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A carotenogenic gene cluster exists on a large plasmid in *Thermus thermophilus*

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Abstract

In *Thermus thermophilus* HB27, the *crtB* gene encoding phytoene synthase was found to exist on the large plasmid, pTT27. One of the carotenoid under-producing mutants, Crt31, carried a derivative of pTT27 (pTT27′) in which deletion and inversion were observed near the *crtB* gene. *T. thermophilus* HB8 also contained a large plasmid which showed homology to pTT27 and the *crtB* gene. These results suggested that genes for carotenoid biosynthesis occurred as a cluster on a large plasmid in *Thermus thermophilus*. This is the first report to show directly that carotenogenesis is plasmid-encoded in microorganisms.

Key words: Extreme thermophile; *Thermus thermophilus*; Large plasmid; Carotenoid biosynthesis

1. Introduction

Thermus thermophilus is a Gram-negative, aerobic microorganism that can grow at temperatures ranging between 50 and 82°C. One unique characteristic of the genus *Thermus* is that most of the *Thermus* species and strains produce carotenoids [1]. Virtala et al. [2] have recently reported that some yellow *Thermus* strains, cured of one or more plasmids, are devoid of pigmentation, suggesting that the carotenogenesis of *Thermus* may be a plasmid-encoded phenomenon. However, no direct evidence that either one or several carotenogenic enzymes are located on a plasmid has been reported yet. In *T. thermophilus* HB27, we have cloned and sequenced the *crtB* gene which encodes one of the carotenogenic enzymes, phytoene synthase, that synthesizes phytoene from geranylgeranyl diphosphate [3]. In this paper, we present direct evidence that the *crtB* gene is located on a large plasmid, pTT27 [4]. The genes for carotenoid biosynthesis are known to occur as a cluster in carotenoid-synthesizing strains of *Erwinia herbicola* [5], *Erwinia uredovora* [6], and *Rhodobacter capsulatus* [7]. In this paper we show that carotenogenic genes also occur as a cluster on pTT27, based on analysis of the plasmid of a carotenoid under-producing mutant of HB27, Crt31 [8]. Moreover, we report that a carotenogenic gene cluster is present on a large plasmid in another *Thermus* strain, *T. thermophilus* HB8.

2. Materials and methods

2.1. Bacterial strains and growth conditions

T. thermophilus HB27 [9], its carotenoid under-producing mutants Crt6, Crt8, Crt10, Crt11, Crt12, Crt16, Crt18, Crt20, Crt23 and Crt31 [8], *T. thermophilus* HB8 [10] and *T. aquaticus* YT1 [11] were used. All the strains were grown in TM medium [12] at 70°C. *T. thermophilus* HB27 *trpB5* carrying the recombinant plasmid pCOP1 [3] was also used. It was grown in TM medium containing 40 μg/ml of kanamycin at 60°C.

2.2. Preparation of DNAs, DNA manipulations and pulsed-field gel electrophoresis

Preparation procedures of intact DNAs in agarose plugs and their digestion were described previously [4]. Plasmid pCOP1 was prepared from HB27 *trpB5* carrying pCOP1 by the standard alkaline lysis method [13] followed by CsCl density gradient centrifugation. Preparation of large plasmids from *Thermus* strains and agarose gel electrophoresis to resolve the plasmids were performed according to the method of Kado and Liu [14]. Restriction endonucleases were supplied by Toyobo. DNA manipulation was performed according to standard methods [13].

Pulsed-field gel electrophoresis (PFGE) was performed on a 1% agarose gel (SeaKem GTG Agarose; FMC BioProducts) in 0.5 × Tris-borate buffer (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.0) at 7.5 V/cm using a CHEF-DR2 apparatus (Bio-Rad Laboratories) at 13–15°C for 18 h. We usually peaked the pulse time for 4 s or 6 s to obtain optimal separation. Phage lambda ladder DNA concatemers (Clontech or Bio-Rad Laboratories) and Lambda *Hin*dIII digest (Takara) were used as size markers.

