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New Host-Vector System for *Thermus* spp. Based on the Malate Dehydrogenase Gene

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A *Thermus thermophilus* HB27 strain was constructed in which the malate dehydrogenase *(mdh)* gene was deleted. The Δ*mdh* colonies are recognized by a small-colony phenotype. Wild-type phenotype is restored by transformation with *Thermus* plasmids or integration vector containing an intact *mdh* gene. The wild-type phenotype provides a positive selection tool for the introduction of plasmid DNA into *Thermus* spp., and because *mdh* levels can be readily quantified, this host-vector system is a convenient tool for monitoring gene expression.

Thermus spp. are gram-negative thermophilic microorganisms that grow at temperatures between 50 and 82°C (1, 11). Plasmid vectors that have been used in *Thermus* spp. have been constructed that encode tryptophan, leucine, or pyrimidine synthesis genes to complement auxotrophic and deleted hosts, providing for positive selection of transformants (4, 12). The host-vector system described here is an improvement on previously reported plasmid vectors for *Thermus* spp. because not only is the *Escherichia coli-Thermus* shuttle vector easily selected and maintained in *Thermus* spp., but malate dehydrogenase activity, encoded by the *mdh* gene present in this vector can also be readily and accurately quantified (2).

Construction of *Thermus thermophilus* HB27 and *Thermus flavus* hosts with deletions of the *mdh* gene. To create the Δ*mdh* strain of *T. thermophilus* HB27, we constructed an integration vector designated pUC-S KmA. The construction is detailed in Fig. 1. A 2.3-kb PCR fragment containing a region spanning three separate genes, succinate coenzyme A ligase (*scsA*), malate dehydrogenase (*mdh*), and purine phosphoribosyltransferase (*gpt*), was amplified from the *T. flavus* chromosome and cloned into pUC18. A 10-ul PCR amplification reaction was conducted in an Idaho Technologies Rapid Air Thermo-Cycler in the presence of 8% glycerol and 1% dimethyl sulfoxide. The PCR amplification cycle was run for 40 cycles at 94°C, 55°C, and 1 min of holding at 72°C. The 984-bp *mdh* gene is located near the center of this 2.3-kb fragment, so that the chromosomal regions that flank *mdh* are 780 bp (5′) and 560 bp (3′). The entire coding sequence of the *mdh* gene was removed by restriction enzymes and replaced with a thermotolerant kanamycin resistance cassette (Km^r) (7). This vector, designated pUC-SKmA, can replicate in *E. coli* but not in *Thermus*. However, homologous recombination between the *scs*A and *gpt* gene sequences allows pUC-SKmA to integrate into the chromosome when it was used to transform *T. thermophilus* HB27 (5). Transformants were screened at 55°C on TT rich medium (6) supplemented with kanamycin (40 μg/ml). Approximately 10^4 kanamycin-resistant transformants per μg of DNA were observed. Two distinct colony types arose after 5 days of incubation. The majority of the colonies were very small (0.1 to 0.5 mm) even after 5 days. A few colonies (60 to 100 CFU) were much larger (1.5 to 2.8 mm), the same size as wild-type *T. thermophilus* HB27 colonies. The two colony types were subcultured, and total DNA (plasmid and chromosomal) was dot blotted onto a nylon membrane.

The dot blot was probed with a digoxigenin-11-dUTP (DIG)-labeled *T. flavus mdh* gene. DNA prepared from the smaller colonies did not hybridize to the *T. flavus mdh* gene, whereas DNA harvested from the larger colonies hybridized to the *mdh* probe. The membrane was also probed with a DIG-labeled Km^r cassette, and both small and large colony types hybridized to it. Because malate dehydrogenase is a key enzyme in the tricarboxylic acid cycle, the colonies resulting from double-crossover integration events (Δ*mdh*) are recognized by this small-colony phenotype. The larger Km^r colonies were single-crossover integration events in which the chromosomal *mdh* is intact and the entire plasmid is in the chromosome. The *T. thermophilus* Δ*mdh* Km^r mutant strain was designated MM8-5. *T. thermophilus* Δ*mdh* Km^r MM8-5 was used as a recipient in further transformation experiments.

