J. Biochem. 2009;146(6)797–803
doi:10.1093/jb/mvp123

A Novel Insertion Sequence Transposed to Thermophilic Bacteriophage φIN93

Isao Matsushita[1],* and Hideshi Yanase[2]

[1]Energy Technology Laboratories, Osaka gas Co., Ltd, 6-19-9, Torishima Konohana-ku, Osaka 554-0051; and [2]Department of Biotechnology, Tottori University, 4-101 Koyamacho-minami, Tottori 680-8552, Japan

Received April 6, 2009; accepted July 28, 2009; published online August 10, 2009

The nucleotide sequence of IStaqTZ2 are available in the DDBJ/EMBL/GenBank databases under the accession number AB063392. A novel insertion sequence (IStaqTZ2) was transposed from the genome of *Thermus thermophilus* TZ2 to that of the thermophilic bacteriophage φIN93. The complete nucleotide sequence of IStaqTZ2 was determined and was found to be 1,258 bp in length and to contain an open reading frame (ORF1179), which is predicted to encode a transposase. IStaqTZ2 was also found to contain two terminal inverted repeats with 48 and 52 bp, respectively. Based on homology analysis, IStaqTZ2 was classified as a member of the IS*256* family.

Key words: Bacteriophage, IS element, IS256 family, Thermus, transposase.

Abbreviations: IS, insertion sequence; EDTA, ethylenediamine tetra acetic acid.

There have been several reports of insertion sequences isolated from *Thermus sp*. Utsumi *et al*. (*1*, *2*) isolated an insertion sequence-like genetic element, ISL*taq1*, from the genome of *T. aquaticus* and found it to be homologous to IS*150* (*3*), which belongs to the IS*3* family. ISL*taq1* has imperfect terminal inverted repeats of 19 bp and does not cause target site duplication. In addition, Bergquist *et al*. (*4*) isolated an insertion sequence, IS1000, from the genome of *T. thermophilus* HB8 and found its nucleotide sequence to be similar to those of IS110 (*5*) and IS492 (*6*). IS1000 also had imperfect terminal inverted repeats of 6 bp and also did not cause target site duplication. Finally, based on a homology analysis, Henne *et al*. (*7*) predicted various insertion elements harboring complete or partial transposase genes in the genome and megaplasmid pTT27 of *T. thermophilus* HB27.

We previously isolated an extremely thermophilic bacteriophage, φIN93, from a lysogenic strain of *T. thermophilus* TZ2 (*8*). Moreover, during an infection experiment, we isolated a φIN93 containing an integrated insertion sequence. A subsequent homology search revealed the insertion sequence to be novel, and to differ from those mentioned above.

MATERIALS AND METHODS

Cell Growth and Phage Infection—Thermus thermophilus TZ2 were grown overnight at 70°C in A-2 medium consisting of 0.1% tryptone, 0.1% yeast extract and Castenholtz basal salts (pH 7.0) (*8*, *9*). To infect *T. thermophilus* TZ2 with φIN93, the culture was incubated for 3.5 h at 70°C with shaking at 220 r.p.m. (OD_{610} around 0.15 measured with a Hitachi U3310 spectrophotometer) and then mixed with φIN93 stock solution to a multiplicity of infection of around 0.4. Thereafter, the culture was incubated under the same conditions until complete lysis had occurred (2.5 h), after which the cell debris was removed by centrifugation at 3,000*g* for 10 min. The resultant φIN93 lysate was stored at 4°C for use as the phage stock. The titre of the phage was assayed using the overlay method of Adams with some modification (*9*, *10*).

Isolation of Phage DNA—φIN93 virions were prepared as described previously (*11*). DNase I and RNase A were added to the φIN93 lysate to final concentrations of 1 μg/ml each and incubated for 30 min at 30°C. Thereafter, 2.5 M NaCl containing 25% polyethylene glycol 8000 was added to the φIN93 lysate to a final concentration of 20% (v/v), and the mixture was incubated for 1 h on ice before being centrifuged at 15,000*g* for 30 min at 4°C using an RPR12-2 rotor in a Hitachi Himac CR20B3 centrifuge. The precipitated pellets were suspended in 2 ml of 10 mM ammonium acetate buffer containing 5 mM MgSO4 (pH 6.0).

To extract the φIN93 DNA, an equal volume of phenol–chloroform (1 : 1 v/v) was added to the purified phage suspension, mixed and centrifuged at 2,800*g* for 10 min at room temperature using a T4SS rotor in a Hitachi Himac CT6E centrifuge. The aqueous phase was then extracted, and the DNA was precipitated in ethanol. The purified DNA was then dissolved in TE buffer (10 mM Tris hydrochloride and 1 mM EDTA, pH 8.0).