2.3. DNA transfer and hybridization

DNA fragments in the agarose gel were transferred onto a nitrocellulose filter (Schleicher and Schuell) by capillary transfer using 20 × SSC (3 M NaCl, 0.3 M tri-sodium citrate at pH 7.0).

A non-radioactive labelling nucleotide, digoxygenin-11-UTP, was used for preparing the DNA probes. The random primer labelling technique, pre-hybridization, hybridization procedures and color development were performed according to the protocol of the DNA labelling and detection kit (Boehringer-Mannheim).

For the hybridization of the *crtB* gene probe, plasmid pCOP1 was digested with *Hin*dIII, and the 1.5 kb *Hin*dIII fragment (see Fig. 2) was

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recovered and labelled. For the hybridization of *Eco*RI linking clones [4], probe plasmids were linearized and divided into each arm, and were then labelled independently. For the hybridization of isolated restriction fragments to PFGE-separated fragments digested by other restriction enzymes, referred to as band hybridization experiments, individual bands were cut from the gels. Each gel slice was heated to 95°C for 10 min to melt the agarose and denature the DNA. Then, labelling reagents were added, and the mixture was incubated at 37°C for 16 h.

3. Results

3.1. CrtB *gene encoding phytoene synthase is located on pTT27*

During our work to construct a physical map of the *T. thermophilus* HB27 chromosome, we detected a large plasmid, pTT27, of about 250 kb [4]. This plasmid consisted of three *Eco*RI fragments (F, G and I) of 99, 88 and 57 kb, respectively (Fig. 1A).

As we had previously cloned the *crtB* gene from HB27, which encoded phytoene synthase [3], we attempted to localize the *crtB* gene on the physical maps of the chromosome or pTT27. The *crtB* gene was used to probe the nitrocellulose membrane onto which the intact total DNA digested by *Eco*RI or *Ssp*I had been transferred. As shown in Fig. 1B, the probe reacted with *Eco*RI fragment I and *Ssp*I fragment D. Thus, the location of the *crtB* gene was determined as shown in Fig. 1A. This is the first and direct evidence to our knowledge, that one of the carotenogenic genes is located on a plasmid in *Thermus* strains and other microorganisms.

In order to determine the exact location of the *crtB* gene on pTT27, we decided to construct a more detailed map of the plasmid. We first tried to prepare pTT27 DNA by lysing cells according to the method of Kado and Liu [14], followed by CsCl density gradient centrifugation. However, although pTT27 was clearly detected in the lysate analyzed by agarose gel electrophoresis (see Fig. 5, lane 1), we could not obtain a sufficient amount of plasmid DNA to conduct further experiments. Hence, we changed our strategy. We tried to construct the detailed map of pTT27 using PFGE. Restriction endonucleases which cut pTT27 into fragments with appropriate sizes and numbers for the construction of the restriction map were first selected. All the *Eco*RI fragments of pTT27 (*Eco*RI fragments F, G and I) generated by PFGE were recovered from the agarose gels and labelled together and hybridized to gel blots of HB27 total DNA digested by *Bcl*I, *Bgl*II, *Not*I, *Pst*I, or *Sca*I (data not shown). Among those restriction endonucleases, *Not*I was found to be useful for the construction of the plasmid map since only five fragments were generated from pTT27 by *Not*I digestion. The estimated sizes of the *Not*I fragments are listed in Table 1.

