An Archaeal 2-Deoxyribose 5-phosphate Aldolase that Exhibits Closer Homology to Bacteria rather than Archaea

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Summary: This study reports on primary structure comparison and some enzymatic characterization of 2-deoxyribose 5-phosphate aldolase from the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1 (*Tk-DeoC*). The *Tk-DeoC* exhibited a significant closer homology to bacterial 2-deoxyribose 5-phosphate aldolases (*DeoC*) than those of archaeal counterparts. Recombinant *Tk-DeoC* catalyzed the conversion of 2-deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate with a highest activity at 95°C and the pH optimum of 4.0. In *T. kodakaraensis* transcription of *deoC* was readily detectable and the corresponding *DeoC* activity was measurable in the cell-free extracts, indicating the constitutive expression of *deoC* in *T. kodakaraensis* cells. However, in contrast to the finding in bacteria where *DeoC*, together with DeoA, DeoB and DeoD, participate in the catabolism of exogenous DNA and presence of nucleotides in the growth medium induces these genes, supplementation of DNA to the growth medium showed no detectable induction of *deoC* transcription.

Introduction

Aldolases are a group of *DeoC*s that facilitate the biological formation and cleavage of carbon-carbon bonds. They catalyze aldol condensation reactions, in which a ketone donor is added to an aldehyde acceptor [1,2]. Based on the mechanism, aldolases can be divided into two distinct classes. Aldolases of Class I form a Schiff-base intermediate between the active center lysine residue and the carbonyl group of the donor substrate [3], while Class II aldolases use a divalent metal cation, such as Zn^{2+}, as a cofactor to form an active metallo-protein complex [4].

A number of distinct types of aldolases in relation to their substrate specificity have been identified. Among these, the most well studied *DeoC* is fructose 1,6-bisphosphate aldolase, where the ketone donor is dihydroxyacetone phosphate and the aldehyde acceptor is glyceraldehyde 3-phosphate. There are also aldolases in which pyruvate is the ketone donor, including 4-hydroxy-2-oxoglutarate aldolase [2]. 2-deoxyribose 5-phosphate aldolase (*DeoC*, E.C 4.1.2.4) is the only *DeoC* that utilizes acetaldehyde as a ketone donor [1]. The *DeoC* catalyzes the reversible aldol condensation reaction between acetaldehyde and the D-glyceral-dehyde-3-phosphate to generate 2-deoxyribose 5-phosphate. The enzymatic properties of *DeoC* have been characterized in mammalian tissues [5] and a number of bacterial species such as *Escherichia coli* [6], *Lactobacillus plantarum* [7], *Salmonella typhimurium* [8], *Bacillus cereus* [9], *Bacillus subtilis* [10] and *Corynebacterium diphtheriae* [11]. Genes encoding *DeoC* proteins have been cloned and sequenced from several bacterial and three archaeal organisms [12-15]. The three-dimensional structures of the *DeoC* from bacteria (*E. coli*, *Thermus thermophilus* and *Thermotoga maritima*) as well as from archaea (*Aeropyrum pernix* and *Pyrobaculum aerophilum*) have recently been reported [13,14,16-18].

At present, the physiological role of *DeoC* seems to vary according to its host cell. It has been suggested that the mammalian *DeoC* participates in the synthesis of deoxyribonucleotides [5]. Inhibition of *DeoC* in mammalian cells led to a specific inhibition of the incorporation of labeled purine and pyrimidine into DNA, while RNA and protein synthesis were unaffected. On the other hand, studies on bacterial *DeoC* proteins support a catabolic role of the *DeoC* [19]. *E. coli* can grow on a variety of

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(deoxy)nucleoside or (deoxy)nucleotides and their derivatives as a sole carbon and energy source. Addition of these substrates to the growth medium causes an induction of the synthesis of a group of *DeoC*s including adenosine deaminase (Add), thymidine phosphorylase (DeoA), purine nucleoside phosphorylase (DeoD), phosphopentomutase (DeoB), as well as *DeoC* [20]. It has been proposed that DeoA and DeoD release the pentose moiety from the (deoxy)ribonucleosides, producing (deoxy)ribose 1-phosphate that is further isomerized to (deoxy)ribose 5-phosphate by DeoB. In the final step, *DeoC* cleaves deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate, allowing further metabolism to obtain carbon and energy. The above mentioned induction of the *DeoC*s is sensitive to glucose repression, supporting the assumption that bacterial cells recognize exogenous (deoxy)nucleoside or (deoxy)nucleotide molecules as a carbon source [21]. Moreover, mutant strains of *E. coli* lacking the Deo proteins failed to grow on these substrates, thus implicating the catabolic nature of this pathway [22].