DNA Sequencing Analysis of the Insert—To clone the insert DNA fragment, the original phage φIN93 DNA and the inserted phage φIN93 DNA were digested with *BamH*I and the digested genomic DNA were separated by 1.0% agarose gel electrophoresis. The newly appeared two DNA fragments were cloned into pUC19 in *Escherichia coli* DH5α. The nucleotide sequences of each clone were determined using an ALF DNA

*To whom correspondence should be addressed. Tel: +81-06-6205-3089, Fax: +81-06-6462-3433,
E-mail: imatsu@osakagas.co.jp

© The Authors 2009. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

sequencer (Amersham Pharmacia Co.) with M-13 primer and T7 primer. From the sequence experiments, the inserted site in φIN93 genome and the partial nucleotide sequence of the insert were determined.

To clone the full length of the insert DNA fragment, DNA fragments were amplified by PCR (Gene Amp PCR System 9700 Applied Biosystems Co.) using a PCR kit (TOYOBO Co.) with the synthetic DNA oligomers 5′-GG TGGTGAAAGGTGGGACC-3′, and 5′-GAGTGAGGCCAA GCTGCT-3′. To determine the nucleotide sequence of the insert, two synthetic oligomers 5′-CTGCCATTCCGC CTGAGGG-3′ and 5′-CGGAGAGCGCCCTGGGATGG-3′ were constructed.

Computer Analysis—Homologous sequences and motifs were sought using GENETYX WIN software (Software Development Co.) with the DDBJ, EMBL and GenBank databases.

Southern Hybridization and PCR Analysis—To analyse the insertion sequence in the genome of host *T. thermophilus* TZ2, Southern hybridization and PCR analysis were carried out.

The genomic DNA of *T. thermophilus* TZ2 was extracted and purified using Isoplant (Nippon gene Co.). The extracted genomic DNA was digested with *BamH*I, *Bgl*II and *Pst*I. These digested DNA were separated by 1.0% agarose gel electrophoresis and transposed to the Hybond –N+ nylon membrane (Amersham Biosciences). Southern hybridization and colour detection with NBT/BCIP was carried out using the Dig High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics GmbH). The probe (185 bp) was amplified by PCR using the synthetic oligomers 5′-CCA GTACCTCCTTCACCTCCG-3′ and 5′-CCTGCGGAGCAC CAACCTG-3′. The probe was random primed labeled with Digoxigenin-11-dUTP using Dig-High-Prime according to the manual. The hybridization temperature is 50°C which is calculated according to GC content and percent homology of probe to target. The probe was washed at 68°C.

PCR was carried out with the synthetic DNA oligomers 5′-CCAGGATACCTTGCGGATC-3′ and 5′-CCGTTCTGCC CACCTCCC-3′ constructed from the nucleotide sequence of the insert and the amplified DNA fragment was sequenced.

Comparison of Lytic and Lysogenic Capacity of Phage—To compare the lytic and lysogenic capacities between φIN93 and an inserted phage φIN93, infection experiment was carried out. *Thermus thermophilus* TZ2 was infected with φIN93 (titre: 8×10^{-10} pfu/ml) or the inserted phage φIN93 (titre: 6×10^{-10} pfu/ml) and incubated at 70°C with shaking at 70 r.p.m. using temperature gradient rocking incubator (TVS126MB ADVANTEC CO.). The growth of the each cell was assessed as a function of the OD_{610}.

RESULTS

Isolation of a Novel Insertion Sequence—To assay the titre of φIN93, phage plaques were formed on an A-2 medium agar plate. To determine whether the φIN93 in each plaque was intact, the phage DNAs were isolated from some plaques, digested with *Bam*HI and subjected to 1.0% agarose gel electrophoresis. Notably, the digestion pattern of the DNA from the plaques differed from the original φIN93 DNA (data not shown): an unknown fragment had been inserted into the DNA harvested from the plaques.

Determination of the nucleotide sequence of the inserted fragment revealed that it was 1,258 bp long and had imperfect terminal inverted repeats, which were 48 and 52 bp, respectively, and showed significant homology, though they were not fully coincident (Figs 1 and 2). The fragment also contained an open reading frame (ORF1179) that could encode a protein of 392 amino acids, and a Shine–Dalgarno sequence (AGGAG G) was present upstream of the initiation codon GTG.