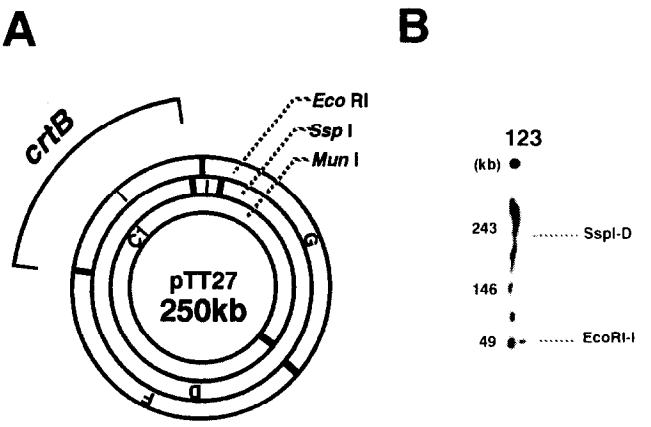

Fig. 1. Physical map of pTT27 (A) and hybridization of the *crtB* probe to PFGE-separated fragments of *T. thermophilus* HB27 total DNA digested with restriction enzymes (B). The 1.5 kb *Hin*dIII fragment (see Fig. 2) was labelled with digoxygenin-11-UTP (DIG) and used to probe the Southern transfer of *T. thermophilus* HB27 DNA digested with *Eco*RI (lane 2) and *Ssp*I (lane 3). Lane 1 contains lambda concatemers. The size of pTT27 was reported to be 240 kb in the previous paper [4]. We have corrected its size to 250 kb according to the fragment sizes obtained in this paper (see Table 1).

Table 1
Hybridization of the arms of linking clones and isolated restriction fragments to the wild-type intact DNA digested by *Eco*RI, *Not*I, or *Eco*RI plus *Not*I

Probe	Fragment hybridized (wild-type)		
	*Eco*RI	*Not*I	*Eco*RI + *Not*I
*Eco*RI linking clone			
VI-1	G (88 kb)	b (62 kb)	b1 (46 kb)
-2	F (99 kb)	b	b2 (17 kb)
VII-3	I (57 kb)	a (65 kb)	a1 (51 kb)
-4	F	a	a2 (15 kb)
VIII-5	G	c (54 kb)	c1 (44 kb)
-6	I	c	c2 (11 kb)
*Eco*RI fragment			
F	F	a,b,d (d1,d2)	NT
G	G	b,c	NT
I	I	a,c	NT
F + G + I	F,G,I	a,b,c,d (d1,d2)	NT
crtB gene	I	a	a1

The sizes of the d1 and d2 fragments were 37 kb. The sizes of *Eco*RI F, G, and I fragments were determined to be 95, 85 and 54 kb, respectively, in the previous paper [4]. NT, not tested.

The *Eco*RI linking clones of pTT27 we obtained [4] were used to construct the *Not*I restriction map of pTT27. Each arm of the *Eco*RI linking clones VI, VII and VIII was labelled independently and hybridized to the gel blots of HB27 total DNA digested by *Eco*RI, *Not*I or *Not*I + *Eco*RI. Fig. 2A shows representative results which are listed in Table 1 together with the results of hybridization experiments subsequently performed with *Eco*RI fragments F, G, I, and the *crtB* gene probes.

The *Not*I cleavage map of pTT27 was thus constructed. Since the other enzymes digested pTT27 into various fragments, we could not construct restriction maps with them. To construct a restriction map of the *crtB* flanking regions, total DNAs were digested with

several enzymes and Southern hybridization with the *crtB* gene probe was performed (data not shown). The closest *Not*I cleavage site to the *crtB* gene probe was identified by this experiment. It was located 18 kb downstream from the right *Hin*dIII site. The *Not*I cleavage map of pTT27 and the cleavage map of the *crtB* region are shown in Fig. 2. As a result, the exact location of the *crtB* gene on the pTT27 map was determined.

