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ORIGINAL PAPER

**Smadar Shulami · Orit Yaniv · Emilia Rabkin
Yuval Shoham · Timor Baasov**

Cloning, expression, and biochemical characterization of 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase from the hyperthermophilic bacterium *Aquifex pyrophilus*

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Abstract 3-Deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase, catalyzes the aldol-type condensation between phosphoenolpyruvate (PEP) and D-arabinose-5-phosphate (A5P) to produce the unusual 8-carbon sugar KDO8P, and inorganic phosphate. A 15.5-kb segment containing the *kdsA* gene from the hyperthermophilic bacterium *Aquifex pyrophilus* was cloned from a genomic library and sequenced. The native *kdsA* gene lacks a typical ribosome binding site, but contains a conserved U,A-rich sequence upstream to the start codon. The purified *kdsA* gene product catalyzes the formation of KDO8P from its natural substrates, PEP and A5P, as determined by ^{1}H NMR analysis. KDO8P synthase showed maximum activity at 80 °C and pH 5.5–6.0 at 10-min reaction assay. At temperatures of 70, 80, and 90 °C, the enzyme exhibited half-lives of 8.0, 2.25, and 0.5 h, respectively. The kinetic constants at 60 °C were $K_m^{A5P} = 70$ μM, $K_m^{PEP} = 290$ μM, and $k_{cat} = 4$ s^{-1}. The isolated enzyme contained 0.19 and 0.26 mol iron and zinc, respectively, per mole of enzyme subunit. Treatment with metal chelators eliminated enzyme activity, and by the addition of several divalent metal ions, the activity was restored and even exceeded the original activity. These results indicate that *A. pyrophilus* KDO8P synthase is a metal-dependent enzyme. A C11A mutant of KDO8P synthase from *A. pyrophulis* retained less than 1% of the wild-type activity and was shown to be incapable of metal binding.

Keywords Aquifex · Biosynthesis of KDO · KDO8P synthase · LPS · Metalloenzyme · Ribosome binding site

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S. Shulami · Y. Shoham
Department of Food Engineering and Biotechnology, Technion-Israel Institute of Technology, 32000, Haifa, Israel

S. Shulami · E. Rabkin · T. Baasov (✉)
Department of Chemistry, Haifa, Israel
E-mail: chtimor@tx.technion.ac.il
Tel.: +972-4-8292590
Fax: +972-4-8295703

O. Yaniv
The Interdepartmental Program in Biotechnology, Haifa, Israel

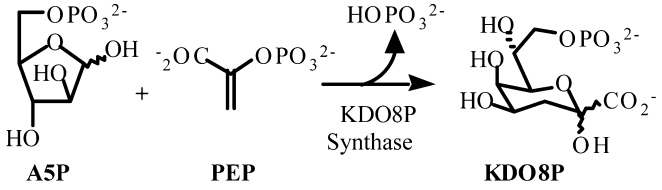
Y. Shoham · T. Baasov
Institute of Catalysis Science and Technology, Haifa, Israel

Introduction

The emergence of bacterial pathogens which are resistant to major classes of commercial antibiotics has created an urgent need for novel antibacterial drugs (Morell 1997). Because the biosynthesis of lipopolysaccharide (LPS) is unique to gram-negative bacteria (Anderson and Unger 1983) and is required for their growth and virulence (Munson et al. 1978; Rick and Osborn 1977) attempts have been made to discover antibacterial agents that inhibit enzymes involved in the synthesis of the LPS (Clements et al. 2002; Jackman et al. 2000). The unusual 8-carbon sugar 3-deoxy-D-*manno*-2-octulosonic acid (KDO) is a site-specific constituent of the LPS and essential for its formation (Raetz 1996). Therefore, much effort has been devoted toward design of synthetic inhibitors against the biosynthesis of KDO (Unger 1981). Indeed, some of the potent inhibitors of CTP:CMP-3-deoxy-D-*manno*-2-octulosonate cytidyltransferase (CMP-KDO synthetase) have shown antibacterial activity in vivo (Goldman et al. 1987; Hammond et al. 1987).

The first committed step in the biosynthesis of KDO is catalyzed by the enzyme KDO 8-phosphate (KDO8P) synthase (EC 4.1.2.16). This enzyme catalyzes an aldol-type condensation of phosphoenolpyruvate (PEP) with D-arabinose 5-phosphate (A5P) to produce KDO8P and inorganic phosphate (Scheme 1; Ray 1980).

Despite extensive mechanistic (Baasov et al. 1993; Dotson et al. 1993, 1995; Du et al. 1999; Hedstrom and Abeles 1988; Liang et al. 1998; Sheffer-Dee-Noor et al.



Scheme 1 The reaction catalyzed by KDO8P synthase

1993) and structural studies (Asojo et al. 2001; Kaustov et al. 2000; Radaev et al. 2000; Wagner et al. 2000) on the reaction catalyzed by KDO8P synthase, which were centered almost exclusively on the *Escherichia coli* enzyme, some mechanistic details and the nature of the reaction intermediates are still uncertain. The most studied KDO8P synthase from *E. coli* does not require metals (Ray 1980); however, it was recently demonstrated that the enzymes from the hyperthermophilic bacterium *Aquifex aeolicus* (Duewel and Woodard 2000) and from the pathogenic bacterium *Helicobacter pylori* (Krosky et al. 2002), require divalent metal cofactor for catalysis. Phylogenetic analyses (Birck and Woodard 2001; Jensen et al. 2002) suggest that KDO8P synthases from other pathogenic bacteria, i.e., *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Campylobacter jejuni*, are also metal-dependent enzymes. Thus, KDO8P synthase is one of the first examples of an enzyme that is metal-dependent in one class of organisms but metal-independent in another. This unique property of KDO8P synthase may further be employed for the development of selective, narrow-spectrum antibiotics against specific classes of pathogenic bacteria.

Aquifex pyrophilus is a hyperthermophilic bacterium that exhibits a maximum growth temperature near 95 °C (Huber et al. 1992) and represents the deepest branching species of the kingdom bacteria (Burggraf et al. 1992). Recently, the LPS from *A. pyrophilus* was isolated and the complete structure of its lipid A was determined (Plotz et al. 2000). This study provided the first demonstration that *A. pyrophilus*, which is phylogenetically ancient on the basis of its 16S rRNA, contains LPS. Quantitative sugar analysis of the isolated LPS revealed that among other sugars it also contains KDO. Thus, although the genes involved in the biosynthesis of *A. pyrophilus* LPS have not yet been isolated, the above observation prompted an investigation to substantiate the presence of these genes in *A. pyrophilus*.

In the present work, we describe the cloning and sequence analysis of a 15.5-kb DNA segment from *A. pyrophilus* containing 15 new genes, and among them is the *kdsA* gene. Specifically, we described the expression and biochemical characterization of the *kdsA* gene product, and its active site mutant C11A. This study demonstrates that *A. pyrophilus* KDO8P synthase requires divalent metal cations for its catalytic activity.

