An archaeal histone is required for transformation of Thermococcus kodakarensis.

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Running Title: Archaeal Histone Deletion Mutants

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Archaeal histones wrap DNA into complexes, designated archaeal nucleosomes that resemble the tetrasome core of a eukaryotic nucleosome. Therefore, all DNA interactions \textit{in vivo} in \textit{Thermococcus kodakarensis}, the most genetically versatile model species for archaeal research, must occur in the context of a histone-bound chromosome. Here we report the construction and properties of \textit{T. kodakarensis} strains that have TK1413 or TK2289 deleted, the genes that encode HTkA and HTkB, respectively, the two archaeal histones present in this Archaeon. All attempts to generate a strain with both TK1413 and TK2289 deleted were unsuccessful arguing that histone-mediated event(s) in \textit{T. kodakarensis} are essential. The HTkA and HTkB amino acid sequences are 84\% identical (56/67) and 94\% (63/67) similar but despite this homology, and their apparent redundancy in terms of supporting viability, the absence of HTkA and HTkB resulted in differences in growth and in quantitative and qualitative differences in genome transcription. A most surprising result was that deletion of TK1413 (\textit{ΔhtkA}) resulted in a \textit{T. kodakarensis} strain that is no longer amenable to transformation whereas deletion of TK2289 (\textit{ΔhtkB}) had no detrimental effects on transformation. Potential roles for the archaeal histones in regulating gene expression, and for HTkA in DNA uptake and recombination are discussed.
INTRODUCTION

The histone fold apparently evolved before the archaeal and eukaryotic lineages diverged ~1.5-1.9 billion years ago and now almost all Eukaryotes, *Euryarchaeia*, *Nanoarchaeia*, *Thaumarchaeia* and some *Crenarchaeia* employ histone fold-based DNA binding to wrap and compact their genomic DNA (2, 9, 14, 21, 28, 30, 38). All transactions in these species that involve chromosomal DNA must therefore be considered in terms of histone-bound chromatin. The presence of histones in *Archaea*, but not in *Bacteria*, was a major distinction first recognized ~20 years ago (26) and research since has established the detailed structure of archaeal histones (3, 19) the composition and architecture of the archaeal nucleosome (11, 24, 20, 22, 24, 30) and the consequences of archaeal histone binding on DNA topology, replication and transcription *in vitro* (7, 37, 43, 44). Archaeal nucleosomes resemble the eukaryotic tetrasome, the structure at the center of the eukaryotic nucleosome formed by ~90 bp of DNA wrapped around a histone (H3+H4)2 tetramer (20, 28, 30). Archaeal histones do not, however, have homologues of the N- and C-terminal amino acid extensions (21) that contain the targets for eukaryotic histone acetylation and methylation and so provide the basis for epigenetic regulation. Consistent with this, scrutiny of archaeal genome sequences has failed to detect recognizable homologues of the eukaryotic histone modification systems and, to date, no archaeal histone modification has been described (5). It is therefore intriguing and important to determine if archaeal histones do, nevertheless, participate in regulating genome functions. In this regard, in species with more than one archaeal histone, differences in their expression do correlate with
differences in laboratory growth rate (4, 25) but how this regulation is achieved and its physiological consequences are unknown.

Archaeal histone binding to DNA in vitro introduces DNA topology constraints, prevents transcription initiation and impedes transcript extension (7, 20, 43, 44).

However, only with the development of genetic approaches has it become possible to investigate how these in vitro observations relate to in vivo functions. Inactivation of the single histone-encoding gene in *Methanosarcina mazei* was possible but the resulting strain exhibited reduced growth, UV sensitivity and global transcriptome changes (41).

Similarly, inactivation of either of the two histone-encoding genes in the distantly related methanogen *Methanococcus voltae* was not lethal but the resulting strains had proteome differences (10). *Thermococcus kodakarensis* is naturally competent for DNA uptake and chromosomal transformation (36) and, with this advantage, *T. kodakarensis* has now been developed into the most genetically versatile model species forarchael research (12). *T. kodakarensis* contains two very similar archaeal histones [Fig. 1; (6)], now designated HTkA and HTkB (encoded by TK1413 and TK2289, respectively) and previously HPkA and HPkB when *T. kodakarensis* was classified as *Pyrococcus kodakarensis* (11). To add to the database and provide additional research tools, we have constructed and determined the phenotypes of *T. kodakarensis* strains with TK1413 or TK2289 precisely deleted. Apparently, at least one archaeal histone is required for viability as we were unable to construct a strain with both TK1413 and TK2289 deleted. As described below, based on differences in growth phenotypes, and the results of microarray hybridization experiments, the absence of each histone results in substantial quantitative and qualitative differences in genome expression. An
unanticipated and surprising discovery is that the deletion of TK1413 (htkA), but not TK2289 (htkB), resulted in a *T. kodakarensis* strain that is no longer naturally amenable to DNA transformation.

**MATERIALS and METHODS**

**Plasmid and *T. kodakarensis* strain constructions.** The *T. kodakarensis* strains and plasmids used in this study are listed in Table 1. Standard molecular biology procedures were used for PCR amplifications, plasmid constructions, *Escherichia coli* DH5α transformation and to select transformants and isolate plasmid preparations from *E. coli*. PCR amplicons generated from *T. kodakarensis* genomic DNA (primer sequences are available in Supplemental Table S1) were cloned into plasmids pTS535 or pTS414, as previously described (32, 33).

