The *thyA* gene from *Bacillus subtilis* exhibits similarity with the phage ϕ 3T thymidylate synthase gene

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The gene encoding thymidylate synthase A (*thyA*) was cloned from a genomic library of *Bacillus subtilis* 168. The sequence of *thyA* was found to be highly similar to that of the phage ϕ 3T thymidylate synthase gene. This similarity is, however, limited to about 862 nucleotides, spanning the coding region and the adjacent 3' region. The flanking sequences are not related. An integrative plasmid containing a DNA fragment with a deletion within the coding region of *thyA* was constructed and used to replace the chromosomal *thyA* gene. Transformants unable to grow without thymidine at 47 °C were obtained. Genetic and physical mapping techniques were used to show that the cloned DNA fragment harbouring Δ *thyA* was integrated at 168° on the *B. subtilis* chromosome.

Keywords: *Bacillus subtilis*, genome sequencing, gene mapping, thymidylate synthase, *thyA*

INTRODUCTION

Unlike all other organisms studied so far, *Bacillus subtilis* grown at temperatures below 37 °C expresses two thymidylate synthases (EC 2.1.1.45), TSaseA and TSaseB. Both enzymes catalyse the synthesis of dTMP from dUMP and are encoded by the *thyA* and *thyB* genes, respectively. These genes are unlinked and map at distant regions on the *B. subtilis* chromosome (Wilson *et al.*, 1966). The *thyA* gene product enables the cells to grow without thymidine at 46 °C, whereas TSaseB directs thymidylate biosynthesis only at temperatures at and below 37 °C (Neuhard *et al.*, 1978). The *thyB* gene maps at about 200° on the *B. subtilis* chromosome (Myoda *et al.*, 1984). Structural characterization of the cloned *thyB* region revealed a *thyB*-*dfrA* operon comprising two ORFs, encoding TSaseB and dihydrofolate reductase, which overlap by one nucleotide and are transcribed from a single promoter (Iwakura *et al.*, 1988).

The *thyA* gene specifying TSaseA has been mapped by PBS1 transduction at 168° close to *citB* on the *B. subtilis* chromosome (Neuhard *et al.*, 1978), but cloning and structural characterization of the gene have not yet been reported. However, hybridization experiments performed

with chromosomal DNA from *B. subtilis* and DNA from *B. subtilis* group III phages revealed close similarity between phage *thyP* genes and *Bacillus thyA* (Stroynowski, 1981a). The high degree of similarity between the *thyP3* gene encoding phage ϕ 3T TSase and *thyA* allows the transformation of *thyA* mutants by DNA from phage ϕ 3T (Tucker, 1969). The phage *thyP3* gene was shown to integrate at two genetically distinct sites. If the *thyP3* gene is contained on a small DNA fragment, the bacterial *thyA* gene is usually replaced by *thyP3*. If the *thyP3* gene lies on a larger fragment, it replaces the region of the SP β prophage, which does not contain *thyP* sequences but exhibits homology to the ϕ 3T regions flanking it (Stroynowski, 1981b). The sequence of the *thyP3* has been reported by Kenny *et al.* (1985).

This paper reports the sequence of the *thyA* gene cloned from chromosomal DNA of *B. subtilis* and discusses the evolutionary origin of the related genes *thyA* and *thyP3*.

METHODS

Bacterial strains and plasmids. A list of strains and plasmids used in this study is given in Table 1. Strains were grown in either LB medium or M9 medium supplemented with casamino acids and thiamine. Additional supplements were added as required. Yeast clone YAC 12-5 was grown in selective URA medium (Azevedo *et al.*, 1993) at 30 °C for at least 2 d.

General techniques. Agarose gel electrophoresis, digestion of DNA with restriction enzyme, ligation, transformation of *Escherichia coli* and Southern hybridization were all standard

Abbreviations: TSase, thymidylate synthase; TSSA medium, Tris-Spizizen salts medium.

The EMBL accession number for the nucleotide sequence reported in this paper is X78560.