In addition, gene analysis using GENETYX WIN revealed the presence of transposase and the mutator family motif (D***GL******VYP) within ORF1179. These findings show the unknown fragment to be a novel insertion sequence (IStaqTZ2) containing a transposase gene. IStaqTZ2 was inserted at nucleotide position 17,819–17,826 in the φIN93 genome and caused duplication of the target site, CCCATGCT (8 bp), and *a Bam*HI site was identified within the inverted repeat of IStaqTZ2. IS*taq*TZ2 present in the genome of φIN93 was apparently stable, as it has never been eliminated in infection experiments. IS*taq*TZ2-inserted phage was termed φIN93-IStaqTZ2.

Classification of IStaqTZ2—When we carried out a search for homologues in the DNA and protein databases, we found that ORF1179 has significant similarity to the transposases of IS256 (*12*), IS1414 (*13*), IS905 (*14*) and ISRm3 (*15*), which are all members of the IS256 family (Fig. 3). We also found a DEE motif within ORF1179 [D***(66)**D****(106)****E*(6)*R] that is similar to those seen in IS256 family members: in parentheses are the numbers of amino acids close to those in IS256 family members (*16*). Likewise, the lengths of the terminal inverted repeats and the target site of IStaqTZ2 are close to those seen in the IS256 family. Thus, IStaqTZ2 was classified as a member of the IS256 family.

Analysis of IStaqTZ2 in the Genome of Thermus sp—To confirm the presence of IStaqTZ2 in the genome of *T. thermophilus* TZ2, Southern hybridization was carried out. In the result, several bands were appeared under the condition described in the MATERIALS AND METHODS section (Fig. 4). This result shows that IStaqTZ2 is present in some locus of the *T. thermophilus* TZ2 genome. The appearance of several thin bands and high molecular bands were thought to be owing to partial digestion of *T. thermophilus* TZ2 genomic DNA.

Furthermore, PCR was carried out using synthetic oligomers constructed from the nucleotide sequence of IStaqTZ2. The length of the amplified DNA fragment was 1,078 bp (Supplementary Figure S1), and its sequence was identical to that of IStaqTZ2, which confirms that IStaqTZ2 is indeed present in the *T. thermophilus* TZ2 genome.

After we had identified IStaqTZ2 and submitted it to the DNA databases, the same insertion sequence was found in the genome of *T. thermophilus* HB8 (accession number: AP008226, the locus; 227,470–228,727, target site; GGAACCGG/the locus; 966,067–967,324,

