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Aquifex aeolicus 3-Deoxy-D-*manno*-2-Octulosonic Acid 8-Phosphate Synthase: A New Class of KDO 8-P Synthase?

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Abstract. The relationship between 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate (KDO 8-P) synthase and 3-deoxy-D-*arabino*-2-heptulosonic acid 7-phosphate (DAH 7-P) synthase has not been adequately addressed in the literature. Based on recent reports of a metal requiring KDO 8-P synthase and the newly solved X-ray crystal structures of both *Escherichia coli* KDO 8-P synthase and DAH 7-P synthase, we begin to address the evolutionary kinship between these catalytically similar enzymes. Using a maximum likelihood-based grouping of 29 KDO 8-P synthase sequences, we demonstrate the existence of a new class of KDO 8-P synthase, the members of which we propose to require a metal cofactor for catalysis. Similarly, we hypothesize a class of DAH 7-P synthase that does not have the metal requirement of the heretofore model *E. coli* enzyme. Based on this information and a careful investigation of the reported X-ray crystal structures, we also propose that KDO 8-P synthase and DAH 7-P synthase are the product of a divergent evolutionary process from a common ancestor.

Key words: Lipopolysaccharide — O-antigen — Shikimate pathway — Maximum likelihood

Introduction

Many comparisons have been made between *Escherichia coli* 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate (KDO 8-P) synthase (P17579) and *E. coli* 3-deoxy-D-*arabino*-2-heptulosonic acid 7-phosphate (DAH 7-P) synthase (Phe sensitive) (P00886). These enzymes comprise fully one-half of all those studied that catalyze the cleavage of the C–O bond of PEP in the course of reaction. Unfortunately, the lack of a defined relationship between these enzymes lead researchers to believe that similarities between them were purely coincidental.

KDO 8-P synthase catalyses the first committed step in the production of KDO, an integral part of the inner core region of the lipopolysaccharide layer (LPS) in Gram-negative (G−) bacteria. KDO acts as a linker between lipid A and the O-antigen region (Levin and Racker 1959). Rick and Osborn (1972) first determined that disruption of KDO 8-P synthase leads to cessation of DNA, RNA, and protein synthesis and eventual cellular death. These and other studies were performed on a strain of *Salmonella typhimurium* which encoded a temperature-sensitive form of KDO 8-P synthase. Traditionally, both KDO 8-P synthase and the LPS were thought to be unique to G− bacteria. Recently however, KDO has been isolated from plant tissue and KDO 8-P synthase activity reported in crude tissue preparations (Sakurai et al. 1996). Homogeneous KDO 8-P synthase from a plant species has not been reported.

DAH 7-P synthase, ubiquitous in bacteria, fungi, and plants, is the first enzyme in the Shikimate pathway. This pathway is responsible for the generation of the intermediate compounds, chorismate and prephenate, which lead to the production of the aromatic amino acids (Phe, Tyr, Trp), catechols, and *p*-aminobenzoic acid (folic acid biosynthesis) (Walsh et al. 1996). Disruption of the Shikimate pathway can lead to rapid death of the organism.

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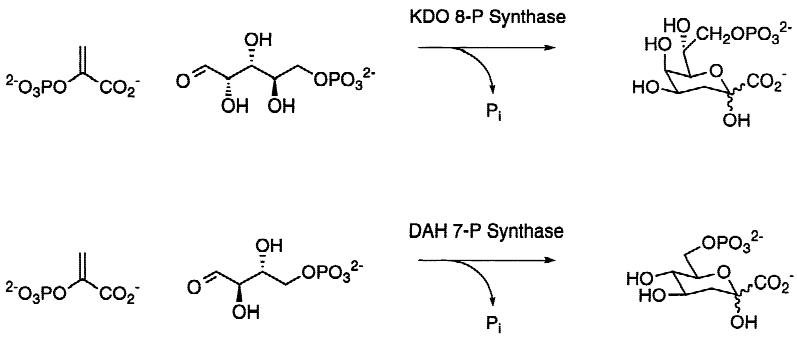

Fig. 1. Proposed chemical mechanisms of KDO 8-P synthase and DAH 7-P synthase.

This phenomenon is exploited by the commercially available herbicide, Roundup, which contains *N*-phosphonomethylglycine (glyphosate), a powerful inhibitor of *enol*pyruvyl shikimate 3-phosphate synthase (Steinrucken and Amrhein 1980), an enzyme downstream of DAH 7-P synthase in the Shikimate pathway. DAH 7-P synthase has not been targeted by antimicrobial research. Neither KDO 8-P synthase nor DAH 7-P synthase has a known counterpart in mammalian physiology (Raetz 1990), making both attractive targets for antimicrobial compounds.

Both KDO 8-P and DAH 7-P synthases catalyze a reaction between phospho*enol*pyruvate (PEP) and a phosphorylated monosaccharide, arabinose 5-phosphate (A5P) in the case of the former (Levin and Racker 1959) and erythrose 4-phosphate (E4P) for the latter (Srinivasan and Sprinson 1959) (Fig. 1). The respective reactions appear to proceed by a common mechanism involving attack from the *si* face of PEP onto the *re* face of the monosaccharide aldehyde, following a proposed attack by water onto the C2 position of PEP (DeLeo et al. 1973; Dotson et al. 1993; Kohen et al. 1993; Onderka and Floss 1969a, b). There is, however, one prominent difference between *E. coli* KDO 8-P synthase and *E. coli* DAH 7-P synthase in the literature: DAH 7-P synthase requires a divalent metal for catalysis (Srinivasan and Sprinson 1959), while KDO 8-P synthase does not (Ray 1980). It has been shown through metal analysis (Stephens and Bauerle 1991) and chemical modification experiments (Stephens and Bauerle 1992) that the metal required for DAH 7-P synthase activity is likely catalytic rather than structural. Based on the paucity of sequence similarity between *E. coli* KDO 8-P synthase and *E. coli* DAH 7-P synthase (18% identity) and the difference in metal requirement, the mechanistic relationship between these two enzymes has been assumed to be primarily coincidental.