However, in comparison with bacteria and mammals very limited literature isavailable about these *DeoC*s in archaea, the third domain of life. The presence of the enzymatic activity in archaea has been reported from *A. pernix*, an aerobic hyperthermophilic archaeon [13]. We have also reported the *DeoC* from an anaerobic hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1 [12].

T. kodakaraensis KOD1 was isolated from a solfatara on Kodakara Island, Kagoshima, Japan [23]. In order to elucidate the metabolic pathways in archaea, we have characterized a variety of enzymes involved in carbon metabolism from this archaeon [12,23-29] and its whole genome has also been sequenced [30]. Here, we report on the sequence analysis and some biochemical properties of *DeoC* protein from this microorganism.

Results and Discussion

Characterization of Tk-DeoC Gene

Whole genome of *T. kodakaraensis* [30] was searched for candidate gene of *DeoC*. An open reading frame (ORF) displaying a strong similarity to previously reported *DeoC* genes was identified. The ORF consisted of 672 bp (ORF Tk2104, positions 1886909 to 1887581 on the entire genome) encoding a protein of 224 amino acids with a calculated molecular mass of 24,505 Da and an isoelectric point of 5.01. A putative ribosomal binding site (5'-GGATG) was identified 5 nucleotides upstream from the initiation codon along with a putative TATA-like element (TAAATA, A-Box) 25 nucleotides upstream of the ribosomal binding site. Adjacent open reading frames were found on the opposite strand, indicating that the gene was not a member of a gene cluster or operon.

The deduced amino acid sequence of *Tk-DeoC* displayed similarity to the previously characterized *DeoC* proteins whose primary structures have been determined, including *T. maritima* (60% identical), *B. subtilis* (54%), *A. pernix* (47%), *S. typhimurium* (33%) and *E. coli* (31%). The residues essential for catalytic activity in the *DeoC* from *E. coli*, K167, K201 and D102 (numbering according to the *E. coli* sequence) [16], were faithfully conserved in *Tk-DeoC* (Fig. 1). Surprisingly *Tk-DeoC* exhibited closer homology (identity) at the amino acid level to bacterial *DeoC*s, *Thermoanaerobacter tengcongensis* (64%), *Bacillus halodurans* (62%), *Clostridium perfringens* (62%), *T. maritima* (60%) and *Caldicellulosiruptor saccharolyticus* (60%), respectively, instead of archaeal counterparts. Among thermophilic and hyperthermophilic archaea, the primary structure of *Tk-DeoC* exhibited a highest homology of 55% with *Methanothermobacter thermautotrophicus*. Other archaea which possess a homologue of *Tk-DeoC* on their genome include *P. aerophilum* and *A. pernix* (Crenarchaeota); *Halobacterium* sp. Strain NRC-1, *Thermoplasma volcanium* and *Thermoplasma acidophilum* (Euryarchaeota). *Tk-DeoC* exhibited a homology ranging from 37 to 47% with these microorganisms. We found that many hyperthermophilic archaea, including the closely related *Pyrococcus* species, did not harbor open reading frames that resembled *Tk*-DeoC. A comparison of the derived amino acid sequences of all thermophilic and hyperthermophilic archaea containing the DeoC on their genome and some of the characterized *DeoC*s from eukaryotes and bacteria is presented as a phylogenetic tree (Fig. 2). *Tk-DeoC* is clustered with thermophilic bacterial counterparts instead of archaeal ones. The amino acids P50, F76, P77, G79, A98, E100, D102, V104, K137, I139, K167, T170, K201, G204 and G205

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T.kodakaraensis     ---------------MNKREIARYIDQTNLKPYATKEDIIKLCDEAIEYG--FYAVCVNP     43
B.subtilis          ---------------MS---LANIIDHTALKPHTQKADILKLIEEAKTYK--FASVCVNP     40
E.coli              ----------MTDLKASSLRALKLMDLTTLNDDDTDEKVIALCHQAKTPVGNTAAICIYP     50
S.typhimurium       ----MPLENVMTDLKASSLRALKLMDLTTLNDDDTNEKVIALCHQAKTPVGNTAAICIYP     56
A.pernix            MPSARDILQQGLDRLGSPEDLASRIDSTLLSPRATEEDVRNLVREASDYG--FRCAVLTP     58
                                      * * *           *   *           *