3.2. *Deletion and rearrangement occurred near the* crtB *gene in a carotenoid under-producing mutant, Crt31*

We have isolated various carotenoid under- and over-producing mutants from *T. thermophilus* HB27 *pro4* and analyzed their characteristics [8]. Among the 22 carotenoid under-producing mutants, twelve mutants were transformed to Crt^+ by the wild-type total DNA, unlike ten of the mutants. It is possible that these ten mutants had lost pTT27, or carried deletion derivatives of pTT27. To verify these assumptions, intact DNAs were prepared from all the under-producing mutants, digested with *Eco*RI, followed by PFGE analysis. In five mutants (Crt10, Crt11, Crt12, Crt23 and Crt31) both *Eco*RI fragments G and I became smaller. Since the sizes of the newly detected G′ or I′ fragments were identical to one another among all the five mutants, only the PFGE patterns of mutant Crt31 are shown in Fig. 3. On the other hand, the PFGE patterns of the other five mutants were identical to those of the parental strain (data not shown).

As the presence of a deletion derivative of pTT27 (pTT27′) in those mutants was evident, we tried to determine the deletion sites of pTT27′ of Crt31 by the same methods as those used to construct the *Not*I cleavage map of pTT27. The results are summarized in Table 2 and Fig. 4. The *crtB* probe hybridized to the *Eco*RI–I′ fragment, indicating that the *crtB* region was not deleted

Table 2
Hybridization of the arms of linking clones and isolated restriction fragments to the Crt31 intact DNA digested by *Eco*RI, *Not*I, or *Eco*RI plus *Not*I

Probe	Fragment hybridized (Crt31)		
	*Eco*RI	*Not*I	*Eco*RI + *Not*I
*Eco*RI linking clone			
VI-1	G′ (77 kb)	b (62 kb)	b1 (46 kb)
-2	F (99 kb)	b	b2 (17 kb)
VII-3	I′ (53 kb)	e (103 kb)	e1 (53 kb)
-4	F	e	a2 (15 kb)
VIII-5	I′	e	e1 (53 kb)
-6	G′	e	e2 (34 kb)
*Eco*RI fragment			
F	F	b,d (d1,d2), e	NT
G	G′, I′	b,e	NT
I	G′, I′	e	NT
F + G + I	F,G′,I′	b,d (d1,d2), e	NT
crtB gene	I′	e	e1

NT, not tested.

