Extremophiles (2012) 16:237–244
DOI 10.1007/s00792-011-0424-x

ORIGINAL PAPER

The third plasmid pVV8 from *Thermus thermophilus* HB8: isolation, characterization, and sequence determination

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Received: 2 November 2011 / Accepted: 13 December 2011 / Published online: 3 January 2012

Abstract The extremely thermophilic bacterium *Thermus thermophilus* is a model organism for structural biology and systems biology, and the so-called "Structural and Functional Whole-Cell Project for *T. thermophilus* HB8" is in progress. The released genomic sequence of the strain HB8 is composed of chromosome, pTT27 megaplasmid, and pTT8 plasmid. In this paper, however, a third plasmid was demonstrated and its sequence was determined. Although this plasmid pVV8 had been reported before, limited information and an unfortunate dropout in the substrain, whose genomic sequence was determined, would have prevented the plasmid from coming to public attention. The intrinsic circular plasmid, which was estimated to be six to ten copies in a cell, is 81151 bp and its G + C content is 68%. Among the identified 91 ORFs, a single gene has been experimentally analyzed before and is known as xylose isomerase. The *phnCDEGHIJKLMX* operon related to phosphonate metabolism, alkaline phosphatase, putative transcriptional regulators, several sets of toxin–antitoxin system, and transposase-like ORFs are also encoded on the pVV8 plasmid. Although association with cell aggregation was the one phenotypic characteristic of the plasmid that had been reported, it was never confirmed. Comparison of *T. thermophilus* HB8 strains suggests that the pVV8 is nonessential for growth.

Communicated by H. Atomi.

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Keywords *Thermus thermophilus* HB8 · Extreme thermophile · Plasmid · Complete sequence · pVV8

Abbreviations

Km	Kanamycin
Hm	Hygromycin
Bm	Bleomycin
ORF	Open reading frame

Introduction

Thermus thermophilus is an extreme thermophile that can grow at temperatures ranging from 50 to 82°C. It is an aerobic, rod-shaped, nonsporulating Gram-negative bacterium, which can also grow in minimal media (Oshima and Imahori 1971;1974; Tanaka et al. 1981). Except for temperature, *T. thermophilus* can be cultured under easily accessible conditions similar to those for model organisms such as *Escherichia coli* or *Bacillus subtilis*. In addition, transformation can be achieved by simply adding transforming DNA to a culture in rich media, as this thermophile exhibits growth-independent natural competence (Hidaka et al. 1994; Koyama et al. 1986). However, *T. thermophilus* is a polyploid organism harboring multiple genomic copies in a cell, similar to *Deinococcus* species and many cyanobacteria (Ohtani et al. 2010). The result of gene disruption should be confirmed with the greatest care. An established genetic engineering system and stable and easily crystallized proteins have added value to *T. thermophilus* as a model organism for functional genomics, structural genomics, and systems biology (Cava et al. 2009; Yokoyama et al. 2000). The "Structural and Functional Whole-Cell Project for *T. thermophilus* HB8," which aims to understand the mechanisms of all biological phenomena

occurring in the HB8 cell by investigating the cellular components at the atomic level on the basis of their 3D structures, is in progress (Yokoyama et al. 2000). Both this target strain and strain HB27 were originally isolated from a natural hot spring in Japan by Oshima and Imahori (1971, 1974).

The genome sequences of three *T. thermophilus* strains, HB8, HB27, and SG0.5JP17-16, are available (Henne et al. 2004; http://gib.genes.nig.ac.jp/single/index.php?spid=Tthe_HB8; http://gib.genes.nig.ac.jp/single/index.php?spid=Tthe_HB27; http://gib.genes.nig.ac.jp/single/index.php?spid=Tthe_SG05JP1716). The genome of the strains HB27 and SG0.5JP17-16 consists of a chromosome and a megaplasmid (pTT27 (0.23 Mbp) for HB27, and pTHTHE1601 (0.44 Mbp) for SG0.5JP17-16), while that of strain HB8 includes a plasmid pTT8 (9.3 kbp) in addition to a chromosome and a megaplasmid pTT27 (0.26 Mbp) (see the website described above). Comparative genomics between HB8 and HB27 have presented that the two chromosomes are highly conserved, whereas the megaplasmids show an elevated plasticity (Brüggemann and Chen 2006). Although comparative analysis with SG0.5JP17-16 has not yet been reported, the pTHTHE1601 megaplasmid is distinctly larger than pTT27. *T. thermophilus* might show wide variations of plasmids. Phenotypically, on the other hand, the HB8 exhibits cell aggregation (biofilm formation) during growth in rich media, while HB27 does not. This cell aggregation of HB8 was reported to be due to pVV8, an unrecognized plasmid with a size of 47 MDa (Mather and Fee 1990). However, information about this plasmid is limited (Mather and Fee 1990; Va'squez et al. 1983), and the released genomic database of HB8 contains no pVV8 sequence. Furthermore, as existence of the megaplasmid pTT27 had been unknown in the few published papers about pVV8, the difference between the two plasmids has been obscure.

