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A Method Using Active-Site Sequence Conservation to Find Functional Shifts in Protein Families: Application to the Enzymes of Central Metabolism, Leading to the Identification of an Anomalous Isocitrate Dehydrogenase in Pathogens

Rajdeep Das and Mark Gerstein*
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ABSTRACT **We have introduced a method to identify functional shifts in protein families. Our method is based on the calculation of an active-site conservation ratio, which we call the "ASC ratio." For a structurally based alignment of a protein family, this ratio is the average sequence similarity of the active-site region compared to the full-length protein. The active-site region is defined as all the residues within a certain radius of the known functionally important groups. Using our method, we have analyzed enzymes of central metabolism from a large number of genomes (35). We found that for most of the enzymes, the active-site region is more AQ: 2 highly conserved than the full-length sequence. However, for three tricarboxylic acid (TCA)-cycle enzymes, active-site sequences are considerably more AQ: 3 diverged (than full-length ones). In particular, we were able to identify in six pathogens a novel isocitrate dehydrogenase that has very low sequence similarity around the active site. Detailed sequence–structure analysis indicates that while the active-site structure of isocitrate dehydrogenase is most likely similar between pathogens and nonpathogens, the unusual sequence divergence could result from an extra domain added at the N-terminus. This domain has a leucine-rich motif similar one in the *Yersinia pestis* cytotoxin and may therefore confer additional pathogenic functions. Proteins 2003;00:000–000.**
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Key words: metabolic enzyme; genome; active site; sequence variation

INTRODUCTION

With the completion of a large number of genome sequences, a major problem for biology is functional annotation on a genome scale—determining a function for all the proteins encoded by a genome.[1,2] Unfortunately, it is very hard to do large-scale functional annotation purely on the basis of sequence.[3] Sequences diverge beyond the point of obvious recognition in terms of functional and structural homologues.[4,5] Furthermore, divergence on the sequence over its entire length has to do with many factors, and it is hard to abstract the specific bit of divergence that relates to functional conservation. This is where structure can play a major role in guiding people to the functionally important residues associated with the active site. Also with the advent of structural genomics, we are now confronted with the production of a large number of crystal and NMR structures. It is very useful to use these structures in a systematic fashion to refine our understanding of protein function. This is the overall aim of this work. We tried to develop a method for assessing active-site sequence conservation in comparison to full-length conservation, and we applied this to a number of pathway enzymes and achieved novel results. AQ: 4

Pathway enzymes have been a topic of wide scientific interest in both the pre- and postgenomic eras. Studies have focused on several aspects of pathway analyses. Particularly in the postgenomic era, metabolic pathways have been studied using sequence information. Such genomic analyses of pathways have been performed in many ways, and various metabolic databases have been constructed.[6–11] In contrast to overall sequence conservation, sequence variability of an enzyme near the functional site may reflect a functional shift. This functional shift can occur in many ways, such as a change in the binding affinity of the substrate or intermediate.[12] Previous studies analyzed protein families in terms of sequence–structure relationships.[13] In this article, we have analyzed a number of enzymes of the central metabolic pathways [i.e., glycolysis, pentose phosphate pathway, and tricarboxylic acid (TCA) cycle] in 35 organisms in terms of sequence variability around active sites. AQ: 5

MATERIALS AND METHODS

Organisms and Databases Used

In this article, we have analyzed 18 enzymes of the central metabolic pathways, in 35 organisms, in terms of sequence variability around active sites. A ribosomal tree, shown in Figure 1(A), lists all the organisms studied in the F1