In the last year, however, two important events have occurred in the study of these enzyme groups. First, representative X-ray crystal structures of both enzymes have become available, the *E. coli* KDO 8-P synthase structure by the Gatti laboratory in collaboration with our own (Radaev et al. 2000) and the *E. coli* DAH 7-P synthase (Phe sensitive) structure by Kretsinger and co-workers (Shumilin et al. 1999). Second, recombinant KDO 8-P synthase from the hyperthermophile *Aquifex aeolicus* (AAC06457) has been overexpressed in *E. coli* and characterized (Duewel et al. 1999). The *A. aeolicus* KDO 8-P synthase, while having many of the same characteristics as the *E. coli* enzyme, requires a divalent metal for catalysis (Duewel and Woodard 2000). This poses an interesting series of questions.

(1) Is *A. aeolicus* KDO 8-P synthase indicative of a class of KDO 8-P synthases that requires a metal for catalysis or is a metallo-KDO 8-P synthase unique to this hyperthermophile?
(2) Is there, in parallel with a metallo-KDO 8-P synthase class, an unrecognized class of DAH 7-P synthases that does not require a metal?
(3) Does the existence of these hypothetical metallo-KDO 8-P synthase and nonmetallo-DAH 7-P synthase classes imply an evolutionary link between KDO 8-P synthase and DAH 7-P synthase?
(4) Did the ancestral KDO 8-P synthase require a metal or was it metal independent?

In this report, we propose the existence of a new class of KDO 8-P synthase enzymes that requires the presence of a divalent metal for catalytic activity and propose a class of DAH 7-P synthases that does not.[1] Furthermore, we theorize that the evolutionary link between KDO 8-P synthase and DAH 7-P synthase is one of divergence and that the ancestral KDO 8-P synthase was a metalloenzyme. The identification of new classes of KDO 8-P and DAH 7-P synthases allows for the opportunity to define further the similarities and differences between them.

Methods

Protein sequences showing similarity to that of *E. coli* KDO 8-P synthase were identified using the Basic Local Alignment Search Tool (BLAST), specifically tblastn (Altschul et al. 1990), primarily from the National Center for Biotechnology Information website (other sources of data are referenced in Table 1). All primary sequence alignments

[1] Class I (enzyme) refers to the putative nonmetallo-KDO 8-P and DAH 7-P synthases, while Class II (enzyme) refers to their putative metal-containing homologues.

Table 1. Compilation of accession numbers and references for all sequences used in the analysis

Organism	Gram +/−, archeae, plant	Included in tree: KDO 8-P synthase (Fig. 2)	Included in tree: DAH 7-P synthase (Fig. 3)	Reference[a]	Accession no. (KDO 8-P synthase/ DAH 7-P synthase)[b]
Actinobacillus actinomycetemcomitans	−	Yes	Yes	University of Oklahoma—NIDR[c]	n.a./n.a.
Actinobacillus pleuropneumoniae	−	Yes		Ward et al. (1998)	O68662/—
Arabadopsis thaliana	Plant	Yes	Yes	TIGR	n.a./P29976.
Aquifex aeolicus	−	Yes		Deckert et al. (1998)	O66496/—
Bacillus subtilis	+		Yes	Kunst et al. (1997)	—/BG10286
Bordetella bronchiseptica	−	Yes		Sanger—Beowolf Genomics	n.a./—
Bordetella pertussis	−	Yes	Yes	Sanger—Beowolf Genomics	n.a./n.a.
Campylobacter jejuni	−	Yes		Sanger—Beowolf Genomics	n.a./—
Caulobacter crescentus	−	Yes		TIGR—DOE	n.a./—
Chlamydia pneumoniae	−	Yes	Yes	Kalman et al. (1999)	Q9Z714/AAD18624
Chlamydia psittaci	−	Yes		TIGR—NIAID	Q46225/—
Chlamydia trachomatis	−	Yes	Yes	Stephens et al. (1988)	P77849/AAC67978
Chlorobium tepidum	−	Yes	Yes	TIGR—DOE	n.a./n.a.
Clostridium acetobutyricum	+		Yes	Genome Therapeutics—DOE	—/n.a.
Corynebacterium glutamicum	+		Yes	LION Bioscience—Degusa	—/n.a.
Deinococcus radiodurans	+		Yes	White et al. (1999)	—/n.a.
Enterococcus faecalis	+		Yes	TIGR—NIAID	—/n.a.
Escherichia coli	−	Yes	Yes	Blattner et al. (1997)	P17579/P00886
Haemophilus influenzae	−	Yes	Yes	Fleischmann et al. (1995)	P45251/P44303
Helicobacter pylori J99	−	Yes		Alm et al. (1999)	AAD05587/—
Helicobacter pylori 26695	−	Yes		TIGR—TIGR	n.a./—
Klebsiella pneumoniae	−	Yes		Washington University Consortium	n.a./—
Mycobacterium avium	+		Yes	TIGR—NIAID	—/n.a.
Neisseria gonorrhoeae	−	Yes		University of Oklahoma—NIAID	CAB44938/—
Neisseria meningitidis	−	Yes	Yes	TIGR—TIGR	CAB44959/n.a.
Pasteurella multocida	−	Yes		University of Minnesota Computational Biology Center—USDA–NRI/Minnesota Turkey Growers Association	n.a./—
Pasteurella haemolytica	−	Yes		LION Bioscience	P95514/—
Pisum sativum	Plant	Yes			n.a./—
Porphyromonas gingivalis	−	Yes		TIGR—NIDR	n.a./—
Pseudomonas aeruginosa	−	Yes	Yes	University of Washington—Cystic Fibrosis Foundation	Q9ZFK4/n.a.
Pyrococcus abyssi	Arch.		Yes	Genoscope	—/n.a.
Pyrococcus furiosus	Arch.		Yes	Utah Genome Center	—/n.a.
Rickettsia prowazekii	−	Yes	Yes	Andersson et al. (1998)	Q9ZE84/n.a.
Salmonella typhi	−	Yes	Yes	Sanger—Wellcome Trust	n.a./n.a.
Shewanella putrefaciens	−	Yes		TIGR—DOE	n.a./—
Solanum tuberosum	Plant		Yes	Zhao and Herrmann (1992)	—/M95201
Staphylococcus aureus	+		Yes	TIGR—NIAID/MGRI	—/n.a.
Streptococcus pyogenes	+		Yes	University of Oklahoma	—/n.a.
Thermotoga maritima	−		Yes	Nelson et al. (1999)	—/n.a.
Vibrio cholerae	−	Yes		TIGR—NIAID	n.a./—
Yersinia pestis	−	Yes	Yes	Sanger—Beowolf Genomics	n.a./n.a.