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

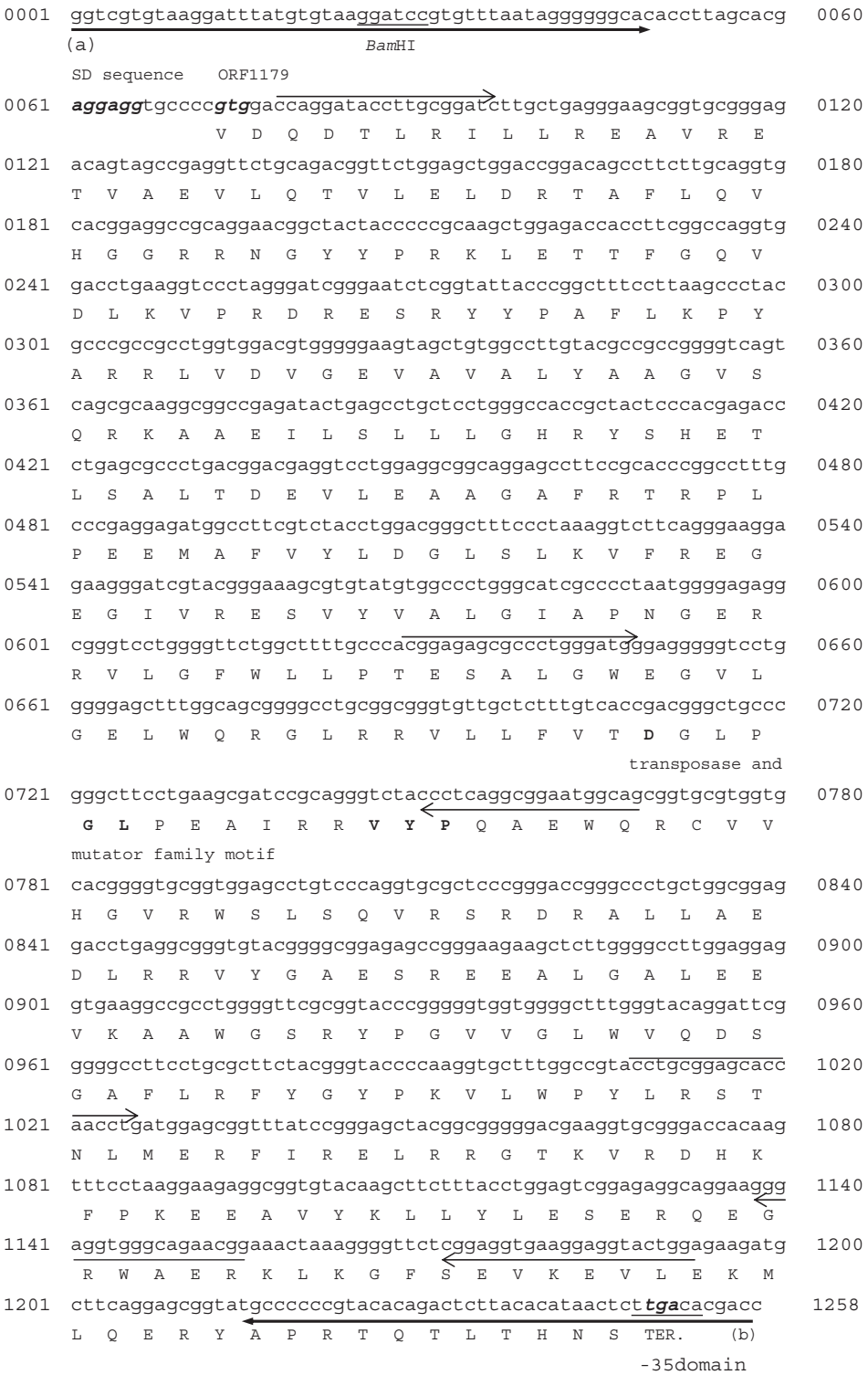

Fig. 1. **Complete nucleotide sequence of IStaqTZ2.** Terminal inverted repeats (48 bp and 52 bp, respectively) are indicated by bold arrows facing each other. The initiation codon GTG, termination codon TGA and Shine–Dalgarno sequence AAGGA are in bold italic. The *BamH*I restriction site and −35 domain (TTGACA) in the *Thermus* promoter are underlined. Transposase and the mutator family motif (D***GL******VYP) are in bold. The synthetic DNA oligomers are indicated by arrows.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

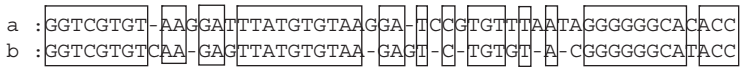

Fig. 2. **Comparison of the terminal inverted repeats.** Identical nucleotide sequences between the terminal inverted repeats are boxed.