In the course of plasmid analyses in *T. thermophilus* HB8, we stumbled across a pVV8-like plasmid with a size of 70–80 kbp corresponding to 47 MDa. The technical difficulty of preparing the plasmid may have delayed the whole picture of pVV8 from being unveiled until now; the method required some practice before it could be performed routinely. In this paper, we demonstrate that the pVV8 is the third circular plasmid in *T. thermophilus* HB8, and also present its features and the results of its sequence analysis.

Materials and methods

T. thermophilus strains, growth conditions and transformation

In this paper, two *T. thermophilus* HB8 strains are described. One is a strain that has been stored in our laboratory since being gifted by Dr. Tairo Oshima (Oshima and Imahori 1971, 1974), and the other is the RIKEN strain whose genome sequence was previously analyzed (Yokoyama et al. 2000). For convenience, we refer to the former strain as a stock strain and the latter as a RIKEN strain. The RIKEN strain was provided by the RIKEN BioResource Center through the National Bio-Resource Project of the MEXT, Japan. BEST24004 lacking the pTT8 plasmid was derived from the stock strain. The Δ*jrn* strain derived from the stock strain as shown in Fig. 3a(i) was constructed previously (Ohtani et al. 2010). *T. thermophilus* was grown at 70°C in TR media, and 1.5% gellan gum with 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ was added to TR media for plates (Hashimoto et al. 2001). Kanamycin (Km; 500 μg/ml), hygromycin (Hm; 100 μg/ml), and/or bleomycin (Bm; 20 μg/ml) were added to the media when needed. Transformation was carried out as described previously (Hashimoto et al. 2001).

Antibiotic-resistant genes

A Km resistance gene (Km^r) (Hoseki et al. 1999) and an Hm resistance gene (Hm^r) (Koyama unpublished) for *T. thermophilus* were kindly donated by Prof. Seiki Kuramitsu (Osaka univ. and RIKEN) and Dr. Yoshinori Koyama (AIST), respectively, whereas a Bm resistance gene (Bm^r) (Brouns et al. 2005) was chemically synthesized by TaKaRa Bio. The Hm^r and Km^r cassette used as a template for PCR had been constructed previously (Ohtani et al. 2010).

Plasmid preparation

The alkali-SDS method (Birnboim and Doly 1979) was used for large-scale plasmid preparation, followed by ultracentrifugation in a CsCl-ethidium bromide gradient (Sambrook et al. 1989). The pVV8 plasmid was gently purified from the thin cell lysate.

Gel electrophoresis

Plasmid or genomic DNAs were analyzed by contour-clamped homogeneous electric field (CHEF) gel electrophoresis in 1.0% agarose gel in TBE buffer (50 mM Tris–borate (pH 8.0) and 1.0 mM EDTA) at 15°C. Gels were stained by ethidium bromide and visualized under UV light.

Screening of pTT8-lacking strain of HB8

The Km^r or Hm^r gene was inserted into a *Bgl*II site of the pTT8 plasmid. *T. thermophilus* HB8 (the stock strain) was transformed by the pTT8 labeled by Km^r. Subsequently,

the resultant Km-resistant HB8 was transformed by the pTT8 labeled by Hm^r and transformants were selected in the presence of both Km and Hm. After the transformant (resistant to both Km and Hm) was grown in TR media in the absence of the antibiotics, the cells were spread on the antibiotics-free TR plate. Among ten thousand colonies analyzed on the drug resistances, five colonies exhibited sensitivity to both Km and Hm. The above cultivation without the antibiotics was performed independently twice, and the drug-sensitive strains were obtained in both trials. Plasmids from the strains were prepared and checked by electrophoresis, showing that all strains lost the pTT8 plasmid. One of them, BEST24004, was used in this study.