Table 1. Strains and plasmids

Strain	Characteristics	Source/Reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	BGSC, Ohio, USA
QB943	<i>ilvA1 pyrD1 thyA1 thyB1 trpC2</i>	BGSC, Ohio, USA
1A634	Em ^R <i>zei82::Tn917</i>	BGSC, Ohio, USA
MW10	<i>his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV</i>	Wolf <i>et al.</i> (1995)
NHT2	<i>thyA thyB trpC2</i>	This work
NHT3	<i>his nprR2 bglT eglS102 thyA⁺ ΔthyA500 Em^R</i>	This work
NHT4	<i>his nprR2 bglT eglS102 ΔthyA500</i>	This work
NHT5	<i>his bglT eglS102</i>	This work
<i>E. coli</i>		
DH5α	F ⁻ <i>endA1 hsdR17 (r⁻m⁺) supE44 thi-1 recA1 gyrA96 relA1 Δ(argF⁻ lacZYA)</i>	Laboratory stock
NHT1	U169 <i>φ80d lacZΔM15 thyA</i> , DH5α derivative	This work
YAC		
YAC 12-5	130 kb YAC insert with <i>thyA</i>	Azevedo <i>et al.</i> (1993)
Plasmid		
pHP13	Cm ^R Em ^R	Haima <i>et al.</i> (1987)
Bluescript SK ⁺	Ap ^R	Mead <i>et al.</i> (1986)
pHV60	Ap ^R Tc ^R Cm ^R , pBR322-pC194 derivative	Michel <i>et al.</i> (1983)
pNHT1	pHP13 with a 3.6 kb <i>BclI</i> fragment containing <i>thyA</i>	This work
pNHT2	Bluescript with a 3.6 kb <i>BclI</i> fragment containing <i>thyA</i>	This work
pNHT3	pHP13 with a 3.1 kb <i>BclI</i> fragment containing <i>ΔthyA</i>	This work
pNHT4	pHV60 with a 3.1 kb <i>BclI</i> fragment containing <i>ΔthyA</i>	This work

procedures (Sambrook *et al.*, 1989). DNA fragments hybridizing with the DNA probes used were detected with a digoxigenin kit from Boehringer Mannheim. Competent *B. subtilis* cells were prepared as described by Cutting & Vander Horn (1990). *B. subtilis* protoplasts were transformed by the method of Chang & Cohen (1979).

Isolation of *thy* mutants and strain construction. DH5α cells (10⁷) were plated onto M9 medium supplemented with aminopterin (0.6 mg ml⁻¹), trimethoprim (15 mg ml⁻¹) and thymidine (50 mg ml⁻¹). Clones resistant to aminopterin and trimethoprim were grown for 90 h at 30 °C. The colonies were streaked on M9 agar with and without thymidine. Strain NHT1 (*thy*) which is unable to grow without thymidine, was selected. Recipient strain NHT2 (*thyA1 thyB1 trpC2*) used for expression of the *thyA* gene was constructed from *B. subtilis* QB943 by conjugation (Cutting & Vander Horn, 1990).

Cloning and sequencing of the *thyA* gene. Chromosomal DNA isolated from *B. subtilis* 168 was digested with *BclI* and sized by agarose gel electrophoresis. DNA fragments of 2–5 kb in size were ligated into pHP13 linearized with *BamHI* and dephosphorylated with alkaline phosphatase. *E. coli* NHT1 cells transformed with the ligation mixture were screened for recombinant plasmids using the blue/white selection system with LB agar containing X-Gal, IPTG and Cm (5 mg ml⁻¹). Transformants exhibiting the white phenotype were replicated onto M9 agar with and without thymidine. Plasmid DNA from clones growing without thymidine was isolated and used to transform *B. subtilis* NHT2 (*thyA thyB trpC2*). At 47 °C, 18 transformants were able to grow without thymidine in Tris-Spizizen salts (TSSA) minimal medium (Cutting & Vander Horn, 1990) supplemented with tryptophan (20 mg ml⁻¹). The clones were characterized by their sensitivity to trimethoprim

(10 mg ml⁻¹) in thymidine-containing TSSA agar. Plasmid DNA from one selected clone was isolated and found to contain an insert of 3.6 kb (pNHT1). DNA sequencing was done by the dideoxy-chain termination method (Sanger *et al.*, 1977). Fragments from plasmid pNHT1 were obtained by using convenient restriction sites and subcloned into appropriately digested and dephosphorylated pHP13 and Bluescript SK⁺ vector plasmids (Fig. 1). Double-stranded recombinant plasmid DNAs were used as templates.