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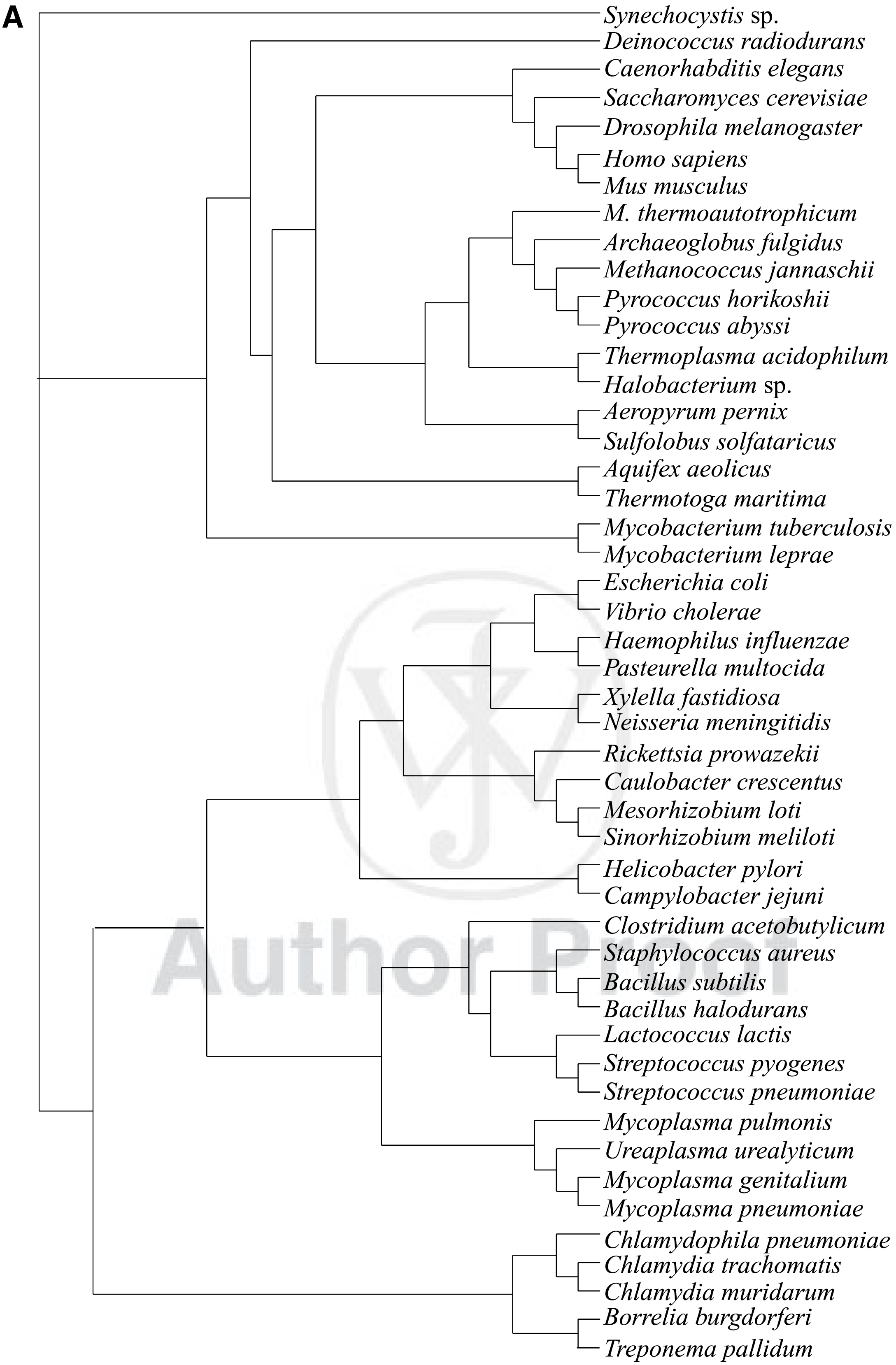


Fig. 1. (**A**) Phylogenetic tree based on rRNA. Phylogenetic tree of 35 organisms studied, based on rRNA sequences. Organisms represent all three kingdoms of life (i.e., archaea, bacteria, and eukarya). (**B**) Average pairwise similarities are shown for the enzymes in central metabolic pathway. For heteromeric enzymes, the subunit that has an active site is considered for the pairwise similarity calculation. We did not calculate the values for the enzymes for which we did not have enough data or information regarding active sites, or if we encountered other problems, as shown by asterisk.