[a] If unpublished data, the organization performing the sequencing is followed by the funding agency, where known.
[b] n.a., not available; —, sequence not used.
[c] DOE, U.S. Department of Energy; JGI–DOE; Joint Genome Institute–U.S. Department of Energy; MGRI, Merck Genome Research Institute; NIAID, National Institute of Allergy and Infectious Diseases; NIDR, National Institute of Dental & Craniofacial Research; Sanger, The Sanger Centre; TIGR, The Institute for Genomic Research; USDA–NRI, U.S. Department of Agriculture–National Research Initiative.

were accomplished using ClustalW (Thompson et al. 1994) on a Silicon Graphics O2 computer. The BLOSUM (Henikoff and Henikoff 1992) series was used for the protein weight matrix. Phylogenetic analyses were completed using PHYLIP (Phylogeny Inference Package, v3.5c) and PAML (Phylogenetic Analysis by Maximum Likelihood, v3.0a).

Sequences of the two proteins (KDO 8-P synthase and DAH 7-P synthase) were aligned using ClustalW. Phylogenetic relationships were calculated by maximum-likelihood analysis using AAML (codeml: seqtype = 2) in the PAML package. To decrease the calculation time, an initial tree search was performed using a parsimony approach. The sequence alignment was bootstrapped using SeqBoot followed by parsimony analysis using ProtPars (100 replicates). The 100 most parsimonious trees were then used as input in the AAML program for maximum-likelihood analysis. The evolutionary model described by Jones (1992) with actual amino acid frequencies was used and the γ distribution was estimated (JTT + F + γ). The differences in

Table 2. Statistical data from the maximum-likelihood analysis of 30 KDO 8-P synthases

Tree no.	−ln likelihood (JTT + F + γ)	SE	Bootstrap proportion	Gamma shape factor (α)
10	−8567.590	—	0.340	0.82549
12	−8570.747	11.335	0.157	0.82871

maximum-likelihood values were evaluated using the method of Kishino and Hasegawa (1989) implemented in AAML. Figures were prepared in part using Drawtree and Drawgram in the PHYLIP package. Unless otherwise stated, all parameters in both ProtPars and AAML were the default values. A molecular clock was not assumed due to the lack of reliable rooting information.

Putative Class II KDO 8-P synthases (*Helicobacter pylori* J99, *Chlamydia psittaci*) were overexpressed and purified as described by Duewel et al. (1999) with the exception of the heat precipitation step. KDO 8-P synthases were treated with 10 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* dipicolinic acid, or 10 m*M* 1,10-phenanthroline to sequester metal away from the enzyme. Typically, excess chelator was removed via Amicon diaflow filtration and enzymatic assays were performed as described previously (Schoner and Herrmann 1976) in both the absence and the presence of 1 m*M* divalent metal (Mg, Zn, Mn, or Fe). An enzyme is classified as metal dependent if chelator renders it inactive (<5% remaining activity) and the subsequent addition of metal to the apoenzyme restores it to full activity. Conversely, a homologue is determined to be metal independent if neither chelator nor exogenous metal results in a greater than 10% change in activity.

Results

Phylogenetic Analysis of KDO 8-P Synthase

Based on BLAST searches conducted using the genomic database, primarily at the NCBI (for others see Table 1), 29 complete and publicly available sequences were identified that demonstrate >40% identity with the well-studied *E. coli* KDO 8-P synthase. Two of these sequences (*Arabadopsis thaliana* and *Pisum sativum*) are from plant species. Although there are no literature reports of homogeneous KDO 8-P synthase isolated from plant tissue, both of these sequences were included in the interest of completeness.

From the maximum-likelihood analysis of 100 possible trees tested, only two have a bootstrap proportion greater than 0.1 (Table 2). Only the most likely tree (tree 10) is shown in Fig. 2. Both of these two phylogenetic trees divide the enzyme sequences into identical classes, with only minor reorganization within the two classes. From Fig. 2, it is readily apparent that two large groups of KDO 8-P synthase (Class I and Class II) exist.

Analysis of KDO 8-P Synthases from H. pylori *J99 and* C. psittaci

Recombinant *H. pylori* J99 and the *C. psittaci* KDO 8-P synthases were expressed and isolated from *E. coli* and assayed for metal dependence. Upon treatment with EDTA, dipicolinic acid, and/or 1,10-phenanthroline, both enzymes are substantially inactivated (Table 3), while the inclusion of divalent metal in the assay mixture dramatically raises the activity of each enzyme.

Phylogenetic Analysis of DAH 7-P Synthase

Twenty-five DAH 7-P synthases were chosen in a manner similar to that described for KDO 8-P synthase. Unlike KDO 8-P synthase, however, DAH 7-P synthase exists in Bacteria, Archea, and Eucarya. Representatives from all of these domains were included in the DAH 7-P synthase analysis. Similarly to the phylogenetic tree generated for KDO 8-P synthase, the DAH 7-P synthases separate into two distinct classes. In the case of DAH 7-P synthase, however, maximum-likelihood analysis generated only a single significant tree. This tree is shown in Fig. 3.