T.kodakaraensis     YRVKLAKDYLREK-NADVKVASVIGFPLGATPTEVKVFEAKRALEDGADELDMVINIGAL     102
B.subtilis          TWVELAAKELKG---TGVDVCTVIGFPLGANTTETKAFETKDAISKGATEVDMVINIAAL     97
E.coli              RFIPIARKTLKEQGTPEIRIATVTNFPHGNDDIDIALAETRAAIAYGADEVDVVFPYRAL     110
S.typhimurium       RFIPIARKTLKEQGTPDIRIATVTNFPHGNDDIDIALAETRAAIAYGADEVDVVFPYRAL     116
A.pernix            VYTVKISGLAEK---LGVKLCSVIGFPLGQAPLEVKLVEAQTVLEAGATELDVVPHLSLG     115
                                    *  ** *         *       ** * * *

T.kodakaraensis     KDKDYEYVKNDIAEVVKVAHERGAKVKVIIETCYLTEEEKV-KACELAKEAGADFVKTST     161
B.subtilis          KDKEDDVVEADIRGVVEAVAGK-ALVKVIIETCLLTDEEKE-RACRLAVSAGADFVKTST     155
E.coli              MAGNEQVGFDLVKACKEACAAANVLLKVIIETGELKDEALIRKASEISIKAGADFIKTST     170
S.typhimurium       IAGNEQVGFDLVKACKDACAAANVLLKVIIETGELKEEALIRKASEISIKAGADFIKTST     176
A.pernix            ----PEAVYREVSGIVKLAKSYGAVVKVILEAPLWDDKTLS-LLVDSSRRAGADIVKTST     170
                                    *** *                     ****  ****

T.kodakaraensis     GFGTGGATVEDVRLMRKVVG-----PEMGVKAAGGIRTYEQALEMIEAG-----------     205
B.subtilis          GFSTGGATKEDIALMRKTVG-----PDIGVKASGGVRTKEDVDTMVEAG-----------     199
E.coli              GKVAVNATPESARIMMEVIRDMGVEKTVGFKPAGGVRTAEDAQKYLAIADELFGADWADA     230
S.typhimurium       GKVPVNATPESARIMMEVIRDMGVSKTVGFKPAGGVRTAEDAQKFLAIADELFGADWADS     236
A.pernix            GVYTKGGDPVTVFRLASLAKP----LGMGVKASGGIRSGIDAVLAVGAG-----------     215
                    *                         * *  ** *

T.kodakaraensis     -ANRIGTSSGVKIVEGAPDE---------                                  224
B.subtilis          -ASRIAPAQAFLS----------------                                  211
E.coli              RHYRFGASSLLASLLKALGHGDGKSASSY                                  259
S.typhimurium       RHYRFGASSLLASLLKALGHGDGKSASSY                                  265
A.pernix            -ADIIGTSSAVKVLESFKSLV--------                                  235
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Fig. 1: Comparison of the primary structure of *Tk-DeoC* with its orthologues from *B. subtilis*, *S. typhimurium*, *E. coli* (bacteria) and *A. pernix* (archaeon). The asterisks at the bottom of the sequence represent the identical amino acid residues, and the essential catalytic residues for *E. coli DeoC* activity [15,16] are shown in bold. Organism names are shown on the left and the amino acid numbering are shown on the right. The accession numbers are as follows. *T.kodakaraensis*, BAD86293; *B.subtilis*, BAA08337; *E.coli*, NP_757311; *S.typhimurium*, NP_463423; *A.pernix*, NP_148609.

(*E.coli* numbering) were conserved among all the organisms shown in Fig.2. The critical residues involved in Schiff base-mediated aldol condensation reaction in *E. coli DeoC* (C47, D102, K167 and K201) are completely conserved in the bacterial as well as archaeal thermophilic *DeoCs*. Close to the active site peptide backbone is reported a small hydrophobic cluster formed by F200, I166 and M185 [14]. Interestingly all the above three amino acids in *Tk-DeoC* are replaced by valine. In other thermophiles or hyperthermophiles they are replaced either by valine or isoleucine.

Production and Purification of Recombinant Tk-DeoC

In order to characterize the protein product of the *Tk-DeoC* gene and to determine whether the *DeoC* was a bona fide *DeoC*, we expressed the gene in *E. coli*. The recombinant protein was produced in a soluble form and was purified to apparent homogeneity by heat treatment at 85°C for 20 min followed by anion exchange and gel filtration chromatographies (Fig. 3). The molecular mass of recombinant *Tk-DeoC* estimated by SDS-PAGE