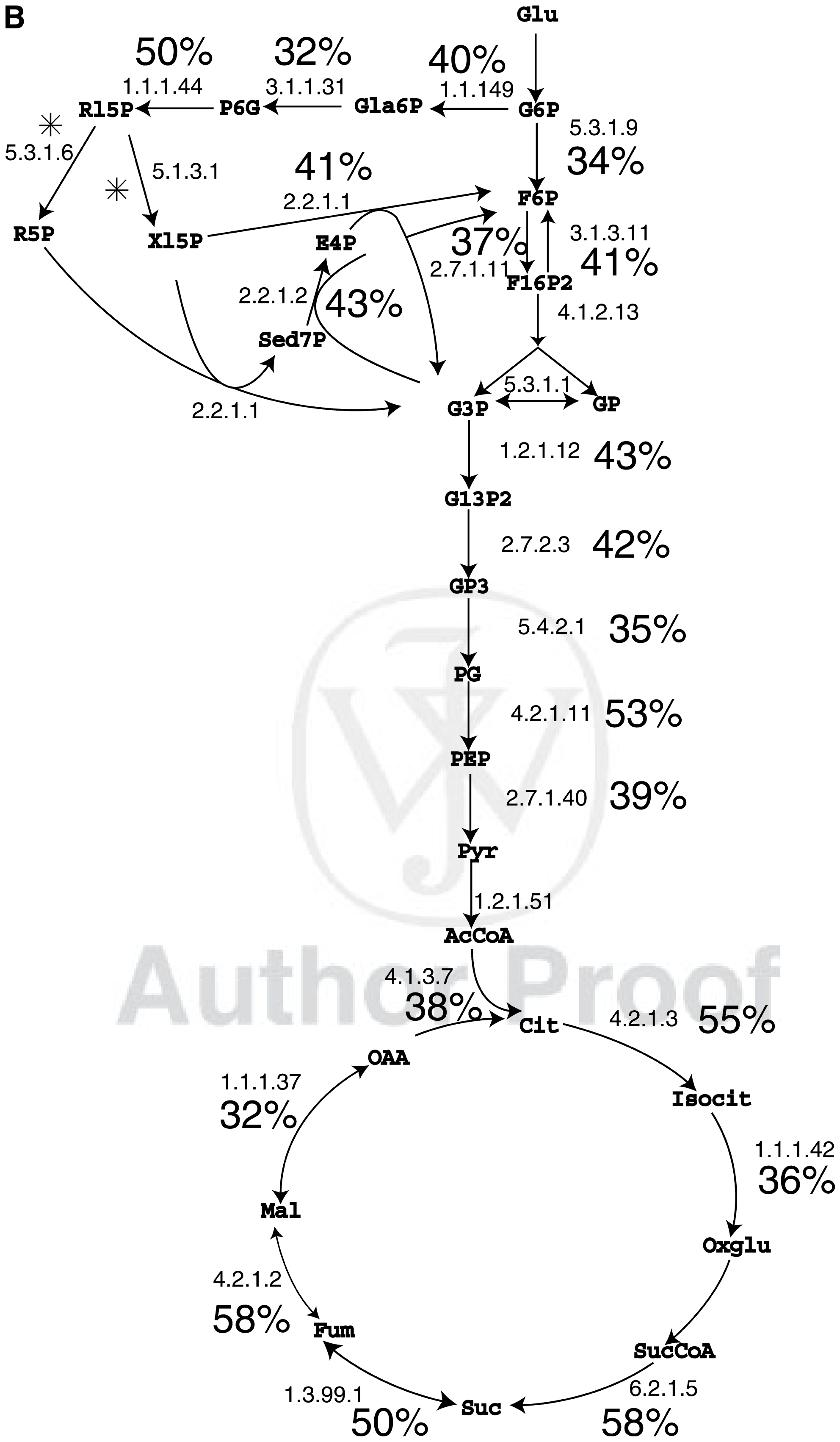


Figure 1. (Continued.)