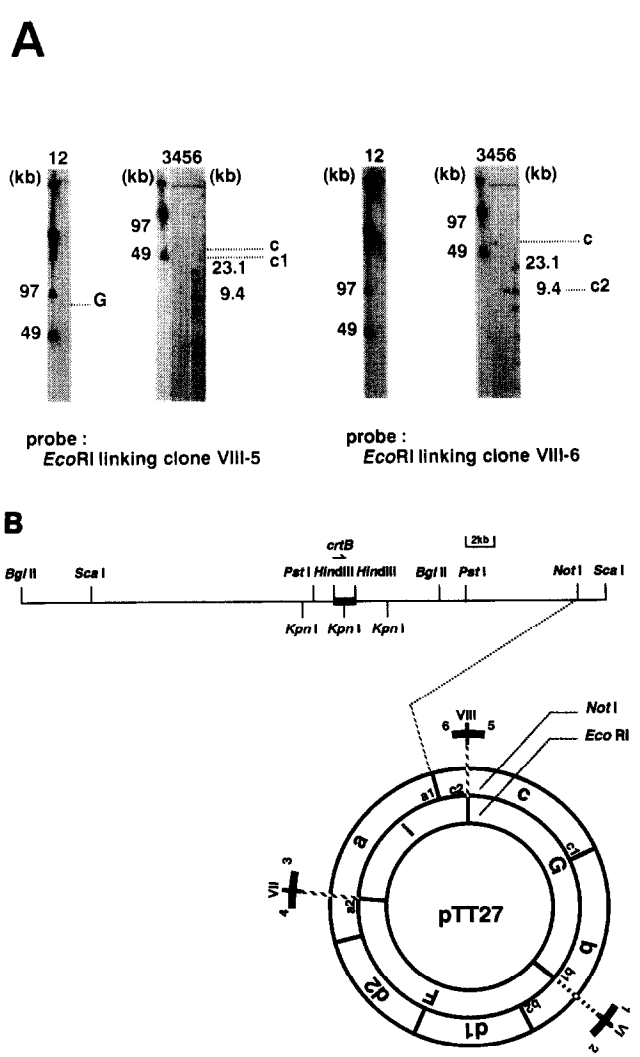

Fig. 2. Construction of the *Not*I cleavage map of pTT27. (A) Hybridization of the arms of *Eco*RI linking clone VIII to PFGE-separated fragments of *T. thermophilus* HB27 DNA digested by restriction enzymes. Each arm was labelled and used to probe Southern transfers of lambda DNA concatemers (lanes 1 and 3) and HB27 DNA digested by *Eco*RI (lane 2), *Not*I (lane 4), and *Not*I + *Eco*RI (lane 5), and lambda *Hin*dIII digest (lane 6). (B) *Not*I cleavage map of pTT27 and restriction map of the *crtB* flanking regions. Linking clones are indicated by bars. The restriction map of the *crtB* flanking region was constructed by hybridization of the *crtB* probe (1.5 kb *Hin*dIII fragment (thick line)) to fragments of HB27 total DNA digested with various restriction enzymes. The arrow represents the extent and directeion of the *crtB* gene.

from pTT27′. One of the *Not*I sites was lost from pTT27′ yielding a new fragment, *Not*I–e of 103 kb. Since the size of *Not*I fragments, b, d1 and d2 was same as that of the corresponding fragments of pTT27, the *Not*I site between fragments a and c of pTT27 was found to be lost from pTT27′. Interestingly, one of the arms of the *Eco*RI linking clone VIII (probe VIII-5) hybridized to the *Eco*RI–I′ fragment and e1 fragment of *Eco*RI + *Not*I digestion of pTT27′, while probe VIII-6 hybridized with the *Eco*RI–G′ fragment and fragment e2 (Fig. 4, Table

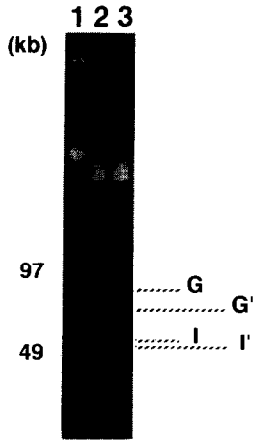

Fig. 3. Comparison of restriction patterns of Crt31 total DNA with that of the wild-type. Total DNAs of Crt31 (lane 3) and wild-type (lane 2) were analyzed by digestion with *Eco*RI followed by PFGE. A relatively long pulse time (10 s) was used to resolve the restriction fragments. Molecular size markers of concatemeric lambda DNA are shown in lane 1.

2). These results indicated that inversion and deletion occurred near the *crtB* gene on pTT27′. A restriction map of the *crtB* flanking regions of pTT27′ was constructed by Southern hybridization according to the same method as that used for pTT27 (Fig. 4B). The *Bgl*II, *Pst*I, *Sca*I and *Not*I sites were lost (shaded region). Thus it became clear that the intact *crtB* gene was located on pTT27′, strongly suggesting that other carotenogenic genes may be present in the inverted or deleted region of pTT27′.

3.3. *HB8 also has a large plasmid which shows homology to pTT27*

Carotenogenic genes, more than one of them, were present on the large plasmid in *T. thermophilus* HB27. To determine whether this is common in the *Thermus* strains, we tried to prepare large plasmids from *T. thermophilus* HB8 and *T. aquaticus* YT1. Many genes have been cloned from HB8 [15–17] and the physical map of its chromosome was constructed [18]. Ray et al. [19] analyzed the carotenoids produced by YT1. We subsequently showed that both HB27 and YT1 produced the same carotenoids [8], although the precise identification of most of them has not been performed yet.