Materials and methods

Materials

A5P was prepared enzymatically according to the procedure of Whitesides (Bednarski et al. 1988). The potassium salt of PEP was prepared in large quantities as previously described (Hirschbein et al. 1982). Metal salts used in this study were obtained from Aldrich and Sigma. All other chemicals were purchased from Aldrich or from Sigma and were used without further purification.

Bacterial strains and plasmids

Escherichia coli strains used were: LE392 for the library amplification of λ-DASHII (Promega, Madison, WI, USA), XL-1 Blue for general cloning in pBluescript KSII (+) (Stratagene, La Jolla, CA, USA) or pGEM-T Easy vector (Promega), and BL21(DE3) (Promega) or Rosetta(DE3) (Novagen, Madison, Wis, USA) for expression via the T7 RNA polymerase expression system with pET9d (Novagen).

Screening of genomic library

A λ-DASHII genomic library of *Aquifex pyrophilus* was screened for the *kdsA* gene both by enzyme activity and hybridization analysis. KDO8P synthase activity was determined using 40 µl of isolated phage lysates in a final volume of 90 µl containing PEP (1.6 mM), A5P (1.6 mM), TRIS-HCl buffer (50 mM, pH 7.3), and 0.2 mM Cd^{2+}. Reactions were incubated at 65 °C for 30 min, stopped with the addition of ice-cold trichloroacetic acid (TCA), and then centrifuged to remove insoluble debris. Under these conditions (65 °C), no activity was detected from the *E. coli* (host) enzyme. The formation of KDO8P was determined by the thiobarbituric acid (TBA) assay as specified by Ray (1980). Hybridization analysis was carried out by using the *kdsA* gene from *E. coli* as a DNA probe. This probe was radiolabeled with [α-^{32}P]dATP (NEN DuPont) by the random-priming method (Feinberg and Vogelstein 1983). Plaque lift was performed with Biodyne A membranes (Pall Geleman Laboratory, MI, USA) and the DNA was fixed by UV radiation. Prehybridization was done in a solution containing 5×SSC (Sambrook et al. 1989), 5×Denhart's solution (Sambrook et al. 1989), and 0.1 mg/ml denatured salmon sperm DNA, for at least 1 h at 65 °C. Hybridization was done in the same solution with about 2×10^7 cpm of the denatured probe, at 65 °C overnight. Filters were washed once in 2×SSC and 0.1% SDS for 30 min at room temperature and then subjected to a stringent wash in 0.1×SSC and 0.1% SDS for 20 min at 65 °C. Filters were visualized in a PhosphoImager (Fuji).

DNA manipulation, sequencing, and analysis

Plasmid DNA was purified with the Wizard DNA Clean-Up system (Promega). Large-scale purification of phage DNA was carried out as outlined by Sambrook et al. (1989) with the following modifications: (1) the purification started with 20 fresh overnight bacterial agar plates with approximately 10^5 plaque-forming units per plate, suspended in 5 ml SM buffer (Sambrook et al. 1989), and (2) participation of the bacteriophage particles was done by ultracentrifugation (Sorvall Discovery^{90}) at 40,000 *g* for 40 min at 4 °C.

Automated sequencing was performed at the DNA sequencing unit of the Weizmann Institute, Rehovot, Israel. DNA sequences were aligned and analyzed using MacVector 7.0 (Oxford Molecular) and by software package of the Genetics Computer Group (GCG, version 9; University of Wisconsin, Madison). Sequence homologies were searched against non-redundant protein databases, using BLASTP (Altschul et al. 1990) or PSI-BLAST

algorithms. The secondary structure and the free energy calculations of RNA stem-loops were analyzed using 'StemLoop' program. The sequence of 12.7-kb containing the *kdsA* gene has been assigned GenBank accession number AY135660.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). The mutagenic primers for C11A were as follows (the mutated nucleotides are shown in bold): 5′-GACTCGCTCTC**G**AT**C**GC**TGC**GGGTCCCGC-3′ and 5′-GCGGGACCC**GCA**GC**G**AT**C**GAGAGCGAGTC-3′. The mutated gene was sequenced to confirm that only the desired mutation was inserted. The mutated gene was overexpressed and purified as the wild-type KDO8P synthase (see below).

Overexpression and purification of the *kdsA* gene product

Based on the DNA sequence of the *kdsA* gene, two PCR primers that allow the in-frame cloning of the gene in the pET vector were designed. The PCR was conducted on chromosomal DNA of *A. pyrophilus*, kindly provided by Prof. Dr. Karl O. Stetter (University of Regensburg, Germany). The N-terminal primer (5′-GATA*CCATGG*AAAAATTTCTGATAATAGCGGGAC-3′) was made to contain an ATG transnational start codon inside a *Nco*I restriction site (CCATGG). The C-terminal primer (5′-CGTA*GGATCC*TCAAACATTAGCGGGTTTTTCG-3′) contained a stop codon (TAG) and a *Bam*HI restriction site (GGATCC) at the end of the gene. Following PCR amplification the gene was cloned into the T7 expression vector pET9d (linearized with *Nco*I and *Bam*HI), resulting in plasmid pET9d-*kdsA*. Cultures [*E. coli* BL21(DE3) (pET9d-*kdsA*)] were grown overnight in Luria-Bertani (LB) broth (Sambrook et al. 1989) with kanamycin (25 μg ml^{-1}) without induction. The overnight cultures (2 l) were harvested, resuspended in 15 ml 50 mM NaCl and 20 mM TRIS-HCl buffer pH 7.5, and disrupted by two passages through a French Press (Spectronic Instruments, Rochester, NY, USA) at room temperature. The cell extract was centrifuged, and the soluble fraction was then heat treated (65 °C, 30 min) and centrifuged again at room temperature. Protamine sulfate was added to the supernatant to a final concentration of 0.26%, and the suspension was stirred for 15 min at room temperature and centrifuged again. The resulting supernatant was applied to an anion-exchange column (HiPrep 16/10Q FF; Pharmacia) equilibrated with 20 mM TRIS-HCl buffer pH 7.5. The column was first washed with 5 column volumes of equilibration buffer and then eluted with 20 column volumes of a linear gradient of 0.05–1.0 M NaCl in 20 mM TRIS-HCl buffer pH 7.5. The enzyme eluted as a distinct peak, which was collected, divided into aliquots of 1 ml, and kept at −80 °C.