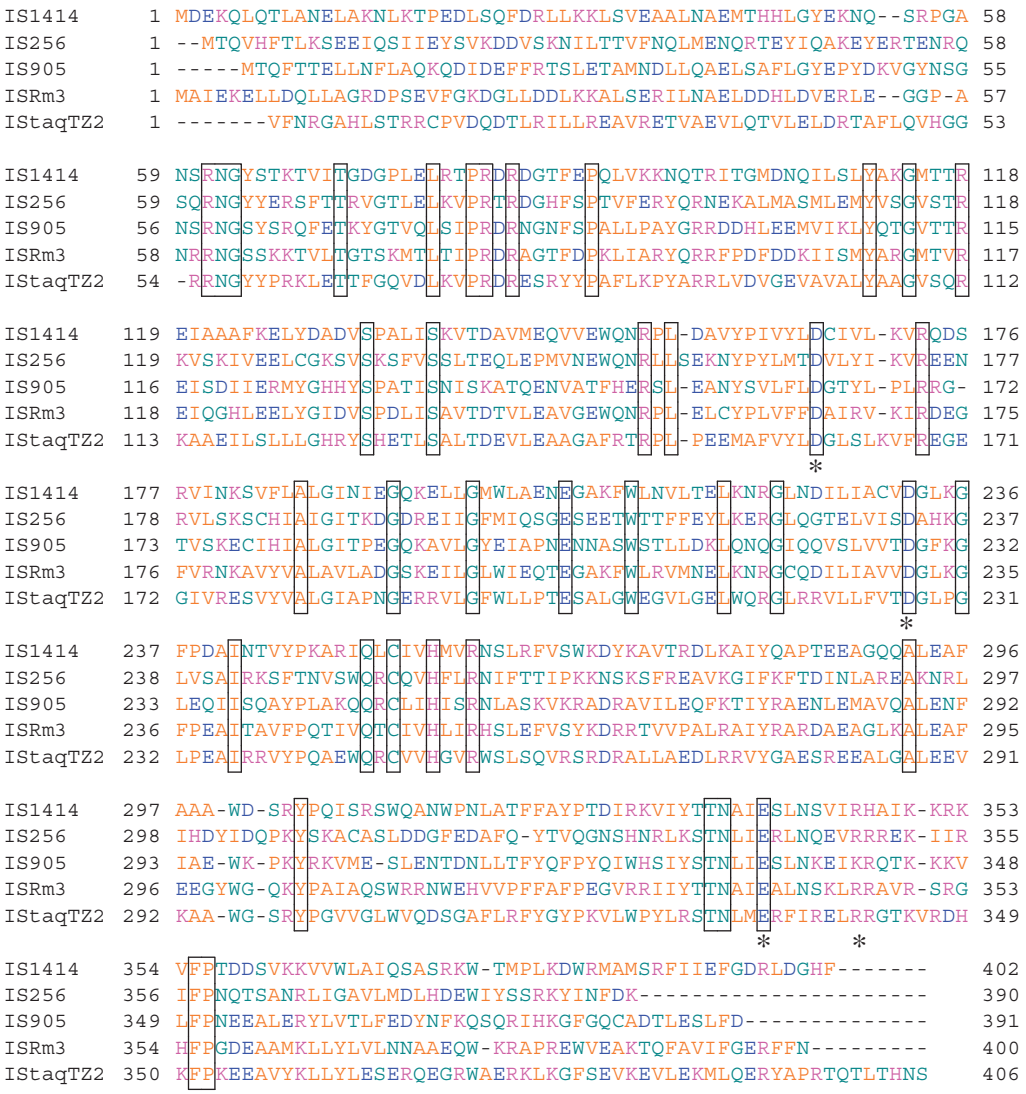

Fig. 3. **Multi-alignment of the protein encoded by ORF1179 and IS256 family transposases.** Identical amino acid residues are boxed. The amino acid residues of the DEE motif (D***(66)**D****(106)****E*(6)*R) are marked with asterisks. Hydrophobic residues are in yellow; acidic residues are in blue; basic residues are in pink; and neutral residues are in green.

target site; GTGGCACC/the locus; 1,210,978–1,212,235, target site; GTTCCTGGC/the locus; 1,750,228–1,751,485, target site; AGGGCCTC), in the megaplasmid pTT27 of *T. thermophilus* HB8 (accession number: AP008227, the locus; 76,013–74,756, target site; CCTTC GTG/the locus; 239,299–240,556, target site; GGAGC TTG) and in the plasmid of *Thermus sp*. 4C (accession number: EF407947, the locus; 891–2,148, target site; TGGGGACG) (*17*), which suggests IStaqTZ2 has been transposed among the host genome, plasmid and phage genome of *Thermus sp*. All of the target sites of these insertions are eight or nine bases but the nucleotide sequences of the target sites are different. So, it shows that the insertion of IStaqTZ2 occurs in a random fashion with eight or nine bases as a target site.

Analysis of IStaqTZ2 in the Genome of φIN93—IS*taq*TZ2 is inserted into ORF36 in the φIN93 genome. Based on the results of a homology search, ORF36 is