Southern hybridization

Genomic DNAs were prepared by a liquid isolation method (Saito and Miura 1963), digested by a restriction enzyme, and used for Southern hybridization analysis. For a probe, the pVV8 plasmid or a 2.2-kbp fragment among *Bgl*II-digested fragments of pVV8 (as indicated by an open arrowhead in Fig. 1b) was used as a template with a DIG high prime DNA labeling kit (Roche). Anti-DIG-alkaline phosphatase Fab fragments and CDP-star were used for detection according to the manufacturer's instructions (Roche).

Construction of the labeled pVV8

The 2.2-kbp *Bgl*II fragment as mentioned above contains a unique *Hin*dIII site. As shown in Fig. 3a(ii), the Hm^r and Km^r marker amplified by PCR, in which a *Hin*dIII site is introduced at both ends by PCR primers, was inserted at the *Hin*dIII site of the 2.2-kbp fragment. The resultant fragment was cloned into the *Bam*HI site of pUC19. To label the pVV8 by the Hm^r and Km^r marker, this pUC19 derivative was used to transform the Δ*jrn* strain after linearization by *Nde*I digestion.

Determination of the pVV8 copy number

The Δ*jrn* strain whose pVV8 was labeled by the Hm^r and Km^r marker was grown in synthetic media (Tanaka et al. 1981) at 55°C until exponential growth (0.8 of OD_{600}) or until the stationary phase (1.7 of OD_{600}). Genomic DNA was prepared from each culture, digested by *Kpn*I, and used for Southern analyses with the Hm^r probe. The signals corresponding to the Hm^r sequence were detected and quantified using the Molecular Imager FX (BIO-RAD).

Plasmid stability

The HB8 stock strain harboring pVV8 with the Hm^r and Km^r marker was subcultured at 70°C in TR media without antibiotics. The *T. thermophilus* was cultivated until the stationary phase for 24 h, and then the cells were spread on antibiotic-free TR plates and diluted 1:1000 in fresh TR media for the next cultivation. This passage was repeated eight times (total for 192 h). The resistances to antibiotics of one hundred of the resultant colonies were checked on a TR plate containing Hm or Km. The presence of the plasmid was defined by plasmid extraction from several of the drug-resistance colonies. Two independent trials were performed.

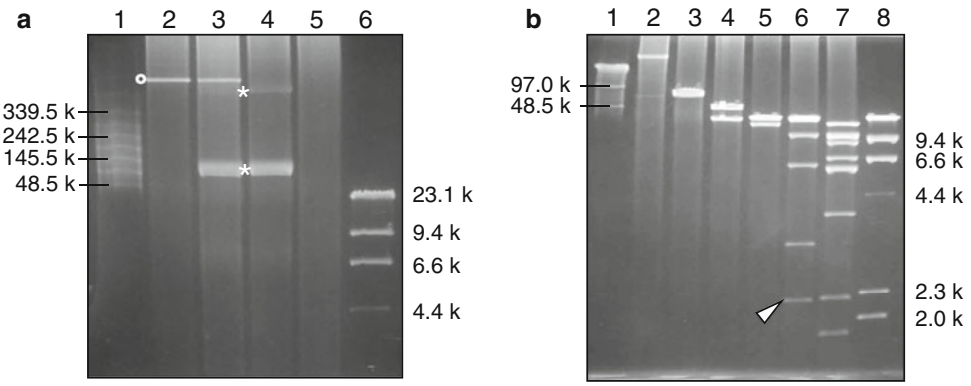

Fig. 1 CHEF gel electrophoresis of *T. thermophilus* plasmids. **a** Plasmids prepared from each strain were subjected to electrophoresis without restriction enzyme digestion: *lane 1* λ ladder marker, *lane 2* BEST24004, *lane 3* the stock strain of HB8, *lane 4* the RIKEN strain of HB8, *lane 5* HB27, *lane 6* λ/*Hin*dIII digest marker. An *open circle* presents a multimer of the pVV8 plasmid. *Asterisks* indicate multimers of the pTT8 plasmid, supported by comparison between *lanes 2* and *3* and analysis by restriction enzymes (data not shown). The pTT27 plasmid is invisible in this plasmid preparation condition (*lane 5*). Electrophoresis was performed at 120 V for 20 h with a switching time of 48 s. **b** Restriction enzyme digestion of pVV8: *lane 1* λ ladder marker, *lane 2* no enzyme, *lane 3 Nde*I, *lane 4 Eco*RV, *lane 5 Eco*RI, *lane 6 Bgl*II, *lane 7 Hin*dIII, *lane 8* λ/*Hin*dIII digest marker. An *open arrowhead* indicates the 2.2-kbp *Bgl*II fragment. Electrophoresis was performed at 90 V for 20 h with a switching time of 12 s. *The numbers along the gel* represent the DNA fragment size (bp) of the marker

