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METHOD PAPER

Transposon mutagenesis of the extremely thermophilic bacterium *Thermus thermophilus* HB27

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Abstract *Thermus thermophilus* is an extremely thermophilic bacterium that grows between 50 and 80 °C and is an excellent model organism not only for understanding life at high temperature but also for its biotechnological and industrial applications. Multiple molecular capabilities are available including targeted gene inactivation and the use of shuttle plasmids that replicate in *T. thermophilus* and *Escherichia coli*; however, the ability to disrupt gene function randomly by transposon insertion has not been developed. Here we report a detailed method of transposon mutagenesis of *T. thermophilus* HB27 based on the EZ-Tn5 system from Epicentre Biotechnologies. We were able to generate insertion mutations throughout the chromosome by in vitro transposition and transformation with mutagenized genomic DNA. We also report that an additional step, one that fills in single stranded gaps in donor DNA generated by the transposition reaction, was essential for successful mutagenesis. We anticipate that our method of transposon mutagenesis will enable further genetic development of *T. thermophilus* and may also be valuable for similar endeavors with other under-developed organisms.

Keywords Thermophile · Genetics · Gap-repair · Extremophile · Recombination

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Introduction

The thermophilic bacterium *T. thermophilus* has become a model organism for understanding life at extreme temperatures (Cava et al. 2009). It is also a rich source of proteins and macromolecular complexes for which structures have been determined including RNA polymerase (Murakami and Darst 2003), the 70S ribosome (Schmeing and Ramakrishnan 2009), and the Cmr complex of the CRISPR-Cas host-defense system (Staals et al. 2013). Thermophilic bacteria are currently being developed as hosts for the production of biotechnologically relevant products and enzymes, and the expansion of available genetic tools for such organisms is instrumental in advancing their full potential (reviewed in Taylor et al. 2011). *T. thermophilus* HB27 is particularly well suited as a model thermophile as its genome is completely sequenced (Henne et al. 2004), it is easily cultivated aerobically in the laboratory, it is sensitive to a wide array of antibiotics (Gregory et al. 2005) and the rate of DNA uptake and transformation are particularly unique. *T. thermophilus* HB27 is naturally competent for transformation with genomic or plasmid DNA with an astonishing transformation efficiency of 10^{-2} transformants per cell (Koyama et al. 1986) and DNA binding and uptake rates of 40 kb s^{-1} per cell (Schwarzenlander and Averhoff 2006; reviewed in Averhoff 2009). Additionally, several genetic tools are in place including an *E. coli-T. thermophilus* shuttle vector (de Grado et al. 1999) and the ability to make targeted gene knockouts by homologous recombination and gene replacement with any of three thermostable antibiotic resistance genes (Hashimoto et al. 2001; Nakamura et al. 2005; Brouns et al. 2005).

A particularly powerful addition to the genetic toolbox would be transposon mutagenesis, as such a capability would enable genome-wide gene disruptions as well as

multiple downstream applications. Transposons provide convenient drug-resistance markers greatly facilitating strain construction and genetic mapping of spontaneous mutations (Kleckner et al. 1991; Miller 1992; Berg and Berg 1996). Transposon mutagenesis has for many years served as one of the most powerful tools in bacterial genetics (reviewed in Hayes 2003). Most modern synthetic transposons used for insertional mutagenesis have been engineered to lack a transposase gene, which is carried externally on a transposon delivery vehicle (Way et al. 1984). Such delivery vehicles are not stably maintained, preventing unwanted subsequent transposition. Although in vivo transposition of the naturally-occurring Tn*916* via conjugation has been demonstrated for *Thermus aquaticus* (Sen and Oriel 1990), and active transposition of endogenous IS elements in *T. thermophilus* has been documented (Gregory and Dahlberg 2008; Swarts et al. 2014), an effective system for in vivo transposon mutagenesis of *Thermus* spp. has not been described. Thus, a major impediment to progress in the genetic analysis of thermophiles is the lack of characterized transposons that function at high temperatures.