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

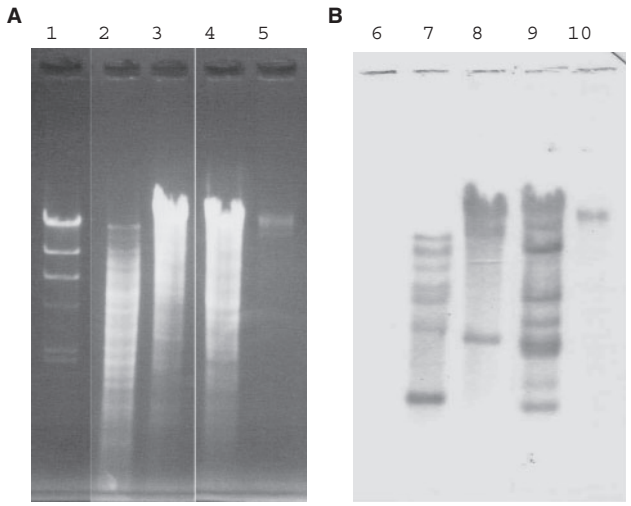

Fig. 4. **Southern hybridization analysis of IStaqTZ2 present in *T. thermophilus* TZ2 genome.** *Thermus thermophilus* TZ2 genome DNA, digested with some restriction enzymes, was subjected to Southern hybridization analysis using the DNA fragment (185 bp) amplified by PCR as a probe. (A) Ethidium bromide staining of 1% agarose gel before capillary transfer of DNA to the Hybond –N+ nylon membrane: lane 1; λDNA/*Hind*III, lane 2; *BamH*I, lane 3; *Bgl*II, lane 4; *Pst*I, lane 5; *T. thermophilus* TZ2 genome DNA. (B) Southern hybridization analysis: lane 6; λDNA/*Hind*III, lane 7; *Bam*HI, lane 8; *Bgl*II, lane 9; *Pst*I, lane 10; *T. thermophilus* TZ2 genome DNA.

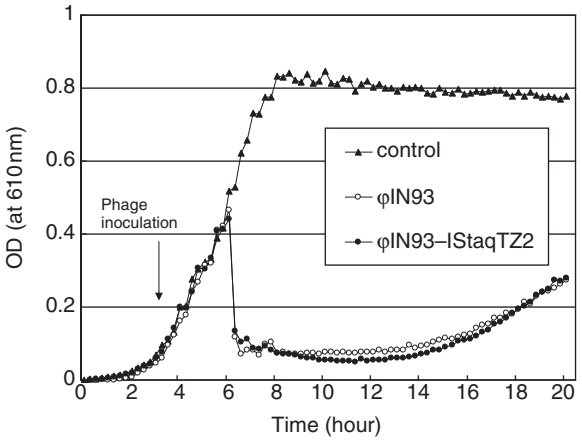

Fig. 5. **Comparison of the growth of *T. thermophilus* TZ2 infected with φIN93 or φIN93-IStaqTZ2.** *Thermus thermophilus* TZ2 was infected with φIN93 or φIN93-IStaqTZ2. The growth curves were indicated as follows: closed triangle, *T. thermophilus* TZ2; open circle, *T. thermophilus* TZ2 infected with φIN93; closed circle, *T. thermophilus* TZ2 infected with φIN93-IStaqTZ2.

predicted to encode a transcriptional regulator that functions in the same way as a lexA repressor (Supplementary Figure S2). ORF36 is therefore considered to be an important gene involved in determining whether the lytic cycle or lysogenic cycle proceeds (under subscription).

To compare the lytic and lysogenic capacities of φIN93 and φIN93-IStaqTZ2, we next carried out an infection experiment. Both the lytic capacity and the lysogenic capacity can be evaluated by the growth of the host cells after phage infection. Under conditions in which the titers of the two phages were about the same, the growth of host *T. thermophilus* TZ2 infected by each phage was assessed as a function of the OD_{610}. We found that the time from each phage's inoculation to lysis of *T. thermophilus* TZ2 or to growth of lysogenic *T. thermophilus* TZ2 was the same, whether the cells were infected with φIN93 or φIN93-IStaqTZ2. So, the growth curves by φIN93 or φIN93-IStaqTZ2 infection were almost coincident. Thus, φIN93-IStaqTZ2 had the same lytic and lysogenic capacities as φIN93 (Fig. 5). On the basis of the results, the gene function of IStaqTZ2-inserted ORF36 seems to be maintained as that of original ORF36.

DISCUSSION

We isolated the specific plaques of φIN93 with IStaqTZ2-inserted genome and confirmed that the IStaqTZ2 was present in *T. thermophilus* TZ2 genome by Southern hybridization and PCR experiment, showing that the insertion sequence, IStaqTZ2, transposed from *T. thermophilus* TZ2 to *Thermus* bacteriophage. This is the first description of transposition in *Thermus sp*. In future experiments, we aim to clarify the efficiency and the conditions under which IStaqTZ2 is transposed from *T. thermophilus* TZ2 genome to φIN93.

From gene analysis of φIN93-IStaqTZ2, ORF36 is splitted by IStaqTZ 2 and is predicted to extend from the initiation codon TTG, overlapping the stop codon (TGA) of the ORF1179, to the stop codon (TGA) of ORF36 (length: 651 bp, 216 amino acid residues). If this is the case, only the four amino acids MAIQ of gp36 are replaced by LT for the N-terminal sequence (Fig. 6). Thus, the gp36 is thought to maintain the functions as a transcriptional regulator. This supposition, however, requires further analysis to confirm its validity.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

ACKNOWLEDGEMENTS

We thank S. Oyama, A. Nakata and M. Moriya for technical assistance and discussion. We are grateful to Professor emeritus H. Kawasaki of Oosaka Prefecture University for valuable discussion.