Sequence determination and analysis

The pVV8 DNA sequence was determined with a genome sequencer FLX system (Roche) by TaKaRa Bio. ORFs on the pVV8 plasmid were identified based on amino acid sequence similarity to proteins registered in the DDBJ/EMBL/GenBank databases and referred to as TTHV001 to TTHV091. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB677526.

Results and discussion

The third plasmid in *T. thermophilus* HB8

Screening of *T. thermophilus* HB8 lacking the pTT8 plasmid was performed without any curing agent as described in "Materials and methods", and several candidates were obtained. One strain among the candidates was confirmed in detail and referred to as BEST24004. As shown in lane 2 of Fig. 1a, in the electrophoretic analysis, a band corresponding to a fragment larger than pTT8 was observed. The band indicated by an open circle was also detected for plasmid preparation from the parental *T. thermophilus* HB8 wild type (lane 3 of Fig. 1a). The strain HB8 also harbors the 0.26-Mbp megaplasmid pTT27 in addition to pTT8. However, in the case of *T. thermophilus* HB27 possessing pTT27 (0.23 Mbp), no band was observed (lane 5 of Fig. 1a), suggesting that this plasmid preparation method would be unsuitable for pTT27. It is not likely that the signal indicated by the open circle might have resulted from pTT27. As shown in Fig. 1b, restriction enzyme analysis of plasmid preparation from BEST24004 strongly supported it. For example, pTT27 of HB8 contains no *Nde*I site (see the website described above), whereas comparison between lanes 2 and 3 of Fig. 1b indicates that the unidentified DNA was cleaved by *Nde*I. These results suggest the possibility that *T. thermophilus* HB8 harbors the third plasmid in addition to pTT8 and pTT27. In lane 2 of Fig. 1b, not only a major band but also a minor band with a size similar to that of the *Nde*I digest (lane 3) can be observed. As pTT8 forms a multimer, as shown in lane 3 of Fig. 1a, it is likely that the unidentified plasmid is also a multimer. The minor band in lane 2 of Fig. 1b would be a monomeric size, and the unidentified plasmid appears to possess a unique *Nde*I site. Judged from lane 3 of Fig. 1b, the size of the unidentified plasmid was estimated to be approximately 70–80 kbp, which was supported by the total size of the *Hin*dIII fragments (lane 7 of Fig. 1b). As described below, the determined sequence agreed with it.

A few papers have reported that *T. thermophilus* HB8 harbors a plasmid with a size of 47 MDa (corresponding to 70–80 kbp), referred to as pVV8 (Mather and Fee 1990; Va'squez et al. 1983). The isolated plasmid was neither pTT8 nor pTT27, and was believed to be this pVV8 judged by the size. However, it is perplexing why there is no sequence of this pVV8 in the released genomic database of *T. thermophilus* HB8.

Presence or absence of pVV8

The *T. thermophilus* HB8, whose genomic sequence was determined, was obtained from RIKEN. Southern analyses with probes for pVV8, which was purified from BEST24004 by ultracentrifugation, were performed on this RIKEN strain and our stock strain of HB8. When pVV8 in its entirety was used as a probe, as shown in Fig. 2a, BEST24004 and its parental stock strain exhibited sharp signals similar to pVV8, whereas the RIKEN strain and HB27 showed thin mixed signals. When only the 2.2-kbp *Bgl*II fragment of pVV8 was used, on the other hand, a signal similar to that of pVV8 was detected in BEST24004 and the stock strain, but no signals were observed in the RIKEN strain and HB27 (Fig. 2b). These results suggested that neither the RIKEN strain nor HB27 would harbor the pVV8 plasmid. This is why the pVV8 sequence was not present in the released HB8 genomic database. As described later, pVV8 contains a sequence similar to a chromosome and pTT27 of HB8 and HB27. These would be the sources of the weak signals in lanes 5 and 6 of Fig. 2a.