TABLE I. Active Residues of the Enzymes

Enzyme	EC No.	PDB ID	Active residues	Reference
Malate dehydrogenase	1.1.1.37	1IB6	R81, R87, N119, H177	20
Isocitrate dehydrogenase	1.1.1.42	1HQS	R110, R120, R144, N106, C118, S104, T30, Y151	22
Fumarate reductase	1.3.99.1	1QLA	R301a, R404a, H369a, F141a	17
Fructose-1,6-biphosphatase	3.1.3.11	1FRP	R243a, K274a	23

analyses: All of the three major kingdoms of life (i.e., archaea, eubacteria, and eukarya) are represented here.

We collected all the sequences of central metabolic enzymes from the KEGG database for 35 organisms.[11] We only selected those sequences that were clearly annotated as functionally active sequence. In some cases, enzymes have multiple sequences and we selected only those isoforms that can be found in at least 10 other organisms for AQ: 6 comparison. We began by selecting one representative structure from the Protein Data Bank (PDB) for each of the 14 enzymes.[14] A list of the chosen PDB structures is T1 shown in Table I for four enzymes that we discuss in this article.

Structural Identification of Active Site

We developed a new method to calculate the active-site AQ: 7 conservation sequence, which we call ASC ratio. Below, we describe the calculation of our ASC ratio, which is essentially the ratio of the active-site sequence similarity to the overall full-length protein, and then we describe how it is employed.

The first step in this procedure is the structural identification of the active-site neighborhood. We got the central position of this from the literature, using the position of biochemically identified functionally important residues.

Table I shows the specific active-site residues that were considered for the enzymes in our study. Next, we define an active-site environment as all the residues that fall within a radius of 10 Å from the active-site residues. An average of about 90 residues falls within this radius. We have used the multipurpose program MOLEMAN to determine the residues in the active-site sphere.[15] Given a certain distance, the program can determine all residues that fall within that radius from the selected residue.

Calculation of ASC Ratio

For each enzyme in question, we gathered all homologs from the KEGG database (see above) and constructed a multiple alignment. We used the program CLUSTAL to AQ: 8 generate multiple alignments of the sequences.[16] Once we determined the residues in the active-site sphere in each representative structure, we mapped those residues onto the sequences from other organisms, using multiple sequence alignment. Finally, we calculated the pairwise sequence similarity between all members of the enzyme for these active-site residues. This part of our analysis is somewhat similar to three-dimensional (3D) cluster analyses used earlier to study a group of protein families.[17] The F2 overall strategy is illustrated in Figure 2. In addition to the active sites, we also computed pairwise full-length sequence similarity from our alignments. The basis of our study is the general understanding that residues that form the active-site environment are under selective pressure, since they are critical to the enzymatic function. Therefore, such residues are likely to be more conserved than the residues in the balance of the sequence. In order to identify sequences for which the active site is modified, we calculated a ratio of active-site similarity to full-length similarity. We call this the ASC ratio, denoted by the symbol R. Since the active-site residues are more likely to be conserved than the residues in the remaining sequences, the ratio of the two quantities, our ASC ratio, is expected to be more than 1. However, if the residues in the active-site sphere are modified, the pairwise active-site similarity becomes lower than the pairwise full-length similarity, and the ASC ratio will be less than 1. We have computed all the ratios from the pairwise similarity values and plotted their distribution. When an organism's enzyme sequences are all extensively modified, the ASC ratio values will tend to cluster at the point where the ASC ratio is less than 1, allowing for easy identification of those organisms.

Detailed Characterization of Functional Shift: Structural Comparison and Identification of New Sequence Motifs

Based on our initial analysis, we determine the enzymes and associated organisms for which we observed modified active sites. In this step, we tried to analyze these enzymes in terms of structure- and sequence-based characterization. For sequence motif detection, we searched Genbank database/PDB sequence database against a query sequence using standard BLAST. We have modeled the structure using the program MODELER.[18] We used the built-in alignment generation option of the program, MALIGN, to generate the alignment for modeling. Finally, we used the program STRUCTAL to compare the homology models.[19]

RESULTS AND DISCUSSION

Enzymes in the central metabolism vary greatly in average pairwise sequence identity. Some of the enzymes in the pathway are highly conserved, with an average sequence identity $\sim$60%, and some are less conserved. This is shown in Figure 1(B). For most of the central metabolic enzymes, we have found that the sequences around an active site are more conserved than the rest of the sequences. However, there are three TCA-cycle enzymes and one glycolytic enzyme for which we have we found large sequence variation near the active site (i.e., a large ASC ratio); these we discuss below.