CONFLICT OF INTEREST

None declared.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

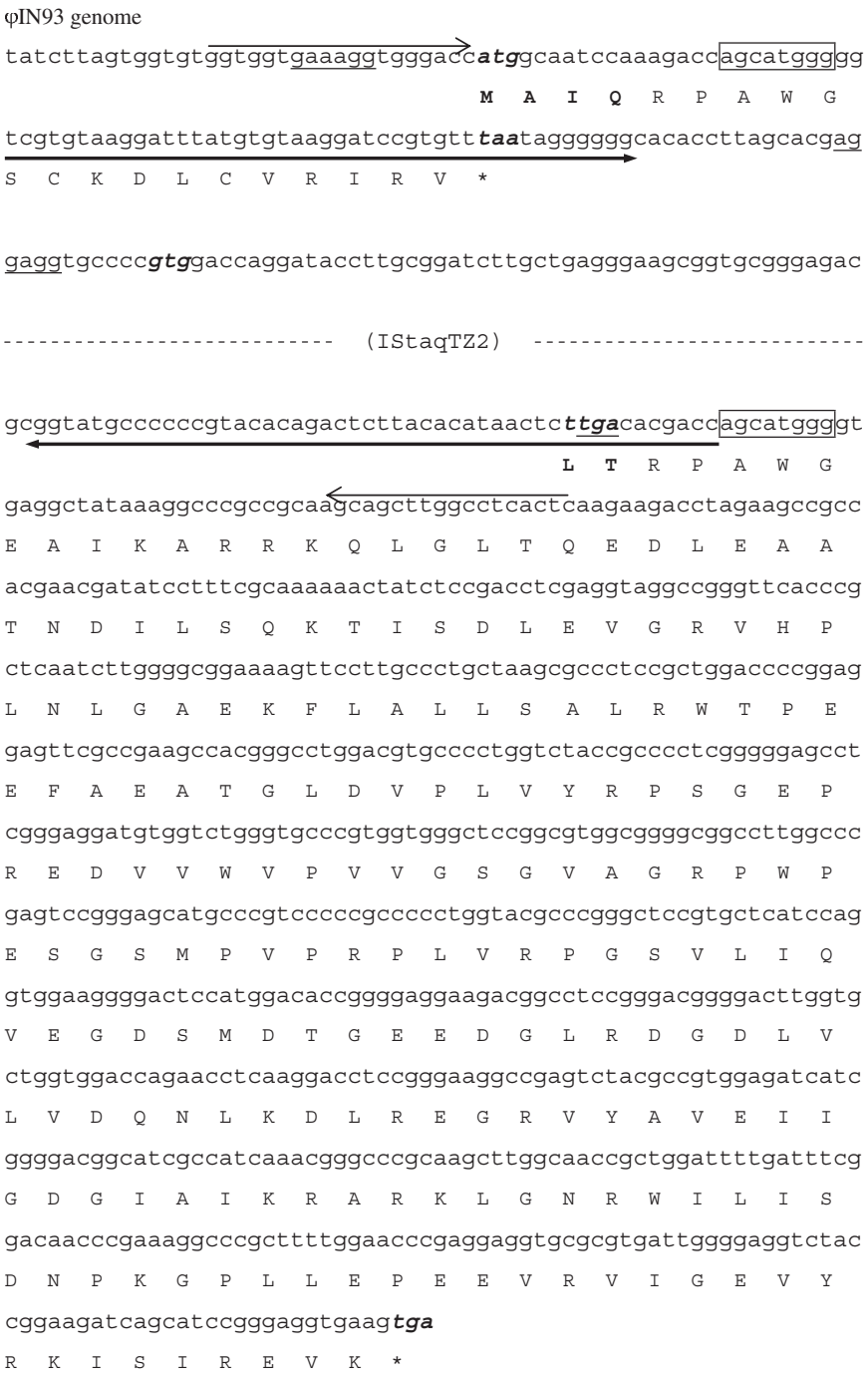

Fig. 6. **Insertion of IStaqTZ2 into ORF36 in the φIN93 genome.** The initiation codons (ATG and TTG) and termination codons (TAA and TGA) are in bold italic. Target site duplicates (AGCATGGG; 8 bps) are boxed. Terminal inverted repeats are indicated by arrows facing each other. The synthetic DNA oligomers are indicated by arrows. The replaced N-terminal amino acids (MAIQ and LT) of gp36 are in bold.