No pVV8 in the RIKEN strain means that the plasmid is not essential for *T. thermophilus* HB8. Moreover, the RIKEN strain showed similar growth to the stock strain harboring pVV8 (data not shown).

Copy number of pVV8

To estimate the copy number of pVV8 on the basis of the chromosomal copy number, an Hm^r and Km^r marker was inserted at the *Hin*dIII site of the 2.2 kbp *Bgl*II region of pVV8 in the Δ*jrn* strain, in which the *jrn* gene on the chromosome was replaced by an Hm^r and Bm^r marker, as shown in Fig. 3a. Because *T. thermophilus* HB8 could harbor some copies of pVV8 in a cell, completed insertions in all the copies were checked by PCR and Southern analysis using a probe against the 2.2-kbp fragment (data not shown). The resultant strain was grown in the synthetic media at 55°C and used for analysis. In Fig. 3b, the signal intensity of the Hm^r gene on pVV8 (lower) was 1.5- to 2-fold that of the Hm^r and Bm^r marker on the chromosome (upper), both during exponential growth and in the stationary phase. *T. thermophilus* is a polyploid bacterium, and the copy number of the chromosome in HB8 has been estimated to be four to five per cell (Ohtani et al. 2010).

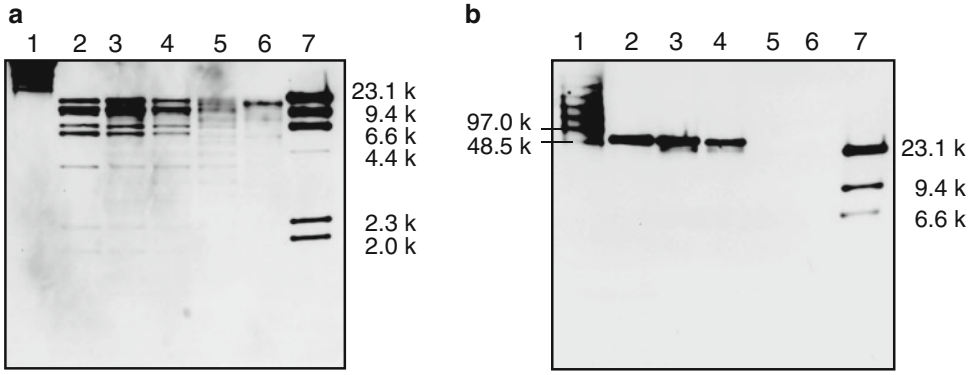

Fig. 2 The presence or absence of pVV8 in *T. thermophilus* strains. Genomic DNAs from each strain were digested, subjected to electrophoresis, and analyzed by Southern hybridization: *lane 1* λ ladder marker, *lane 2* purified pVV8 plasmid, *lane 3* BEST24004, *lane 4* the stock strain of HB8, *lane 5* the RIKEN strain of HB8, *lane 6*, HB27, *lane 7* λ/*Hin*dIII digest marker. *The numbers along the gel* represent the DNA fragment size (bp) of the markers. **a** DNAs were digested by *Hin*dIII, and pVV8 in its entirety was used as a template for a probe. Electrophoresis was performed at 90 V for 15 h with a switching time of 12 s. **b** DNAs were digested by *Nde*I, and the 2.2-kbp *Bgl*II fragment of pVV8 was used as a template for a probe. Electrophoresis was performed at 120 V for 20 h with a switching time of 48 s