Deletion of *thyA* and integration into the *B. subtilis* chromosome. Plasmid pNHT1 was cut at the unique *XbaI* site and progressively digested with *Bal31* nuclease. The ligation products were used to transform *E. coli* DH5α cells, and plasmid pNHT3, containing a 500 bp deletion within the coding region of *thyA*, was obtained (Fig. 3). The 3.1 kb DNA fragment containing the deleted *thyA* gene was recloned into integration vector pHV60 and designated pNHT4. Plasmid pNHT4 was used to transform *B. subtilis* MW10. Primary transformants selected by their Cm^R phenotype were found to be prototrophic. After five passages in LB medium containing thymidine (50 mg ml⁻¹) and without antibiotic selection, Cm^S clones, unable to grow without thymidine at 47 °C, were obtained. The clones were found to be resistant to trimethoprim.

Isolation of YAC DNA. DNA from *Saccharomyces cerevisiae* clone 12-5 was prepared following the method of P. Serron (INRA, Jouy-en-Josas, France; unpublished). High molecular mass yeast DNA was separated on 1.0% (w/v) low melting point agarose (SeaKem) gel by pulse field electrophoresis for 20 h at 200 V. After electrophoresis, the YAC DNA band was cut out and the agarose removed by treatment with agarase (Boehringer Mannheim) following the procedure recommended by the supplier.

Fig. 1. Strategy for sequencing the *thyA* region. The 2.2 kb *BclI*–*EcoRV* fragment used for preparing subclones for sequencing is indicated by a heavy line. Positions of selected restriction sites are indicated. The arrows indicate the partial sequences determined by the dideoxy method.

Mapping procedures. Mapping of the *thyA* deletion was performed by PBS1 transduction (Cutting & Vander Horn, 1990) with lysates prepared from a set of strains with silent Tn917 insertions (Vandeyar & Zahler, 1986). The recipient was strain NHT4 ($\Delta thyA \Delta eglS \Delta hglTS$) and *Thy*⁺ transductants were scored for hydrolysis of CM-cellulose and resistance to Em.

RESULTS

Identification of a *thyA* clone by complementation of *thyA* mutants

A gene library of *B. subtilis* 168 constructed in the *E. coli*/*B. subtilis* shuttle vector pHP13 was used to transform *E. coli* NTH1 (*thy*) cells. Plasmid DNA from clones able to grow in minimal medium without thymidine was isolated and used to transform *B. subtilis* NHT2 (*thyA1 thyB1*). Transformants were selected by their ability to grow in minimal medium without thymidine at 47 °C. The clones were characterized further by their sensitivity to trimethoprim (10 mg ml⁻¹) in thymidine-containing TSSA agar.

As noted in the Introduction, the *B. subtilis thyA* gene directs thymidylate biosynthesis at high temperatures. Temperature-resistant thymidylate biosynthesis and enhanced trimethoprim sensitivity were used for scoring the *ThyA*⁺ phenotype.

Plasmid pNHT1 DNA was isolated from one selected clone. Single and double digests with a number of restriction endonucleases yielded the restriction map of the 3.6 kb *BclI* insert of plasmid pNHT1 (Fig. 1).

Sequence analysis

The entire sequence of a 2.2 kb fragment containing the *thyA* gene of *B. subtilis* 168 was determined on both strands and the sequencing strategy is depicted in Fig. 1. Compilation of the sequence data revealed an ORF comprising 836 nucleotides and encoding a 279 residue enzyme, deduced by direct translation. The molecular mass of the deduced gene product is 32807 Da.

A Shine–Dalgarno sequence, AGaAGGA, is evident 12 nucleotides upstream of the putative AUG start codon. Putative –10, TAAArT, and –35, TTGcgA, regions separated by 17 nucleotides are located 5' of the start codon. A potential stem–loop structure was identified about 100 nucleotides downstream of the translation stop

signal, TAA. The free energy value of this putative ρ -independent termination signal was calculated as ΔG (25 °C) = –17.4 kcal (–72.8 kJ).