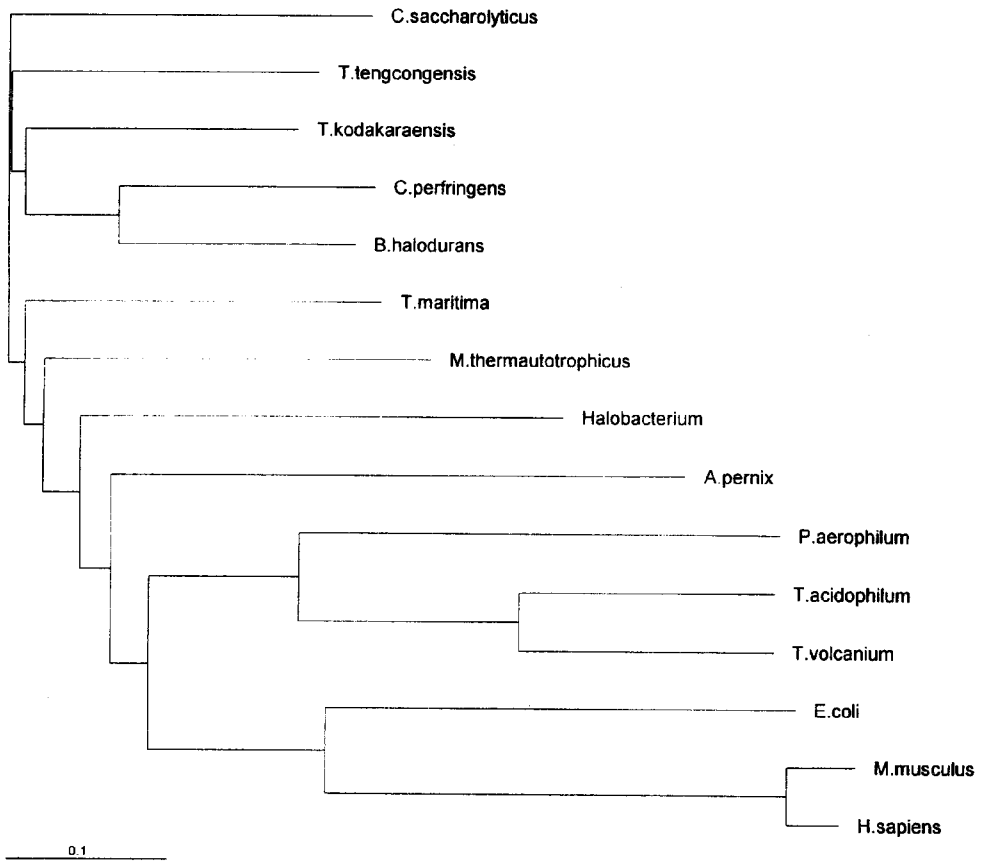

Fig. 2: Phylogenetic tree obtained from sequence comparison of *Tk-DeoC* and *DeoC* from other organisms. Multiple sequence alignments were conducted using ClustalW program. Tree topology and evolutionary distance estimations were done by the neighbour-joining method. The names at the termini are those of the organisms from which the *DeoC* originated. The accession numbers are as follows. *T. tengcongensis*, NP_622626; *C. perfringens*, NP_562968; *C. saccharolyticus*, YP_001181086; *B.halodurans*, NP_242218; *T. maritime*, NP_229359; *A. pernix*, NP_148609; *P. aerophilum*, NP_559158; *Halobacterium* sp., NP_280582; *M. thermautotrophicus*, NP_275957; *T. acidophilum*, NP_394155; *T. volcanium*, NP_110694; *E. coli*, NP_757311; *M. musculus*, NP_766321; *H. sapiens*, AAH56234.

agreed with that calculated from the deduced amino acid sequence. Furthermore, the N-terminal 10 amino acid residues of the purified protein were identical to the deduced amino acid sequence of the gene, confirming that we had obtained purified *Tk-DeoC*.

The molecular mass of *Tk-DeoC* was determined by gel filtration chromatography. The protein eluted at a retention volume of 14.42 ml equivalent to a molecular mass of 53 kDa calculated from the standard curve obtained from the retention volume of the standard markers (data not shown). Taking into account the molecular mass of the subunit (25 kDa), this result indicated that *Tk-DeoC* exists as a dimer like mesophilic bacterial counterparts. It is note worthy that the other characterized thermophilic *DeoC*s, from *A. pernix* (archaea) and *T. thermophilus* (bacteria), exist in a tetrameric form [13,17].

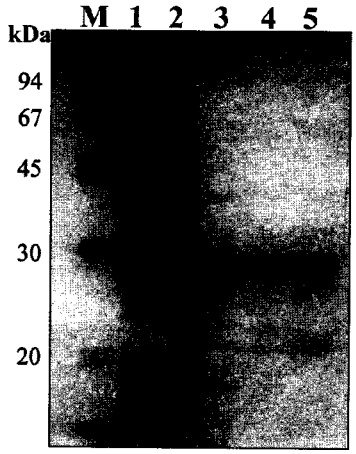

Fig. 3: SDS-PAGE of purified recombinant *Tk-DeoC*. Lane M, molecular mass markers; lane 1, soluble fraction of the host cells carrying the pET-21a plasmid; lane 2, soluble fraction of the host cells carrying the pET-*deoC* plasmid; lane 3, soluble fraction after heat treatment at 85°C for 20 min; lane 4, eluate after ion exchange chromatography with Resource Q; lane 5, purified *Tk-DeoC* after gel-filtration chromatography with Superdex 200 HR 10/30.