A potentially effective way to circumvent the need for a thermostable transposase is to perform transposition of transposase-deficient elements in vitro, with transposase enzyme provided in *trans* (Morisato and Kleckner 1987). Several commercially available transposase systems have been developed. For this study, we utilized the customizable EZ-Tn5 system from Epicentre Biotechnologies. This system, based on Tn*5* (Reznikoff 2002), is comprised of donor DNA flanked by a pair of inverted repeats, hyperactive 19-bp mosaic ends (ME), which are recognized in vitro by transposase (Goryshin et al. 2000; reviewed in Reznikoff 2008). This in vitro approach yields insertions that are stably integrated in the chromosome since there is no transposase expressed in the cell. Importantly, Tn*5* shows no target sequence bias (Reznikoff 2002) which is ideal for random transposon mutagenesis. Here we report a detailed method for transposon mutagenesis of *T. thermophilus* HB27 using the EZ-Tn5 system, with a modification to the manufacturer's protocol that was absolutely essential for successful implementation. We believe this will be extremely useful for expanding the capacity of *T. thermophilus* as a model organism and that our protocol variation may be advantageous for other organisms for which transposon mutagenesis has thus far been recalcitrant.

Materials and methods

Bacterial strains, plasmids and growth conditions

The EZ-Tn5 pMOD-3 <R6Kγ*ori*/MCS> transposon construction vector (catalog number TNP10623) and electrocompetent *E. coli* TransforMax EC100D *pir*-116 cells (catalog number EC6P095H) were obtained from Epicentre Biotechnologies (Madison, WI). *T. thermophilus* HB27 (ATCC BAA-163) was grown in ATCC medium 1598 (Thermus enhanced medium, TEM) at 65 °C and was supplemented with 2.8 % BD Bacto Agar for growth on solid media. *T. thermophilus* HB8 was obtained from ATCC (27634) and plasmid pTT8 was isolated using a Qiagen Miniprep kit from 6 ml of culture. Kanamycin was purchased from Fisher Scientific and used at 30 or 50 μg/ml for *T. thermophilus* and *E. coli*, respectively. Hygromycin B was from Sigma and used at 50 μg/ml. Cultures were archived in 25 % glycerol and stored at −80 °C.

Preparation of *T. thermophilus* chromosomal DNA

T. thermophilus chromosomal DNA was isolated from a 3 ml saturated overnight culture as follows. Pelletted cells were lysed with 25 μl of lysozyme solution (5 mg lysozyme dissolved in 0.5 ml 250 mM Tris–HCl, pH 8) on ice for 15 min. The lysate was treated with 50 μl STEP solution (1 % SDS; 50 mM Tris–HCl, pH 7.6; 50 mM EDTA; 1 mg/ml proteinase K) at 65 °C for 15 min, followed by RNase treatment (5 μl; Sigma-Aldrich R4642) at 37 °C for 30 min. Tubes were chilled on ice 5 min before adding 110 μl Protein Precipitation Solution (Promega catalog number A7951), vortexed vigorously and centrifuged. These large fragments of DNA were precipitated with isopropanol, washed with 70 % ethanol and resuspended in TE buffer.

Transposon generation and transposition

The *slpA* promoter and *kat* gene were amplified as a unit from plasmid pMK18 (de Grado et al. 1999) with primers slpA-kat-2 and slpA-kat-3 (see Online Resource 1 for primers used in this study). This was inserted using *Pst*I and *Bam*HI into the MCS of EZ-Tn5 pMOD-3 <R6Kγ*ori*/MCS> (Epicentre Biotechnologies, Madison, WI) an *E. coli* plasmid containing both the Tn*5* mosaic ends and the R6Kγ*ori*. The transposon was generated by PCR amplification with primers PCRFP and PCRRP in a 50 μl reaction using high-fidelity Phusion polymerase, then purified with a Qiagen PCR cleanup kit and estimated to be 100 ng/μl by gel electrophoresis. The in vitro transposition reaction was carried out with 1 μl EZ-Tn5 transposase according to the manufacturer's instructions using 1 μg each of purified transposon DNA and fragmented *T. thermophilus* HB27 DNA in a 20 μl volume. The reaction was incubated at 37 °C for 2 h, followed by addition of 1X Stop Solution and incubation at 70 °C for 10 min. The entire reaction volume was used to transform *T. thermophilus*. In the case of pTT8, we used 500 ng each of pTT8 and transposon DNA in a 50 μl transposition reaction.

