5 M mannitol, 10% [vol/vol] glycerol) and suspended in 1.6 ml of the same ice-cold solution. The cells were now electrocompetent and ready for immediate use. Cells were kept on ice and used within 30 min although aliquots could be stored at −80°C. Linearized plasmid DNA (1 μl, or ~50 ng) was added to 60 μl of electrocompetent cells, and the mixture was transferred to a prechilled cuvette (1-mm gap width) and incubated for 1.5 min on ice. The cuvette was then placed inside the electroporator (Bio-Rad GenePulser Xcell), and the following parameters were used for electroporation: voltage, 2,100 V, resistance, 200 W; time, 5 ms; number of pulses, 1. After electroporation 1 ml of recovery medium (SOC2 medium containing 0.5 M sorbitol and 0.38 M mannitol) was added to the cuvette, and the mixture was transferred to a 2-ml Eppendorf tube and incubated for 3 h at 37°C, after which cells were serially diluted and plated on appropriate selective medium.

Whole-spore ELISA. An ELISA was used to detect surface-exposed proteins as described previously (48). Microplate wells were coated with 50 μl of a suspension of pure spores (2×10^8 spores/well) and left overnight at 4°C. Plates were blocked with 2% (wt/vol) bovine serum albumin (BSA) for 1 h at 37°C. Rabbit polyclonal antibodies recognizing either the heterologous antigen expressed on the spore surface or the whole *B. subtilis* spore were used as primary antibodies with incubation for 2 h at room temperature (RT). Anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (1:5,000 in phosphate-buffered saline [PBS] plus 0.05% Tween 20) was used as a secondary antibody with 1 h of incubation at RT. TMB (3,3′,5,5′-tetramethylbenzidine) was used as the substrate.

Antibodies. Polyclonal antibodies to VP28 and TcdA$_{26-39}$ were raised in rabbits using four subcutaneous injections (1 mg/dose, every 14 days). Recombinant proteins were complexed with Freund's adjuvant and serum purified using protein A chromatography. VP26 polyclonal antibodies were raised in mice using purified recombinant VP26 (rVP26) protein (four intraperitoneal doses at 14-day intervals; 10 μg/dose, with Freund's adjuvant).

Animal studies. All animal work was performed under UK Home Office project license PPL 70/8276. For immunogenicity studies mice (C57 black, female, 7 weeks old) were dosed with preparations of pure spores of PY79 or SH14. Immune responses to TcdA$_{26-39}$ in serum and fecal samples were determined as described previously (4). For longevity studies, BALB/c mice (female, 7 to 8 weeks old) were used. Mice ($n = 5$) were administered a single dose of pure spores (2×10^9) of SH14 or SH250 by oral gavage. At times thereafter, freshly voided fecal samples were collected (3 to 4 pellets) and homogenized, and for

SH14 feces, serial dilutions were plated on DSM and on DSM agar plates with trimethoprim (6 μg/ml) and thymine (50 μg/ml). SH250 fecal dilutions were plated on DSM plates containing chloramphenicol (5 μg/ml). Individual SH14 colonies were randomly checked for the presence of the *thy* insertions using PCR.

Conjugation to streptavidin. Polyclonal antibodies (rabbit; 100 μg) raised to rTcdA$_{26-39}$ protein were biotinylated using a Lightning-Link Rapid biotin conjugation kit, type A (Innova Biosciences). Purified spores (1×10^9) of strain PY79 or spores expressing CotB-SA (SH16) in 200 μl of PBS were mixed with 1 μg of biotinylated antibody and incubated overnight at 4°C. Spores were then washed four times with PBS and suspended in 1 ml of PBS. A total of $\sim 3 \times 10^8$ conjugated spores were used to coat microplate wells, which were then probed with an anti-rabbit IgG-horseradish conjugate (1:5,000 in PBS plus 0.05% Tween 20) with 1 h of incubation at RT, followed by three washes before TMB (3,3′,5,5′-tetramethylbenzidine) color development. As a control, PY79 spores were taken through the same procedure. For immunofluorescence, 5×10^6 treated spores of SH16 or PY79 were added to microscope slides and allowed to air dry. After three washes with PBS, slides were blocked with PBS containing 2% (wt/vol) BSA plus 0.05% (vol/vol) Tween 20 for 45 min at 37°C. Biotinylated anti-TcdA$_{26-39}$ antibodies (1:300 dilution; 200 μl) were added to slides, incubated for 30 min at RT, and then washed three times with PBS–0.05% (vol/vol) Tween 20. Rabbit fluorescein isothiocyanate (FITC)-conjugated serum (F0382 at a 1:200 dilution; Sigma) was added, and slides were incubated for 30 min at RT. Image analysis was done with an EVOS FL LED microscope.