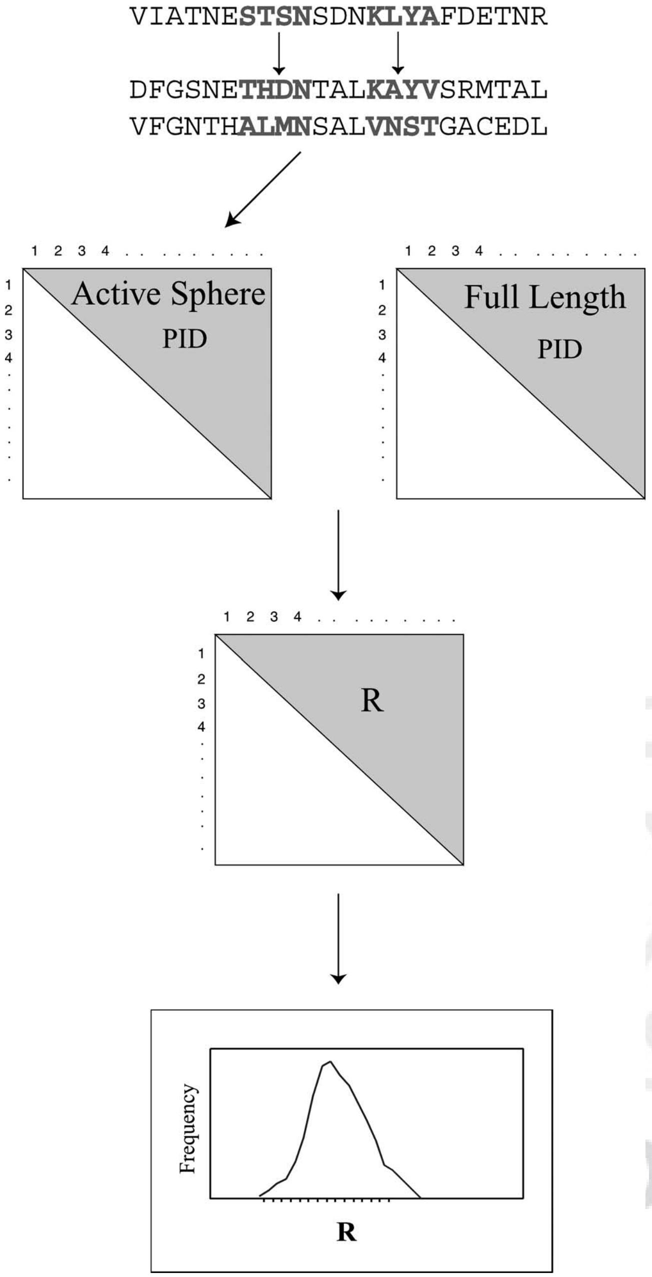


Fig. 2. Strategy of structure-based sequence comparison. We determined the residues that fell within the sphere of a 10-Å radius around the active residues using MOLEMAN.[10] Then we mapped those residues onto sequences from other organisms, using multiple sequence alignment. The red-colored residues in the sequence are the residues that fall in the active-site sphere of the known structure; these are mapped onto the sequences of other organisms. Corresponding residues in other sequences are also shown in red. We computed pairwise sequence similarity matrices for the active-sphere residues and for the overall full-length sequence proteins shown.