Transformation of *T. thermophilus*

For gap repair of DNA, drop dialysis was performed on nitrocellulose filters (Millipore, 0.025 µm) for 15 min at room temperature against 200 ml distilled water. This was incubated with the PreCR Repair Mix from New England Biolabs (catalog number M0309) at 37 °C for 20 min. Transformation of *T. thermophilus* HB27 with chromosomal DNA was then performed as described (Koyama et al. 1986) using 1.5 ml culture, allowing 3 h of outgrowth after addition of DNA, and plating all cells on selective media. Standard molecular biology techniques and reagents were employed for additional procedures.

Rescue Cloning

Genomic DNA from transposon mutants was isolated and 1 µg was fragmented by restriction digestion with either *Aat*II or *Sac*II in a 20 µl volume at 37 °C for 2 h. Enzymes were inactivated at 65 °C for 20 min prior to adding T4 DNA ligase and 10X buffer in a 23 µl reaction volume to allow for self-ligation and circularization of plasmid clones. Ligase was inactivated at 70 °C for 10 min and the plasmid was desalted by drop dialysis, as described above, prior to transforming electrocompetent *E. coli* TransforMax EC100D *pir*-116 cells.

Results

In vitro transposition and transformation

Our overall approach for transposon mutagenesis (Fig. 1) exploits the natural competence of *T. thermophilus* and its highly efficient natural transformation and homologous recombination system using chromosomal DNA (Koyama et al. 1986). The transposon is generated by PCR to fuse the mosaic ends and an *E. coli* plasmid replication origin to a selectable marker as described in the "Materials and methods" (Fig. 2a). For this study, our transposon contained the thermostable *kat* gene controlled by the *slpA* promoter, which is known to confer kanamycin resistance in both *E. coli* and *T. thermophilus* (Lasa et al. 1992). The resulting transposon is incubated with *T. thermophilus* chromosomal DNA and in vitro transposition is catalyzed with purified transposase. The in vitro transposition reaction generates insertions at random with little or no sequence preference (Reznikoff 2002). *T. thermophilus* is transformed with mutagenized DNA selecting antibiotic resistance and generates mutants that have undergone recombination with the chromosome via double cross over events at regions of homology flanking the transposon (Fig. 2b). The recombination events generate insertional inactivation of the target gene and could potentially affect expression of downstream genes. Sites of insertion are identified by rescue cloning, in which the transposon and surrounding genomic sequences are extracted and cloned in *E. coli* by virtue of the plasmid replication origin. Insertion sites are then readily identified by sequencing.

Initial attempts at transposon mutagenesis were unsuccessful following the manufacturer's protocol. Although KmR colonies were obtained, rescue cloning (described below) was inefficient and, when possible, sequencing of some clones (primers SqFP and SqRP; Fig. 2a) gave conflicting results indicating insertion at discontiguous regions of the *T. thermophilus* chromosome. This made reliable identification of true insertion sites impossible. Transposon mutant JC852 lacked the expected 9-bp target site duplication (Fig. 2c), while mutant JC848 also lacked a 9-bp duplication and, instead, carried a 4-bp deletion. The mechanism by which EZ-Tn5 transposase inserts into target DNA involves a 9-bp staggered cut and, in *E. coli*,