Toxin subtraction assays. *C. difficile* strain RT176 (*tcdA*$^+$ *tcdB*$^+$) was grown in TY broth (3% [wt/vol] tryptose, 2% [wt/vol] yeast extract, and 0.1% [wt/vol] sodium thioglycolate) for 24 h at 37°C. The cell-free supernatant was filter sterilized and kept at 4°C until assay. The minimum lethal concentration (MLC) of supernatant required to cause 100% toxicity to HT29 cells was determined using a 2-fold dilution and addition of the diluted lysate to HT29 cells, followed by assessment of toxicity using a cell-rounding assay (4). For the assay, 10^9 pure spores of SH16 conjugated to TcdA$_{26-39}$ polyclonal antibodies (see above) were added to 200 μl of 2% McCoy's medium containing the toxin MLCs (typically, a 1/4,000 dilution). The mixture was incubated for 5 min at RT, and then cytotoxicity was assessed using HT29 cells followed by incubation for 24 h. As a control, PY79 spores that had been mixed with TcdA$_{26-39}$ antibodies (see above) were used in parallel.

Enzyme activity. Protease activity was determined using a universal protease assay using casein as a substrate, as described by Sigma-Aldrich (https://www.sigmaaldrich.com/life-science/learning-center/life-science-video/universal-protease.html). Casein agar was 1% (wt/vol) casein, 1% (wt/vol) skim milk, and 1.2% (wt/vol) agar (technical, no. 2; Oxoid). Amylase activity in liquid was measured as described previously (49). Production of active amylase was tested by applying suspensions of spores (volume of 20 μl) to agar plates carrying only soluble starch (1%, wt/vol), beef extract (0.3%, wt/vol), and three antibiotics (10 μg/ml trimethoprim, 30 μg/ml chloramphenicol, and 30 μg/ml erythromycin). Antibiotics were used to prevent any bacterial growth on the plates, ensuring that activity arose from dormant spores only. Plates were incubated for 48 h at 37°C, after which the plate was flooded with Lugol solution (Sigma) for 2 min to reveal zones of starch degradation. Units of amylase activity were determined as described by Bernfield (50).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02334-17.

SUPPLEMENTAL FILE 1, PDF file, 7.5 MB.

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REFERENCES

1. Nicholson WJ, Munakata N, Horneck G, Melosh HJ, Setlow P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64:548–572. https://doi.org/10.1128/MMBR.64.3.548-572.2000.
2. Pan JG, Kim EJ, Yun CH. 2012. *Bacillus* spore display. Trends Biotechnol 30:610–612. https://doi.org/10.1016/j.tibtech.2012.09.005.
3. Duc LH, Hong HA, Fairweather N, Ricca E, Cutting SM. 2003. Bacterial spores as vaccine vehicles. Infect Immun 71:2810–2818. https://doi.org/10.1128/IAI.71.5.2810-2818.2003.
4. Permpoonpattana P, Hong HA, Phetcharaburanin J, Huang JM, Cook J, Fairweather NF, Cutting SM. 2011. Immunization with *Bacillus* spores expressing toxin A peptide repeats protects against infection with *Clostridium difficile* strains producing toxins A and B. Infect Immun 79:2295–2302. https://doi.org/10.1128/IAI.00130-11.
5. Lee S, Belitsky BR, Brinker JP, Kerstein KO, Brown DW, Clements JD, Keusch GT, Tzipori S, Sonenshein AL, Herrmann JE. 2010. Development of a *Bacillus subtilis*-based rotavirus vaccine. Clin Vaccine Immunol 17:1647–1655. https://doi.org/10.1128/CVI.00135-10.
6. Fu LL, Li WF, Du HH, Dai W, Xu ZR. 2008. Oral vaccination with envelope protein VP28 against white spot syndrome virus in *Procambarus clarkii* using *Bacillus subtilis* as delivery vehicles. Lett Appl Microbiol 46:581–586. https://doi.org/10.1111/j.1472-765X.2008.02355.x.
7. Nguyen AT, Pham CK, Pham HT, Pham HL, Nguyen AH, Dang LT, Huynh HA, Cutting SM, Phan TN. 2014. *Bacillus subtilis* spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against white spot syndrome. FEMS Microbiol Lett 358:202–208. https://doi.org/10.1111/1574-6968.12546.
8. Ning D, Leng X, Li Q, Xu W. 2011. Surface-displayed VP28 on *Bacillus subtilis* spores induce protection against white spot syndrome virus in crayfish by oral administration. J Appl Microbiol 111:1327–1336. https://doi.org/10.1111/j.1365-2672.2011.05156.x.
9. Valdez A, Yepiz-Plascencia G, Ricca E, Olmos J. 2014. First *Litopenaeus*