Discussion

It is apparent from the KDO 8-P synthase phylogenetic tree (Fig. 2) that two classes of enzyme exist. We propose that the unifying trait of Class I is the lack of a metal requirement, while that of Class II is the requirement of a divalent metal for catalysis. The KDO 8-P synthases from *E. coli* (Ray 1980), *Salmonella typhimurium* (Taylor et al. 2000), *Neisseria gonorrhoeae* (Sheflyan et al. 2000), *A. aeolicus* (Duewel and Woodard 2000), *H. pylori* J99, and *C. psittaci* have all been characterized. The enzymes from *E. coli*, *S. typhimurium*, and *N. gonorrhoeae* have all been shown to catalyze the condensation reaction in the absence of any metal cofactor. It has been reported that KDO 8-P synthase from *A. aeolicus* requires a divalent metal for activity. Here we report that, based on a preliminary characterization, the enzymes from *H. pylori* J99 and *C. psittaci* also require a divalent metal for catalytic activity. As shown in Fig. 2, the three known nonmetallo-KDO 8-P synthases (*E. coli*, *S. typhimurium*, and *N. gonorrhoeae*) are all members of Class I in the phylogenetic tree. Conversely, the metallo-KDO 8-P synthases from *A. aeolicus*, *H. pylori* J99, and *C. psittaci* all belong to Class II. Beyond the obvious difference of metal requirement, the two classes of KDO 8-P synthase appear to be very similar in most other respects. Those enzymes fully characterized (*E. coli*, *A. aeolicus*, *S. typhimurium*, and *N. gonorrhoeae*) all share very similar biochemical properties, the only significant difference being the optimal temperature of the *A. aeolicus* KDO 8-P synthase. This is hardly surprising, as *A. aeolicus* is a hyperthermophilic organism, whereas the others are mesophiles. As the other proposed Class II KDO 8-P synthases are mesophiles, temperature optimum can be reasonably disregarded as the Class II uni-

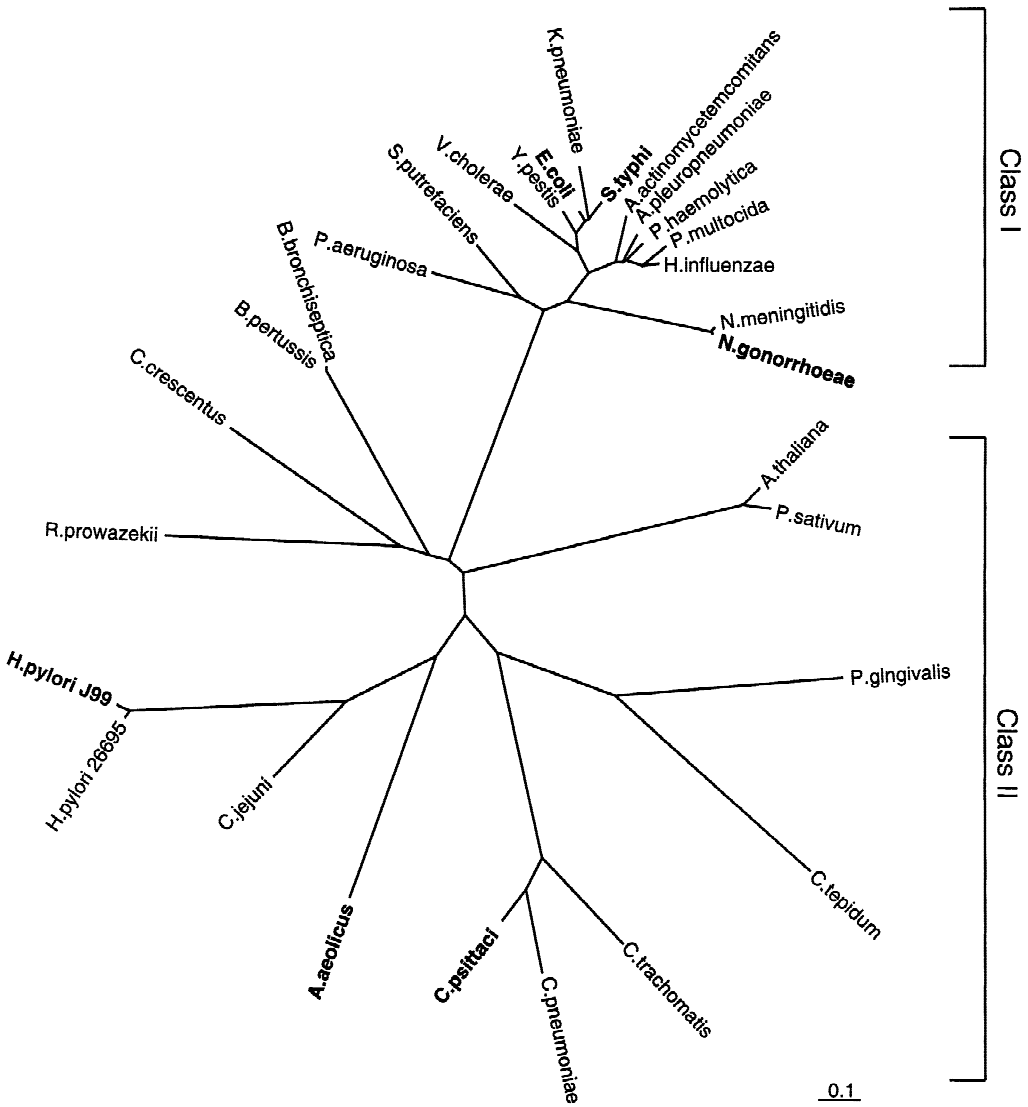

Fig. 2. Phylogenetic tree generated by maximum-likelihood analysis from the sequences of 29 KDO 8-P synthase sequences from various organisms. The first group (Class I) is predicted to maintain the characteristics of the model *E. coli* enzyme (i.e., no metal requirement), while the second group (Class II), including the *A. aeolicus* enzyme, is predicted to require a divalent metal for catalysis. Enzymes for which in vitro metal binding data are known are shown in *boldface* type.

