

8. Cloning of the Thymidylate Synthetase Gene of the Phage Phi-3-T

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INTRODUCTION

Phi-3-T is a *Bacillus subtilis* temperate bacteriophage, isolated by Tucker (11) from the soil. Tucker has shown that infection by this phage causes, by lysogenic conversion of thy⁻ *B. subtilis* clones, prototrophy. The phage carries genetic information specifying the thymidylate synthetase activity, the *thyP* gene.

Recently, we have reported purification of *B. subtilis* DNA segments achieved by *EcoRI* cleavage followed by gel electrophoresis (6). Since a still further purification can be achieved by cloning the segments (8), we have chosen to use that approach for the isolation of the *thyP* gene of the Phi-3-T. We describe here the successful cloning of that gene on the *Escherichia coli* plasmid pSC101 (3). The gene complements the thymine deficiency in *E. coli*, indicating its correct transcription and translation in this new host. The promoter of the gene is likely to be contained within the cloned segment. The hybrid plasmid transforms *B. subtilis* 100-fold less efficiently than the intact phage DNA. The excised segment, however, displays the same transforming activity (1,000 times less than the intact phage DNA) whether it comes from the phage or from the hybrid plasmid. *B. subtilis* clones transformed with the hybrid plasmid DNA do not contain detectable pSC101 sequences, but do show sequences homologous to part of the Phi-3-T genome.

METHODS AND RESULTS

Isolation of Tc^r thy⁺ *E. coli* Transformants

The *EcoRI* cleavage pattern of the Phi-3-T DNA is shown in Fig. 1. In agreement with the data of Wilson et al. (12), 20 bands can be seen; four

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FIG. 1. Agarose gel electrophoresis of *EcoRI*-cleaved DNA. From left to right: Phi-3-T, pFT23, pFT24, pFT25, pFT33, and Phi-3-T. Five-hundred nanogram-cleaved DNA in 15 μ l of digestion mixture containing 10% sucrose and 0.1% bromophenyl blue dye was loaded on a 4-mm thick 0.6% agarose slab gel. Electrophoresis was performed for approximately 8 hr at 2 V/cm in the TRIS-borate-EDTA buffer containing 500 ng/ml of ethidium bromide (6). Photograph was taken under the short wavelength ultraviolet light.

additional bands have been allowed to migrate out of the gel. A more detailed study has shown that approximately 30 segments can be resolved. Their sizes, determined using *EcoRI*-cleaved SPP1 DNA as a standard (4), are displayed in Table 1. The sum of their molecular weights approximates 7.2×10^7 daltons and is somewhat lower than the $82.6 \pm 5.3 \times 10^6$ daltons measured by electron microscopy that used pSC101 as a standard. The discrepancy probably results from one or more of the following factors: repetition within the phage genome (see below), incomplete resolution during gel electrophoresis, and the use of different molecular weight standards for the two measurements.

Kinetics of inactivation of *thyP* gene-transforming activity on cleavage with *EcoRI* enzyme is shown in Fig. 2. Nearly a 1,000-fold inactivation of fully cleaved DNA was observed. This finding prompted us to attempt the cloning of the *thyP* gene from an incompletely digested DNA sample, in which only 10% of the original transforming activity was lost. Such a sample was mixed with *EcoRI*-cleaved pSC101 DNA in a ratio 10 to 1, with a total DNA concentration of 11 μ g/ml. Treatment with the T4 ligase resulted in appearance of approximately 40% circles in addition to a number of long, linear molecules. Ligated samples were used to transform thymine-requiring *E. coli* cells, selecting either for tetracycline resistance or thymine independence. The results of the transformation are displayed in Table 2.

Approximately 8% of the Tc^r clones contain sequences that are complementary to Phi-3-T cRNA. This value probably underestimates the pro-

TABLE 1. Molecular weight of ϕ 3T DNA segments after *EcoRI* cleavage

Band	MW (10^{-6})
1	9.4
2	7.9
3	5.4
4	4.9
5a,b	4.5, 4.4
6	3.9
7a,b	3.2, 3.1
8	2.9
9	2.5
10a,b,c	2.07, 2.05, 2.05
11	1.96
12a,b	1.70, 1.70
13	1.45
14	1.25
15	1.05
16	.90
17	.70
18	.67
19	.65
20	.46
21	.41
22	.26
23	.24
24	.19
Total	71.86

portion of hybrid plasmids, since the very small inserts might have been undetected by the technique used (5). Two of the Tc^r clones hybridizing Phi-3-T cRNA also showed a thy^+ phenotype. Two more thy^+ clones have been obtained by selecting that phenotype directly; it is interesting to note that the frequency of occurrence of the directly selected thy^+ character is lower than when the Tc^r is selected first (Table 2).