Construction of *Thermus* vectors containing *mdh* as a reporter gene. The malate dehydrogenase (*mdh*) gene from *T. flavus* was amplified by PCR and cloned into *Thermus-E. coli* expression vector pTEXI. The expression vector pTEXI is capable of replication in both *Thermus* spp. and *E. coli*, and the promoter (J17) employed in this expression vector functions in both

TABLE 1. Malate dehydrogenase activity of *Thermus* vector constructs[a]

Strain	Promoter expressing *mdh*	MDH activity (U/mg)	
		25°C	50°C
HB27	Wild type	2.5 ± 0.12	19.9 ± 0.25
MM8-5/pSJ17mdhA*	J17	0.6 ± 0.01	5.5 ± 0.17
HB27/pTEX1-mdh	J17	3.7 ± 0.06	29.6 ± 1.39
MM8-5	None	0.0	0.5 ± 0.01
MM8-5/pTEXI-mdh	J17	4.2 ± 0.13	32.6 ± 0.72
MM8-5/pTEX1-D50-3	D50-3	0.3	2.7 ± 0.07
MM8-5/pTEX1-P2-300	P2-300	3.0 ± 0.07	23.2 ± 0.99

[a] Activity values recorded are averages of three replicate samples from three separate experiments, for a total of nine data points. Standard deviation is less than 5%. One unit of activity is defined as the amount of enzyme needed to convert 1 μmol of NADH to NAD in 1 min. *, integrative vector.

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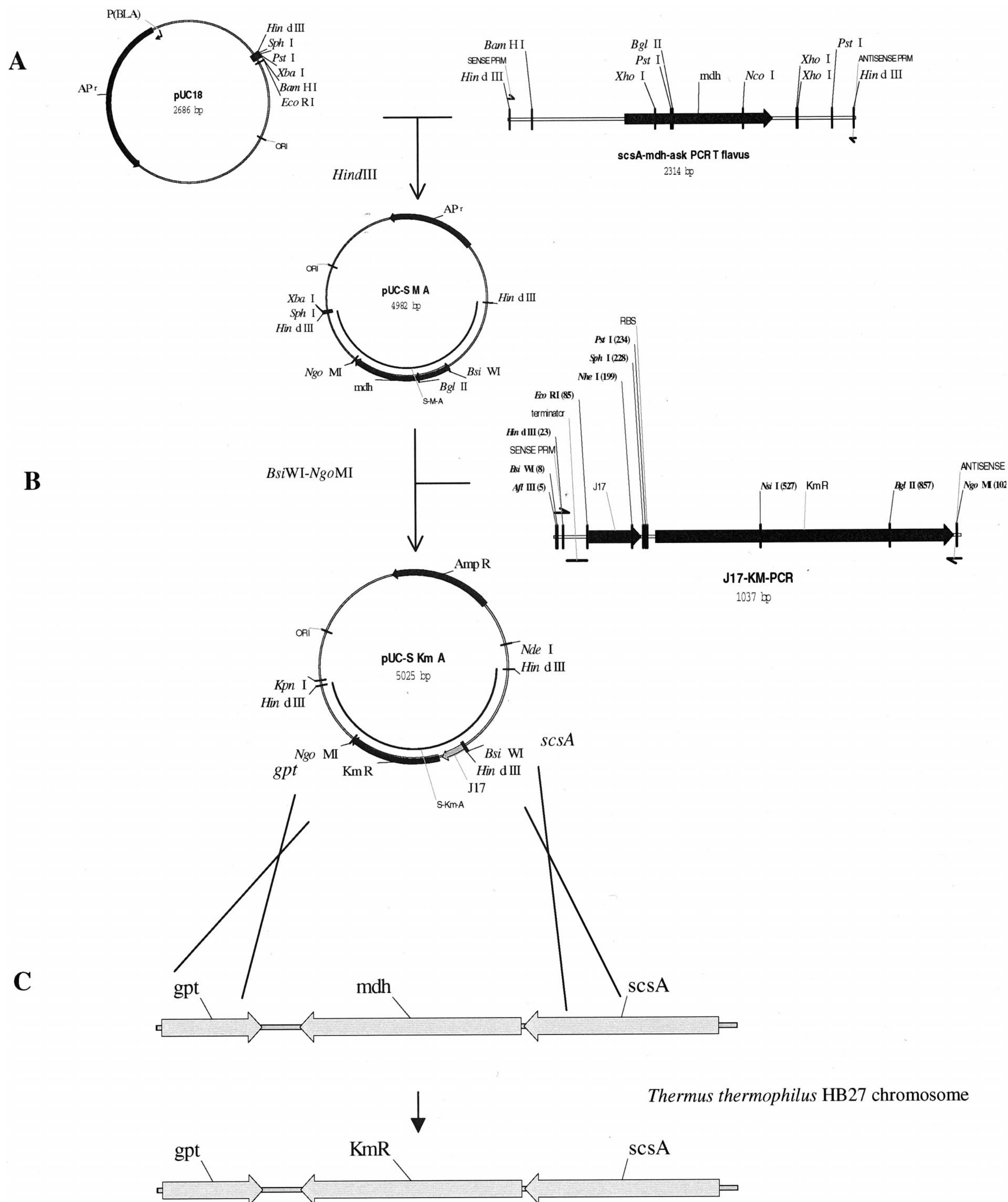