Enzymatic Properties of Tk-DeoC

Purified *Tk-DeoC* was dialyzed against 50 mM bicine buffer (pH 8.0) containing 10 mM EDTA and used for further analysis. Activity measurements were performed in a linked assay coupled with alcohol dehydrogenase (monitoring the amount of acetaldehyde) or with triosephos-phate isomerase and glycerophosphate dehydrogenase (monitoring the amount of glyceraldehyde 3-phosphate). *Tk-DeoC* exhibited significant *DeoC* activity in both systems and no activity was observed when either *Tk*-DeoC or the substrate 2-deoxyribose 5-phosphate was omitted.

As in the case of the *E. coli DeoC*, the presence of citrate was found to enhance the activity of *Tk-DeoC*. Activities were much higher in citrate buffer than those observed in acetate buffers at equivalent pH, with a 2.8-fold activation at the optimal pH 4.0 (Fig. 4A). The specific activity of *Tk-DeoC* under optimal conditions was 235 U/mg. The *DeoC* catalyzed the cleavage reaction following Michaelis-Menten kinetics with a K_m value of 0.81 ± 0.07 mM towards 2-deoxyribose-5-phosphate and a k_{cat} value of 116 ± 4 s^{-1} subunit^{-1} at 95°C.

Thermostability of the recombinant protein was examined at pH 4.0 and 7.0. The protein was found to be stable at high temperatures. The *DeoC* retained over 80% of its activity after 120 min at 90°C. The half-life of the *DeoC* was 40 min in boiling water at pH 7.0 while it was 20 min at pH 4.0 (Fig. 4B).

Tk-DeoC exhibited a specificity towards the substrate 2-deoxyribose 5-phosphate (dR5P). *DeoC* activities towards the following substrates were also investigated; 2-deoxyribose (dR), ribose 5-phosphate (R5P), arabinose 5-phosphate (A5P), fructose 1,6-bisphosphate ($F1,6P_2$) and fructose 6-phosphate (F6P). R5P and A5P correspond to molecules with a hydroxyl group on the C2 of dR5P and therefore the ketone donor moiety is derived from glycol aldehyde. In the case of $F1,6P_2$ and F6P, the ketone donor moiety is derived from the three carbon molecules, dihydroxy-acetone phosphate and dihydroxyacetone, respectively. On the other hand, the aldehyde acceptor molecule has been changed to glyceraldehyde in the case of dR. We observed aldolase activity only with the substrate dR5P, indicating that *Tk-DeoC* was a *bona fide* archaeal *DeoC* with strict substrate specificity.

Transcription of Tk-deoC in T. Kodakaraensis

We further investigated whether the *Tk-deoC* gene was transcriptionally active in *T. kodakaraensis* KOD1. As transcription levels of