An amino acid sequence alignment between the translated sequence of the ORF designated *thyA* and that of the *thyP3* gene of phage $\phi 3T$ (Kenny *et al.*, 1985) revealed extensive similarity between the two products. Out of a total of 279 residues, 271 were found to be identical (97.1 %). In contrast, only 38.3 % identity was found with the amino acid sequence encoded by the *B. subtilis thyB* gene (Iwakura *et al.*, 1988). Comparison of nucleotide sequences between *thyA* and phage $\phi 3T$ DNA indicates extensive identity (96.3 %) within a sequence of 862 bp comprising the entire coding region and a short 3' stretch. Remarkably, neither the 5' regulatory sequences nor the inverted repeat structure located 3' of the coding region are conserved in the $\phi 3T$ DNA. Moreover, no significant DNA homology was detected between phage and *Bacillus* DNA in the regions flanking the TSase gene (Fig. 2).

To confirm that the cloned 3.6 kb *BclI* fragment containing *thyA* was derived from *B. subtilis* chromosomal DNA, we used different DNA fragments from the central portion of *thyA* (nucleotides 833–1198) as well as from the 5' (nucleotides 12–361) and 3' (nucleotides 2203–3000) flanking regions to probe *BclI*-digested chromosomal DNA from *B. subtilis* 168 and its derivatives MW10, NHT2 and NHT5. All probes recognized the 3.6 kb *BclI* fragment, suggesting that it was indeed *B. subtilis* DNA (Fig. 3).

Mapping of the cloned *thyA* gene

To facilitate chromosomal mapping of the cloned DNA fragment, permanent deletions within the *thyA* gene were created by gene replacement (Stahl & Ferrari, 1984). To construct an integrative plasmid carrying a defective *thyA* gene, plasmid pNHT2 containing the 3.6 kb *BclI* fragment carrying the entire *thyA* sequence was linearized with *XbaI*. The linearized plasmid was treated with *Bal31* and religated. Plasmid pNHT3, carrying a deletion of about 500 bp within the *thyA* gene ($\Delta thyA500$) was used to reclone the deleted insert into integrative vector pHV60.

Transformation of MW10 with the integrative plasmid pNHT4 carrying the deleted *thyA* gene with the flanking sequences ($\Delta thyA500$) yielded transformants with a Cm^R

Fig. 2. Nucleotide sequence of the *Bcl*I-*Xba*I fragment and the predicted amino acid sequence encoded by *thyA*. The Shine-Dalgarno sequence (SD) and putative -35/-10 promoter boxes are underlined. Convergent horizontal arrows indicate a stem-loop structure. Short vertical arrows label the sequence between 314 and 1372 used in alignment with the *thyP3* gene and its surrounding sequences. Identical nucleotides and amino acid residues are indicated by bold letters. The sequence between 459 and 1319, exhibiting 96.3% identity with phage ϕ 3T DNA, is labelled by filled triangles.

Fig. 3. Southern hybridization of *B. subtilis* chromosomal DNA and DNA from YAC 12-5 with probes prepared from the *thyA* gene and its flanking regions. Chromosomal DNA (1–2 mg) digested with appropriate restriction enzymes was subjected to agarose gel electrophoresis, transferred to a nylon membrane and hybridized to different fragments isolated from pNHT2 as described in Methods. (a) Cross-hybridization with the 0.8 kb *EcoRV*–*HindIII* fragment from the 3' flanking region of *thyA*. Lanes: 1, *B. subtilis* 168, *BclI*; 2, pNHT1, *EcoRI*–*SallI*; 3, MW10, *BclI*; 4, NHT3, *BclI*; 5, NHT4, *BclI*; 6, NHT5, *BclI*; 7, YAC12-5, *BclI*. (b) Cross-hybridization with the 365 bp *Sau3A*-fragment derived from the central portion of *thyA* (nt 742–1197). Lanes 1, *B. subtilis* 168, *BclI*; 2, pNHT1, *EcoRI*–*SallI*; 3, YAC12-5, *BclI*; 4, MW10, *BclI*; 5, NHT4, *BclI*; 6, NHT5, *BclI*; 7, NHT4, *BclI*–*EcoRV*; 8, *B. subtilis* 168, *BclI*–*EcoRV*; 9, NHT5, *BclI*–*EcoRV*; 10, NHT3, *BclI*–*EcoRV*. Cross-hybridization with the 349 bp *Sau3A* fragment derived from the 5' flanking region of *thyA* (nucleotides 12–361) yielded the same hybridizing fragments as obtained with the central 365 bp fragment (not shown). Note: this picture has been electronically imaged.