FIG. 1. Construction of *T. thermophilus* Δ*mdh* Km^r MM8-5. (A) The region surrounding the *mdh* gene from *T. flavus* was amplified from chromosomal DNA using PCR primers forward. (5′-ACAACAAAGCTTCGGGCAAAGGGGGAACGGAGGTCCT-3′) and reverse (5′-ACAACAAAGCTTGAG CCTTTTGACCTCGTCCTGGGG-3′). These primers were designed to amplify the sequence of the malate dehydrogenase gene and the regions immediately flanking the malate dehydrogenase gene from *T. flavus* according to published DNA sequences (8–10). Restriction sites were added to the PCR primers to give 5′ and 3′ HindIII sites. The 2.3-kb PCR product containing the *mdh* gene and flanking regions was cloned into the HindIII site of pUC18. The resulting plasmid is 4,982 bp and was designated pUCS-M-A. (B) Plasmid pUCS-M-A was digested with *Bsi*WI and *Ngo*MI, removing the all but the first 20 bp of the *mdh* gene sequence. A kanamycin resistance cassette was amplified using plasmid pTEXJ17 as the template and using PCR primers forward (5′-ACAACACGTACGGATTACGCCAAGCTTCATGGCCTAA-3′) and reverse (5′-ACAACAGCCGGCTCGTTCAAA ATGGTATGCGTTTTG-3′). Restriction sites were added to the PCR primers to give a 5′ *Bsi*WI and 3′ *Ngo*MI site. The cassette contains a strong constitutive *Thermus* promoter (J17) upstream of the themostable kanamycin nucleotidyltransferase cassette (Km^r). To prevent transcription readthrough from the native *mdh* promoter, a transcription termination sequence was cloned upstream of the J17 promoter. The kanamycin resistance cassette was digested with *Bsi*WI and *Ngo*MI and ligated into *Bsi*WI-*Ngo*MI-digested pUCS-M-A. The *mdh* gene was replaced with a kanamycin resistance cassette, and the resulting plasmid was designated pUC-SKmA. (C) After transformation of plasmid pUC-SKmA into *T. thermophilus* HB27, a double-crossover homologous recombination event replaces *mdh* with the Km^r determinant. The Δ*mdh* Km^r *T. thermophilus* HB27 strain subsequently isolated was designated MM8-5. RBS, ribosome-binding site; PRM, primer.