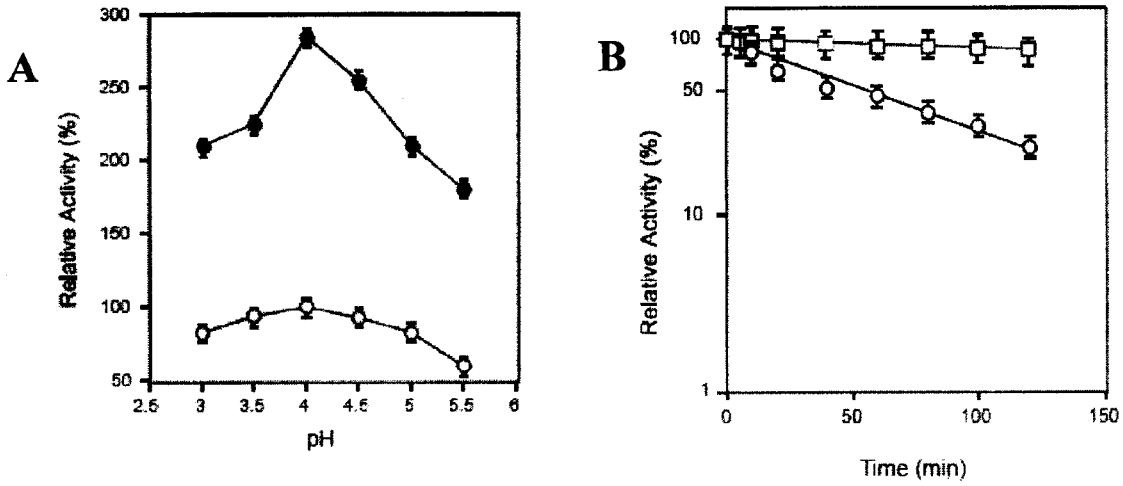

Fig. 4: Effect of pH and temperature on the 2-deoxyribose 5-phosphate cleavage activity of *Tk-DeoC*. (A) *Tk-DeoC* activity as a function of pH. Activity was examined as described in the experimental section. Following buffers were used: citrate buffer (closed circles), acetate buffer (open circles). (B) Thermostability of *Tk-DeoC*. *Tk-DeoC* was heated in Tris-HCl buffer (pH 7.0) at 90°C (squares), and in the boiling water (circles) for various intervals of time and the residual activity was examined as described in the experimental section.

bacterial *DeoC* genes have been previously reported to be inducible in the presence of exogenous DNA [10,19], so we also examined transcription levels of *Tk-deoC* in cells grown in the presence and absence of sheared calf thymus DNA (0.5 mg/ml) in the medium. The presence of DNA did not have any significant effect on the growth rate of the cells. Total RNA of the respective cells were isolated in the early exponential growth phase. Northern blot analysis indicated that *Tk-deoC* was indeed transcribed in *T. kodakaraensis*. However, in contrast to the observations in bacterial cells, we could not detect any significant difference in transcription of *Tk-deoC* in cells grown with or without exogenous DNA (Fig. 5). We also measured *DeoC* activity in the cell free extracts of cells grown in the presence and absence of exogenous DNA and found no significant difference between the activity levels (0.9 U/mg) in these cells (data not shown).

Discussion

The primary structure of *Tk-DeoC* displayed highest homology to the bacterial *DeoC*s rather than archaeal ones. The same is true for the quarternary structure. Mesophilic bacterial *DeoC*s are reported to exist in a dimeric form similar to *Tk-DeoC* whereas the thermophilic archaeal and bacterial *DeoC*s from *A. pernix* and *T. thermophilus* are reported to exist as tetramers [13,17]. It is suggested that oligomeric state of *DeoC* contributes to the thermostability of the *DeoC*s. *Tk-DeoC* exists in a dimeric form like the *DeoC* from *E. coli* [16] instead of a tetrameric form of *DeoC* from thermophiles [14,17].

The biochemical characterization of *Tk-DeoC* displayed a strict substrate specificity towards dR5P, indicating that the *DeoC* was an authentic *DeoC*. *Tk-DeoC* displayed highest *DeoC* activity at pH 4.0 whereas the DeoCs from *P. aerophilum*, *T. maritima* and *E. coli* exhibited highest activities at pH 6.0, 6.5 and 7.7, respectively. Among the characterized DeoCs, *Tk*-DeoC is the enzyme that displayed highest DeoC activity in highly acidic conditions (pH 4.0). Apart from this, *Tk*-DeoC is the most thermostable enzyme among the characterized DeoCs till now with no significant loss of DeoC activity even after 120 min of heating at 90°C and a half life of 40 min at 100°C. The enzyme activity of DeoC from the hyperthermophile *T. maritima* was almost completely lost even after 10 min of heating at 100°C [14]. We also found that the *Tk-deoC* gene was transcribed and DeoC activity was detected in the cell-free extracts, indicating that the protein was present in *T. kodakaraensis* cells.

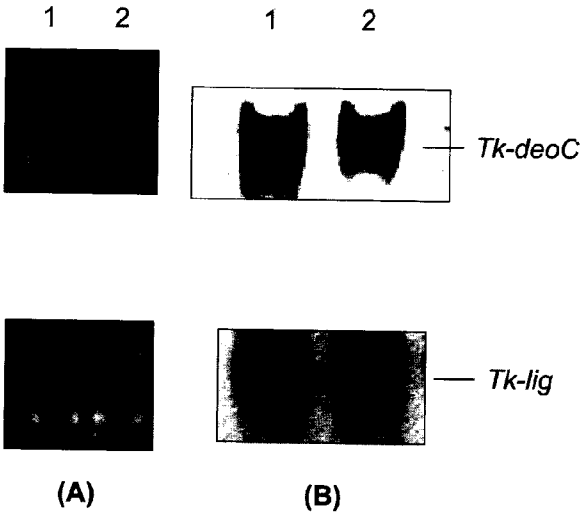

Fig. 5: Northern blot analysis of RNA from *T. kodakaraensis* KOD1 grown in the absence (lane 1) and presence (lane 2) of exogenous DNA. *Tk-deoC* (upper pannel) and DNA ligase (lower panel) genes from *T. kodakaraensis* KOD1 were used as probes. Each lane contains 15 μg of total RNA. (A) Ethidium bromide stained 1% agarose gel. (B) Detection of the RNA bands hybridized with the probes.