vannamei WSSV 100% oral vaccination protection using CotC::Vp26 fusion protein displayed on *Bacillus subtilis* spores surface. J Appl Microbiol 117:347–357. https://doi.org/10.1111/jam.12550.
10. EFSA. 2008. The maintenance of the list of QPS microorganisms intentionally added to food or feed. EFSA J 923:1–48.
11. Hong HA, Duc le H, Cutting SM. 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 29:813–835. https://doi.org/10.1016/j.femsre.2004.12.001.
12. Amalaradjou MA, Bhunia AK. 2013. Bioengineered probiotics, a strategic approach to control enteric infections. Bioengineered 4:379–387. https://doi.org/10.4161/bioe.23574.
13. Sorokulova IB, Beliavskaia VA, Masycheva VA, Smirnov VV. 1997. Recombinant probiotics: problems and prospects of their use for medicine and veterinary practice. Vestn Ross Akad Med Nauk 3:46–49. (In Russian.)
14. Potot S, Serra CR, Henriques AO, Schyns G. 2010. Display of recombinant proteins on *Bacillus subtilis* spores, using a coat-associated enzyme as the carrier. Appl Environ Microbiol 76:5926–5933. https://doi.org/10.1128/AEM.01103-10.
15. Nguyen VA, Huynh HA, Hoang TV, Ninh NT, Pham AT, Nguyen HA, Phan TN, Cutting SM. 2013. Killed *Bacillus subtilis* spores expressing streptavidin: a novel carrier of drugs to target cancer cells. J Drug Target 21:528–541. https://doi.org/10.3109/1061186X.2013.778262.
16. Shimotsu H, Henner DJ. 1986. Construction of a single copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene 43:85–94. https://doi.org/10.1016/0378-1119(86)90011-9.
17. Guerout-Fleury AM, Frandsen N, Stragier P. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene 180:57–61. https://doi.org/10.1016/S0378-1119(96)00404-0.
18. Bloor AE, Cranenburgh RM. 2006. An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. Appl Environ Microbiol 72:2520–2525. https://doi.org/10.1128/AEM.72.4.2520-2525.2006.
19. Iwanicki A, Piatek I, Stasilojc M, Grela A, Lega T, Obuchowski M, Hinc K. 2014. A system of vectors for *Bacillus subtilis* spore surface display. Microb Cell Fact 13:30. https://doi.org/10.1186/1475-2859-13-30.
20. Nicholson WL. 2002. Roles of *Bacillus* endospores in the environment. Cell Mol Life Sci 59:410–416. https://doi.org/10.1007/s00018-002-8433-7.
21. Mauriello EM, Cangiano G, Maurano F, Saggese V, De Felice M, Rossi M, Ricca E. 2007. Germination-independent induction of cellular immune response by *Bacillus subtilis* spores displaying the C fragment of the tetanus toxin. Vaccine 25:788–793. https://doi.org/10.1016/j.vaccine.2006.09.013.
22. Cohen SS, Barner HD. 1954. Studies on unbalanced growth in *Escherichia coli*. Proc Natl Acad Sci U S A 40:885–893. https://doi.org/10.1073/pnas.40.10.885.
23. Goulian M, Bleile BM, Dickey LM, Grafstrom RH, Ingraham HA, Neynaber SA, Peterson MS, Tseng BY. 1986. Mechanism of thymineless death. Adv Exp Med Biol 195:89–95. https://doi.org/10.1007/978-1-4684-1248-2_15.
24. Ahmad SI, Kirk SH, Eisenstark A. 1998. Thymine metabolism and thymineless death in prokaryotes and eukaryotes. Annu Rev Microbiol 52:591–625. https://doi.org/10.1146/annurev.micro.52.1.591.
25. Rolfe R. 1967. On the mechanism of thymineless death in *Bacillus subtilis*. Proc Natl Acad Sci U S A 57:114–121. https://doi.org/10.1073/pnas.57.1.114.
26. Neuhard J, Price AR, Schack L, Thomassen E. 1978. Two thymidylate synthetases in *Bacillus subtilis*. Proc Natl Acad Sci U S A 75:1194–1198. https://doi.org/10.1073/pnas.75.3.1194.
27. Hong HA, Hitri K, Hosseini S, Kotowicz N, Bryan D, Mawas F, Wilkinson AJ, van Broekhoven A, Kearsey J, Cutting SM. 2017. Mucosal antibodies to the C terminus of toxin A prevent colonization of *Clostridium difficile*. Infect Immun 85:e01060-16. https://doi.org/10.1128/IAI.01060-16.
28. Walsh GA, Power RF, Headon DR. 1993. Enzymes in the animal-feed industry. Trends Biotechnol 11:424–430. https://doi.org/10.1016/0167-7799(93)90006-U.