Table 3. *In vitro* activity of KDO 8-P synthase

Source of recombinant KDO 8-P synthase	Activity as isolated (k_{cat}; min^{-1})	Activity in the presence of 10 m*M* EDTA (min^{-1})	Activity in the presence of 1 m*M* $MnCl_2$ (min^{-1})
H. pylori J99	20.	0.82	58
C. psittaci	5.7	0.03	85

Table 4. Statistical data from the maximum-likelihood analysis of 30 DAH 7-P synthases

Tree no.	−ln likelihood (JTT + F + γ)	SE	Bootstrap proportion	Gamma shape factor (α)
73	−16,405.84	—	0.525	0.27197

fying trait. That these in vitro data correlate well with the in silico phylogenetic data strongly supports the evolutionary division of Class I and Class II KDO 8-P synthases suggested in this report.

Having identified two classes of KDO 8-P synthase, we also propose a class of DAH 7-P synthase whose activity is not dependent on a metal cofactor. Figure 3 illustrates the phylogenetic tree generated for 28 DAH 7-P synthase sequences. Again, the bifurcation of the tree into two distinct groups is readily evident. It is tempting to assign the *E. coli* DAH 7-P synthase, which is known to require a divalent metal for activity, to Class II and to assume that the other group is Class I DAH 7-P synthases. Support for this hypothesis can be found in the literature. Specifically, the DAH 7-P synthase from *Bacillus subtilis* Marburg 168 has been initially character-

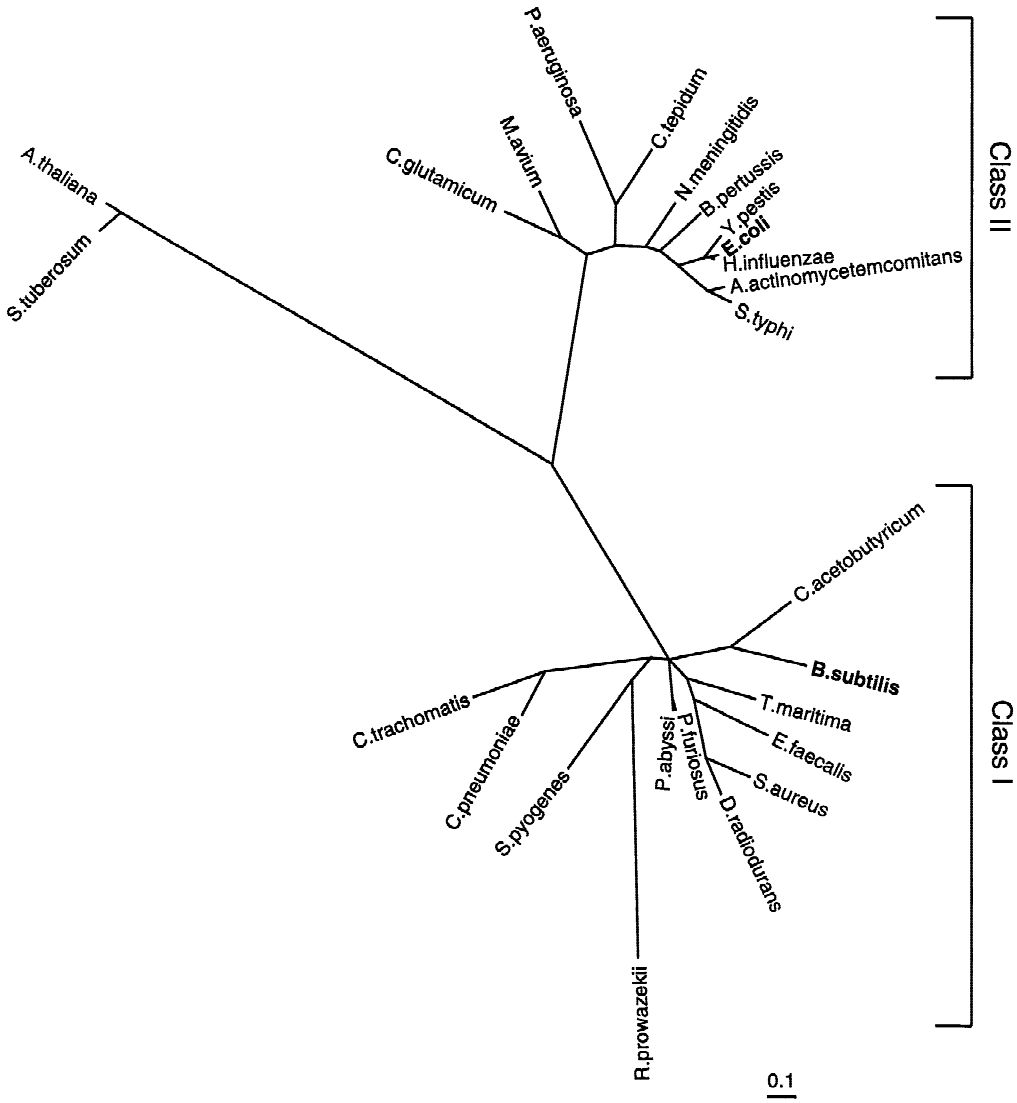

Fig. 3. Phylogenetic tree generated by maximum-likelihood analysis from 25 DAH 7-P synthase sequences. The group labeled Class I (including the *B. subtilis* enzyme) is postulated not to require a metal, while Class II enzymes (including the *E. coli* enzyme) are predicted to have a metal requirement. Enzymes for which in vitro metal binding data are known are shown in *boldface* type.

ized as a nonmetallo-DAH 7-P synthase. (Llewellyn et al. 1980) There exist several confounding factors, however, that make the assignment of the DAH 7-P synthase classes as metallo and nonmetallo less conclusive than in the case of KDO 8-P synthase. First, the *B. subtilis* protein, unlike that from *E. coli*, exhibits both DAH 7-P synthase and chorismate mutase activities. Second, while the feedback inhibitor of the *E. coli* DAH 7-P synthases is the ultimate pathway product (Phe, Tyr, or Trp), the *B. subtilis* enzyme is sensitive to an intermediate (prephenate) in the Shikimate pathway (Llewellyn et al. 1980). Therefore, it is possible that the difference between Class I and Class II DAH 7-P synthases is not metal requirement but, rather, allosteric effector.