Plasmids incapable of autonomous replication in *T. kodakarensis* were constructed and introduced into *T. kodakarensis* TS517 (ΔpyrF; ΔtrpE::pyrF; ΔTK0664) (32). Recombination of the plasmid into the chromosome resulted in transformants capable of growth on plates containing artificial sea water (ASW) medium supplemented with vitamins, elemental sulfur (S˚) and 19 amino acids (AA) but lacking tryptophan (ASW+S˚+AA-trp medium). Transformants (intermediate strains, Figures 2 and 3) in which two homologous recombinations had replaced the chromosomal DNA with the desired plasmid DNA, including the [TK0254 (trpE)+TK0664] cassette, were identified via diagnostic PCRs. Expression of TK0664 resulted in sensitivity to 6-methylpurine (6MP). Dilutions of the intermediate strains, grown in ASW-yeast extract-tryptone plus sulfur (ASW-YT-S˚) medium, were plated on ASW+S˚+AA containing 6MP.
Spontaneously 6MP-resistant mutants were screened for the precise markerless deletion of TK1413 or TK2289. Two such 6MP\(^R\) isolates were designated \(T.\ kodakarensis\) LC124 and LC125 (Table 1) and used in the subsequent studies. Plasmids capable of autonomous replication and expression of cloned genes in \(T.\ kodakarensis\) were generated from the pLC70 [Table 1; (32)] which, when introduced into \(T.\ kodakarensis\) strains, resulted in transformants that were both tryptophan prototrophs and resistant to mevinolin. Plasmid pTS706 was generated from pTS414 by replacing TK1167 (\(rpoL\)) with TK1413 (\(htkA\)). Plasmids pTS707 and pTS708 were generated by using the Quikchange procedure (Agilent Technologies, Santa Clara, CA, USA) to introduce the desired sequence changes into TK1413 in pTS706 (Table 1; Fig. 1).

**Southern blots.** Genomic DNA preparation, restriction enzyme digestions, digoxigenin (DIG)-labeled probe preparation, DNA transfer, and development of blots using anti-DIG antibodies coupled to alkaline phosphatase were performed as described (23, 31). An oligonucleotide with the sequence underlined in Fig. 1B was synthesized, \([^{32}P]\)-labeled by incubation with T4 polynucleotide kinase and \([^{32}P]\)-\(\gamma\)-ATP and used as the probe in Southern blots.

**Growth curves.** Cultures of \(T.\ kodakarensis\) TS517, LC124, and LC125 were grown in ASW-YT-S\(^\circ\) medium under an atmosphere of 95% \(N_2\) plus 5% \(H_2\) at 85\(^\circ\)C. Changes in optical density at 600 nm (OD\(_{600}\)) were measured using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA).

**RNA extraction and microarray analyses.** RNA extractions and microarray hybridizations were performed as previously described (16). \(T.\ kodakarensis\) cultures...
were grown at 85°C in ASW-yeast extract-tryptone plus sodium pyruvate (ASW-YT-Pyr) medium, cells harvested during exponential growth (OD$_{660}$ ≈ 0.2) and RNA preparations were isolated using RNeasy Midi kits (Qiagen). The microarrays (Array Tko2) were manufactured by Takara Bio (Otsu, Japan) and contained target sequences corresponding to all 2306 open reading frames (ORFs) annotated in the *T. kodakarensis* KOD1 genome. Two copies of each sequence were present at different locations on each microarray providing two data sets for each microarray hybridization experiment. The data files contain the individual signal intensity ratios for each target sequence plus the average ratio and SD values.

RESULTS

*T. kodakarensis* is viable with either TK1413 (*htkA*) or TK2289 (*htkB*) deleted.

Plasmids pLC124 and pLC125 were constructed such that transformation and recombination into the *T. kodakarensis* TS517 genome resulted in deletion of the target gene (TK1413 or TK2289), insertion of the two-gene (TK0254+TK0664) cassette, and a duplication of genes that flanked the inserted cassette (Figs. 2 and 3). Representative transformants, selected by growth on ASW-medium lacking tryptophan, were confirmed by diagnostic PCRs (see below) to have the desired genome organization. As these intermediate strains lacked TK1413 or TK2289, they established that neither histone was essential for *T. kodakarensis* growth under laboratory conditions. Plating on ASW+S$^+$AA containing 10 µM 6MP selected spontaneously 6MP$^+$ clones. Diagnostic PCRs (see below) and sequencing confirmed the absence of the (TK0254 +TK0664) cassette and the precise and markerless deletions of TK1413 or TK2289 in two such
isolates, designated *T. kodakarensis* LC124 and LC125 respectively (Table 1), that were used in the subsequent studies.