Enzyme activity assay and determination of kinetic constants

The standard assay was performed at 60 °C in a final volume of 200 μl reaction buffer consisting of 100 mM HEPES buffer pH 7.0, 0.48 mM Cd^{2+}, 2 mM PEP, 2 mM A5P, 0.2 mM EDTA, and appropriately diluted enzyme (typically 0.3 μM). An aliquot of the enzyme (10 μl) was first preincubated with EDTA (10 μl, 4 mM) for 15 min at room temperature. To this solution was added the reaction buffer (160 μl) containing Cd^{2+} (0.48 mM) and the resulting mixture was incubated for 10 min at 60 °C. The reaction was initiated by adding the mixture (20 μl) of both substrates, PEP (20 mM) and A5P (20 mM). After incubation at 60 °C (typically for 10 min) the reaction was quenched by adding TCA to a final concentration of 5%. The amount of KDO8P was determined by the TBA assay as specified by Ray (1980). One unit of activity is defined as the amount of enzyme required to produce 1 μmol KDO8P per minute. Protein concentration was determined by the Bradford method with BSA as a standard (Bradford 1976). Michaelis-Menten kinetic constants were determined by measuring the initial velocities at saturated concentration of one substrate (0.2 mM) and varying the concentrations of the second substrate (0.2–15 K_m). Data analysis was carried out with GRAFIT 5.0 (Leatherbarrow 2001). UV-visible spectra were recorded on a Biochrom 4060 spectrophotometer (Pharmacia).

Biochemical characterization

pH-dependent studies were carried out in 100 mM buffers of sodium cacodylate (pH 1.5–5.5), HEPES (pH 6–7.5), and TRIS-HCl (pH 7.5–9.5). The effect of temperature on the reaction rate was determined by performing the standard reaction in 100 mM HEPES buffer, pH 7.0, for 10 min at temperatures ranging from 60 to 90 °C. Thermal stability was determined after incubating the purified enzyme solutions (0.1 mg/ml protein in 100 mM HEPES buffer pH 7.0) at 60, 70, 80, and 90 °C for various time intervals and cooling them at 4 °C. The residual activity was measured after preincubation of the enzyme for 15 min at room temperature and under the standard assay conditions described above.

Preparation of EDTA-treated KDO8P synthase and metal content analysis

The native KDO8P synthase enzyme as isolated (2.4 mg/ml) was treated with 2 mM EDTA for 3 h at room temperature, and then extensively dialyzed (three buffer changes) at 4 °C against 20 mM buffer TRIS-HCl pH 7.0 (without EDTA) containing Chelex 100 (25 g/l, Na^+ form, 100–200 mesh; BioRad Laboratories, Hercules, CA). Additional sample of the native enzyme was prepared without EDTA treatment. The concentration of the metals Mn, Cd, Zn, Cu, Fe, Ni, Co, Mg, and Ca, in both EDTA-treated and EDTA-untreated enzymes (40 μM), were determined by high-resolution inductively coupled plasma mass spectrometry (ICP-MS) on a Perkin-Elmer Optima 3000DV. Samples of the dialysate buffer (from the last change) were also analyzed for metal content and the observed concentrations were subtracted from those obtained in the enzymes samples.

Effect of metal chelators and divalent metals on KDO8P synthase activity

The effect of chelators on KDO8P synthase activity was determined as follows: 10 μl enzyme (2 μM) was incubated with 10 μl EDTA or 1,10-phenanthroline at final concentration of 10–500 μM for 15 min at room temperature. The assays were performed at 60 °C with the same final concentration of the chelator. The effect of various divalent metals was measured by preincubating the enzyme with 0.2 mM EDTA for 15 min at room temperature, then reaction buffer (100 mM HEPES pH 7.0, 160 μl), various metals concentrations, and EDTA (2 mM, 20 μl) were added, and incubated for an additional 10 min at 60 °C. The reactions were initiated by adding 20 μl mixture of the substrates A5P (40 mM) and PEP (40 mM).

Synthesis of KDO8P

A solution of PEP (0.1 M) in a 0.1 M TRIS-HCl buffer pH 7.3, containing A5P (0.11 M), Cd^{2+} (0.1 mM), and 3 U purified *A. pyrophilus* KDO8P synthase in a total volume of 4 ml, was incubated at 60 °C. The reaction progress was monitored by ^{31}P NMR. Aliquots (200 μl) of the reaction mixture were withdrawn at various times, diluted with D_2O (300 μl), and analyzed by ^{31}P NMR. Completion of the reaction was deduced by disappearance of the PEP peak centered at −0.2 ppm ($\delta/^{31}P$) and the increase of the KDO8P peaks at 4.3, 4.7, and 5.0 ppm ($\delta/^{31}P$), assigned as the

α- and β-furanose and α-pyranose anomers, respectively. Then, the mixture was incubated with ice-cold TCA (10%, 2 ml) and the precipitated protein was removed by centrifugation. After adjusting the pH to 7.0, the solution was subjected to ion-exchange chromatography on AG 1X8 (100–200 mesh, HCO_3^- form) and the sugar was eluted with a linear gradient (0–0.6 M) of triethylammonium bicarbonate buffer pH 7.5. Fractions containing KDO8P (assayed by the TBA method as specified above) were collected, the buffer was removed by evaporation under reduced pressure, and the resultant oily material was passed through a column of Dowex 50 W (K^+ form) to obtain the purified KDO8P as the tripotassium salt. Fractions containing KDO8P were collected and lyophilized. The ^{1}H NMR spectrum of the isolated material [potassium salt, pD 7.4, 25 °C, D2O, 400 MHz (referenced to HOD at 4.63 ppm): δ α-pyranose, 1.76 (dd, 1H, J = 13.4 and 12.8 Hz, Hax), 1.70 (dd, 1H, J = 13.4 and 6.0 Hz, Heq); δ β-pyranose, 1.58 (dd, 1H, J = 13.3 and 12.7 Hz, Hax), 2.15 (dd, 1H, J = 13.4 and 5.8 Hz, Heq); δ α-furanose, 1.89 (dd, 1H, J = 14.4 and 3.2 Hz, Hax); 2.40 (dd, 1H, J = 14.3 and 7.2 Hz, Heq); δ β-furanose, 2.12 (dd, 1H, J = 13.4 and 7.4 Hz, Hax), 2.20 (dd, 1H, J = 13.3 and 7.2 Hz, Heq)] was similar to that previously described for KDO8P (Baasov and Jacob 1990; Kohen et al. 1993).

Results

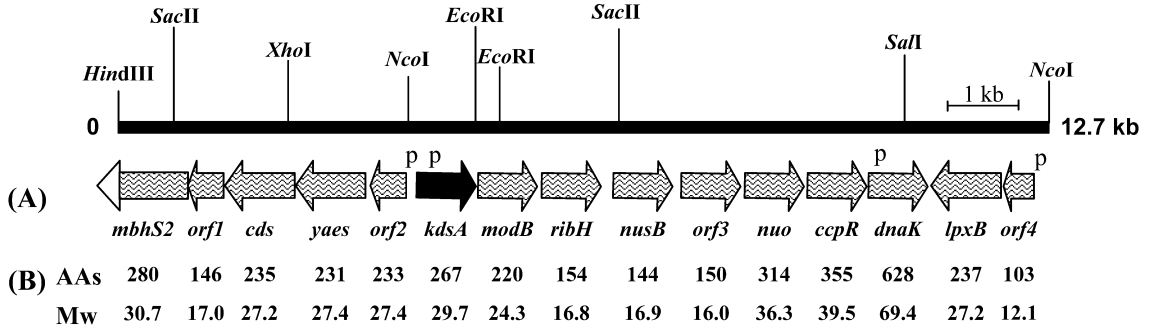
Cloning the *kdsA* gene from *Aquifex pyrophilus*

Our strategy for cloning the *kdsA* gene included two approaches. In the first approach, a genomic library of *A. pyrophilus* constructed in λ-DASH II was screened for KDO8P synthase activity at 60 °C. More than 1,000 phage lysates were tested for enzyme activity, however, no positive clones were detected. Taking into account the sizes of an average insert and the bacterial chromosome, we expected to identify the target gene after screening 100–150 phage lysates. Based on these initial negative results we concluded that the *kdsA* gene is either not well-represented in the library or is not expressed in *Escherichia coli*.