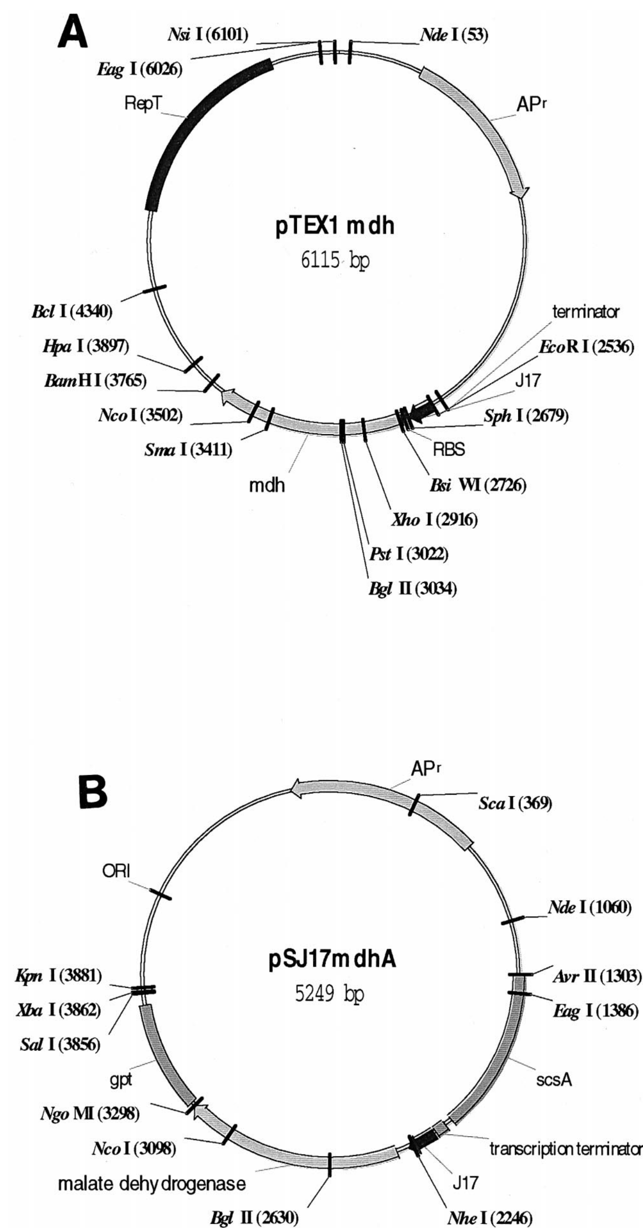

FIG. 2. (A) Expression vector pTEX1-mdh contains the *T. flavus* malate dehydrogenase gene downstream of the strong constitutive *Thermus* promoter J17. The *mdh* gene from *T. flavus* was amplified from chromosomal DNA using PCR primers forward *mdh* (5′-ACACAGAATTCGCATGCTCAAGAAGGCCCTGGGCTAA-3′) and reverse *mdh* (5′-ACACACGGATCCTGCGCCAGCATGGGGTGGTATAAA-3′). Restriction sites were added to the PCR primers to give 5′ *Eco*RI and 3′ *Bam*HI sites. Plasmid pTEX1 was digested with *Eco*RI and *Bam*HI, and an *Eco*RI-*Bam*HI-digested *mdh* PCR product was ligated into the vector to create pTEX1-*mdh*. (B) Integrative vector pSJ17mdhA has the J17 promoter-*mdh* gene cassette flanked by the *scsA* and *gpt* chromosomal regions.

bacterial hosts. J17 is a constitutive promoter isolated in our lab from *T. thermophilus* chromosomal DNA. The expression vector containing the *mdh* gene, designated pTEXI-mdh, is diagramed in Fig. 2A.

The J17 promoter from pTEXI-J17mdh was replaced by two constitutive *Thermus* promoters isolated in our laboratory (D50-3 and P2-100). These promoters have low and medium levels of expression, respectively, in *Thermus* spp. relative to J17. The resulting plasmids were designated pTEX1-D50-3 and pTEX1-P2-100. An integrative vector was constructed to examine the expression of the *mdh* gene under control of the J17 promoter present as a single integrated copy. This construct was designated pSJ17mdhA and is shown in Fig. 2B. pSJ17mdhA contains pUC19 sequences and can replicate in *E. coli*. pSJ17mdhA does not replicate in *Thermus* spp. as a plasmid but integrates into the chromosome by a double-crossover event. pSJ17mdhA has the J17 promoter-*mdh* gene cassette flanked by the *scsA* and *gpt* chromosomal regions. A transcription termination sequence from the *T. flavus* phenylalanyl tRNA synthetase operon (3) was cloned upstream of the cassette to prevent transcription readthrough from the native succinateCoA ligase/malate dehydrogenase operon promoter.