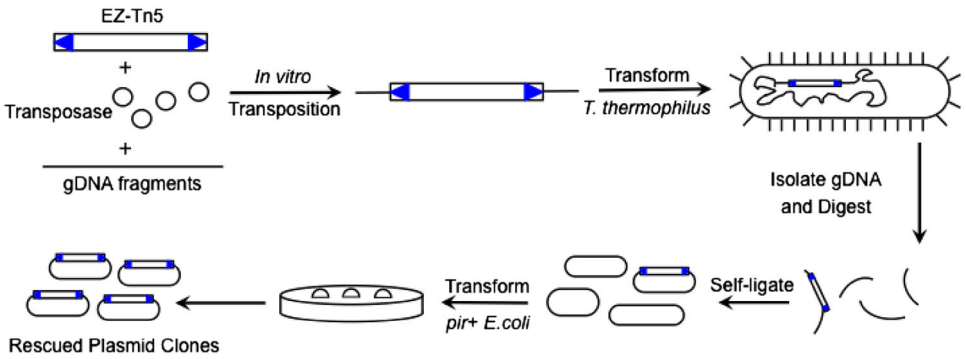

Fig. 1 Overall approach for transposon mutagenesis and rescue cloning. PCR-generated transposon shown as a *box* with *blue arrows* indicating the inverted repeat mosaic ends (see Fig. 2 for additional details). Transposon and genomic DNA (gDNA) were incubated with transposase in vitro; this was used to transform *T. thermophilus*. DNA isolated from these mutants was digested using a restriction enzyme and was self-ligated and used to transform *pir* + *E. coli*. Rescued plasmid clones containing the transposon and surrounding genomic DNA were isolated and sequenced

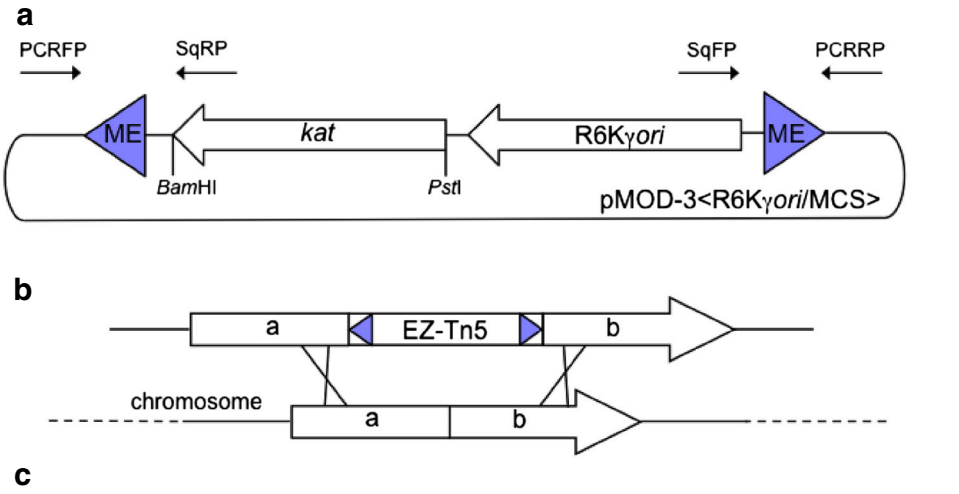

Fig. 2 Schematic diagram of EZ-Tn5 transposon generation and mutagenesis. **a** Orientation of thermostable kanamycin resistance (*kat*) gene and primers used for PCR amplification of transposon and for sequencing rescued clones. Mosaic ends (ME) in blue; R6K*γori*, replication origin in *pir* + *E. coli* cells including TransforMax EC100D *pir*-116. **b** Insertion of transposon in *T. thermophilus* by homologous recombination. In vitro-generated mutant chromosomal DNA was used to transform *T. thermophilus*, selecting kanamycin resistance. Double crossover events at genomic regions of homology generate a collection of insertion mutants in vivo. **c** Insertion site duplication event. The sequence of the 19-bp mosaic ends recognized by EZ-Tn5 transposase is shown in blue. Numerals 1 through 9 indicate the 9-bp direct repeat target site duplication. The 9-bp gap is illustrated

these single stranded gaps are repaired in vivo by the DNA replication and repair machinery. We speculated that the 9-bp single stranded gap might be recombinogenic in *T. thermophilus* and that the overhangs may have been removed by nuclease activity.