Example 1: Isocitrate Dehydrogenase

The most interesting result is observed for isocitrate dehydrogenase. This is a TCA-cycle enzyme, which catalyzes the following reaction:

$$\text{Isocitrate} + \text{NADP+} \rightarrow \text{2-Oxoglutarate} + CO_2 + \text{NADPH}$$

Distribution of ASC ratios shows that 6 organisms have AQ: 9 modified isocitrate dehydrogenase; therefore, the distribution of ASC ratios falls in the region where the value is less than 1. Figure 3(A) shows the distribution of the ASC F3 ratios and the corresponding list of 6 organisms in the box. They are all known pathogens. We have compared 33 sequences of isocitrate dehydrogenase from different organisms. The probability of pathogenic sequences, as a sample of the entire distribution of ASC ratios, having a mean ASC ratio of 0.52 was tested using normal statistics. The result shows that the probability p is very significant (i.e., less than 0.00001).

Homology modeling–based structural comparison of the active site

We compared the structure of the active site by comparing model structures of 4 pathogenic isocitrate dehydrogenases with the representative structure. We have modeled the structure of the 30-residue cluster using homology modeling, as described in the Methods section. Table II T2 shows the root-mean-square deviation (RMSD) difference between the modeled isocitrate dehydrogenase and the representative structure. The pairwise RMSD values are quite low and indicate that the active site of the pathogenic isocitrate dehydrogenase is most likely similar to the known structure. It should be noted here that we are comparing modeled structures derived from low homology and are thus likely to observe a very large deviation. Therefore, a small RMSD would mean a similarity in structures.

Sequence motifs

Finally, we analyzed the sequence of the modified isocitrate dehydrogenase in terms of sequence motifs. It is interesting to note that all the pathogenic sequences are ~300 residues longer than the class sequence length. It is possible that there may be a domain addition to all the 6 pathogenic sequences. From the multiple sequence alignment, it is observed that the extra stretch of sequence occurs on the N-terminal side of the enzyme. When we searched for similar sequences in the sequence database, we observed a 90-residue-long stretch of sequence in this extra domain, with high sequence similarity to a leucine-rich domain of an effector, YopM, of *Yersinia pestis* (a bubonic plague pathogen). The stretch of sequences is extremely rich in leucine. Although the antihost function of this effector is unknown, YopM is believed to be an important cytotoxin for bacterial virulence.[20] Figure 4 F4 shows the sequence alignment of the sequence YopM with the N-terminal domain of 4 pathogenic isocitrate dehydrogenases. Other evidence that the modified isocitrate dehydrogenase may have additional function comes from the observation that *Mycobacterium tuberculosis* has two isocitrate dehydrogenase sequences, Rv3339c and Rv0066c, the first of which is a standard isocitrate dehydrogenase sequence, and second of which is the modified sequence. AQ: 10 Therefore, the presence of the two sequences may indicate an extra function of modified sequence.

It should be noted here that our phylogenetic clustering of the organisms based on protein sequences also groups