The plasmids were prepared from three strains according to the method of Kado and Liu [14]. In YT1 several plasmids were observed (Fig. 5). In HB8, a new plasmid with a size almost equivalent to that of pTT27 was observed in addition to the other plasmids already identified, pTT8 [20] and pVV8 [21] (Fig. 5). Southern hybridization with the *crtB* gene probe and pTT27 probe was performed. The large plasmid of HB8 clearly reacted with both probes (only the results of hybridization with the *crtB* gene probe are shown in Fig. 5). These results strongly suggested that the carotenogenic genes were

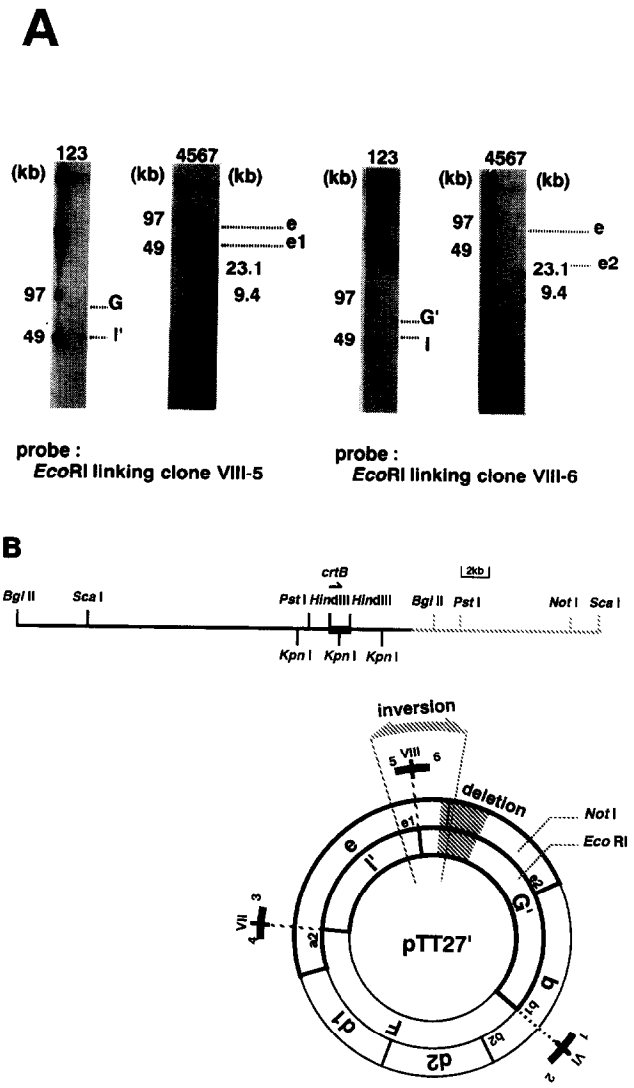

Fig. 4. Analysis of pTT27′. (A) Hybridization of *Eco*RI linking clone VIII-5 and VIII-6 probes to PFGE-separated fragments of *T. thermophilus* HB27 DNA digested by restriction enzymes. Probes were used to detect Southern transfers of lambda DNA concatemers (lanes 1 and 4), HB27 DNA digested by *Eco*RI (lane 2), and Crt31 DNA digested by *Eco*RI (lane 3), *Not*I + *Eco*RI (lane 5), and *Not*I (lane 6), and lambda *Hin*dIII digest (lane 7). (B) Physical map of pTT27′. Linking clones are indicated by bars. Inversion is indicated by a thick and shaded arrow, and the shaded region on the plasmid indicates the deleted region.

also present on a plasmid in HB8. In contrast, no positive signals were observed on plasmids from YT1.