This problem was solved by filling in the 9-bp gaps after the in vitro transposition reaction to eliminate any single stranded DNA prior to transformation. To this end, we drop-dialyzed the transposon and initiated DNA repair using the PreCR Repair Mix which includes DNA polymerase and ligase activities. The reaction containing 1 µg of transposon-mutagenized, gap-repaired DNA was used to transform 1.5 ml of *T. thermophilus* and the entire cell volume was plated on one TEM kanamycin plate. Whereas no colonies arose on the no-DNA control plate over the course of 1 week, colonies were visible on the experimental plate after 1 day while slower growing mutants continued to appear over several days. Most of 186 total mutants were picked after 4 or 5 days of growth and each was streaked once on selective media, then once on non-selective media, and grown in liquid TEM. Saturated cultures were then used to prepare chromosomal DNA and frozen glycerol stocks.

Rescue cloning and identification of chromosomal mutants

One of the advantages of the EZ-Tn5 transposon system is the incorporation of the R6Kγ origin of replication. This plasmid ori is active only in *E. coli* strains expressing the Π protein, the product of the *pir* gene. This arrangement allows the excision of the transposon and surrounding genomic DNA by restriction digest, circularization by ligation, and replication in *E. coli*. The transposition site can then be identified by sequencing the cloned genomic fragment. From the 186 transformants obtained, 66 were examined and successfully cloned in *E. coli*.

Electrocompetent *E. coli* TransforMax EC100D *pir*-116 cells were transformed by electroporation with 5 µl circularized, rescued plasmid clones, selected on LB kanamycin plates and grown in liquid LB kanamycin. Plasmids were isolated using a Qiagen Miniprep kit and digested with *Kpn*I or *Hin*dIII to estimate their size for optimal sequencing results. Clones were sequenced with primers SqFP and SqRP which anneal within the transposon and sequence out toward the cloned chromosomal insertion site in either direction (Fig. 2a). Sequencing indicated that the gap-filled transposon reaction worked as anticipated, having generated both the 19-bp ME and the 9-bp target site duplication. Sequence results were used to search the *T. thermophilus* HB27 genome (GenBank accession number NC_005835.1; Henne et al. 2004) using BLAST (Altschul et al. 1990) to identify sites of transposon insertion. Importantly, results from both sequencing primers were in agreement in all cases and clearly indicated a single insertion site for each transposition event, in contrast to our initial efforts using non-repaired DNA. Insertions at 44