The grouping of highly diverse species in the DAH 7-P synthase phylogeny is perhaps surprising. While these results appear to be statistically valid, it is unexpected that Archael species would be monophyletic with both G− and G+ Bacteria and Eucarya. Also, it appears that there is a stronger than expected relationship between G+ eubacteria and proteobacteria on the Class I portion of the tree; Class II contains only G− eubacteria. More expected, though, is the large distance between the plant and the bacterial DAH 7-P synthases. This was, of course, not the case in the KDO 8-P synthase phylogeny. Although these relationships may point to interesting phenomena such as lateral gene transfer, that these groupings are related to a different common trait among the Class I enzymes, other than metal independence, seems just as likely. Work currently progressing in our laboratory is aimed toward clarifying the relationship among the DAH 7-P synthases.

While it has been suggested that KDO 8-P synthase and DAH 7-P synthase are mechanistically related, the existence of Class II KDO 8-P synthases and Class I DAH 7-P synthases suggests a much closer evolutionary

link between the two enzymes than has been previously thought.[2] Unfortunately, the extremely low sequence similarity between the 29 KDO 8-P synthases and the 25 DAH 7-P synthases (<5% identity) limits the value of statistical techniques in establishing the relationship between them. Therefore, we have chosen a necessarily qualitative assessment of their connection. Although the lack of primary sequence similarity between the KDO 8-P synthases and the DAH 7-P synthases may at first glance seem to indicate the convergent evolution of these two important enzymes, other observations potentially indicate divergent evolution. In an attempt to relate enzyme families, a set of six criteria for judging the likelihood of divergent rather than convergent evolution has been put forth (Matthews et al. 1981). These criteria include the similarity of DNA sequence, protein sequence, three-dimensional structure, enzyme–substrate interaction, and catalytic mechanism and that the segments essential for catalysis occur in the same sequence (i.e., not transposed). This is, of course, not as rigorous an assessment of the relationship between two enzyme families as statistical techniques would be. KDO 8-P synthase and DAH 7-P synthase appear to fall within the bounds of divergence as described by these criteria.

First, while neither the DNA nor the protein sequences of *E. coli* KDO 8-P and DAH 7-P synthase necessarily suggest a strong relationship, even a cursory examination of the X-ray crystal structures reveals startling similarities. Both *E. coli* KDO 8-P synthase and DAH 7-P synthase fold into a $(\beta/\alpha)_8$ tertiary structure (TIM barrel); their structures are superimposable with a C_α RMS deviation of 1.83 Å (Radaev et al. 2000). Crystallographic data on *A. aeolicus* KDO 8-P synthase shows that it folds into a TIM barrel whose backbone is virtually indistinguishable from that of the *E. coli* enzyme. The high level of tertiary structure similarity between KDO 8-P synthase and DAH 7-P synthase suggests divergence from a common ancestor.

Second, many of the active-site residues are the same in KDO 8-P synthase and DAH 7-P synthase. The residues responsible for metal binding in *E. coli* DAH 7-P synthase (Phe sensitive) are Cys, His, Glu, and Asp (Shumilin et al. 1999). Similarly, a Cys, a His, a Glu, and an Asp in *A. aeolicus* KDO 8-P synthase appear to be involved in metal binding. These residues correspond to *E. coli* KDO 8-P synthase N26, H202, E239, and D250 (all numbering refers to the *E. coli* KDO 8-P synthase); the latter three amino acids are highly conserved in all KDO 8-P synthases. Residue 26, which aligns with the abovementioned Cys in *A. aeolicus* KDO 8-P synthase and *E. coli* DAH 7-P synthase, is Asn in *E. coli* KDO 8-P synthase and all proposed Class I KDO 8-P synthases. As in *A. aeolicus* KDO 8-P synthase, this position is occupied by a Cys in all Class II KDO 8-P synthases and DAH 7-P synthases. This again speaks to the similarity of structure, especially of active-site structure, between the KDO 8-P synthases and the DAH 7-P synthases as well as to the point that essential residues occur in the same order across all classes of both enzymes.

The binding sites for both PEP and A5P or E4P (Figs. 4a and b, respectively) are also similar in *E. coli* KDO 8-P synthase and DAH 7-P synthase. In the case of PEP, the phosphate moiety is bound in *E. coli* KDO 8-P synthase by A116, K138, and R168 (Radaev et al. 2000) and by homologous residues in DAH 7-P synthase (Shumilin et al. 1999). The carboxylate moiety is bound by K55/K60/K138 in *E. coli* KDO 8-P synthase and Arg, Lys, and Lys in DAH 7-P synthase. Of the six residues involved in binding PEP in both enzymes, five are identical, with the sixth representing a conservative substitution (K → R). In addition, all six residues occur in the same order and with very similar spacing [A–x(22)–K–x(30,48)–K (standard PROSITE nomenclature) (see Bucher and Bairoch 1994) for the phosphate binding site and [KR]–x(5)–K–x(78,89)–K for the carboxylate binding site]. In binding the monosaccharide phosphate, an active-site loop is involved in both KDO 8-P synthase and DAH 7-P synthase. This small loop is responsible for positioning the monosaccharide phosphate, and hence the entire monosaccharide, so as to allow for the proper distance between PEP and the monosaccharide carbonyl for efficient reaction. In the *E. coli* enzymes, the sequence ^{60}KANRS is predicted to bind to the terminal phosphate of the five-carbon monosaccharide in KDO 8-P synthase, while the sequence ^{97}KP–RT is responsible for the same function with the four-carbon analogue in DAH 7-P synthase. The substitution of Pro in DAH 7-P synthase for Ala and Asn in KDO 8-P synthase allows this slightly shortened loop to turn more sharply in DAH 7-P synthase than in KDO 8-P synthase, bringing the remaining sequence approximately 1.8 Å closer to the center of the active site and PEP. This shift in the DAH 7-P synthase active site then allows for the correct positioning of the slightly shorter E4P. The only other difference (Ser to Thr) is a very conservative substitution. Similarity of the binding sites for both substrates is indicative both of similarity of enzyme–substrate interaction and of nontransposed active site residues.