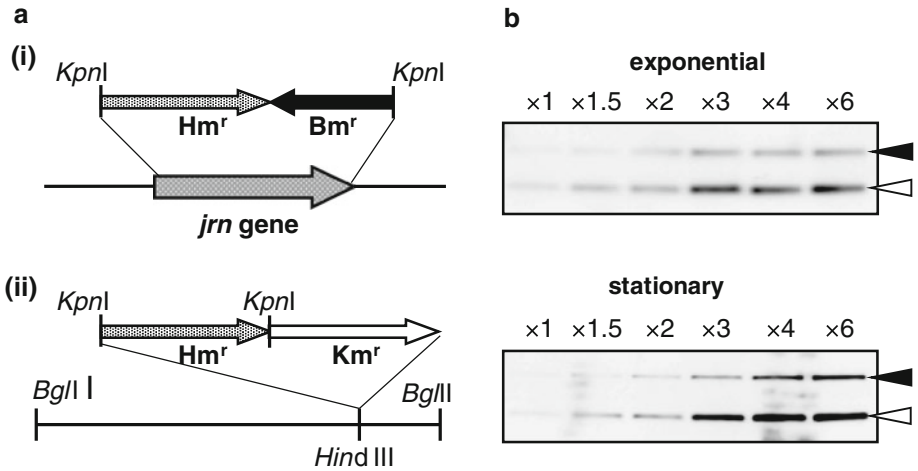

Fig. 3 Copy number of the pVV8 plasmid. **a** Constructs for estimation of copy number of pVV8. (*i*) The *jrn* gene-null mutant in *T. thermophilus* HB8 (stock strain). The gene on the chromosome was deleted by replacement with the Hm^r and Bm^r marker, as described previously (Ohtani et al. 2010). A *Kpn*I site is located at both ends of the marker. (*ii*) The Hm^r and Km^r marker-labeled pVV8. In the above Δ*jrn* strain, the Hm^r and Km^r marker was inserted into the *Hin*dIII site of the 2.2-kbp *Bgl*II region of pVV8. A *Kpn*I site is located at both ends of the Hm^r gene. **b** Genomic DNA from the Δ*jrn* strain harboring pVV8 labeled by the Hm^r and Km^r marker was prepared during exponential growth or in the stationary phase, digested by *Kpn*I, and was used for Southern analyses with a probe for the Hm^r marker. In the figure, ×1.5 to ×6 mean that 1.5- to 6-fold amounts of the digested DNAs were applied for electrophoresis. *Closed and open arrowheads* indicate the Hm^r and Bm^r signal on the chromosome and the Hm^r signal on pVV8, respectively

From the result in Fig. 3b, therefore, the copy number of pVV8 is ratable to be 6–10 per cell.

Cell aggregation

T. thermophilus HB8 exhibits cell aggregation (biofilm formation) when grown in rich media, whereas the strain HB27 does not. The aggregation phenotype has been published to be associated with pVV8 (Mather and Fee 1990). As the phenotype was a unique reported feature that could identify the plasmid as pVV8 with certainty, it was examined. The Hm^r- and Km^r-labeled pVV8 plasmid prepared from the strain for estimation of the copy number was used to transform the strain HB27. No aggregation was observed in the resultant transformants, whose pVV8 plasmid was confirmed (data not shown). This was a probable result, however, because the RIKEN strain without pVV8 presented the aggregation phenotype. Actually, it has been reported that the *galE* gene encoding uridine diphosphate-galactose-4′-epimerase on the chromosome is

important for the phenotype, backed up by more plausible evidence (Niou et al. 2009). Unfortunately, the link between the aggregation phenotype and pVV8 seems to be unlikely.

Plasmid stability

As pVV8 has been reported from several laboratories, *T. thermophilus* HB8 would have harbored the plasmid originally. The fact that the RIKEN strain lacks the pVV8 plasmid suggests that this plasmid might drop out easily from the cell during cultivation. Therefore, the stability of the Hm^r and Km^r-labeled pVV8 was analyzed in the HB8 stock strain as described in "Materials and methods". However, even after passage of culture for 8 days, all of one hundred checked colonies exhibited both drug resistances, suggesting that the plasmid was stably harbored. The plasmids from several colonies were extracted similarly in quantity in each passage (data not shown), supporting conservation of its copy number. In consequence, the pVV8 plasmid seems to be stable throughout the culture. In the case of the RIKEN strain, the HB8 strain lacking the unnoticed plasmid was speculated to have been selected in the laboratory by chance. Unfortunately, the strain was considered as the standard without ascertainment of the presence of pVV8, because of the scarcity of published information.