**Differences in transformation and recombination frequencies.** Construction of *T. kodakarensis* LC125 (ΔTK2289) was routine (12, 33) Transformation of *T. kodakarensis* TS517 with 1 µg of pLC125 DNA generated >10³ tryptophan-independent transformants and genomic screening confirmed the desired cassette integration in all transformants so evaluated. Spontaneous 6MPR isolates were readily and abundantly obtained with the expected loss of the (TK0254+TK0664) cassette and the presence of the ΔTK2289 markerless-deletion. In contrast, transformation of *T. kodakarensis* TS517 with 1 µg of pLC124 resulted in ~100-fold fewer tryptophan-independent transformants and in ~90% of these transformants, genomic screening revealed that a single recombination event had integrated the entire pLC124 sequence into the *T. kodakarensis* genome adjacent to TK1413. In the remaining ~10% of transformants, the pLC124 sequence was either integrated at a site remote from TK1413 or the cells contained two genomes, one with the desired (TK0254+TK0664) integration and so with TK1413 deleted and a second genome that had not undergone recombination and retained TK1413. By subjecting such isolates to repeated single-colony isolations, clones were obtained in which genome segregation had occurred and so contained only the genome with the (TK0254+TK0664) cassette and ΔTK1413 deletion. Plating on 6MP-containing medium did then select 6MPR clones that had lost the (TK0254+TK0664) cassette but such mutants occurred at a 100- to 1000-fold lower frequency than routinely observed. Apparently, spontaneous recombinations between the duplicated sequences flanking
the (TK0254+TK0664) cassette occurred at a much-reduced frequency in the absence
of HTkA.

**PCR and Southern blot confirmation of the *T. kodakarensis* LC124 and LC125 genome structures.** The genome structures of *T. kodakarensis* LC124 (ΔTK1413) and LC125 (ΔTK2289) were confirmed by three different diagnostic PCRs and by Southern blot hybridizations (Figs. 2 and 3). PCRs with primers that hybridized on either side of TK1413 or TK2289 confirmed deletion of the target gene from its wild-type location and eliminated the presence of a second genome that retained the target gene at the wild-type locus (Figs. 2C and 3C). Failure to obtain an amplicon in PCRs using one primer that hybridized within TK1413 or TK2289 and a second primer that hybridized to a sequence adjacent to TK1413 or TK2289 further confirmed deletion of the target gene from the wild-type locus (Fig. 2C). Failure to obtain an amplicon with a primer pair that would generate an amplicon from within TK1413 or TK2289 eliminated the possibility of retention of TK1413 or TK2289 at any location within the *T. kodakarensis* genome.

Genomic DNA preparations from *T. kodakarensis* TS517, LC124 and LC125 were also subjected to Southern blot analyses with probes that hybridized to sequences within TK1413 and TK2289 and/or to genomic sequences directly adjacent to these genes. PvuII digestion of *T. kodakarensis* TS517 DNA generates a 2,905 bp fragment that contains TK1413 (Fig. 2A). An amplicon probe, generated from within TK1413 hybridized to this PvuII fragment in *T. kodakarensis* TS517 DNA (Fig. 2D; black arrow) but this fragment was not present in PvuII digests of *T. kodakarensis* LC124 DNA. With the sequence similarities of TK1413 and TK2289 (Fig. 1B), this probe also hybridized to
an ~1 Kbp PvuII fragment that contains TK2289 (Fig. 2D; asterisk) present in both \emph{T. kodakarensis} TS517 and LC124 DNAs, but not in \emph{T. kodakarensis} LC125 DNA (not shown). Southern blots using the oligonucleotide underlined in the TK1413 sequence in Fig. 1B as the probe (designated e; see Table S1), chosen as likely to minimize cross-hybridization with TK2289, confirmed that TK1413 was absent in \emph{T. kodakarensis} LC124 but present on a the 2,905 bp PvuII fragment in \emph{T. kodakarensis} LC125 and TS517 DNAs (Fig. 2E). As indicated in Fig. 3A, EcoRI digestion of \emph{T. kodakarensis} TS517 DNA generates a 5,534 bp restriction fragment that contains TK2289 (Fig. 3E; black arrow) and this was reduced to 5,330 bp in \emph{T. kodakarensis} LC125 DNA by the \(\Delta\)TK2289 mutation (Fig. 3E; grey arrow). Both these fragments hybridized to the amplicon probe generated with the primer pair (k - m) that contains TK2289 and sequences directly adjacent to TK2289. With the histone sequence conservation (Fig. 1A), this probe also hybridized to an ~11 Kbp EcoRI fragment present in both \emph{T. kodakarensis} TS517 and LC125 that contains TK1413 (Fig. 3E; asterisk).

\textbf{Failure to generate a strain with both TK1413 and TK2289 deleted.} \emph{T. kodakarensis} LC124 (\(\Delta\)TK1413) and \emph{T. kodakarensis} LC125 (\(\Delta\)TK2289) retain the \(\Delta\)pyrF; \(\Delta\)trpE::pyrF; \(\Delta\)TK0664 mutations (Table 1) and so are tryptophan auxotrophs and should be amenable to a second round of gene deletion by a repetition of the tryptophan selection-6MPR counter-selection protocol (12, 33). However, despite repeated attempts, we were unable to generate a \emph{T. kodakarensis} strain with both TK1413 and TK2289 deleted. Transformation of \emph{T. kodakarensis} LC125 with plasmid pLC124 DNA (Fig. 2) did generate tryptophan-independent transformants but TK1413 was still present in all of the ~100 transformants screened. The (TK0254+TK0664) cassette was integrated
either via a single entire-plasmid inserting event or via non-homologous recombination at a remote site. All attempts to transform *T. kodakarensis* LC124 with pLC125 DNA were unsuccessful and this led to the discovery that deletion of TK1413 resulted in a *T. kodakarensis* strain that was no longer amenable to transformation.