Finally, as mentioned in the Introduction, the proposed mechanisms of KDO 8-P synthase and DAH 7-P synthase are largely the same. In both cases, an activated water molecule is believed to attack at C2 of PEP followed by (or concurrent with) an attack of C3 of PEP on

[2] Jensen and colleagues noted the existence of two classes of DAH 7-P synthase (Subramaniam et al. 1998) but failed to place KDO 8-P synthase into the proper context with respect to DAH 7-P synthase. This resulted in grouping all KDO 8-P synthases with Class I DAH 7-P synthase. The division was attributed to a difference between "narrow-substrate specificity" and "broad-substrate specificity" KDO 8-P synthases; this division has been shown to be erroneous (Sheflyan et al. 2000).

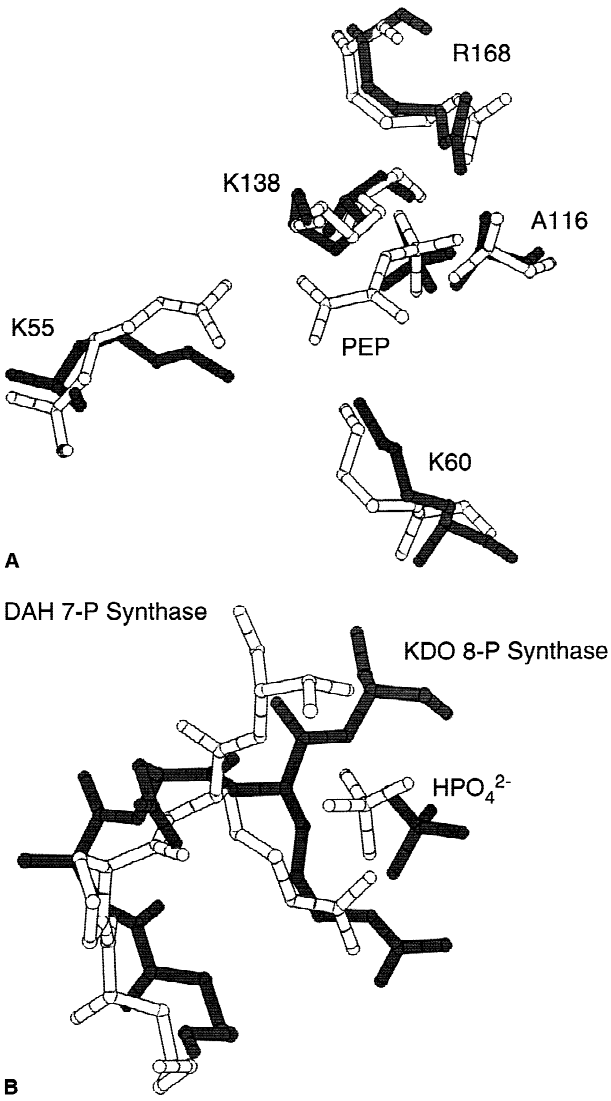

Fig. 4. **A** Superposition of the PEP binding sites of *E. coli* KDO 8-P synthase (*shaded* residues) and DAH 7-P synthase (*white* residues). PEP is present in the DAH 7-P synthase structure, while only a sulfate is present in the KDO 8-P synthase structure; both are labeled PEP. Residue numbering is that of *E. coli* KDO 8-P synthase. **B** Superposition of the monosaccharide phosphate binding sites of *E. coli* KDO 8-P synthase (*shaded* residues) and DAH 7-P synthase (*white* residues). The two phosphate groups shown represent the terminal phosphate of A5P or E4P, respectively, and are approximately 1.8 Å apart.

C1 of A5P or E4P. In both KDO 8-P synthase and DAH 7-P synthase, the phosphate is cleaved at the C–O bond, rather than the more typical P–O bond (DeLeo and Sprinson 1968; Dotson et al. 1995; Hedstrom and Abeles 1988), and the reaction stereochemistry (Dotson et al. 1993; Kohen et al. 1993; Onderka and Floss 1969b) is identical in both enzyme systems.

In the case of these two enzyme families, four of the six criteria for divergent evolution are met: they have similar three-dimensional structures, similar enzyme–substrate interactions (both substrates and metal), proposed active-site residues occurring in the same order (metal and substrate binding sites), and similar (proposed) catalytic mechanisms. Taken together, the above data provide a high level of evidence that these two enzymes share some distant common ancestor.[3]

It is also apparent from the maximum-likelihood analysis that the Class I KDO 8-P synthases are more closely related to one another than the Class II enzymes. The Class I KDO 8-P synthases would then represent a relatively recent offshoot of an ancestral Class II KDO 8-P synthase and, having had less time to evolve, do not show the same degree of divergence. This is well demonstrated in Fig. 2, wherein the Class II KDO 8-P synthases have consistently longer branch lengths than their Class I counterparts.