In the second approach, the genomic library was screened by hybridization analysis using the *kdsA* gene from *E. coli* as a DNA probe. In this procedure, about one out of 150 plaques gave a positive signal. The DNA from one of the positive clones was isolated and restriction analysis indicated that it contains a DNA segment of about 15.5 kb. To locate the position of the *kdsA* gene in this segment, the labeled *kdsA* gene from *E. coli* was used to probe a Southern blot of an *Eco*RI digest of the phage DNA. The probe hybridized specifically with a 4.5-kb DNA fragment that was subsequently cloned. To facilitate sequencing of the segment, the DNA was digested with various restriction enzymes and the appropriate fragments were cloned and sequenced. Sequence analysis of the segment revealed 15 ORFs and among them was the *kdsA* gene (Fig. 1). Based on the proximity of the initiation and stop codons of adjacent genes together with absence of any obvious transcriptional initiations signals, the genes appear to be organized in several potential novel operons. It is worth nothing that these operons are composed of genes from different biosynthesis pathways, similar to what is found in the related strain *A. aeolicus* (Deckert et al. 1998). Out of the 15 ORFs, 11 can be assigned based on their similarity to proteins with known functions. Four ORFs shared no homology to known genes from other bacteria, and were assigned as ORF1–4. The BLAST analysis of the various ORFs is shown in Table 1.

One ORF at the end of the segment is incomplete, but its coding region shared homology to the small subunit of a membrane-bound hydrogenase (Brugna-Guiral et al. 2003). One gene probably encodes for cytochrome *c* peroxidase that catalyzes the detoxification of H_2O_2. Another gene is similar to coproporphyrinogen III oxidase, an enzyme involved in hemes (integral part of electron transport chains) biosynthesis. The gene product designated as DnaK has the same DNA sequence as the previously identified *dnaK* gene (encodes for heat-shock protein) from *A. pyrophilus* (Gribaldo et al. 1999). No other genes involved in the biosynthesis of KDO are located adjacent to the *kdsA* gene (Fig. 1). At 10 kb downstream from *kdsA* we identified a putative protein with high similarity to lipid A disaccharide synthase (LpxB) from *E. coli*. This enzyme catalyzes the condensation of lipid X with UDP-2,3-diglucosamine as part of the lipid A biosynthesis (Raetz 1996).

Fig. 1A, B Genetic map of the 12.7-kb segment from *Aquifex pyrophilus* containing the *kdsA* gene. **A** The relative position and direction of transcription of the 15 putative ORFs. The sequence of *mbhS2* at the end of the segment is not complete. *P* denotes proposed promoter regions. **B** The number of amino acids and the calculated molecular weights (in thousands) of the putative proteins



Sequence analysis of the *kdsA* gene

The *kdsA* gene encodes for a 267-amino acid protein with a calculated molecular weight of 29,696 Da.

Table 1 Homologies of the putative ORFs of the 12.7-kb chromosomal segment from *Aquifex pyrophilus*

Gene homologous	Length (bp)	Homologous gene product[a]	Identity (%)/amino acid overlap	Organism/accession number
mbhS2	840	Hydrogenase small subunit	84/283	*Aquifex aeolicus*/D70383
orf1	438	Unknown	72/125	*Aquifex aeolicus*/F70473
cds	705	Phosphatidate cytidylyl transferase	56/235	*Aquifex aeolicus*/F70408
yaes	693	Undecaprenyl pyrophosphate synthetase	44/236	*Escherichia coli*/F64741
orf2	699	Unknown	50/218	*Aquifex aeolicus*/G70407
kdsA	801	KDO8P synthase	87/262	*Aquifex aeolicus*/AAC06457
modB	660	Molybdenum transport system permease	42/112	*Rhodobacter capsulatus*/D36914
ribH	462	Riboflavin synthase beta subunit	93/154	*Aquifex aeolicus*/F70312
nusB	432	Transcription termination factor	81/135	*Aquifex aeolicus*/G70312
orf3	450	Unknown	82/151	*Aquifex aeolicus*/H70312
nuo	942	NADH dehydrogenase	74/311	*Aquifex aeolicus*/A70313
ccpR	1,065	Cytochrome*c* peroxidase	51/317	*Pseudomonas aeruginosa*/1535515
dnaK	1,884	Heat shock protein 70	54/604	*Escherichia coli*/D10765
lpxB	711	Lipid A disaccharide synthetase	30/187	*Escherichia coli*/M19334
orf4	309	Unknown	64/99	*Aquifex aeolicus*/A70424

[a]The homologous gene products are those showing the highest score among proteins with an experimentally defined function. In the absence of relevant biochemical data, the most similar putative protein was reported