**Deletion of TK2289 (htkB) reduces growth.** As shown in Fig. 4, when grown in nutrient rich ASW-YT-S medium, cultures of *T. kodakarensis* LC125 (ΔTK2289) reached only ~75% of the final cell density of *T. kodakarensis* TS517 cultures whereas *T. kodakarensis* LC124 (ΔTK1413) cultures grew to almost the same final cell densities as *T. kodakarensis* TS517. Despite this difference in growth in liquid culture, the three strains formed colonies on Gelrite-solidified media with the same plating efficiency.

Inactivation of the histone-encoding gene in *M. mazei* resulted in a strain with increased UV-sensitivity (41) but there were no detectable differences in the UV sensitivities of *T. kodakarensis* TS517, LC124 and LC125 (results not shown).

**Loss of HTkA or HTkB results in quantitative and qualitative changes in the *T. kodakarensis* transcriptome.** The consequences of HTkA and HTkB loss on the abundance of transcripts *in vivo* were documented and quantified by comparing the steady state levels of transcripts in *T. kodakarensis* TS517 versus LC124 and in *T. kodakarensis* TS517 versus LC125. Fluorophore-labeled cDNAs, generated from RNA preparations isolated from exponentially growing cultures, were incubated with *T. kodakarensis* microarrays that carried duplicated spots of sequences generated from all 2,306 ORFs annotated in the *T. kodakarensis* genome (32). Reproducible and meaningful results were obtained for transcripts of 2,138 ORFs (~96%) in comparisons between *T. kodakarensis* TS517 and LC124 and for 2,153 ORFs (~97%) in...
comparisons between *T. kodakarensis* TS517 and LC125 (Table 2). Deletion of TK1413 resulted in a ≥2-fold change in the abundance of transcripts of 65 ORFs (~3% of transcripts measured) with transcripts of 30 ORFs increasing and transcripts of 35 ORFs decreasing in abundance in the absence of HTkA. Deletion of TK2289 resulted in a ≥2-fold change in the abundance of transcripts of 87 ORFs (~4% of transcripts measured) with transcripts of 56 ORFs increasing and transcripts of 31 ORFs decreasing in abundance in the absence of HTkB.

Thirteen of the ORFs that were transcribed at higher levels in the absence of HTkA appear to be components of four operons (Table 2). Transcripts of three of these operons were also more abundant in the absence of HTkB, consistent with both histones normally decreasing transcription or the stability of these transcripts *in vivo*. Increases in the abundance of the same eight monocistronic transcripts also occurred in the absence of either HTkA or HTkB but there were also increases and decreases in operon and single-gene transcript abundances that occurred specifically in the absence of HTkA or HTkB (Table 2). Notably, in the absence of HTkA, transcription of an operon (TK0161-TK0166) that is predicted to encode several membrane and cell surface components (6) was reduced, possibly playing a role in the loss of transformability of *T. kodakarensis* LC124. Similarly, in the absence of HTkB, the decreased transcription of several genes predicted to encode translation factors and ribosomal proteins seems notable as a potential factor in the reduced growth of *T. kodakarensis* LC125 cultures.

Transcription of several ORFs encoding proteins associated with purine metabolism increased in the absence of either histone, and transcription of one ORF
(TK1591) predicted to encode a transcription regulator was increased in the absence of HTkB but not in the absence of HTkA.

Deletion of TK1413 had no detectable effect on transcripts of TK2289 but, intriguingly, deletion of TK2289, resulted in a decreased abundance of TK1413 transcripts and of transcripts of TK0560 that encodes the unrelated chromatin protein Alba (29). The decrease in TK1413 transcripts however must not reduce HTkA production below that needed for viability.

**T. kodakarensis LC124 has lost competency for transformation.** The development of *T. kodakarensis* as a model system was founded on this species being naturally competent for DNA uptake and transformation (36). It was therefore important to explore the observation that transformation of *T. kodakarensis* TS517 with plasmid pLC124 DNA resulted in $\sim 10^3$ fewer tryptophan-independent transformants than routinely observed with similar donor DNAs, including pLC125, that direct the integration of the (TK0254+TK0664) cassette at other chromosomal locations (Table 3). Despite the low transformation frequency, *T. kodakarensis* LC124 ($\Delta$TK1413) was constructed but all subsequent attempts to obtain chromosomal transformants of *T. kodakarensis* LC124 were unsuccessful. Several different donor DNAs were used that generated large numbers of tryptophan-independent transformants using the identical protocol and reagents with *T. kodakarensis* TS517 and LC125 (12). One possibility was that *T. kodakarensis* had acquired an unusual sensitivity to some step in the protocol but this was not the case. *T. kodakarensis* LC124, exposed to the transformation protocol, formed colonies on non-selective media with the same plating efficiency as *T. kodakarensis* TS517 and LC125.
T. kodakarensis strains can also be transformed by autonomously replicating plasmids [pLC70 and derivatives (32); Table 1]. These plasmids are maintained in the T. kodakarensis cytoplasm by a replication machinery derived from plasmid pTN1 that was isolated from Thermococcus nautilus (40). Routinely, such plasmid transformations result in ~2-fold more transformants per µg of donor DNA than chromosomal transformations (Table 3). Transformation of T. kodakarensis LC125 with pLC70 and pLC71 generated transformants at the same frequency as transformation of T. kodakarensis TS517. In contrast, transformation of T. kodakarensis LC124 with pLC70 and pLC71 resulted in ~10⁴-fold fewer transformants (Table 3). However, once established in T. kodakarensis LC124, these plasmids were maintained as cytoplasmic replicons with the same copy number and stability as in T. kodakarensis TS517 and LC125.