Plasmids pTEXI-mdh, pTEXI-D50-3, and pTEXI-P2-100 and the integrative vector pSJ17mdhA were transformed into MM8-5. Transformants were easily detected by the restoration of cultures to the wild-type or larger and faster-growing colonies by the expression of the malate dehydrogenase gene located on these expression vectors. Typically, *T. thermophilus* strain MM8-5 takes 4 to 5 days to form small visible colonies at 55°C in TT supplemented with 40 μg of kanamycin per ml. *T. thermophilus* MM8-5 transformants that received an expression vector carrying the *mdh* gene yielded larger colonies in 2 to 3 days.

Expression vector pTEXI-mdh and the alternative promoter pTEX derivatives are very stable in *T. thermophilus* MM8-5. After more than 20 generations of growth under nonselective conditions, pTEX plasmids were detected in all of the colonies examined (100 for each species). This result is expected because *T. thermophilus* MM8-5 cells that possess expression vectors containing the *mdh* gene grow more rapidly than plasmid-free cells that lack a functional *mdh* gene.

Malate dehydrogenase activity of *Thermus* constructs. The levels of malate dehydrogenase (2) being produced by plasmid and integrative expression vectors were evaluated in both *T. thermophilus* HB27 and MM8-5. Crude lysates prepared from each culture were assayed for enzyme activity at two temperatures (25 and 50°C), and the results are shown in Table 1. *T. thermophilus* MM8-5 had slight to no malate dehydrogenase activity, confirming complete deletion of the *mdh* gene from the chromosome of this strain. The activity observed in assays performed at 50°C reflect a slight amount of background due to the conversion of NADH to NAD by unidentified components of cell lysates rather than the malate dehydrogenase-dependent conversion of oxaloacetate and NADH to malate and NAD.

The malate dehydrogenase activity of crude extracts assayed at 50°C is on average nine times higher than the activity levels measured at 25°C. The data in Table 1 clearly indicate that promoters D50-3, P2-300, and J17 have different strengths, resulting in enzyme levels in MM8-5 strains that are 0.28, 1.16, and 1.65 times the level in wild-type *T. thermophilus* HB27, respectively. Since each promoter is evaluated here in identical genetic constructs that differ only by the promoter driving expression of the *mdh* gene, these levels should serve to accurately quantify the strength of these promoters. Other strains whose enzyme activity is listed in Table 1 all use the same promoter, J17, to express the *mdh* gene in various backgrounds.

MM8-5/pS-J17mdh-A contains a single copy of the *mdh* gene integrated into the chromosome under the control of the J17 promoter. MM8-5/pTEXI-mdh contains the *mdh* gene under the control of the J17 promoter on a plasmid vector, and HB27/pTEXI-mdh contains two separate sources of the *mdh* gene, a wild-type *mdh* gene on the chromosome and the *mdh* gene under the control of the J17 promoter on a plasmid vector. Since MM8-5/pTEXI-mdh yields 32.6 U of malate dehydrogenase per mg of protein, it is unexpected that HB27/pTEXI-mdh, which contains two separate copies *mdh* gene, a wild-type *mdh* gene on the chromosome as well as the *mdh* gene on a plasmid vector, shows nearly the same activity (29.6 U/mg). Plasmid instability may have contributed to homologous recombination between the two *mdh* gene copies.

We describe the construction of a *T. thermophilus* Δ*mdh* strain with a deletion of the entire DNA sequence encoding the *mdh* gene and its use as a host for *Thermus* plasmids expressing an intact *mdh* gene. The tricarboxylic acid cycle in *Thermus* spp., like most microorganisms, plays a central role in metabolism. Malate dehydrogenase catalyzes the dehydrogenation of malate to oxaloacetate using NAD^+ as a cofactor and is a key enzyme in the tricarboxylic acid cycle. This host-expression vector system offers a strong positive selection tool for the introduction of plasmid DNA into *T. thermophilus*, and *mdh* can be used as a reporter gene to quantify promoter strength in *T. thermophilus*. The growth rate advantage of mdh^+ versus Δ*mdh* cells enriches for cells that retain *mdh*-containing plasmids, which has the effect of stabilizing these plasmids in Δ*mdh* hosts.

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