Molecular Structure of Hybrid Plasmids

Plasmids from the clones hybridizing cRNA are denoted as "pFT," followed by the clone number. The *EcoRI* restriction pattern of the four DNAs extracted from the Tc^r thy^+ transformants is shown in Fig. 1. Molecular weights of *EcoRI*-released segments, together with those resulting from the action of *Bam* and *Sma* restriction enzymes, are shown in Table 3.

A paradox is immediately apparent: although the four plasmids all carry the thy^+ character, the only *EcoRI* segment that all four have in common is the pSC101 moiety of the hybrid (6.2×10^6 dalton segment, Table 3). The 4.5×10^6 dalton segment (A, Table 3) of the plasmids pFT23, 24, and 25, with a mobility identical to that of the segment 5a of the Phi-3-T, is missing

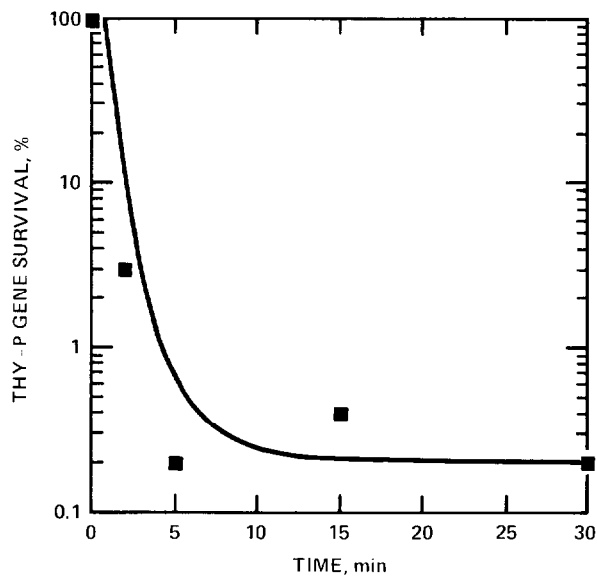


FIG. 2. Kinetics of inactivation of the *thyP* gene on cleavage of Phi-3-T DNA with *EcoRI* endonuclease. Enzymatic degradation at a DNA concentration of 100 $\mu\text{g/ml}$ and transformation of *B. subtilis* strain SB591 (*thy*⁻) were performed as described previously (6).

in the plasmid pFT33. The latter contains instead a 5.4×10^6 dalton segment A', which differs from the segment A not only in size but also by embodying a *Bam* and *Sma* site. The two small segments (B and C, Table 3), which co-electrophorese with the segments 18 and 23 of the phage, are also not present in all of the plasmids. Which of the segments, then, carries the *thyP* gene?

An answer was obtained by electron microscope analysis of the heteroduplexes between different hybrid plasmid DNAs. The 5.4×10^6 dalton segment A' of the pFT33 contains a 4.5×10^6 dalton stretch of DNA that is homologous to the total length of segment A of pFT23, 24, and 25. In

TABLE 2. Isolation of *Thy*⁺ *Tc*^r *E. coli* transformants^a

Selection	Tc ^r	Thy ⁺	Thy ⁺ Tc ^r
Plated cells	10 ⁷	10 ⁸	10 ⁸
Transformants	447	1	2
Hybridize ϕ 3T cRNA	31	N.D. ^b	2
Tc ^r Thy ⁺ phenotype	2	0	2

^a Recipient *E. coli* strain was W5443 (see legend of Fig. 3 for the genotype).

^b N.D. = not determined.