**Constitutive plasmid expression of HTkA is toxic.** Plasmid pTS706 was constructed to determine if the transformation defect of T. kodakarensis LC124 could be suppressed by plasmid expression of HTkA. Plasmid pTS706 was derived from pLC70 (Table 1) and so should replicate autonomously in T. kodakarensis and had TK1413 positioned appropriately downstream from P₉₄₉₅, a constitutive promoter that has been used to express many genes in T. kodakarensis (31, 32, 33, 35, 42). Despite several attempts, incubation of T. kodakarensis LC124 with pTS706 never resulted in selectable transformants. Surprisingly, this was also the case in all attempts to transform T. kodakarensis TS517 and LC125 with pTS706 DNA. To determine if it was HTkA synthesis that was inhibiting the growth of transformants, the second codon (GCC) of TK1413 was changed to a translation stop codon (TGA). When the resulting plasmid
pTS707 (Table 1) was used as the donor DNA, transformants were obtained with *T. kodakarensis* TS517, LC125 and LC124 although transformants of *T. kodakarensis* LC124 were obtained at a much lower frequency than transformants of *T. kodakarensis* TS517 and LC125 (Table 3). DNA binding by archaeal histones is dependent on a universally conserved arginine residue (R20 in TK1413; Fig. 1) and variants with an isoleucine at this location do not bind DNA (27, 39). Plasmid pTS708 was therefore generated with codon 20 of TK1413 changed from AGG to ATT to synthesize the HTkA (R20I) variant but incubation of *T. kodakarensis* TS517, LC124 and LC125 with pTS708 DNA failed to produce transformants. Apparently, therefore, ectopic expression of an HTkA variant incapable of DNA binding was still toxic although it remains possible that the HTkA (R20I) monomers might assemble into toxic DNA-binding heterodimers with HTkB monomers synthesized from the chromosomal copy of TK2289.

DISCUSSION

The results obtained demonstrate that loss of the either HTkA or HTkB can be accommodated by *T. kodakarensis* but, as it was impossible to generate a strain with both TK1413 and TK2298 deleted, the presence of at least one archaeal histone seems essential for viability. Given that homodimers of either HTkA or HTkB are sufficient for viability, the histone requirement must only be for functions common to both histones. The results obtained however also argue for HTkA and HTkB have unique functions. In the absence of HTkA but not of HTkB, *T. kodakarensis* is no longer amenable to transformation whereas the loss of HTkB but not HTkA reduces growth in liquid culture.
As discussed, these different phenotypes may correlate with differences in HTkA-
versus HTkB-dependent gene expression.

Comparing the abundances of transcripts in *T. kodakarensis* LC124 (ΔTK1413) and *T. kodakarensis* LC125 (ΔTK2289) with those in the parental strain *T. kodakarensis* TS517, revealed that HTkA and HTkB participate in both transcription activation and repression *in vivo* (Table 2). For most ORFs where an increase in transcription occurred, this occurred in the absence of either HTkA or HTkB consistent with both histones normally negatively regulating transcription of these genes. Based on *in vitro* studies, promoter binding by HTkA and HTkB most likely limits access of the transcription apparatus and so limits initiation of transcription of these genes (7, 43, 44).

Using high-resolution nucleosome position technology (1) and with the availability of *T. kodakarensis* LC124 and LC125, this prediction can now be tested. In the absence of HTkA and HTkB reductions occurred in the abundances of transcripts of ~30 ORFs, with little overlap in the genes so regulated by HTkA versus HTkB. Transcription of these ORFs most likely is stimulated *in vivo* by HTkA and/or HTkB, either directly through histone interactions with the transcription machinery or by histone construction of a chromatin configuration(s) that promote local transcription (43).

The most striking discovery of this research is that deletion of TK1413 results in a *T. kodakarensis* strain that is no longer amenable to transformation. Apparently, therefore, HTkA but not HTkB plays a role in transformation, possibly directly in DNA integration and/or indirectly through the expression of other genes required for transformation. A direct role for HTkA in recombination would be consistent with the difficulty encountered in constructing *T. kodakarensis* LC124. Recombination deleting
the (TK0254+TK0664) cassette from the intermediate strain genome, a strain that lacks
TK1413, occurred at a frequency \(10^3\) -fold lower than usually observed (12, 32). Also
consistent with a direct role for HTkA in recombination, all attempts to integrate
selectable genes by single or double cross-over recombination into the genome of \(T.
\text{kodakarensis}\) LC124 have failed. However, as transformation of \(T. \text{kodakarensis}\) LC124
with autonomously replicating plasmids is also severely reduced, deletion of TK1413
may also negatively effect other steps required for transformation. Possibly, the DNA
uptake mechanism generates DNA fragments that require HTkA for protection (8, 42),
or deletion of TK1413 increases the expression of a nuclease or decreases the
synthesis of components of the DNA uptake system. Hinting at the latter possibility,
transcripts of an operon (TK0161-TK0166) that is predicted to encode membrane and
surface-located proteins are less abundant in \(T. \text{kodakarensis}\) LC124 (\(\Delta\)TK1413) than in
the parental strain \(T. \text{kodakarensis}\) TS517 (Table 2). In contrast, the loss of HTkB
(\(\Delta\)TK2289) had no detectable effects on transcripts of this operon.