In contrast to the bacterial deoC, we did not observe induction of deoC transcription and enzyme activity in the presence of exogenous DNA. It is unlikely that this is due to the lack of uptake of DNA by the cells. We have constructed a gene disruption system in *T. kodakaraensis* KOD1 [31]. In these studies, we have observed that this strain could be transformed with exogenous DNA under normal growth conditions, indicating the capability of exogenous DNA uptake in *T. kodakaraensis* cells.

Although we have clearly revealed the presence of 2-deoxyribose 5-phosphate aldolase in a hyperthermophilic archaeon, our data at present do not allow us to suggest a particular physiological role for the *Tk-DeoC*. Recently, an abundant number of complete genome sequences from various archaeal strains have been determined. Therefore, using the sequences of the Deo proteins from *E. coli*, we searched for orthologues of the Deo proteins (DeoA~D) in the genomes of various thermophilic and hyperthermophilic archaea. To our surprise, we found that many hyperthermophilic archaea, including the closely related *Pyrococcus* species, did not harbor open reading frames that resembled *Tk-deoC*. Among hyperthermophilic archaea, *deoC* orthologues were present only in the Crenarchaeota *A. pernix* and *P. aerophilum*. Corresponding genes were found in some thermophilic strains, including *M. thermoautotrophicum*, *T. acidophilum* and *T. volcanium*, but were not present in *Sulfolobus* species.

Most strains harbored one of the *deoA* or *deoD* orthologues, with a tendency of *deoD* in Crenarchaeota and *deoA* in Euryarchaeota. The assignment of *deoD* orthologues in the complete genome sequences displays a variation among these strains, including uridine phosphorylase, 5-methylthioadenosine phosphorylase and purine nucleoside phosphorylase (*deoD*). *deoA* and *deoD* may functionally complement one another in archaea, as both encode putative nucleoside phosphorylases. Interestingly, in the process of genome sequencing of *T. kodakaraensis*, we found that this strain possesses both orthologues. We could not find either gene in *M. thermoautotrophicum*, *T. acidophilum* and *T. volcanium*, the three coincident euryarchaeota strains that harbored *deoC* orthologues. Another notable point was the fact that *deoB* orthologues were not found in any of the genomes. Regardless of the direction, if the pathway between nucleosides and glyderaldehyde 3-phosphate/ acetaldehyde were to be present in archaea, a phosphopentomutase would be

necessary to link the DeoA/ DeoD and DeoC activities. We have identified and characterized a phosphopentomutase in *T. kodakaraensis* which has a novel structure [12]. The primary structure of the enzyme is similar to the phosphoglucomutase/ phosphomannomutase but it exhibited a phosphopentomutase activity instead of phosphohexomutase activity. In recent studies, a number of archaeal enzymes that are functionally equivalent to their bacterial and eukaryotic counterparts have been found to harbor distinct primary structure [12, 28, 32]. There is also the possibility that the Deo proteins in archaea have distinct physiological roles other than those identified in Bacteria and Eucarya. Gene disruption of the *deo* orthologues in *T. kodakaraensis* will undoubtedly shed further light on this issue.

It is generally accepted that all living organisms on earth are descended from a common ancestor or ancestral gene pool and there is horizontal gene transfer along with the genetic transfer from parents to offspring or vertical gene transfer. The higher homology of *Tk*-DeoC towards bacterial counterparts rather than archaeal ones further supports this evolutionary relationship. Over the course of time the thermophilic archaea may have been the first branch to diverge from the ancestral line.

Experimental

Strains and Plasmids

T. kodakaraensis KOD1 was isolated from a solfataric hot spring at a wharf in Kodakara Island, Kagoshima, Japan [23]. *E. coli* strain DH5α was used for subcloning of the gene fragments and DNA manipulations. *E. coli* strain BL21 (DE3) CodonPlus-RIL (Stratagene, La Jolla, CA, USA) was used as a host and pET-21a vector (Novagen, Madison, WI, USA) was employed for gene expression.

DNA Manipulation

Restriction enzyme and DNA polymerase were purchased from Toyobo (Osaka, Japan) and Takara Shuzo (Kyoto, Japan). Genomic and plasmid DNAs were isolated using QIAGEN DNA isolation kits (QIAGEN, Hilden, Germany). DNA ligation reactions were performed using DNA ligation kit (Toyobo, Japan). QIAEX Gel Extraction Kit (QIAGEN) was used to recover DNA fragments from agarose gels.