ThyA⁺ phenotype. Southern analysis revealed that this phenotype may result from a single cross-over event between homologous regions in which Δ *thyA* and *thyA*⁺ are separated by the integrated plasmid DNA sequence. Two DNA sequences hybridizing with the cloned *thyA* gene were detected in strain NHT3 (see Fig. 3b, lane 10) indicating the presence of both the deleted and wild type *thyA* gene copies on its chromosome. To excise the plasmid DNA, Cm^R ThyA⁺ transformants were cultivated for about 10 generations in antibiotic-free medium. One cured Cm^S clone, designated NHT4 (*thyA*), with a stable ThyA[–] phenotype was selected. As verified by Southern hybridization, this clone contains only the Δ *thyA*500 allele (Fig. 3).

Strain NHT4 (Δ *thyA*500) was used as host in phage PBS1-mediated transduction. With lysates prepared from 1A637 (λ ei82::Tn917) containing a silent Tn917 insertion at 177°, 29% cotransduction of the *thyA* marker was observed. The integrated Δ *thyA*500 gene was found to be closely linked to the *eglS* marker (170°, Wolf *et al.*, 1995).

Lysates from phage PBS1 grown on MW10 (*thyA*⁺ Δ *eglS*) were used to infect NHT4 (*thyA* *eglS*⁺). In this case 40% cotransduction was observed between the markers, suggesting that Δ *thyA*500 was integrated at the *thyA* locus at about 168° on the *B. subtilis* chromosome (Neuhard *et al.*, 1978).

In addition, DNA prepared from YAC 12-5 carrying the *thyA* locus (Azevedo *et al.*, 1993) was used to probe DNA fragments containing either the coding region or the adjacent 5' and 3' non-coding regions of the cloned putative *thyA* gene. Southern hybridization (Fig. 3) revealed that the cloned insert from pNHT1 is located on the 130 kb *B. subtilis* DNA segment carried by YAC 12-5, thus confirming the results obtained by transduction.

DISCUSSION

A DNA fragment restoring the ThyA⁺ phenotype in *E. coli* and *B. subtilis* was isolated from a plasmid library of *B. subtilis* 168. Several lines of evidence suggest that it contains the *thyA* gene located at around 168° on the *B. subtilis* chromosome. (i) Transformation of *thyA* and *thyB* mutants of *B. subtilis* with the cloned DNA fragment enabled them to grow at 47 °C in minimal medium without thymidine. This property is characteristic for strains carrying the *thyA* wild type gene. (ii) Transformants exhibited the same enhanced sensitivity to antifolates as *thyA*⁺ strains. (iii) All DNA fragments containing either *thyA* or its flanking regions hybridized with *B. subtilis* chromosomal DNA and that of YAC 12-5 carrying a 130 kb fragment with the *thyA* locus of *B. subtilis*. (iv) A gene replacement technique was used to integrate the cloned DNA fragment deleted within the coding region into the chromosome. The phenotype of the resulting strain was found to be ThyA[–] and the mutation introduced was mapped at the *thyA* locus at 168° by PBS1-mediated transduction.

The sequence of the ORF shows only limited similarity with *thyB* but is 96% identical with the phage ϕ 3T TSase (*thyP3*) gene. It has been shown previously that the TSase genes *thyA* and *thyP3* are related. Results of Southern hybridization and the ability of *thyP3* DNA to recombine with *thyA* sequences suggest that the genes might have evolved from a common ancestor. Moreover, the phenotype of *B. subtilis thyA* strains transformed with *thyP3* consistently resembles that of ThyA⁺ strains (sensitivity to trimethoprim in the presence of thymine and to high temperature, Stroynowski, 1981b).