Sequence analysis

The sequence of pVV8 purified from BEST24004 was determined. The pVV8 is a circular plasmid consisting of 81151 bp and its G + C content is 68%. Based on amino acid sequence similarity, ninety-one ORFs (TTHV001 to TTHV091) were speculated as shown in Table 1. However, two of them, TTHV014 and TTHV043, contain frameshift mutation and nonsense mutation, respectively. Among the ORFs, only the TTHV085 gene has been previously analyzed and published as xylose isomerase (Dekker et al. 1991). TTHV084 encoding xylulokinase, and TTHV087 to TTHV089 encoding ABC-type D-xylose transporter-related protein are adjacently located on the plasmid. The pVV8 plasmid contains at least eight transposase-like ORFs (containing resolvase) similar to those on the chromosome and the pTT27 megaplasmid, and TTHV031 (xylose isomerase domain-containing protein/AP endonuclease), TTHV035 (*surE*), TTHV049 (hypothetical protein), TTHV057 (hypothetical protein), and TTHV058 (PilT domain protein) are almost the same as TTHB071, TTHB070, TTHB236, TTHB234, and TTHB233 on pTT27, respectively. This would be the reason for the weak signals in lanes 5 and 6 of Fig. 2a. Although the *phn* genes related to phosphonate metabolism are uncommon in

Table 1 ORFs encoded on the pVV8 plasmid

ORF	Nucleotide position (5′–3′)		No. of aa	Putative function
TTHV001	240	1322	360	Hypothetical protein
TTHV002	1329	1910	193	Hypothetical protein
TTHV003	2042	3001	319	ParA/cobyrinic acid ac-diamide synthase
TTHV004	3001	3903	300	ParB-like partition protein
TTHV005	7268	3900	1122	Type III restriction enzyme, res subunit
TTHV006	7369	8535	388	Filamentation induced by cAMP protein, Fic
TTHV007	8528	11353	941	Putative adenine-specific DNA methylase
TTHV008	11414	11683	89	HicA, YcfA family protein
TTHV009	11683	12150	155	HicB family protein
TTHV010	12197	12619	140	Helix-turn-helix domain protein
TTHV011	12623	13180	185	Hypothetical protein
TTHV012	13233	16487	1084	ATPase (AAA+ superfamily)-like protein
TTHV013	16571	16762	63	Hypothetical protein
TTHV014	16762	17895	–[a]	Transposase IS4 family protein
TTHV015	17985	18962	325	LacI family transcriptional regulator
TTHV016	19025	19804	259	Phosphonate ABC transporter, ATP-binding protein, PhnC
TTHV017	19801	20706	301	Phosphonate ABC transporter, periplasmic phosphonate-binding protein, PhnD
TTHV018	20675	21496	273	Phosphonate ABC transporter, permease protein, PhnE
TTHV019	21506	21925	139	Phosphonate metabolism protein, PhnG
TTHV020	21922	22467	181	Phosphonate metabolism protein, PhnH
TTHV021	22455	23486	343	Phosphonate metabolism protein, PhnI
TTHV022	23470	24351	293	Phosphonate metabolism protein, PhnJ
TTHV023	24248	25108	286	Phosphonate C-P lyase system protein, PhnK
TTHV024	25120	25833	237	Phosphonate C-P lyase system protein, PhnL
TTHV025	25799	26935	378	Phosphonate metabolism protein, PhnM
TTHV026	26922	27401	159	Phosphonate metabolism protein, probable acetyltransferase, PhnX
TTHV027	27401	28057	218	ABC transporter-like protein
TTHV028	28092	28508	138	Extracellular solute-binding protein
TTHV029	28530	29333	267	Metallophosphoesterase
TTHV030	29579	30052	157	Efflux ABC transporter permease