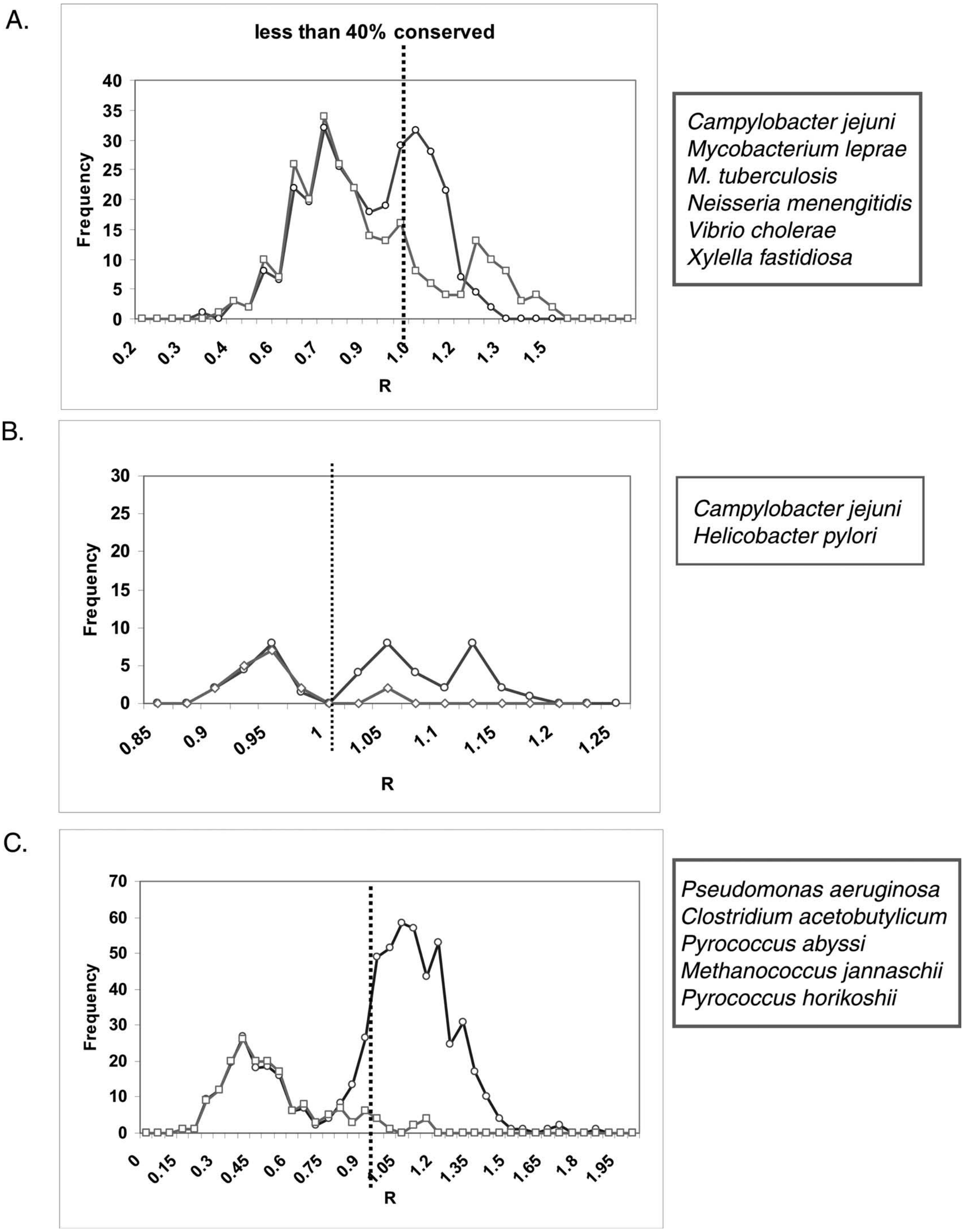
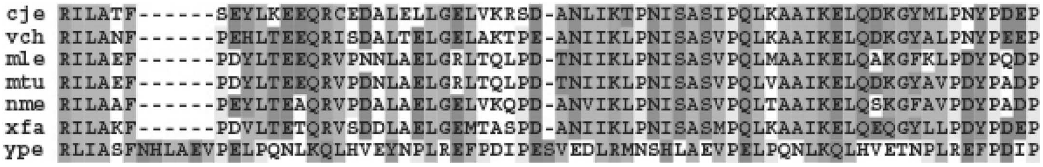


Fig. 3. Distribution of *R*-values for three enzymes in the TCA cycle. The *x* axis shows *R*-values and the *y* axis shows frequency. Overall distribution is shown in the blue line, and the red line represents the distribution that corresponds to the 6 organisms with modified sequences. The dashed bar represents the line where *R* is 1; the active-site similarity is equal to the overall sequence similarity. **A**, **B**, and **C**, respectively, represent the distributions corresponding to isocitrate dehydrogenase, fumerate reductase, and malate dehydrogenase.