Attempts to confirm, by complementation, that it was the absence of HTkA that
was responsible for the loss of transformation, by expression of TK1413 from the
replicating plasmid pTS706, led to the discovery that such HTkA synthesis was toxic to
\(T. \text{kodakarensis}\). Regardless of the recipient strain, with or without a chromosomal
copy of TK1413 and/or TK2289, incubation of \(T. \text{kodakarensis}\) strains with pTS706
never resulted in transformants. Transformants were readily obtained with pTS707 that
has a translation terminating codon at position two of TK1413, consistent with the
toxicity of pTS706 being a consequence of the plasmid-directed synthesis of HTkA. As
transformation with pTS708 was also impossible, synthesis of HTkA (R20I) was also
toxic and homodimers of HTkA(R20I) would not bind DNA (27, 39). Plasmid expression of HTkA(R20I), however, might still confer toxicity through DNA binding if they assembled into [HTkA(R20I)+HTkB] heterodimers (20).

*T. kodakarensis* grows rapidly to high cell densities, tolerates air exposure, has a high plating efficiency, natural competency and many genetic tools are now available (12). Given these advantages, *T. kodakarensis* is now used widely as a model system for archaeal research, in programs that investigate topics ranging from DNA replication (17, 18, 37) to hyperthermophily (13) to biofuel production (15, 34). The discovery that HTkA but not HTkB plays a critical role in genetic manipulations of *T. kodakarensis* adds a surprising facet and feature that is likely relevant to many such *T. kodakarensis*-based investigations.

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FIGURE LEGENDS

FIG 1. Alignment of the *T. kodakarensis* histone sequences (6) using clustalW.
Alignments of (A) the amino acid and (B) the encoding nucleic acid sequences of HTkA (TK1413) and HTkB (TK2289). Between the sequences identical amino acids and nucleotides are indicated by asterisks, and similar amino acids by colons. The codon changes introduced into TK1413 in plasmid pTS706 to generate plasmids pTS707 and pTS708 are indicated by underlining. An oligonucleotide with the sequence double-underlined in TK1413 was synthesized, [³²P]-labeled and used as the probe in Southern blots (see Fig. 2D).

FIG 2. Construction and confirmation of *T. kodakarensis* LC124 (ΔTK1413).
(A). Plasmid pLC113 was generated from pTS535 (Table 1) by cloning amplicons from *T. kodakarensis* genomic DNA upstream and downstream of the (TK0254-TK0664) cassette. Plasmid pLC124, generated by deletion (Δ) of TK1413 from pLC113, was used as donor DNA to transform *T. kodakarensis* TS517. The genome structure of intermediate strain generated was confirmed, as illustrated, and recombination between the duplicated sequences deleted the cassette to yield *T. kodakarensis* LC124 with the genome as shown. The positions of primers (a to g; see Table S1 for sequences) used to generate diagnostic amplicons (panel C) and probes in Southern blots (panels D-F) are indicated by arrows above the *T. kodakarensis* TS517 genome. The 2,905 bp PvuII restriction fragment to which the probes hybridized is indicated (B). Expanded illustration of the primer locations (heavy arrows) and the amplicons generated. (C)
Ethidium bromide stained, agarose gel electrophoretic separation of the amplicons generated using the primer pairs indicated (a - b; c - d) from *T. kodakarensis* LC124, LC125 and TS517 genomic DNAs. (D, E, F) Southern blots of PvuII digested *T. kodakarensis* TS517, LC124 and LC125 DNA probed with DIG-labeled amplicons (c - d; f - g) or [³²P]-labeled oligonucleotide primer e. In (D) the 2,905 bp PvuII fragment that contains TK1413 in *T. kodakarensis* TS517 is indicated by an arrow. This fragment is not present in *T. kodakarensis* LC124. The PvuII fragment present in both genomic DNA that contains TK2289 and so cross-hybridizes with the probe is noted by an asterisk. In (F), as the probe includes sequences immediately adjacent to TK1413 it hybridized the PvuII fragment in *T. kodakarensis* LC124 DNA that contains the ΔTK1413 deletion (grey arrow). As the probe contains the entire TK1413 sequence it cross-hybridized to the ~1 Kbp PvuII fragment present that contains TK2289 (asterisk) in both *T. kodakarensis* TS517 and LC124 DNA.

