The thyA gene from Bacillus subtilis exhibits similarity with the phage φ3T thymidylate synthase gene

Nguyen Hoai Tam and Rainer Borriss

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 φ3T 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 ΔthyA 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–drfA 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 φ3T TSase and thyA allows the transformation of thyA mutants by DNA from phage φ3T (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 SPB prophage, which does not contain thyP sequences but exhibits homology to the φ3T 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
procedures (Sambrook et al., 1989). DNA fragments hybridizing with the DNA probes used were detected with a digoxygenin 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 (10^9) were plated onto M9 medium supplemented with amonio-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
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<tr>
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<td></td>
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</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>BGSC, Ohio, USA</td>
</tr>
<tr>
<td>QB943</td>
<td>ileA1 tyrD1 thyA1 thyB1 trpC2</td>
<td>BGSC, Ohio, USA</td>
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<td>1A634</td>
<td>Em^R zei82::Tn917</td>
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<td>MW10</td>
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<td>Wolf et al. (1995)</td>
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<td>This work</td>
</tr>
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<tr>
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<td>NHT5</td>
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<td>thyA, DH5α derivative</td>
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<td>130 kb YAC insert with thyA</td>
<td>Azevedo et al. (1993)</td>
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<td>Cm^R Em^R</td>
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<td>Bluescript SK^-</td>
<td>Ap^R</td>
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<td>Ap^R Tc^R Cm^R, pBR322-pC194 derivative</td>
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<td>pNHT4</td>
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<tr>
<td>YAC 12-5</td>
<td>130 kb YAC insert with thyA</td>
<td>Azevedo et al. (1993)</td>
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**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 pHPl3 linearized with *BamH*1 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 thyminosine was isolated and used to transform *B. subtilis* NHT2 (thyA thyB trpC2). At 47 °C, 18 transformants were able to grow without thyminosine 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 pHPl3 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^R 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. Sellar (INRA, Jouy-en-Josas, France; unpublished). High molecular mass yeast DNA was separated on 1–0.5% (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.
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 (ΔthyA degI5 hyp/T3) 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 pHPl3 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, TAaAT, 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 φ3T (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 φ3T 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 φ3T 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 (ΔthyA500) 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 (ΔthyA4500) yielded transformants with a CmR.
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Fig. 2. Nucleotide sequence of the Bcll-XbaI 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 φ3T DNA, is labelled by filled triangles.
ThyA+ phenotype. Southern analysis revealed that this phenotype may result from a single cross-over event between homologous regions in which ΔthyA 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 gene copies on its chromosome. Moreover, the pheno-
cotype 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.

Lysates from phage PBS1 grown on MW10 (thyA+ΔeglS) were used to infect NHT4 (thyA eglS+). In this case 40% cotransduction was observed between the markers, suggesting that ΔthyA500 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 pNHT2 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 φ3T T Sac (thyP3) gene. It has been shown previously that the T Sac 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 φ3T 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 φ3T DNA fragments carrying thyP3 integrated exclusively into the thyA site, whereas large DNA molecules preferred to recombine with the SPP prophage (Stroynowski, 1981b).

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

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–SalI; 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–SalI; 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 observed with the central 365 bp fragment (not shown). Note: this picture has been electronically imaged.
various bacterial TSases revealed that the *B. subtilis* TSaseA and φ3T 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* thyA and phage φ3T 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 φ3T 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 φ3T and p11 can complement a thymine deficiency in their host. The thyP3 gene of phage φ3T 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 SPβ prophage due to phage φ3T sequences flanking it (Stroynowski, 1981a). The cryptic temperate bacteriophage SPβ of *B. subtilis* 168, which is a close relative of φ3T, does not contain a thyA gene but does carry sequences surrounding thyP3 in φ3T, suggesting that φ3T and p11 might have acquired the *B. subtilis* thyA gene by a recombination event.

Alternatively, an original thy coding region might have been deleted from the SPβ 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 φ3T 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**


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