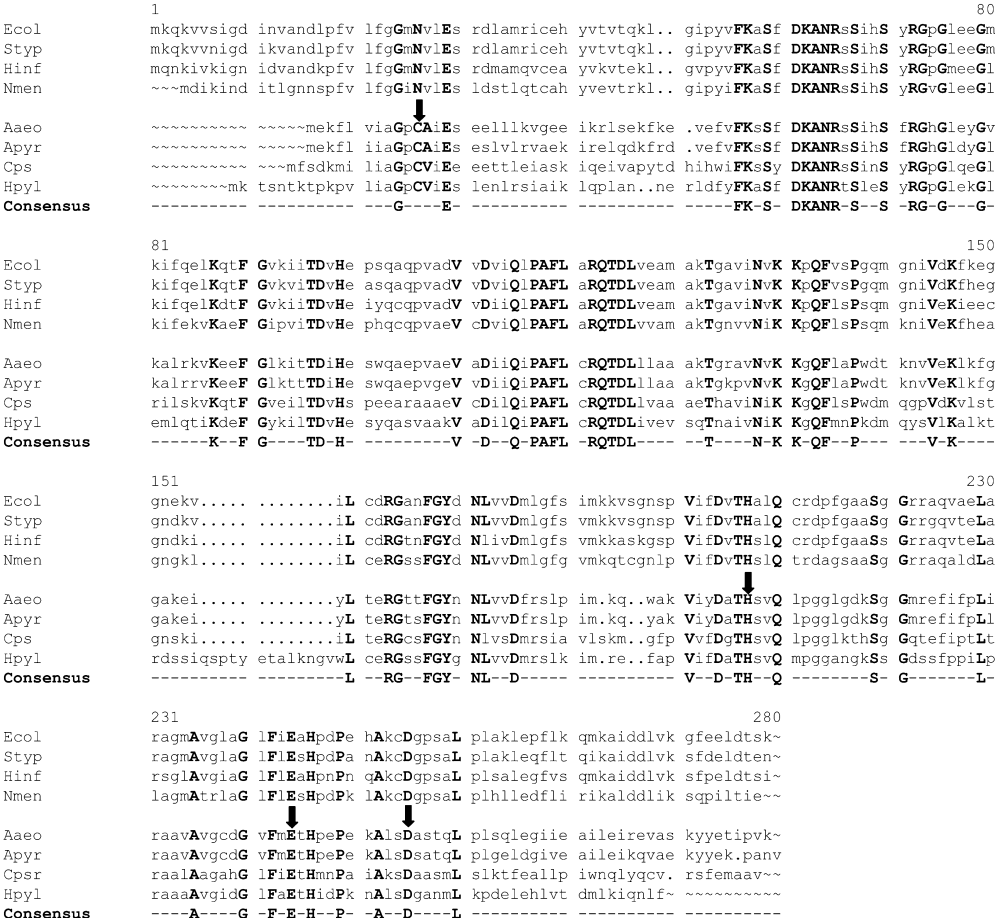


Fig. 2 Representative sequence alignment of metal-independent and metal-dependent KDO8P synthases. The alignment was made with the PILEUP and PRETTY programs (Genetics Computer Group) by using plurality of 8 for the consensus sequence. The four metal-independent KDO8P synthase enzymes (Birck and Woodard 2001) are as follows (data base accession numbers are in parentheses): *Eco*l, *Escherichia coli* (P17579); *Styp*, *Salmonella typhimurium* (Q8XGR9); *Hinf*, *Haemophilus influenza* (P45251); *Nmen*, *Neisseria meningitidis* (CAB84726). The four metal-dependent KDO8P synthases (Birck and Woodard 2001) are: *Aaeo*, *Aquifex aeolicus* (E70308); *Apyr*, *Aquifex pyrophilus* (AY135660); *Cps*, *Chlamydophila psittaci* (Q46225); *Hpyl*, *Helicobacter pylori* J99 (E71985). *Arrows* indicate the four suggested residues (Cys11, His185, Glu222, and Asp233 for *A. pyrophilus* and *A. aeolicus*) involved in metal binding

According to the BLAST results KDO8P synthase from *A. pyrophilus* has the highest homology to the enzyme from *A. aeolicus* (Deckert et al. 1998) (87% identity in 262 amino acids overlap) and 45% identity (262 amino acids overlap) to the KDO8P synthase from *E. coli* (Fig. 2). The typical Shine-Delgarno sequence (AGGAGG) was not found upstream to the putative start codon of the *kdsA* gene. Instead, a high U,A-rich sequence (UAUUAUAUUAA) was found several bases upstream to the start codon. The fact that the *kdsA* gene lacks a typical *E. coli* ribosome binding sequence may explain the failure to detect KDO8P synthase activity while screening the genomic library. Upstream to the ATG start codon of the *kdsA* gene a potential −35

Table 2 Purification steps of KDO8P synthase from *Aquifex pyrophilus*

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity[a] (U/mg)	Yield (%)	Purification (fold)
Crude soluble extract[b]	22	3.3×10^2	2.9×10^2	0.9	100	1
Heat treatment	20	85	2.1×10^2	2.5	74	3
Protamine sulfate	17	51	1.4×10^2	2.7	48	3
Anion exchange chromatography	15	14	1.0×10^2	7.2	35	8

[a]Activities were determined under assay conditions (see Materials and methods)
[b]The extract was obtained from 1.8 l overnight culture ($OD_{600} = 2.5$)

region (TTCCGA) with three of six bases matching the consensus for promoter recognized by vegetative sigma factor (σ^A) of *E. coli* (Lonetto et al. 1992), is separated by 21 bp from the potential −10 region (TAAAAT), with five of six bases matching the σ^A consensus.

Expression and purification of *A. pyrophilus* KDO8P synthase in *E. coli*

The *kdsA* gene was expressed using the T7 RNA polymerase expression vector (pET9d) in *E. coli* strain BL21(DE3) without induction. Attempts to add the inducer IPTG at 10 μM resulted in arrest of cell growth. At 37 °C, the overall expression was reasonable, however, only about 50% of the protein was soluble. Under conditions that promote better protein folding (Schein 1989; i.e., lower growth temperature, 18 °C) the soluble fraction was considerably higher but the amount of the total protein was significantly reduced. Since the codon usage by *A. pyrophilus* (www.kazusa.or.jp/codon) differs from that of *E. coli*, it is possible that translation limits the expression in the *E. coli* host. To solve this potential problem we used the Rosetta (DE3) strain containing the rare tRNA codons AUA, AGG, AGA, CUA, CCC, and GGA. However, no significant differences were found between the conventional BL21(DE3) host to the Rosetta (DE3) cells. We therefore expressed the *kdsA* gene in *E. coli* strain BL21(DE3) at 37 °C without induction. The overall purification procedure (Table 2) included two steps, heat treatment and anion exchange chromatography, and resulted in about 10–20 mg of purified enzyme (>95% purity by inspection; Fig. 3) from a 2-l culture.

Biochemical characterization of KDO8P synthase

To establish that the recombinant protein under investigation does in fact catalyze the formation of KDO8P by direct coupling of PEP with A5P, the enzymatic reaction was performed on a preparative scale and the reaction product was isolated and characterized by NMR. The 400 MHz ^{1}H NMR spectrum of the isolated product was in complete agreement to that previously described for KDO8P (Baasov and Jacob 1990; Kohen et al. 1993). The enzyme was most active at pH 5.5–6.0 and retained about 40% of its activity at pH 4.5 and 7.5

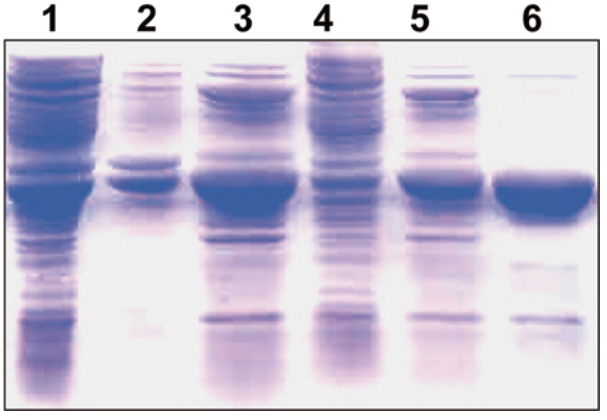


Fig. 3 SDS-PAGE of different purification steps. *Lane 1* Total soluble extract following cell disruption, *lane 2* insoluble extract fraction after cell disruption, *lane 3* the soluble fraction after heat treatment, *lane 4* the insoluble fraction after heat treatment, *lane 5* the soluble fraction after protamine sulfate, *lane 6* the major protein fraction after anion exchange chromatography

(Fig. 4A). The optimal temperature in a 10-min reaction at pH 7.0, was 80 °C (Fig. 4B) and the Arrhenius plot-calculated activation energy was 14.1 kcal/mol, which is characteristic of typical enzymatic reactions. Thermoinactivation experiments revealed half-lives of 0.5, 2.25, and 8 h at 90, 80, and 70 °C, respectively (Fig. 5).