TABLE 3. Molecular weights of DNA segments from the hybrid plasmids

Plasmid	<i>Eco</i> RI	<i>Bam</i>	<i>Sma</i>	Intact
pFT23	6.2	11.7	11.7	11.7
	A 4.5			
	B 0.67			
	C 0.24			
pFT24	6.2	10.8	10.8	10.8
	A 4.5			
pFT25	6.2	11.7	11.7	11.7
	A 4.5			
	B 0.67			
	C 0.24			
pFT33	6.2	8.3	8.0	12.6
	A' 5.4	4.4	4.6	
	B 0.67			
	C 0.24			

addition, it contains a 9×10^5 dalton insert, which on denaturation and re-naturation appears as a hairpin, with the stem approximately 100 base pairs long. Such structure can arise from the presence of palindromic sequences bordering the 9×10^5 dalton insert. We are presently investigating the origin of that insert. Thus, the sequences of segment A appear common to all of the hybrid plasmids, and presumably contain the *thyP* gene.

The *thy*⁺ Character is Plasmid-borne

Three lines of evidence demonstrate that the *thy*⁺ gene is harbored on a plasmid. First, DNA prepared by the standard, clear lysis procedure (2), containing less than 1% of linear molecules, can transform Tc^s*thy*⁻ *E. coli* to Tc^r or Tc^r*thy*⁺ phenotype in a manner indistinguishable from that displayed by pSC101 transforming to Tc^r character (Fig. 3). Approximately 1,000 transformants, selected either for Tc^r or *thy*⁺, were tested by replica plating and found always to carry the unselected marker as well. The efficiency of transformation, calculated from the linear part of the curve (slope = 1) shown in Fig. 3, is displayed in Table 4.

Second, *E. coli* transformants of the Tc^r*thy*⁺ phenotype can eliminate the plasmid using ethidium bromide treatment, followed by the ampicillin selection in the presence of tetracycline. All the Tc^s clones lost the *thy*⁺ character simultaneously. Both markers could be reintroduced into the so-treated strain, at a frequency identical to that of the parent, by transformation with the hybrid plasmid DNA. Since the Tc^r character is known to be plasmid-borne, and our experiments have established linkage of the *thy*⁺ marker

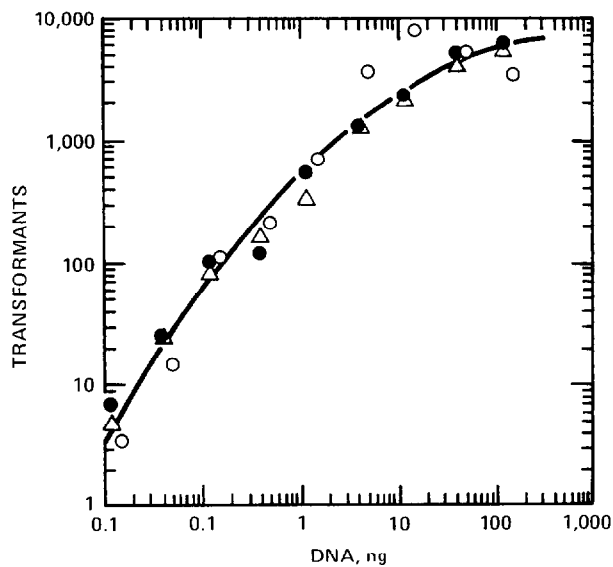


FIG. 3. Transformation of *E. coli* with pSC101 [Tc^r (○)] and pFT24 [Tc^r (△); Tc^r thy⁺ (●)] DNA. *E. coli* strain W5443 (*thr-1 leu-6 thi-1 supE44 lacY1 r⁻ m⁻ thy⁻ Str^r tonB trp⁻*) was rendered competent and transformed as described previously (7).

with the antibiotic resistance, then the thy⁺ character must be carried by the plasmid.

Third, all the hybrid plasmid DNAs transform thymine-requiring *B. subtilis* strains to prototrophy. A representative experiment is shown in Fig. 4. Efficiencies of transformation, averaged from a number of experiments, are presented in Table 4. Intact Phi-3-T DNA was used as a control and displayed approximately a 100-fold higher efficiency than did pFT25 DNA. This low efficiency of plasmid DNA is not due to its supercoiled configura-

TABLE 4. Transforming efficiencies of hybrid plasmids

DNA	Cells	Efficiency (cge) ^a
pSC101	<i>E. coli</i> W5443	10 ⁻⁵
pFTs		10 ⁻⁵
φ3T	<i>B. subtilis</i> SB591	10 ⁻⁴
φ3T/ <i>Bam</i>		10 ⁻⁵
φ3T/RI		10 ⁻⁷
pFTs		10 ⁻⁶
pFTs/ <i>Bam</i>		10 ⁻⁶
pFTs/RI		10 ⁻⁷

^a Colonies per genome equivalent.