Cloning and Characterization of the Tk-deoC Gene

DNA sequencing was performed using ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and a Model 310 capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). Open reading frame search and molecular mass calculations were performed using DNASIS software (Hitachi Software, Yokohama, Japan). Multiple alignment and phylogenetic analysis was performed using the Clustal W program provided by DNA Data Bank of Japan (DDBJ). Search for orthologue genes in various genomes was performed with CMR blast at http://tigrblast.tigr.org/cmr-blast/.

Expression of Tk-deoC Gene in E. coli

The *Tk-deoC* gene was amplified by polymerase chain reaction (PCR) and inserted into pUC118. An *Nde*I site was introduced in the 5'-flanking region of the gene. After confirming the sequence, the *Nde*I-*Eco*RI restriction fragment was inserted into the pET-21a expression vector at the corresponding sites. The resulting plasmid, pET-deoC, was used to transform *E. coli* strain BL21(DE3)CodonPlus-RIL. *E. coli* cells carrying pET-deoC were grown overnight at 37°C in LB medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%; pH7.0) containing 50 μg/ml ampicillin. The culture was inoculated (1%) into fresh LB medium containing ampicillin and the cultivation was continued until OD_{660} reached 0.5. Heterologous expression of the archaeal gene was induced by the addition 0.2 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for another 4 h at 37°C.

Purification and Molecular Mass Determination

Recombinant *Tk*-DeoC was purified as described previously [12]. Apparent molecular mass of the purified protein was calculated by gel filtration on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) pre-equiliberated with 50 mM Bicine buffer (pH 8.0) containing 150 mM sodium chloride. The void volume was determined with Blue dextran (2000 kDa) and a standard calibration curve was obtained using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and albumin (67 kDa) (Amersham Pharmacia Biotech).

Determination of N-Terminal Amino Acid Sequence

In order to determine the N-terminal amino acid sequence, the band corresponding to *Tk*-DeoC on SDS-PAGE gels was electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was stained with amido black solution (5% amido black, 40% methanol, 10% acetic acid) for 5 min. The membrane was de-stained and the sequence determination was performed using protein sequencer (Model 491 cLC, Applied Biosystems).

DeoC Assay

To determine the *DeoC* activity at 60°C or higher, a discontinuous enzyme assay coupled with alcohol dehydrogenase was adopted. The assay mixture (200 μl) containing 20 mM sodium citrate buffer (pH 4.0), 1 mM EDTA, 10 mM 2-deoxyribose 5-phosphate was pre-incubated at the respective temperature for 1 min and the reaction was initiated by the addition of *Tk-DeoC*. After incubation for 1 min, the mixture was cooled in ice water to stop the reaction. The pH of the mixture was adjusted to 8.0 with 100 μl of 1 M Tris-HCl. Following this, 0.4 mM NADH and 2 U alcohol dehydrogenase were added to the reaction mixture which was adjusted to a final volume of 1 ml. After the reaction was allowed to proceed at 30°C for 3 min, the production of acetaldehyde was determined from the decrease in absorbance at 340 nm using an Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech). Two control experiments were carried out, one with all the components except *Tk-DeoC* and the other with all the components except for the substrate. Product formation was proportional to the incubation time under these conditions. One unit corresponds to the production of 1 μmol of acetaldehyde or glyceraldehyde 3-phosphate/ min.

RNA Isolation and Northern Blot Analysis

For isolation of RNA from strain KOD1, cells were grown in ASW-YT medium consisting of 1.25-fold dilution of artificial sea water (0.8 x ASW), 5.0 g/l yeast extract, 5.0 g/l tryptone and 0.2 g/l elemental sulfur (pH 6.6) with or without addition of 500 mg/l sheared calf thymus DNA (Wako Pure Chemicals, Osaka, Japan). Cells were harvested at the early log phase when OD_{660} was approximately 0.1. RNA was isolated using RNeasy Midi Kit (QIAGEN). For Northern blot analysis, 15 μg of total RNA was denatured by heat treatment at 65°C for 15 min and separated by 1% agarose gel electrophoresis and transferred to a nylon membrane (Hybond^{TM}-N+; Amersham Pharmacia Biotech) by capillary blotting. Digoxigenin (DIG)-labeling of DNA fragments, hybridization and washing of the membranes were performed as recommended by the manufacturer (Roche Diagnostics, Basel, Switzerland). A DNA fragment corresponding to the entire *Tk-deoC* coding region was used as a probe.

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