The purified KDO8P synthase, as isolated, was first tested by the standard assay in which the reaction mixture did not contain EDTA and Cd^{2+}. Under these conditions enzyme specific activity was about 3 U/mg. To investigate if the enzyme requires a metal cofactor, the activity was determined in the presence of different concentrations of EDTA and 1,10-phenanthroline (Fig. 6A). With both metal chelators, the activity was inhibited in a dose-dependent manner, suggesting that the enzyme requires a metal cofactor for catalysis.

To test the effect of exogenous metal cations on enzyme activity, the enzymatic reactions were carried out in the presence of 0.2 mM EDTA and various concentrations of metals (Fig. 6B). Under these conditions, Zn^{2+} did not significantly affect activity, while the presence of Mn^{2+}, Co^{2+}, and Cd^{2+} at concentrations above 0.4 mM stimulated the activity of KDO8P synthase, resulting in specific activity higher than that of the isolated enzyme. Of these three metals, the addition of Mn^{2+}

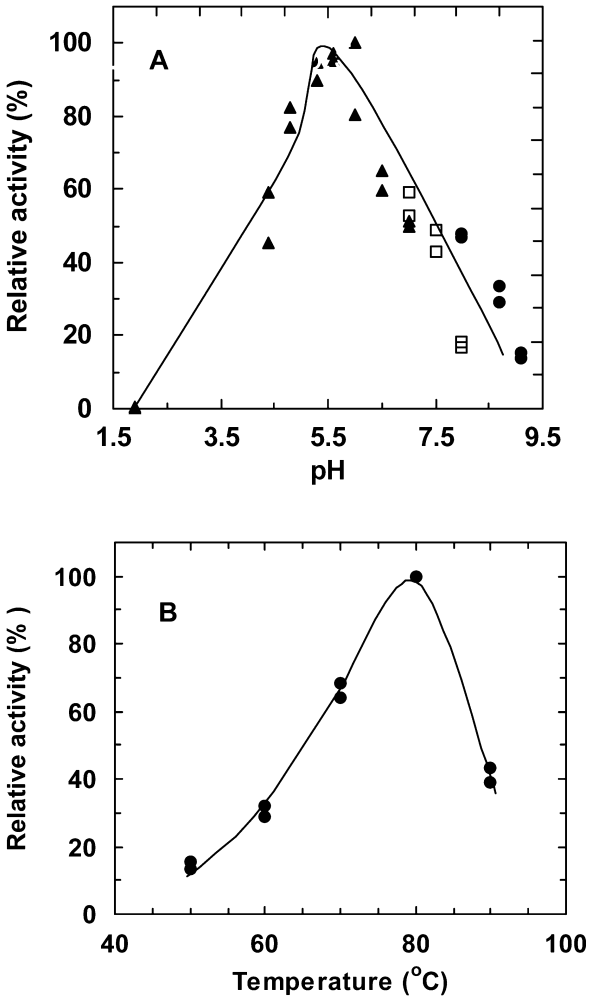


Fig. 4A,B Effect of pH and temperature on KDO8P synthase activity. **A** The enzymatic reactions were carried out for 20 min at 60 °C in 100 mM of different buffer solutions at the pH range of 1.7–9.3. The buffers used were sodium cacodylate (*filled triangles*), HEPES (*open squares*), and TRIS-HCl (*filled circles*). The actual pH of the assay mixture was determined at the reaction temperature of 60 °C. **B** The effect of temperature on KDO8P synthase activity was determined by performing the standard reaction in 100 mM HEPES buffer, pH 7.0, for 10 min at temperatures ranging from 50 to 90 °C. All the experiments were performed at least three times within average standard deviation of 8%

resulted in the highest activity (over 15 U/mg). To further examine whether the metal ion is essential for catalytic activity, EDTA-treated enzyme was extensively dialyzed against metal-free buffer to remove the EDTA. The resultant apoenzyme retained about 1% of its original activity (3 U/mg) and addition of 0.05 mM Cd^{2+} gave an active enzyme with a specific activity of 7.5 U/mg.

Metal content analysis of the purified KDO8P synthase using ICP-MS revealed substoichiometric amounts of iron and zinc (0.19 and 0.26 mol, respectively, per mole of enzyme subunit), traces of manganese but no detectable amounts of cobalt, nickel, and copper. Similar analysis of the EDTA-treated enzyme showed less

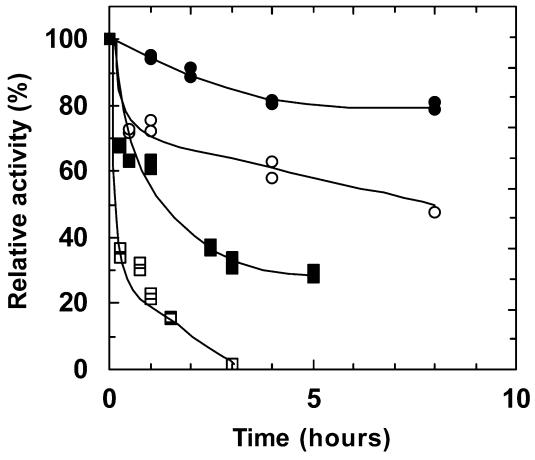


Fig. 5 Thermostability of KDO8P synthase. The enzyme (0.1 mg/ml) was incubated in 100 mM HEPES buffer, pH 7.0, at 90 (*open squares*), 80 (*filled squares*), 70 (*open circles*), and 60 °C (*filled circles*). At various time intervals, aliquots of the samples were removed and assayed for residual activity under the standard assay conditions

than 0.08 and 0.06 mol of iron and zinc, respectively, per mole of enzyme subunit. The presence of iron in the native protein is probably responsible for the pinkish color observed for the wild-type enzyme solution (above 4 mg/ml), as it reflected by a broad absorption band centered at 575 nm ($\epsilon_{575} = \sim 800\ M^{-1}\ cm^{-1}$; Fig. 7).

Kinetics constants were determined in the presence of 0.2 mM EDTA, 0.48 mM Cd^{2+}, at pH 7.0, 60 °C, at saturated concentration of one substrate (0.2 mM) and various concentrations (0.2–15 K_m) of the second substrate. Under these conditions, typical Michaelis-Menten curves were obtained resulting in a k_{cat} of 4 s^{-1}, $K_m = 290 \pm 40$ μM for PEP, and $K_m = 70 \pm 8$ μM for A5P.