The homology between *B. subtilis thyA* and ϕ 3T *thyP3* is limited to the region of 836 nt encoding *thyA* and 28 nucleotides of the 3' flanking sequence (Fig. 2). Presuming that the probability of recombination is positively correlated with the degree of homology shared by both sequences, this finding explains why smaller ϕ 3T DNA fragments carrying *thyP3* integrated exclusively into the *thyA* site, whereas large DNA molecules preferred to recombine with the SP β prophage (Stroynowski, 1981b).

The 279 amino acid *thyA* gene product differs from the *thyP3* gene product at eight positions only. Alignment of

Fig. 4. Dendrogram of bacterial TSases. Sequences of TSases encoded by *B. subtilis* *thyA*, *B. subtilis* phage ϕ 3T, *B. subtilis* *thyB*, *E. coli* *thyA* (Belfort et al., 1983), *L. casei* *thyA* (Maley et al., 1979) and *E. coli* phage T4 *thy* (Chu et al., 1984) were compared using the CLUSTAL program PC/gene release 6.6, IntelliGenetics). Values for the percentage identity between the different TSases were taken from the pairwise similarity scores.

various bacterial TSases revealed that the *B. subtilis* TSaseA and ϕ 3T TSase are clearly distinguishable from other bacterial TSases. Whereas the TSases encoded by *B. subtilis* *thyB*, *E. coli* *thyA* and *Lactobacillus casei* *thyA* genes exhibited striking similarity to each other (57–62%), only 27–31% sequence identity was found between this homogeneous group of TSases to *B. subtilis* TSaseA and phage ϕ 3T TSase (Fig. 4). This extraordinary similarity may be explained in two ways. (i) The chromosome of *B. subtilis* strain 168 used for cloning contains an integrated *thyP3* gene which has been slightly modified during evolution in its *Bacillus* host. (ii) Alternatively, phage ϕ 3T may have acquired the region encoding TSaseA from the *B. subtilis* genome by recombination. We can only speculate about which of these evolutionary routes was followed. Temperate *B. subtilis* phages ϕ 3T and ρ 11 can complement a thymine deficiency in their host. The *thyP3* gene of phage ϕ 3T integrates into the *B. subtilis* chromosome at two distinct sites; at the *thyA* locus at 168° due to its homology to the host *thyA* gene, and in the SP β prophage due to phage ϕ 3T sequences flanking it (Stroynowski, 1981a). The cryptic temperate bacteriophage SP β of *B. subtilis* 168, which is a close relative of ϕ 3T, does not contain a *thy* gene but does carry sequences surrounding *thyP3* in ϕ 3T, suggesting that ϕ 3T and ρ 11 might have acquired the *B. subtilis* *thyA* gene by a recombination event.

Alternatively, an original *thy* coding region might have been deleted from the SP β prophage within the *B. subtilis* chromosome. *B. subtilis* is unique in the possession of two functional TSase genes. Sequence comparison of the genes indicated that the *thyB* gene is more related to other bacterial TSases than to the *B. subtilis* *thyA* gene (Fig. 4). The striking similarity found between *thyA* and *thyP3* might be due to an early evolutionary event in which the *B. subtilis* host has acquired a second *thy* gene from an integrated phage.

At present, neither possibility can be ruled out. This study shows that the regions flanking the bacterial structural

thyA gene do not share any similarity with the phage ϕ 3T DNA except a short region of 28 nucleotides adjacent to the TAA codon. The phage and bacterial *thy* genes are flanked by putative regulatory sequences, including promoter and terminator motifs which are apparently not related to each other.