**FIG 3.** Construction and confirmation of *T. kodakarensis* LC125 (ΔTK2289).

(A) Plasmid pLC114 was constructed by cloning amplicons from *T. kodakarensis* genomic DNA upstream and downstream of the (TK0254-TK0664) cassette. Plasmid pLC125, generated by deletion (Δ) of TK2289 from pLC114, was used as donor DNA to transform *T. kodakarensis* TS517. The genome structure of intermediate strain generated was confirmed, as illustrated, and recombination between the duplicated sequences deleted the cassette to yield *T. kodakarensis* LC125 with the genome as shown. The positions of primers (h to m; see Table S1) used to generate diagnostic
amplicons (panels C and D) and the probe used in the Southern blot (panel E) are indicated by arrows above the *T. kodakarensis* TS517 genome. The 5,534 and 5,330 bp EcoRI restriction fragments to which the probe hybridized in digests of *T. kodakarensis* TS517 and LC125 genomic DNAs are indicated. (B) Expanded illustration of the primer locations (heavy arrows) and the amplicons generated. (C, D) Ethidium bromide stained, agarose gel electrophoretic separations of the amplicons generated with the primer pairs (h - i; j - l; see Table S1) from *T. kodakarensis* LC124, LC125 and TS517 genomic DNAs. (E) Southern blot of EcoRI digested *T. kodakarensis* TS517 and LC125 DNA. The amplicon generated by primer pair (k - m) was DIG-labeled and used as the probe. The 5,534 EcoRI fragment present in *T. kodakarensis* TS517 DNA (black arrow) is shorted to 5,330 bp by the ΔTK2289 mutation as indicated in the *T. kodakarensis* LC125 DNA (grey arrow). The probe also cross-hybridized to an ~11 Kbp EcoRI fragment present in both genomes that contains TK1413.

**FIG 4.** Comparison of growth of *T. kodakarensis* TS517, LC124 and LC125. The growth of *T. kodakarensis* TS517 (○), LC124 (△) and LC125 (□) cultures in ASW-YT-S° medium at 85°C followed by measuring the increases in optical density at 600 nm. The curves show the average values, with errors, obtained in three independent experiments from a total of 9 cultures of each strain.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS517</td>
<td>ΔpyrF; ΔtrpE::pyrF; ΔTK0664</td>
<td>(29)</td>
<td></td>
</tr>
<tr>
<td>LC124</td>
<td>ΔpyrF; ΔtrpE::pyrF; ΔTK0664; ΔTK1413</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>LC125</td>
<td>ΔpyrF; ΔtrpE::pyrF; ΔTK0664; ΔTK2289</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant feature</th>
<th>Source</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTS535</td>
<td>pUC118 with MCS1-(P&lt;sub&gt;TK2279&lt;/sub&gt;-TK0254; P&lt;sub&gt;hisD&lt;/sub&gt;-TK0664)-MCS2</td>
<td>(29)</td>
<td></td>
</tr>
<tr>
<td>pLC113</td>
<td>pTS535::TK1412-TK1413-TK0664-TK0254-TK1412-TK1414-TK1415</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pLC114</td>
<td>pTS535::TK2286-TK2287-TK2288-TK0664-TK0254-TK2286-TK2287-TK2288-TK2290</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pLC124</td>
<td>pTS535::TK1412-TK0664-TK0254-TK1412-TK1414-TK1415</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pLC125</td>
<td>pTS535::TK2286-TK2287-TK2288-TK0664-TK0254-TK2286-TK2287-TK2288-TK2290</td>
<td>This study</td>
<td></td>
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<tr>
<td>pUDHisD</td>
<td>pUC118 derivative; Δ hisD::trpE</td>
<td>(25)</td>
<td></td>
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<tr>
<td>pTS503</td>
<td>pUC118; P&lt;sub&gt;hisD&lt;/sub&gt;-TK1827-trpE-TK1827 with TAG at codon 5 of TK1827</td>
<td>(29)</td>
<td></td>
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<tr>
<td>pLC70</td>
<td>P&lt;sub&gt;TK2279&lt;/sub&gt;-TK0254; P&lt;sub&gt;gap&lt;/sub&gt;-PF1848</td>
<td>(28)</td>
<td></td>
</tr>
<tr>
<td>pLC71</td>
<td>pLC70 Δp24</td>
<td>(28)</td>
<td></td>
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</tbody>
</table>
pTS414 pLC70::PhmtB-rpoL-HA (28)
pTS706 pLC70::PhmtB-TK1413
This study
pTS707 pLC70::PhmtB-TK1413 with TGA at codon 2 of TK1413 (A2STOP)
This study
pTS708 pLC70::PhmtB-TK1413 with ATT at codon 20 of TK1413 (R20I)
This study
TABLE 2. Microarray comparisons of RNA abundances in (left) T. kodakarensis LC124 versus T. kodakarensis TS517 and (right) T. kodakarensis LC124 versus TS517.