Characterization of the C11A mutant of KDO8P synthase

Based on the X-ray crystal structures of KDO8P synthase from *A. aeolicus*, four amino acids (Cys11, His185, Glu222, and Asp233) have been suggested to be involved in metal binding (Duewel et al. 2001; Wang et al. 2001). Sequence alignment revealed that these four amino acids are located at identical positions in KDO8P synthase from *A. pyrophilus*. To assess the role of Cys11 on metal binding and catalysis of the *A. pyrophilus* enzyme, we replaced Cys11 to Ala by site-directed mutagenesis. The purified C11A mutant, as isolated, showed < 1% of the wild-type activity. The activity of this mutant was not stimulated by the addition of exogenous metal cations such as Mn^{2+}, Co^{2+}, and Zn^{2+}, and only Cd^{2+} at a concentration above 1 mM stimulated the activity of the C11A mutant by twofold (data not shown).

ICP-MS analysis of the mutant protein (as isolated) showed a significant reduction in the amount of iron and zinc (0.04 and 0.09 mol, respectively, per mole of

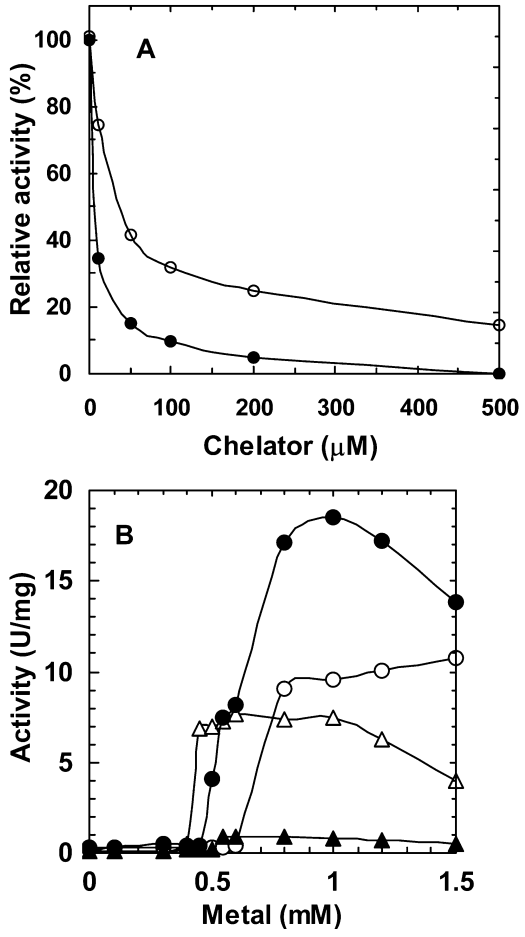


Fig. 6A, B Effect of metal chelators on KDO8P synthase activity. **A** An appropriately diluted enzyme was incubated with EDTA (*filled circles*) or 1,10-phenanthroline (*open circles*) at final concentrations of 10–500 μM, for 15 min at room temperature. After preincubation, the assays were performed in 100 mM HEPES, pH 7.0, and the same final concentration of the chelator. The activities are expressed as the percentage of the initial activity with no chelator. **B** Effects of divalent metals on KDO8P synthase activity. The reactions were performed under standard assay conditions (100 mM HEPES, pH 7.0, 0.2 mM EDTA, 0.2 mM PEP, 0.2 mM A5P, 60 °C) in the presence of various concentrations of $MnCl_2$ (*filled circles*), $CoCl_2$ (*open circles*) $CdCl_2$ (*open triangles*), and $ZnCl_2$ (*filled triangles*). All the experiments were performed at least three times within average standard deviation of 8%

enzyme subunit). In contrast to the wild-type KDO8P synthase a concentrated solution of the C11A mutant (above 10 mg/ml) was completely colorless, as it reflected from the lack of 575 nm peak in its absorption spectrum (Fig. 7). This result further supports the ICP-MS data that the binding of iron by this mutant is significantly reduced.

Discussion

In the present study we have cloned, purified to homogeneity, and biochemically characterized the *kdsA* gene product from *Aquifex pyrophilus*. KDO8P synthase is a

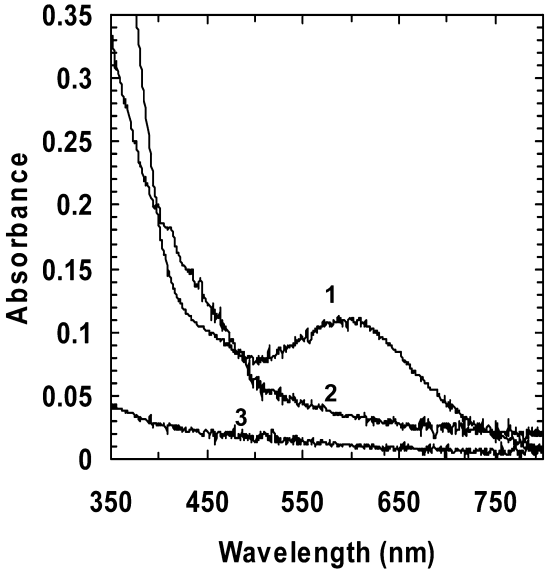


Fig. 7 Absorption spectra of the wild-type and C11A mutant of *A. pyrophilus* KDO8P synthase. Spectra of the wild-type KDO8P synthase (160 μM) and of C11A mutant (320 μM), both as purified, are shown in *curve 1* and *curve 2*, respectively. *Curve 3* is of the wild-type KDO8P synthase (40 μM) after treatment with EDTA (10 mM) and removal of excess EDTA by dialysis. All spectra were acquired at 35 °C in 50 mM TRIS-HCl buffer, pH 7.5

key enzyme in the synthesis of KDO, a key sugar component of LPS. Since KDO is not present in mammalian cells, the enzymes involved in the synthesis of KDO are attractive targets for the development of potential new antimicrobial drugs.

Cloning and sequence analysis of the *kdsA* gene

Screening of *A. pyrophilus* genomic library for enzyme activity failed to produce positive clones. However, screening the library by hybridization resulted in positive clones at a frequency of about one out of 150 plaques. Taking into account the size of the chromosome and the average insert size, this frequency is what one would expect. These results indicate that the native *kdsA* gene is probably not expressed efficiently in the *Escherichia coli* host. This poor expression could be a result of rare codons in the target gene and due to the lack of the classical canonical ribosome binding site (RBS) sequence, AGGAGG. Since the *kdsA* gene was expressed reasonably well in the pET expression system, the codon usage does not seem to be the main obstacle.

Sequence analysis of the 12.7-kb segment, containing the *kdsA* gene, revealed several interesting features. Fifteen putative ORFs, among them the *kdsA* gene, could be identified and based on their arrangement they appear to be organized in several operons. This fact by itself is not surprising for bacterial genomes, however, the composition of these potential operons is unexpected, since they constitute genes with no obvious common biosynthetic role. For example, the *kdsA* gene

is probably co-transcribed with at least six more ORFs, and none of them are involved in the biosynthesis of KDO or LPS. This is in contrast to what is usually found in bacterial operons that are made of genes with common overall function. In enteric bacteria the genes involved in the biosynthesis of LPS (such as the *lpx* and *rfa* genes) are grouped in clusters (Schnaitman and Klena 1993). It seems that it is not only genes from the same biosynthetic pathway that are not clustered, but rather genes that are expected to be required in stoichiometric amounts. For example, the gene for the α subunit of riboflavin synthase (*rib*H) appears to be co-transcribed in an operon lacking the β subunit (*rib*C; Fig. 1). Another interesting feature of the sequence is the absence of any obvious secondary (hairpin loop) structures for transcriptional termination at the end of the potential operons. This situation was also reported for other prokaryotes including *A. aeolicus*, a close relative of *A. pyrophilus* (Washio et al. 1998). Within the 15.5-kb chromosomal segment from *A. pyrophilus*, part of the identified ORFs is organized similarly to the corresponding genes of *A. aeolicus*, for example, *kdsA* through *modB*, *ribH* through *ccpR*, and *lpxA* through *orf4* (Fig. 1).