Table 1 continued

ORF	Nucleotide position (5′–3′)		No. of aa	Putative function
TTHV031	30091	30855	254	AP endonuclease
TTHV032	31181	32089	302	Glycerol-3-phosphate ABC transporter permease
TTHV033	32079	32891	270	Sn-glycerol-3-phosphate transport system permease UgpE
TTHV034	32903	34222	439	Glycerol-3-phosphate ABC transporter substrate-binding protein
TTHV035	34297	35031	244	Multifunctional protein, SurE
TTHV036	35024	35749	241	Glycosyltransferase
TTHV037	37083	36298	261	Putative transposase
TTHV038	37448	37161	95	Hypothetical protein
TTHV039	37748	39016	422	Alkaline phosphatase
TTHV040	39027	42464	1145	Endonuclease/exonuclease/ phosphatase
TTHV041	43011	42613	132	Hypothetical protein
TTHV042	43355	42978	125	Hypothetical protein
TTHV043	43846	43373	–[*]	Uncharacterized protein family UPF0150
TTHV044	43987	44214	75	Hypothetical protein
TTHV045	44256	44615	119	Hypothetical protein
TTHV046	45072	44626	148	Hypothetical protein
TTHV047	45218	45069	49	Hypothetical protein
TTHV048	45217	45624	135	Hypothetical protein
TTHV049	47051	46086	321	Hypothetical protein
TTHV050	47469	48206	245	Hypothetical protein
TTHV051	49771	48203	522	Hypothetical protein
TTHV052	49810	49908	32	Hypothetical protein
TTHV053	50095	49943	50	Hypothetical protein
TTHV054	50810	50085	241	Hypothetical protein
TTHV055	51173	50829	114	Hypothetical protein
TTHV056	51222	51560	112	Hypothetical protein
TTHV057	51557	51787	76	Hypothetical protein
TTHV058	51784	52200	138	PilT protein domain protein
TTHV059	53091	52702	129	PilT domain-containing protein
TTHV060	53323	53078	81	Hypothetical protein
TTHV061	56147	55629	172	Transposase
TTHV062	56662	56147	171	Transposase
TTHV063	57167	56724	147	Hypothetical protein/C-terminus of putative DNA methylase
TTHV064	57201	57761	186	Resolvase/N-terminal domain
TTHV065	57752	59017	421	Transposase, IS605 OrfB family
TTHV066	59736	59302	144	PilT protein domain protein
TTHV067	60011	59733	92	Toxin–antitoxin system, antitoxin component, PHD family

Table 1 continued

ORF	Nucleotide position (5′–3′)		No. of aa	Putative function
TTHV068	60677	62263	528	Transposase, IS605 OrfB family
TTHV069	62289	63269	326	Hypothetical protein
TTHV070	63266	63754	162	Hypothetical protein
TTHV071	64146	63751	131	PilT protein domain protein
TTHV072	64393	64139	84	SpoVT/AbrB domain-containing protein
TTHV073	64759	64418	113	Hypothetical protein
TTHV074	66063	65677	128	Hypothetical protein
TTHV075	66697	66053	214	SOS-response transcriptional repressor, LexA
TTHV076	66886	67191	101	Protein of unknown function DUF433
TTHV077	67188	67646	152	Hypothetical protein
TTHV078	67734	68000	88	Hypothetical protein
TTHV079	67952	68194	80	Hypothetical protein
TTHV080	68187	69407	406	Transposase
TTHV081	70135	69359	258	Short-chain dehydrogenase/ reductase SDR
TTHV082	71165	70128	345	Oxidoreductase domain-containing protein
TTHV083	72169	71168	333	Peptidase M24
TTHV084	73635	72166	489	Xylulokinase
TTHV085	74795	73632	387	Xylose isomerase[b]
TTHV086	75917	74796	373	ROK family protein
TTHV087	76692	75946	248	ABC transporter-like protein
TTHV088	77903	76689	404	Permease protein, ABC-type xylose transporter
TTHV089	78979	77957	340	D-xylose ABC transporter periplasmic substrate-binding protein
TTHV090	80227	79007	406	Transposase
TTHV091	80456	81103	215	Transcriptional regulator, XRE family

[a] TTHV014 and TTHV043 contain frameshift mutation and nonsense mutation, respectively

[b] TTHV085 has been experimentally analyzed before and is known as xylose isomerase (Dekker et al. 1991)

Thermus sp., the *phnCDEGHIJKLMX* operon (TTHV016 to TTHV026) is encoded on pVV8.

The pVV8 plasmid seems to possess at least three sets encoding the toxin–antitoxin system, TTHV008–TTHV009 (*hicAB*-like), TTHV057–TTHV058, and TTHV066–TTHV067, implying that these systems would maintain the plasmid stably in *T. thermophilus* HB8 cells. Although *parAB* genes (TTHV003 and TTHV004) and restriction and modification enzymes (TTHV005 and TTHV007) were identified, the precise replication origin was undefined.

However, we have experimentally defined the replication origin of the pVV8 plasmid (in preparation).

Acknowledgments We thank Prof. Seiki Kuramitsu (Osaka Univ. and RIKEN) for the Km^r gene, Dr. Yoshinori Koyama (AIST) for the Hm^r gene, and Mr. Mitsuru Sato for technical assistance.

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