Table 1 Transposon mutants in *T. thermophilus* HB27

Gene ID	Description	# of isolates	Strain
TTC0043	*lysN* (Miyazaki et al. 2004); α-aminoadipate aminotransferase (Nishida and Nishiyama 2012)	1	JC943
TTC0051	hypothetical protein	4	e.g. JC939
TTC0083	*livF*; branched chain amino acid transport	1	JC913
TTC0155	thiosulfate reductase precursor	1	JC1098
TTC0285	UDP-N-acetylglucosamine 2-epimerase	1	JC1054
TTC0600	*livF*; branched-chain amino acid transport ATP-binding protein	1	JC949
TTC0779	acyl-CoA dehydrogenase	1	JC1093
TTC0980	mannose-6-phosphate isomerase	2	e.g. JC915
TTC1005	serine protease-like protein	1	JC848
intergenic TTC1044-1045	between *ccdA*, cytochrome C-type biogenesis protein and cytochrome c	1	JC1099
TTC1052	*soxB*; sulfur oxidation protein	1	JC1076
TTC1071	ABC transporter ATP-binding protein	1	JC1078
intergenic TTC1099-1100	between long-chain-fatty-acid-CoA ligase and *rpsI*, 30S ribosomal subunit protein S9	1	JC1073
TTC1100	*rpsI*; 30S ribosomal subunit protein S9	1	JC1094
TTC1120	small heat shock protein	1	JC1030
TTC1168	oligoendopeptidase F protein	1	JC1049
TTC1268	transporter	1	JC1097
TTC1270	*dppB*; dipeptide transport system permease	1	JC1095
intergenic TTC1305-1306	Shine Dalgarno of *infA*; translation initiation factor IF-1	1	JC1077
TTC1361	*phoR*; phosphate regulon sensor protein	1	JC1090
TTC3024	*rrsB*; 16S rRNA	8	e.g. JC914
TTC1388	mannose-6-phosphate isomerase	1	JC1064
TTC1396	acetyl-lysine deacetylase	1	JC1040
TTC1401	high-affinity iron permease	1	JC852
TTC1482	ABC transporter permease	1	JC942
TTC1547	3-isopropylmalate dehydratase large subunit	1	JC1044
TTC1604	*parB*; chromosome partitioning protein	1	JC1041
TTC1661	hypothetical protein	1	JC1067
intergenic TTC1674-1675	between permease and uracil permease	1	JC1102
TTC1675	uracil permease	1	JC1080
TTC1682	hypothetical protein	1	JC1096
TTC1707	formate-tetrahydrofolate ligase	1	JC1070
TTC1711	6-carboxyhexanoate-CoA ligase	1	JC1038
TTC1757	2-oxoisovalerate dehydrogenase subunit α	1	JC940
TTC1831	hypothetical protein	1	JC1091
TTC1845	general secretion pathway protein F	1	JC1032
TTC1852	*prfB*; peptide chain release factor 2 (RF2)	1	JC1063
TTC3048	*rrsA*; 16S rRNA	12	e.g. JC920
TTC1914	NADH-quinone oxidoreductase chain G	1	JC1101
TTC1948	*hrcA*; heat-inducible transcription repressor	1	JC1069
TT_P0024	alkaline phosphatase	1	JC1103
TT_P0042	*bgl*; β-glucosidase (Ohta et al. 2006)	1	JC1071
TT_P0142	putative plasmid stability protein Y4JK	1	JC1029
TT_P0166	hypothetical protein	1	JC1042

Identification of insertions sites in the chromosome and megaplasmid as annotated in GenBank, unless indicated otherwise

distinct genetic loci were identified among the 66 clones including, in some cases, multiple insertional events in the same gene (Table 1). All clones resulted from independent transposition events, having different insertion sites within the particular gene (see Online Resource 2 for all insertion site sequences).

The mutants listed in Table 1 highlight several interesting insertional events including the disruption of *rpsI* encoding ribosomal protein S9 (JC1094). S9 is characterized by an N-terminal globular domain and an extended C-terminal tail, which winds deeply through the internal structure of the 30S subunit and makes extensive contact with 16S rRNA. Insertion of the transposon generates an altered S9 C-terminus including an extension of the protein by 58 amino acids; thus we expect that the extended form of S9 will most likely fail to assemble into 30S subunits due to both loss of sequence-specific contacts with 16S rRNA and steric clashes involving the tail extension (Online Resource 3). It is likely that ribosomes from this strain lack S9 which is consistent with S9 being a nonessential protein (Bubunenko et al. 2007). In wild-type ribosomes the C-terminal residues of S9 contact the anticodon of the tRNA in the P site of the 30S (Selmer et al. 2006) and help to maintain reading frame. Alterations of the S9 C-terminus increase translational frameshifting (Näsvall et al. 2009) and these ribosomes exhibit altered translational initiation (Arora et al. 2013). Thus, the absence of functional S9 in this strain is likely to result in

erroneous translation, albeit at non-lethal levels. A similar type of insertion was identified (JC1063) in which the C-terminal 18 amino acids of RF-2 (protein release factor 2, *prfB*) have been swapped for 8 different amino acids. However, RF-2 is an essential protein involved in stop codon recognition and release of the peptide chain from the ribosome (reviewed in Youngman et al. 2008); therefore, this short truncation and alteration is not anticipated to interfere with overall function of RF-2.