An altered ribosome binding site?

Interestingly, the gene *kdsA* lacks the well-known canonical RBS sequence, AGGAGG [Shine-Dalgarno (SD) sequence], and contains a U,A-rich region (UAUAUUAA) 8 bp upstream to the start codon. Representative recombinant genes from *A. pyrophilus* that lack the conserved RBS and possess a U,A-rich region upstream to the initiation codon are shown in Fig. 8.

This observation may explain our inability to identify positive phages based on KDO8P synthase activity, and prompted us to search for other consensus sequences that may replace the classic RBS. *A. aeolicus* is a close relative of *A. pyrophilus* and its complete genome sequence has been published (Deckert et al. 1998). A close examination of the upstream sequence of 807 putative initiation start codons in *A. aeolicus* reveals that about 10% of them lack the classic SD sequence, but contain the sequence UAAAAU four to nine bases upstream to the start codon. The absence of the complementary sequence (ATTTTA) in the two 16S rRNA genes of *A. aeolicus* (Deckert et al. 1998), may indicate the existence of an alternative translation initiation mechanism. Efficient translation in the absence of the SD initiation signal was already demonstrated, and it has been proposed that specific sequences might be directly "recognized" by ribosomes (Tzareva et al. 1994). Further work is necessary to verify the direct role of this sequence in the initiation of translation in these bacteria.

Fig. 8 Alignment of the regions upstream to the start codon of four recombinant genes from *A. pyrophilus*. *Bold letters* indicate the U,A-rich region with the consensus UAAAAU upstream to the start codon (AUG). Data base accession numbers are in *parentheses*

		−25 +1
alanyl-tRNA synthase	(AF027500)	AAAAACUAUAAUU**UAAACU**UUAGCU **AUG**
Pyridoxal phosphate synthase	(X74277)	ATCAAGGGCUUU**UAAAAU**UAAAAAU **AUG**
Glutamate racemase	(AF212972)	AAAUUCCCGA**UAAAAU**AAACUCCCU **AUG**
Alanine racemase	(AF212103)	CCAUAGGAAAAUUU**UAAAAU**AGCUC **AUG**
KDO8P synthase	(AY135660)	GACCAGUAU**UAUAUUAA**CAGAUACC **AUG**
		start codon

Biochemical characterization of KDO8P synthase from *A. pyrophilus*

The recombinant *kdsA* gene product catalyzed the formation of KDO8P from its natural substrates, PEP and A5P, as was proved by ^{1}H NMR analysis of the enzymatic reaction products. This result indicates that *A. pyrophilus* is capable of producing KDO8P, the precursor of KDO in gram-negative bacteria, and further confirms the presence of KDO in the LPS of *A. pyrophilus* (Plotz et al. 2000). The observed optimal temperature (80 °C), and the half-life (2.25 h) at this temperature, clearly demonstrates that the recombinant KDO8P synthase is extremely thermostable.

Several lines of evidence obtained in this study substantiate that *A. pyrophilus* KDO8P synthase requires divalent metal cofactor for catalytic activity. First, both EDTA and 1,10-phenanthroline inactivate the isolated enzyme in a concentration-dependent manner (Fig. 6A). Second, several divalent metal cations restore a broad range of activities by incubating with the EDTA-inactivated enzyme (Fig. 6B). These data clearly demonstrate that inactivation by EDTA is a direct effect of the chelation of a metal cofactor and not a result of non-specific binding of EDTA to the enzyme's active site residues that normally accommodate carboxylate and phosphate groups of the substrates PEP and A5P. Typically the isolated enzyme contained approximately 0.2 and 0.3 mol of iron and zinc, respectively, per mole of enzyme subunit. These results are consistent with the specific activity of 3 U/mg, and suggest that the metal binding site in the isolated enzyme is only partially occupied with metal cations.

Kinetics constants were determined in the presence of 0.2 mM EDTA, 0.48 mM Cd^{2+}, at pH 7.0, 60 °C, resulting in a k_{cat} of 4 s^{-1}, $K_m = 290$ μM for PEP, and $K_m = 70$ μM for A5P. These values are somewhat different from the constants obtained for the enzyme from *A. aeolicus* at 60 °C ($k_{cat} = 0.38$ s^{-1}, $K_m^{PEP} = 43$ μM, $K_m^{A5P} = 8$ μM; Duewel et al. 1999), although the specificity constants (k_{cat}/K_m) for both substrates in both enzymes are similar. The observed difference in kinetic

constants may reflect different optimum temperatures and/or different intrinsic catalytic properties of these two enzymes.

Based on the 3D structure of *A. aeolicus* enzyme in complex with exogenous Cd^{2+}, four amino acids residues involved in metal binding were identified (Duewel et al. 2001; Wang et al. 2001). The corresponding four residues in the *A. pyrophilus* enzyme are His185, Glu222, Asp233, and Cys11. At this stage, our understanding as to the contribution of each of these residues to metal binding and catalysis is incomplete. Initial characterization of C11A mutant of KDO8P synthase from *A. pyrophilus* demonstrated that Cys11 has an essential role for both metal binding and catalysis. Very recently, the 3D structure of an H185G mutant of *A. aeolicus* KDO8P synthase was determined (Wang et al. 2002). This study suggested that His185 is necessary for the correct binding of PEP in the active site.

It is noteworthy that three out of the four metal binding residues, His185, Glu222, and Asp233 are completely conserved in all KDO8P synthases currently sequenced (Birck and Woodard 2001), implying that they play an essential role in both metal-dependent and metal-independent KDO8P synthases. The fourth residue, Cys11, is conserved only in the metal-dependent enzymes (Birck and Woodard 2001), while in the metal-independent enzymes, a conserved Asn replaces Cys (Fig. 2) . Therefore, it is tempting to speculate that a single amino acid replacement, Cys to Asn, could eliminate the requirement for metals in metal-dependent enzymes. In fact, our preliminary results indicate that in the *A. pyrophilus* KDO8P synthase, the C11N replacement converts the enzyme to be metal-independent. Similarly, the reciprocal replacement (N26C) in the metal-independent *E. coli* KDO8P synthase, resulted in metal-dependent activity (Shulami et al., unpublished results). Further studies are underway to provide more rigorous characterization of the *A. pyrophilus* KDO8P synthase and its active site mutants.

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