29. Tran TT, Mamo G, Mattiasson B, Hatti-Kaul R. 2010. A thermostable phytase from *Bacillus* sp. MD2: cloning, expression and high-level production in *Escherichia coli*. J Ind Microbiol Biotechnol 37:279–287. https://doi.org/10.1007/s10295-009-0671-3.
30. Kinoshita N, Akiyoshi H, Endo H. 1969. Isolation and characterization of substance in yeast extract which inhibits growth of thymine-less strains of *Escherichia coli*. J Gen Microbiol 59:393–400. https://doi.org/10.1099/00221287-59-3-393.
31. Blanchard KC. 1941. The isolation of *p*-aminobenzoic acid from yeast. J Biol Chem 140:919–926.
32. Davis BD. 1951. Inhibition of *Escherichia coli* by p-aminobenzoic acid and its reversal by *p*-hydroxybenzoic acid. J Exp Med 94:243–254. https://doi.org/10.1084/jem.94.3.243.
33. Amyes SG, Smith JT. 1974. Trimethoprim action and its analogy with thymine starvation. Antimicrob Agents Chemother 5:169–178. https://doi.org/10.1128/AAC.5.2.169.
34. Stepanek JJ, Schakermann S, Wenzel M, Prochnow P, Bandow JE. 2016. Purine biosynthesis is the bottleneck in trimethoprim-treated *Bacillus subtilis*. Proteomics Clin Appl 10:1036–1048. https://doi.org/10.1002/prca.201600039.
35. Schirch L. 1982. Serine hydroxymethyltransferase. Adv Enzymol Relat Areas Mol Biol 53:83–112.
36. Ponce-de-Leon MM, Pizer LI. 1972. Serine biosynthesis and its regulation in *Bacillus subtilis*. J Bacteriol 110:895–904.
37. Ferla MP, Patrick WM. 2014. Bacterial methionine biosynthesis. Microbiology 160:1571–1584. https://doi.org/10.1099/mic.0.077826-0.
38. Vogel-Scheel J, Alpert C, Engst W, Loh G, Blaut M. 2010. Requirement of purine and pyrimidine synthesis for colonization of the mouse intestine by *Escherichia coli*. Appl Environ Microbiol 76:5181–5187. https://doi.org/10.1128/AEM.00242-10.
39. Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B, Cox E, Remon JP, Remaut E. 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. Nat Biotechnol 21:785–789. https://doi.org/10.1038/nbt840.
40. Fu X, Xu JG. 2000. Development of a chromosome-plasmid balanced lethal system for *Lactobacillus acidophilus* with *thyA* gene as selective marker. Microbiol Immunol 44:551–556. https://doi.org/10.1111/j.1348-0421.2000.tb02533.x.
41. Zeigler DR, Pragai Z, Rodriguez S, Chevreux B, Muffler A, Albert T, Bai R, Wyss M, Perkins JB. 2008. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. J Bacteriol 190:6983–6995. https://doi.org/10.1128/JB.00722-08.
42. Harwood CR, Cutting SM. 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, England.
43. Nicholson WL, Setlow P. 1990. Sporulation, germination and outgrowth, p 391–450. In Harwood CR, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, UK.
44. Ikemura H, Takagi H, Inouye M. 1987. Requirement of pro-sequence for the production of active subtilisin E in *Escherichia coli*. J Biol Chem 262:7859–7864.
45. Dubnau D, Davidoff-Abelson R. 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. J Mol Biol 56:209–221.
46. Cutting SM, Vander-Horn PB. 1990. Genetic analysis, p 27–74. *In* Harwood CR, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, England.
47. Sun QY, Ding LW, He LL, Sun YB, Shao JL, Luo M, Xu ZF. 2009. Culture of *Escherichia coli* in SOC medium improves the cloning efficiency of toxic protein genes. Anal Biochem 394:144–146. https://doi.org/10.1016/j.ab.2009.07.023.
48. Permpoonpattana P, Phetcharaburanin J, Mikelsone A, Dembek M, Tan S, Brisson MC, La Ragione R, Brisson AR, Fairweather N, Hong HA, Cutting SM. 2013. Functional characterization of *Clostridium difficile* spore coat proteins. J Bacteriol 195:1492–1503. https://doi.org/10.1128/JB.02104-12.
49. Harwood CR, Coxon RD, Hancock IC. 1990. The *Bacillus* cell envelope and secretion, p 327–390. In Harwood CR, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, England.
50. Bernfield P. 1955. Amylases a and b. Methods Enzymol 1:149–158. https://doi.org/10.1016/0076-6879(55)01021-5.
51. CLSI. 2010. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline, 2nd ed. CLSI document M45-A2. Clinical Laboratory and Standards Institute, Wayne, PA.