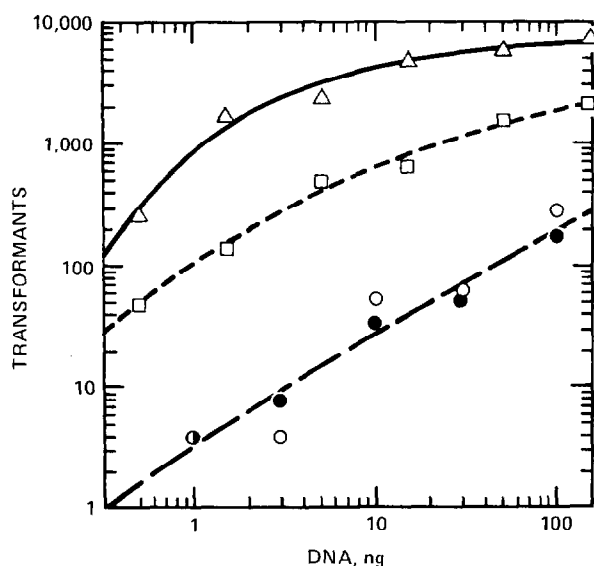


FIG. 4. Transformation of *B. subtilis* to thymine independence with Phi-3-T (Δ), pFT24 (\circ), *Bam*-cleaved Phi-3-T (\square), and pFT24 (\bullet) DNAs. The lines represent exponential functions, best fitting by the least squares test, to the experimental points.

tion: the cleavage with *Bam* endonuclease, which linearizes the molecule by introducing a cut about 2×10^5 daltons from the *EcoRI* site in the pSC101 part of the hybrid, does not change its biological activity. Similar treatment of the Phi-3-T DNA leads to a 10-fold decrease in transforming activity. Interestingly, the *EcoRI* treatment decreases biological efficiency of hybrid plasmids approximately 10-fold while the activity of the phage DNA is impaired 1,000-fold (Fig. 2). Therefore, the *EcoRI* segment embodying the *thyP* gene displays the same transforming efficiency regardless of the vector (Phi-3-T phage or the hybrid plasmid) by which it was carried or its previous host.

Hybrid Plasmids Contain Phi-3-T Sequences

Several lines of evidence demonstrate the presence of Phi-3-T sequences in the hybrid plasmids. As mentioned previously (Table 2), *E. coli* clones that harbor these plasmids hybridize RNA synthesized *in vitro* by the *E. coli* RNA polymerase on the Phi-3-T DNA template (Phi-3-T cRNA). In addition, all except one of the segments released by *EcoRI* cleavage from hybrid plasmids show electrophoretic mobility identical to some Phi-3-T segments (see above).

Further evidence for the presence of the *thyP* gene on the hybrid plasmids is that DNA of the latter can transform *thy*⁻ *B. subtilis* strains to a state of

thymine independence. These transformants acquire not only the thy⁺ character, but also the ability to hybridize Phi-3-T cRNA.

Figure 5 shows that the colony hybridization technique (5) can be used with *B. subtilis* clones: SB168 (a standard *B. subtilis* strain), following lysis by Phi-3-T, hybridizes the Phi-3-T cRNA. Specificity of staining is demonstrated by the absence of hybridization with the pSC101 cRNA. Interestingly, SB168 (not carrying the prophage) also hybridizes the phage cRNA; the strain is not immune to the phage and does not release it on mitomycin induction (not shown). SB591, a thymine-requiring derivative of SB168, has lost the capacity to bind the Phi-3-T cRNA; the mutagenesis has apparently deleted sequences complementary to the phage. However, SB591 transformed with either the Phi-3-T DNA or the hybrid plasmid DNA does hybridize Phi-3-T cRNA. The low extent of hybridization displayed by clones of Phi-3-T DNA-transformed SB591 and their sensitivity to Phi-3-T phage indicate that only a limited amount of the phage genome (including the *thyP* gene) has been inserted in the chromosome. It is interesting to note that SB591 transformed to thymine prototrophy with DNA extracted from SB19 (a strain related to SB168) also hybridizes Phi-3-T cRNA.