Given knowledge of the phylogenetic relationship between Class I and Class II KDO 8-P synthases, features of the primary sequence alignment (Fig. 5) become easier to interpret. Perhaps the most interesting regions of the alignment are those that both demonstrate a high level of conservation unique to each class of KDO 8-P synthase and show marked differences between the two classes (intraclass versus interclass similarity). For example, C38 (*E. coli* KDO 8-P synthase numbering) is known to be essential for activity of the *E. coli* homologue (Salleh et al. 1996). In all other Class I KDO 8-P synthases, a Cys occupies the analogous position. In the Class II homologues, however, an Ala (or Gly in one case) aligns with this position. Similarly, the metal binding site includes one Cys residue, which corresponds to C11 (*E. coli* residue 26) in the *A. aeolicus* sequence. Again, this residue is completely conserved in all Class II KDO 8-P synthases but is uniformly absent in their Class I brethren; an Asn exists in its place. Due to the small number of these sites, it seems reasonable that most, if not all, are involved either with the metal's catalytic role in the Class II KDO 8-P synthases or with compensating for the lack of metal in the Class I KDO 8-P synthases. It is worth noting that while there are several residues or pairs of residues that meet this criterion, no multiple residue string falls into this category. Thus little or no structural alteration was needed to affect the change from Class II to Class I; only specific amino acid functional groups were transformed. Consequently, overall interclass similarities remain significant, while those residues most likely involved in the evolution from the ancestral Class II to the modern Class I KDO 8-P synthases are highlighted.

The existence of two classes of both KDO 8-P synthase and DAH 7-P synthase has not been considered previously. The difference in metal requirement and lack of obvious sequence similarity between *E. coli* KDO 8-P synthase and DAH 7-P synthase were often thought to outweigh the mechanistic similarities of the reactions

[3] Indeed, it has even been proposed that all $(\beta/\alpha)_8$ enzymes may have diverged from a very distant common ancestor (Farber and Petsko, 1990).

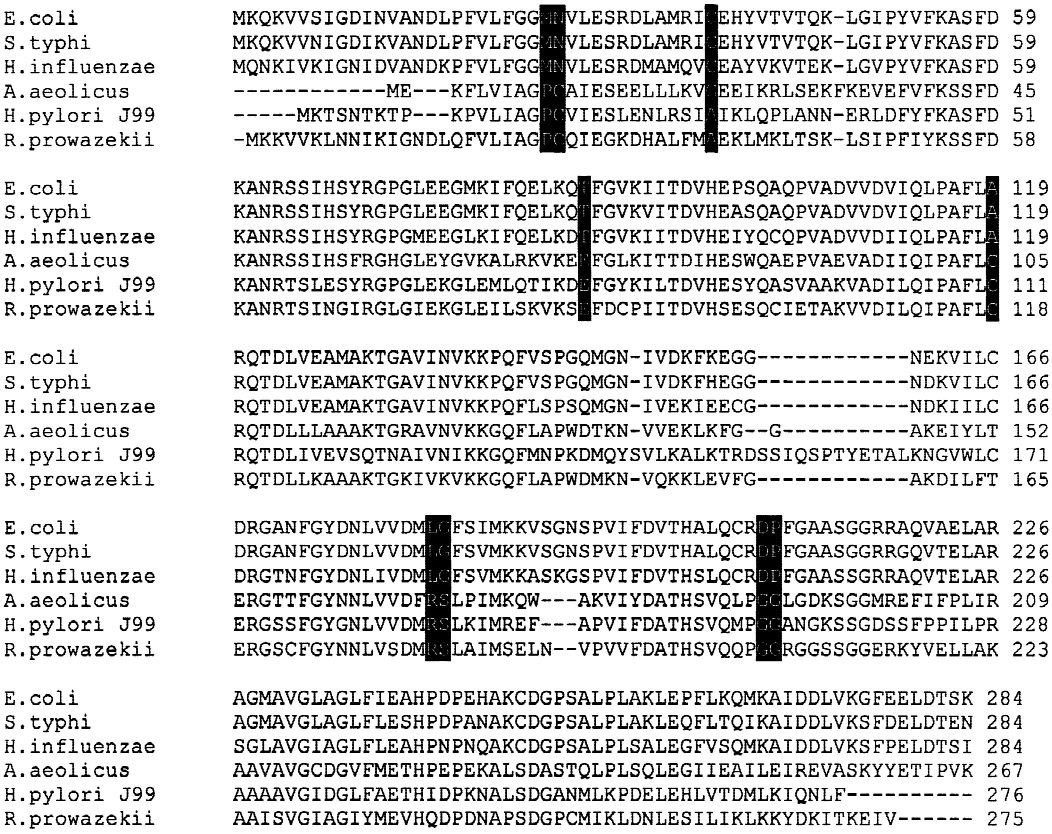

Fig. 5. A representative alignment of three Class I and three Class II KDO 8-P synthases. Residues that are identical intraclass and show a marked difference interclass are indicated by *shading.*

catalyzed by these two enzymes. Now, with the characterization of the KDO 8-P synthase from *A. aeolicus, H. pylori* J99, and *C. psittaci* and the structural information available for both *E. coli* enzymes and the KDO 8-P synthase from *A. aeolicus*, the existence of Class II KDO 8-P synthases and Class I DAH 7-P synthases seems not only plausible but likely. Furthermore, these data support a scenario wherein KDO 8-P synthase and DAH 7-P synthase have more in common than once thought; the data suggest that KDO 8-P synthase and DAH 7-P synthase may actually share a common ancestor. To provide experimental evidence of these predictions, we have cloned the KDO 8-P synthase gene from several diverse species of bacteria and are in the process of purifying and characterizing the recombinant proteins for metal requirement. We are in the preliminary stages of crystallizing the *B. subtilis* protein as well as overexpressing several DAH 7-P synthases to further the comparison of Class I DAH 7-P synthases with their Class II counterparts. By establishing both a second class of KDO 8-P synthase and a divergent relationship between KDO 8-P and DAH 7-P synthases, we can begin to shed light on the relationship between these unique enzymes and further the knowledge of both groups.

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