Another mutant, JC1077, was found to have a transposon insertion at the Shine Dalgarno (SD) sequence (Shine and Dalgarno 1974), or ribosome binding site of *infA* encoding translation initiation factor IF-1. IF-1 is an essential protein (Cummings and Hershey 1994) which binds to the ribosomal A site during initiation of protein synthesis in order to direct initiator tRNA binding to the P site (reviewed in Laursen et al. 2005). *T. thermophilus infA* is imbedded in an operon containing downstream ribosomal protein and RNA polymerase genes that are essential for growth. As a consequence of the mechanism of Tn*5* transposition, the target site sequence that contains the SD of *infA* in JC1077 is duplicated downstream of the transposon, providing a SD for *infA*. It is interesting nonetheless that this large insertion between the native promoter and the SD apparently does not confer any significant polar effects on IF-1 nor the essential genes downstream.

T. thermophilus HB27 also contains a ~230-kb megaplasmid pTT27 (Accession number NC_005838.1) that is generally characterized as harboring genes that aid growth at high temperature or are involved in UV protection and DNA repair (Brüggemann and Chen 2006). It also contains eight of the ten CRISPR loci in the HB27 genome. We identified four insertions into pTT27 (designated "TT_P", Table 1). Mutant JC1071 has an insertion in the *bgl* gene, encoding β-glucosidase (TT_P0042), and has the great potential to serve as a host strain when combined with a plasmid-encoded *bgl* gene acting as a reporter (Ohta et al. 2006).

Transposon mutagenesis of plasmid pTT8

T. thermophilus HB8 harbors the 9322 bp cryptic plasmid pTT8 (Hishinuma et al. 1978), the sequence of which has been determined (Accession number NC_006463.1; Takayama et al. 2004), and that is absent from the HB27 strain. It is predicted to contain fourteen open reading frames, most of which are annotated as hypothetical proteins. Thus, it would be useful to generate mutants of these genes to gain a better understanding of their function. We also wished to demonstrate, as a proof of principle, that we could mutagenize pTT8 with the EZ-Tn5 transposon and simultaneously create a plasmid that replicates in *T. thermophilus* and *E. coli* by virtue of the R6Kγ*ori* within the transposon.

It has been shown that a fragment of pTT8 corresponding to the replication origin is active in the HB27 strain (Takayama et al. 2004; Fujita et al. 2012). We amplified the *hph* gene (primers hphKpnI and hphPstI) from plasmid pMHPnqosGFP (Cava et al. 2008), which confers resistance to hygromycin B in *T. thermophilus* and *E. coli*, cloned it into pMOD-3 <R6Kγ*ori*/MCS> and selected transformants on LB hygromycin B plates. The transposon was generated by PCR as described in the "Materials and methods". Unlike the chromosomal mutagenesis we did not repair the single stranded gaps as we directly transformed *E. coli* TransforMax EC100D *pir*-116 cells; the gaps are expected to be repaired in vivo in *E. coli*. Transformants were selected on LB hygromycin B plates, purified once by streaking on selective media, grown in liquid LB with hygromycin B, and plasmids were isolated from 4.5 ml culture using a Qiagen Miniprep kit.

Sequencing of two clones demonstrated that the transposition reaction worked as anticipated and included the 9-bp target site duplication. These mutants were determined to be in gene TTHC004 and between open reading frames TTHC009 and TTHC010, all of which are annotated as hypothetical proteins. Both plasmids were used to successfully transform *T. thermophilus* HB27, selecting hygromycin B resistance, thus demonstrating their ability to replicate in both *E. coli* and *T. thermophilus*. Each plasmid was isolated from *T. thermophilus* HB27, subjected to restriction digestion, and was determined to be identical to that isolated from *E. coli*. Notably a lower plasmid yield was obtained from *T. thermophilus* HB27 compared to *E. coli*, consistent with the known copy number of eight per cell in *T. thermophilus* HB8 (Hishinuma et al. 1978).