Additional evidence for the presence of Phi-3-T sequences in the hybrid plasmids has been obtained using the hybridization technique developed by Southern (10). Phi-3-T cRNA binds to all *Eco*RI-cleaved segments of hybrid plasmids except the one corresponding to the pSC101 part of the molecules. Conversely, the cRNAs synthesized on each of the hybrid plasmid templates (including pFT33) hybridize to segment 5a of the phage. They also stain Phi-3-T segments 18 and 23, but not pFT24 cRNA; this is expected since pFT24 does not carry these latter segments (Table 3).

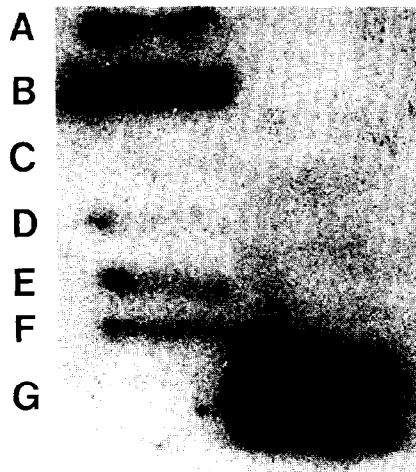


FIG. 5. Autoradiograph of *B. subtilis* colonies to which Phi-3-T (left) or pSC101 (right) cRNA was hybridized. An *E. coli* strain harboring pSC101 was used as control. Bacteria were streaked on nitrocellulose filter disks, incubated overnight, and prepared for hybridization according to the procedure of Grunstein and Hogness (5), as modified by J. Lis and L. Prestidge (personal communication). The filter was then cut into halves, one half was hybridized with Phi-3-T cRNA, the other with pSC101 cRNA. Hybridization and autoradiography were performed as described previously (5). **A:** SB168. **B:** SB168 (Phi-3-T). **C:** SB591^{thy-}. **D:** SB591 transformed with Phi-3-T DNA to thy⁺. **E:** SB591 transformed with pFT23. **F:** SB591 transformed with pFT24 DNA. **G:** *E. coli* C600(pSC101).

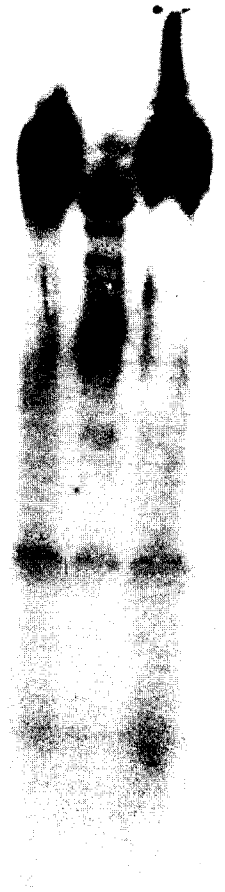
pSC 101

5a



FIG. 6. Autoradiograph of *Eco*RI-cleaved pFT24 (*right*) and Phi-3-T (*left*) DNA, separated by gel electrophoresis and hybridized with the pFT24 cRNA. Electrophoretic separation was as described in the legend of Fig. 1, except that the segments smaller than 3.5×10^6 daltons have been allowed to migrate out of the gel. Hybridization and autoradiography were performed as described previously (10).

FIG. 7. Autoradiograph of *Eco*RI-cleaved pFT23 (*left*), Phi-3-T (*center*), and pFT23 plus carrier calf thymus (*right*) DNA, separated by gel electrophoresis and hybridized with pFT23 cRNA. Film was overexposed in order to show staining of shorter segments.



Hybridization of pFT24 cRNA to the phage and the plasmid DNA is displayed in Fig. 6.