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REFERENCES

- Azevedo, V., Alvarez, E., Zumstein, E., Damiani, G., Sgaramella, V., Ehrlich, S. D. & Serror, P. (1993). An ordered collection of *Bacillus subtilis* DNA segments cloned in yeast artificial chromosomes. *Proc Natl Acad Sci USA* **90**, 6047–6051.
- Belfort, M., Maley, G., Pedersen-Lane, J. & Maley, F. (1983). Primary structure of the *Escherichia coli* *thyA* gene and its thymidylate synthase product. *Proc Natl Acad Sci USA* **80**, 4914–4918.
- Chang, S. & Cohen, N. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol & Gen Genet* **168**, 111–115.
- Chu, F. K., Maley, G. F., Maley, F. & Belfort, M. (1984). Intervening sequence in the thymidylate synthase gene of bacteriophage T4. *Proc Natl Acad Sci USA* **81**, 3049–3053.
- Cutting, S. M. & Vander Horn, P. (1990). Genetic analysis. In *Molecular Biological Methods for Bacillus*, pp. 27–74. Edited by C. R. Harwood & S. M. Cutting. Chichester: J. Wiley & Sons.
- Haima, P., Bron, S. & Venema, G. (1987). The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. *Mol & Gen Genet* **209**, 335–342.
- Iwakura, M., Kawata, M., Tsuda, M. & Tanaka, T. (1988). Nucleotide sequence of the thymidylate synthase B and dihydrofolate reductase genes contained in one *Bacillus subtilis* operon. *Gene* **64**, 9–20.
- Kenny, E., Atkinson, T. & Hartley, B. S. (1985). Nucleotide sequence of the thymidylate synthetase gene (*thyP3*) from the *Bacillus subtilis* phage ϕ 3T. *Gene* **34**, 335–342.
- Maley, G. F., Bellisario, R. L., Guarino, D. U. & Maley, F. (1979). The primary structure of *Lactobacillus casei* thymidylate synthetase. *J Biol Chem* **254**, 1301–1304.
- Mead, D. A., Szczwsna-Skorupa, E. & Kemper, B. (1986). Single-stranded DNA 'blue' T7-promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Eng* **1**, 67–74.
- Michel, B., Niaudet, B. & Ehrlich, S. D. (1983). Intermolecular recombination during transformation of *Bacillus subtilis* competent cells by monomeric and chimeric plasmids. *Plasmid* **10**, 1–10.
- Myoda, T. T., Lowther, St. V., Funanage, V. L. & Young, F. E. (1984). Cloning and mapping of the dihydrofolate reductase gene of *Bacillus subtilis*. *Gene* **29**, 135–143.
- Neuhard, J., Price, A. R., Schack, L. & Thomassen, E. (1978). Two thymidylate synthetases in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **75**, 1194–1198.

- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Stahl, M. L. & Ferrari, E. (1984).** Replacement of the *Bacillus subtilis* subtilisin structural gene with an *in vitro*-derived deletion mutation. *J Bacteriol* **158**, 411–418.
- Stroynowski, I. T. (1981a).** Distribution of bacteriophage ϕ 3T homologous deoxyribonucleic acid sequences in *Bacillus subtilis* 168, related bacteriophages, and other *Bacillus* species. *J Bacteriol* **148**, 91–100.
- Stroynowski, I. T. (1981b).** Integration of the bacteriophage ϕ 3T-coded thymidylate synthetase gene into the *Bacillus subtilis* chromosome. *J Bacteriol* **148**, 100–108.
- Tucker, R. G. (1969).** Acquisition of thymidylate synthetase activity by a thymine-requiring mutant of *Bacillus subtilis* following infection by the temperate phage ϕ 3. *J Gen Virol* **4**, 489–504.
- Vandeyar, M. A. & Zahler, S. A. (1986).** Chromosomal insertions of Tn917 in *Bacillus subtilis*. *J Bacteriol* **167**, 530–534.
- Wilson, M. C., Farmer, J. L. & Rothman, F. (1966).** Thymidylate synthesis and aminopterin resistance in *Bacillus subtilis*. *J Bacteriol* **92**, 186–196.
- Wolf, M., Geczi, A., Simon, O. & Borriess, R. (1995).** Genes encoding xylan and β -glucan hydrolysing enzymes in *Bacillus subtilis*: characterization, mapping and construction of strains deficient in lichenase, cellulase and xylanase activity. *Microbiology* **141**, 281–290.
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