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>LC124/T. kodakarensis TS517</th>
<th>LC124/T. kodakarensis TS517</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Oxoacid:ferredoxin oxidoreductases, gamma subunit</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Hypothetical protein</td>
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<td>2.0</td>
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<tr>
<td>Probable formate transporter</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>ABC-type multidrug transport system, hypothetical protein</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Predicted hypothetical protein</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Hypothetical membrane protein</td>
<td>2.0</td>
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</tr>
<tr>
<td>Hypothetical membrane protein</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>S-layer-like array protein</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Histone-like protein</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Indole-3-glycerol phosphate synthase</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Replication factor</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Hypothetical membrane protein</td>
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<tr>
<td>Orotate phosphoribosyltransferase</td>
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<td>Methyl-accepting chemotaxis protein</td>
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<td>Glycerate kinase-related protein</td>
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<td>2-Dehydro-3-deoxyphosphoheptonate aldolase</td>
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<td>ATPase component</td>
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<td>Aspartate carbamoyltransferase, catalytic subunit</td>
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<td>Translation elongation factor EF-1, alpha subunit</td>
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<td>Transketolase, N-terminal section</td>
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<td>2.0</td>
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<tr>
<td>synthase, glutamine amidotransferase component</td>
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<td>2.0</td>
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<td>Phosphoribosylaminoimidazole-succinocarboxamide synthase</td>
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<td>2.0</td>
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<td>Phosphoribosylaminoimidazole carboxylase, catalytic subunit</td>
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<td>Adenylosuccinate synthase</td>
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<td>Inosine-5'-monophosphate dehydrogenase</td>
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<td>Predicted acetyltransferase, isoleucine patch superfamily</td>
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<tr>
<td>PP-loop-A synthase</td>
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</table>

Footnote: Average values from two independent microarrays. Genes in experiments that increased or decreased at least 2-fold in T. kodakarensis LC124 or LC125 when compared with their abundances in T. kodakarensis TS517. The microarrays had amplions from all 2,306 T. kodakarensis ORFs (27) spotted twice at different locations.
are listed even when the expression of some genes did not change by 2-fold. Colors highlight operons and genes with the same transcription response to the absence of HTkA or HTkB.
TABLE 3. Transformation of *T. kodakarensis* TS517, LC124 and LC125 with replicative and non-replicative plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Autonomous Replication</th>
<th>Gene target</th>
<th>TS517&lt;sup&gt;3&lt;/sup&gt; (ΔTK1413)</th>
<th>LC124&lt;sup&gt;3&lt;/sup&gt; (ΔTK1413)</th>
<th>LC125&lt;sup&gt;3&lt;/sup&gt; (ΔTK2289)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUDHisD</td>
<td>-</td>
<td>TK0244</td>
<td>1±0.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0</td>
<td>1±0.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>pLC124</td>
<td>-</td>
<td>TK1413</td>
<td>1±1 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1±1 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>pLC125</td>
<td>-</td>
<td>TK2289</td>
<td>1±0.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0</td>
<td>NA&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTS503</td>
<td>-</td>
<td>TK1827</td>
<td>1±0.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0</td>
<td>1±0.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>pLC70</td>
<td>+</td>
<td>-</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1±1</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>pLC71</td>
<td>+</td>
<td>-</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1±1</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTS414</td>
<td>+</td>
<td>-</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1±1</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>pTS706</td>
<td>+</td>
<td>-</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>pTS707</td>
<td>+</td>
<td>-</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1±1</td>
<td>2±1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTS708</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Foot note:
1. Plasmid capable (+) or incapable (-) of autonomous replication in *T. kodakarensis*
2. Gene that the plasmid was constructed to delete.
3. Transformants recovered after incubation of the *T. kodakarensis* strain (10<sup>12</sup> cells) with 1 µg of the plasmid DNA.
4. Gene target already deleted.
Figure 1

A.

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
</table>
HTkA | MAELPIAFVDRLIKAGAAGVSEDAAAAYAEYLXEIAISLKKKAVDPAHAGKRTKAEIKLAIKA |

*************** ********* *** **:******** :* ******************:

HTkB | MAELPIAFVDRLIKAGAARVSEEAAKVLAEHLEEKALE1AKRAVALAQHAGKRTKAEIKLAIK5 |

B.

TGA in pTS707

TK1413 | ATGGCCGAGCTTCCGATTGGCCGGTTGACAGGCTGAGGCTGAGGTCACTGA 68 |

************************************************************************* ** ** *   * ****** **

TK2289 | ATGGCCGAGCTTCCGATTGGCCGGTTGACAGGCTGAGGCTGAGGTCACTGA 68 |

************************************************************************* ** ** *   * ****** **

TK1413 | GAGCTTCCGATTGGCCGGTTGACAGGCTGAGGCTGAGGTCACTGA 136 |

***   *** ***   ***   ***   ***   ***   ***   ***   ***   ***   ***   ***

TK2289 | GAGCTTCCGATTGGCCGGTTGACAGGCTGAGGCTGAGGTCACTGA 136 |

CCCTCGCCCAGCACGCCGGCAGAAAGACCGTCAAGGCTGAGGACATCAAGCTTGCCATCAAGAGCTGA 204 |

Figure 1
A. plasmid pLC113

T. kodakarensis TS517

2,905 bp

Intermediate strain

T. kodakarensis LC124

B. Amplicon → a—b
c—d
E. Probe → e—d

C. Probe → e—d

D. Probe → f—g

Figure 2
Figure 3

A. plasmid pLC114

B. Amplicon

C. Probe

D. E. TS517 LC125

T. kodakarensis TS517

Intermediate strain

T. kodakarensis LC125

M 1000 2000 4000 6000

Figure 3
Figure 4

OD 600 nm vs. Time (min)

- TS517
- LC124
- LC125

0 60 120 180 240 300 360 420 480 540 600 660 720 780