A somewhat puzzling observation is that all pFT cRNAs hybridize to several additional bands in the Phi-3-T restriction pattern: 3, 6, 7, 10, 12, and 14 (Fig. 7 shows an example), and suggest to us the existence of several regions of internal homology within the phage genome. Incomplete degradation, another possible explanation, seems to be ruled out by the fact that a 10-fold increase in either the amount of *EcoRI* or the time of incubation did not change the described pattern. Similarly, the presence of multiple species in each of the plasmids is ruled out since the cleaved plasmids appear to hybridize both their own and Phi-3-T cRNA exclusively to segments detected by electrophoresis (Table 3), even when cleaved calf thymus DNA is added as a carrier prior to electrophoresis so that trace amounts of postulated contaminating segments are not lost (Fig. 7).

Still further evidence for the presence of Phi-3-T sequences in hybrid plasmids is obtained by electron microscope inspection (not shown) of the heteroduplex molecules between the phage and the plasmid DNAs. A double-stranded region without any detectable imperfection is formed between the pFT24 and the Phi-3-T DNAs: its length is 4.5×10^6 daltons—exactly that measured by gel electrophoresis (Table 3) for the insert.

An important conclusion can be drawn from these data. The inserts are homologous with the Phi-3-T DNA (except for pFT33) along their entire length. Therefore, the hybrid plasmids contain only the phage sequences added to the pSC101. Since these plasmids confer the *thy*⁺ phenotype to *E. coli*, the gene that functions in the *E. coli* environment is the *thyP* gene of the Phi-3-T.

Promoter of the *thyP* Gene in Hybrid Plasmids

Is the *thyP* under control of a Phi-3-T promoter within the cloned segment in the hybrid plasmids, or does it depend on the read-through initiated on the promoter of the vector? The following evidence indicates that the former might be true:

1. The 4.5×10^6 dalton segment of the pFT23, which contains the *thyP* gene, is oriented in a direction opposite to that of the corresponding segment of pFT24, as revealed by the heteroduplex analysis (not shown). Nevertheless, both plasmids display the same *thy*⁺ character.
2. The insert from the pFT24 was cloned in the Co1E1-amp plasmid (9) and its biological activity was not impaired, regardless of its orientation.

DISCUSSION

EcoRI cleavage of the Phi-3-T DNA leads to a 1,000-fold decrease of the transforming activity of the *thyP* gene. Nevertheless, the cleavage does not occur within the gene, since segment 5a of the phage can transform both the

E. coli and the *B. subtilis* thymine-requiring strains to prototrophy; therefore, its genetic information must be intact. It seems more likely that the loss of biological activity is caused by the decrease in size, on cleavage, of the DNA carrying the *thyP* gene, a phenomenon we have observed for chromosomal markers of *B. subtilis* (6). This conclusion is supported by the fact that the cleavage of hybrid plasmids with the *Bam* restriction enzyme (which does not change the size of DNA) does not affect its transforming activity; on the other hand, treatment with the *EcoRI* enzyme (decreasing the size of DNA) also provokes a loss of biological activity.

In each of approximately 100 *B. subtilis* clones transformed to thymine independence by the hybrid plasmid DNA, Phi-3-T, but no pSC101, sequences were detected by hybridization. In addition, attempts to transform *B. subtilis* to Tc^r using the hybrid DNA, selecting directly for the resistance or by replica plating of thy⁺ transformants, have consistently failed. It can therefore be concluded that there is excision of DNA sequences contained in the hybrid plasmids during transformation of *B. subtilis* cells. Experiments addressing that question in more detail are in progress.

We have demonstrated that a gene which functions in a Gram-positive organism (*B. subtilis*) can also function in a Gram-negative one (*E. coli*). Only one example of this nature has been previously reported: the ampicillin gene of the *Staphylococcus aureus* plasmid conferred resistance to *E. coli* when cloned in the pSC101 plasmid (1). The *thyP* gene of the phage Phi-3-T does not belong to the category of antibiotic resistance genes (known to be promiscuous), but is rather a gene involved in the biosynthetic processes of its host. Our finding has, therefore, a more general implication: it is quite likely that most of the genes remain functional on exchange between two prokaryotic species. This notion is supported by the fact that two other *B. subtilis* genes, *leu* and *ura*, have been demonstrated to be functional in *E. coli* (N. Y. Chi, *manuscript in preparation*). In addition, our evidence suggests that the *thyP* gene uses its own promoter in *E. coli*. This might make possible the experimental assessment of an analog of the universality of the genetic code: namely, the universality of genetic control.

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