REFERENCES

1. Utsumi, R., Ikeda, M., Horie, T., Yamamoto, M., Ichihara, A., Taniguchi, Y., Hashimoto, R., Tanabe, H., Obata, K., and Noda, M. (1995) Isolation and Characterization of the IS*3*-like Element from *Thermus aquaticus*. *Biosci. Biotech. Biochem.* **59**, 1707–1711
2. Tanabe, H., Kauta, Y., Yoshioka, S., and Utsumi, R. (1998) Binding of the Protein from *Thermus aquaticus* ISLtaq1 to Its Inverted Repeat in vitro. *Biosci. Biotech. Biochem.* **62**, 401–403
3. Schwartz, E., Kroeger, M., and Rak, B. (1988) IS150: distribution, nucleotide sequence and phylogenetic relationships of a new E. coli insertion element. *Nucleic Acids Res.* **16**, 6789–6802
4. Ashby, M.K. and Bergquist, P.L. (1990) Cloning and sequence of IS1000, a putative insertion sequence from *Thermus thermophilus* HB8. *Plasmid* **24**, 1–11

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

5. Bruton, C.J. and Chater, K.F. (1987) Nucleotide sequence of IS110, an insertion sequence of *Streptomyces coelicolor* A3. *Nucleic Acids Res.* **15**, 7053–7065
6. Bartlett, D.H. and Silverman, M. (1989) Nucleotide sequence of IS492, a novel insertion sequence causing variation in extracellular polysaccharide production in the marine bacterium Pseudomonas atlantica. *J. Bacteriol.* **171**, 1763–1766
7. Henne, A., Bruggemann, H., Raasch, C., Wiezer, A., Hartsch, T., Liesegang, H., Johann, A., Lienard, T., Gohl, O., Martinez-Arias, R., Jacobi, C., Starkuviene, V., Schlenczeck, S., Dencker, S., Huber, R., Klenk, H.P., Kramer, W., Merkl, R., Gottschalk, G., and Fritz, H.J. (2004) The genome sequence of the extreme thermophile *Thermus thermophilus*. *Nat. Biotechnol.* **22**, 547–553
8. Matsushita, I., Yamashita, N., and Yokota, A. (1995) Isolation and characterization of bacteriophage induced from a new isolation of *Thermus aquaticus*. *Microbiol. Cult. Coll.* **11**, 133–138
9. Brock, T. D. and Freeze, H. (1969) *Thermus aquaticus* gen. nor. and sp. nor., a nonspolulating extreme thermophile. *J. Bacteriol.* **98**, 289–297
10. Adams, M.H. (1950) Methods of study of bacterial viruses. *Methods Med. Res.* **2**, 1–73
11. Yamato, K.R., Alberts, B.M., Benzinger, R., Lawwhorne, L., and Treiber, G. (1970) Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. *Virology* **40**, 734–744
12. Byrne, M.E., Rouch, D.A., and Skurray, R.A. (1989) Nucleotide sequence analysis of IS*256* from the *Staphylococcus aureus* gentamicin-tobramycin-kanamycin-resistance transposon Tn4001. *Gene* **81**, 361–367
13. McVeigh, A., Fasano, A., Scott, D.A., Jelacic, S., Moseley, S.L., Robertson, D.C., and Savarino, S.J. (2000) IS*1414*, an *Escherichia coli* insertion sequence with a heat-stable enterotoxin gene embedded in a transposase-like gene. *Infect. Immun.* **68**, 5710–5715
14. Dodd, H.M., Horn, N., and Gasson, M.J. (1994) Characterization of IS*905*, a new multicopy insertion sequence identified in lactococci. *J. Bacteriol.* **176**, 3393–3396
15. Wheatcroft, R. and Laberge, S. (1991) Identification and nucleotide sequence of *Rhizobium meliloti* insertion sequence ISRm3 similarity between the putative transposase encoded by ISRm3 and those encoded by *Staphylococcus aureus* IS*256* and *Thiobacillus ferrooxidans* IST2. *J. Bacteriol.* **173**, 2530–2538
16. Craig, N.L., Cragie, R., Gellert, M., and Lambowitz, A.M. (2002) Mobile DNA II. American Society for Microbiology Press, Washington, DC
17. Ruan, L. and Xu, X. (2007) Sequence analysis and characterizations of two novel plasmids isolated from *Thermus sp*. 4C. *Plasmid* **58**, 84–87

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012