4. Discussion

Gantotti and Beer [22] have indicated that the pigmentation of *Erwinia herbicola* was controlled by a large plasmid. Virtala et al. [2] and Nordstrom [23] have suggested that pigmentation is plasmid-encoded in *Thermus* spp. However, there is no direct evidence to show that the genes responsible for pigmentation are actually pres-

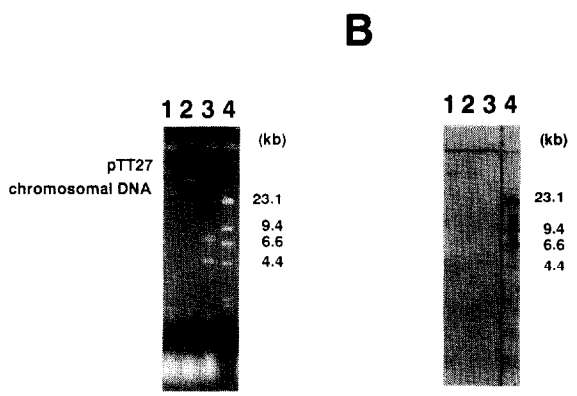

Fig. 5. Hybridization of the *crtB* probe to plasmid extracts prepared from *T. thermophilus* HB27 (lane 1), HB8 (lane 2) and *T. aquaticus* YT1 (lane 3). Plasmid extracts were prepared as described in section 2 and run on a 0.7% agarose gel in 0.5 × TBE at 120 V for 2.5 h. Lambda *Hin*dIII digest is shown in lane 4.

ent on plasmids in any microorganisms. In this paper, we have clearly shown that carotenogenesis, at least a part of the entire carotenogenic pathway, is plasmid-encoded in *T. thermophilus*. The location of the *crtB* gene was determined on the physical map of pTT27. The fact that one of the carotenoid under-producing mutants, Crt31, carried pTT27′ on which inversion and deletion occurred downstream of the *crtB* gene, suggested that other carotenogenic genes may be present in the rearranged region. These results and the fact that there was another truncated open reading frame (ORF-B) the deduced amino acid sequence of which showed a similarity to that reported for some of the carotenogenic genes of *Erwinia* spp. and *Rhodobacter capsulatus* next to the *crtB* gene [3], indicated that carotenogenic genes were present on pTT27 as a cluster, as reported in *Erwinia* spp. [5,6] and *R. capsulatus* [7]. Cloning and analysis of the flanking regions of the *crtB* gene to confirm the existence of other carotenogenic genes are in progress in our laboratory.

In *T. thermophilus* HB8 a large plasmid other than the already known two plasmids, pTT8 and pVV8, was newly detected in this study. The size of the plasmid is almost equivalent to that of pTT27, and it showed homology to pTT27 and the *crtB* gene. It is most likely that a carotenogenic gene cluster is present on the large plasmid in *T. thermophilus* HB8. In contrast, no positive signal was observed in the plasmids prepared from *T. aquaticus* YT1 by hybridization with either the *crtB* gene probe or pTT27 probe. The *crtB* gene encodes phytoene synthase which synthesizes phytoene from geranylgeranyl diphosphate [3]. Phytoene is a colorless carotenoid which is subsequently converted to colored carotenoids by the sequential reactions of carotenogenic enzymes. Since phytoene was identified as one of the carotenoids produced by YT1 [19] and we have shown that both YT1 and HB27 produced the same carotenoids [8], it was assumed that YT1 harboured the *crtB* gene. The lack of positive signal in hybridization experiments could be due to the lower degree of homology between the *crtB* gene of HB27 and that of YT1. Many auxotrophic mutants of HB27 were transformed to prototrophy by the wild-type HB27 chromosomal DNA at high efficiencies, although the transformation efficiencies with the YT1 chromosome were very low (unpublished data). We used labelled chromosomal DNA of HB27 to probe the total DNA of YT1 digested with restriction enzymes. As only a few fragments weakly hybridized (data not shown) it is possible that a carotenogenic gene cluster is also present on one of the plasmids in *T. aquaticus* YT 1.

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