Discussion

Here, we have described in detail the successful transposon mutagenesis of the extremely thermophilic bacterium *T. thermophilus* HB27 based on the Epicentre EZ-Tn5 mutagenesis kit. Most notably, we found that modification of the protocol to include a step to repair single stranded DNA gaps in the transposon was absolutely essential. We hypothesize that the presence of single stranded regions of DNA in this instance may have been recombinogenic in *T. thermophilus* during transformation. DNA uptake in *T. thermophilus* occurs initially with the binding of double stranded DNA followed by translocation to the inner membrane channel where one strand of DNA is degraded while the other strand proceeds to the cytoplasm where it may recombine with the genome (reviewed in Averhoff 2009). The competence protein DprA is required for high transformation frequency in *T. thermophilus*

(Friedrich et al. 2002) and the DprA homolog in *S. pneumoniae* is known to promote loading of RecA onto ssDNA (Mortier-Barrière et al. 2007). Thus, the presence of internal regions of single strandedness on donor DNA may promote more than one recombination event per transformation, consistent with our observation that discontiguous regions of DNA were identified in initial rescue cloning attempts.

Our method enabled the identification of transposon insertions at sites throughout the chromosome and megaplasmid including several genes that had been targeted more than once. Specifically, the hypothetical protein TTC0051 was identified four times; mannose-6-phosphate isomerase was identified twice; and both copies of the 16S rRNA gene, *rrsA* and *rrsB*, were identified twelve and eight times, respectively. In all cases these were independent, non-clonal isolates with distinct insertion sites (Online Resource 2). As there is no apparent sequence motif common to the insertion sites, we speculate this may relate to the manner of preparation of genomic DNA for in vitro transposition and/or that these regions may have a more accessible structure in vivo if they are highly expressed, as would be expected for rRNA genes.

We believe that the transposon mutagenesis described here will help to expand the development of *T. thermophilus* as a model thermophile. This opens the door to classic genetic approaches such as promoter probing which could, as an example, be done under variable growth conditions using a promoterless version of the *bgl* gene as a reporter. Adding to this is the ability to generate by transposition transcriptional or translational fusions that would shed light on specific gene expression or regulation. Transposon mutagenesis of the chromosome may also enable a more comprehensive understanding of how frequently or likely polar inactivation occurs in this organism. Also promising is the potential to explore the biotechnologically relevant CRISPR-Cas systems (Shen 2013) that primarily reside on pTT27, since the megaplasmid is also targeted with this approach. Owing to the natural competence of *T. thermophilus* with chromosomal DNA there is the opportunity to combine transposon insertions with mutations in other strains, simply by transformation. Similarly, we have the capacity to investigate the functions of the hypothetical genes of plasmid pTT8, as our hygromycin B-resistant derivatives may be combined with various chromosomal mutants and screened for phenotypic interactions.

Significantly, the approach and methods described here have potential for use with other extremophiles. In particular, additional genetic tools are desired for engineering the biotechnologically relevant *Thermoanaerobacter* and *Thermoanaerobacterium* spp., which have recently been shown to be naturally competent and transformable with genomic DNA (Shaw et al. 2010). The method may also aid in the genetic development of archaea that are transformable, such as *Methanococcus voltae* (Bertani and Baresi 1987), *Pyrococcus furiosus* (Lipscomb et al. 2011), *Sulfolobus* spp. (reviewed in Atomi et al. 2012), and *Halobacterium* spp. (Cline et al. 1989). Although transformation with genomic DNA has not been formally demonstrated for some of these organisms, the general approach and the requirement for gap repair may prove insightful in future applications.

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