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cje RILATF------SEYLKEEQRCEDALELLGELVKRSD-ANLIKTPNISASIPQLKAAIKELQDKGYMLPNYPDEP
vch RILANF------PEHLTEEQRISDALTELGELAKTPE-ANIIKLPNISASVPQLKAAIKELQDKGYALPNYPEEP
mle RILAEF------PDYLTEEQRVPNNLAELGRLTQLPD-TNIIKLPNISASVPQLMAAIKELQAKGFKLPDYPQDP
mtu RILAEF------PDYLTEEQRVPDNLAELGRLTQLPD-TNIIKLPNISASVPQLVAAIKELQDKGYAVPDYPADP
nme RILAAF------PEYLTEAQRVPDALAELGELVKQPD-ANVIKLPNISASVPQLTAAIKELQSKGFAVPDYPADP
xfa RILAKF------PDVLTETQRVSDDLAELGEMTASPD-ANIIKLPNISASMPQLKAAIKELQEQGYLLPDYPDEP
ype RLIASFNHLAEVPELPQNLKQLHVEYNPLREFPDIPESVEDLRMNSHLAEVPELPQNLKQLHVETNPLREFPDIP
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Fig. 4. Sequence alignment of novel isocitrate dehydrogenase with the cytotoxin from *Yersinia pestis*. Organisms are abbreviated as follows: Cje, *Campylobacter jejuni*; Hpy, *Helicobacter pylori*; Vch, *Vibrio cholerae*; Mle, *Mycobacterium leprae*; Mtu, *M. tuberculosis*; Xfa, *Xylella fastidiosa*.

TABLE II. Comparison of Representative Structure and Modeled Structure for Isocitrate Dehydrogenase Based on C-α Atoms

Organisms	RMSD
Campylobacter jejuni	0.76
Mycobacterium leprae	0.23
Mycobacterium tuberculosis	0.54
Neisseria meningitidis	1.25

the pathogenic organisms in one cluster, as shown in F5 Figure 5. Interestingly the structure of similar isocitrate dehydrogense has been solved recently showing similarity in the active site.[21]

Example 2: Malate Dehydrogenase

$$\text{Malate} + \text{NAD+} \rightarrow \text{Oxaloacetate} + \text{NADH}$$

The distribution of ASC ratios for malate dehydrogenase is shown in Figure 3(C). This figure shows that 5 organisms—*Pseudomonas aeruginosa*, *Clostridium acetobutylicum*, *Pyrococcus abyssi*, *Methanococcus jannaschii*, and *Pyrococcus horikoshii*—all have modified malate dehydrogenase sequences. However, previous studies show that *M. jannaschii* has two sequences for malate dehydrogenase, MJ0490 and MJ1425.[22] Although two sequences are annotated to be malate dehydrogenase, MJ1425 is linked to methylpterin biosynthesis. Therefore, it is likely that the enzyme in these organisms has a dual role: catalyzing conversion of malate and biosynthesis of the cellular component. In our analyses, we have used sequences from 35 organisms. The probability of a sample of sequences of this size having a mean ASC ratio of 0.45 (mean ASC ratio for the modified organisms) was tested using normal statistics and is less than 0.00001.

Example 3: Fumarate Reductase

Fumarate reductase, usually associated with organisms with anaerobic respiration, catalyzes the following reaction:

$$\text{Fumarate} + \text{FADH2} \rightarrow \text{Succinate} + \text{FAD}$$

This enzyme consists of three subunits: A, B, and C. Subunit A binds to fumarate, and B and C bind to the Fe-S cluster and membrane, respectively. Comparison of the active-site residue cluster shows that while the Fe-S cluster-binding B and flavin-binding C subunits are similar across organisms, the binding site of the fumarate in subunit A is different for *Helicobacter pylori* and *Campylobacter jejuni* compared with the rest of the organisms. These two mucosal pathogens are known to have fumarate respiration.[23,24] The distribution of ASC ratios is shown in Figure 3(B). In order to see if there is any structural difference in the active site of the two pathogenic fumarate reductases, we modeled the active-site structures for the two organisms. Table II shows that the pairwise RMSD from that of the known structure is low enough to argue that the active-site structure in the two pathogenic fumarate reductases is similar to the known structure.

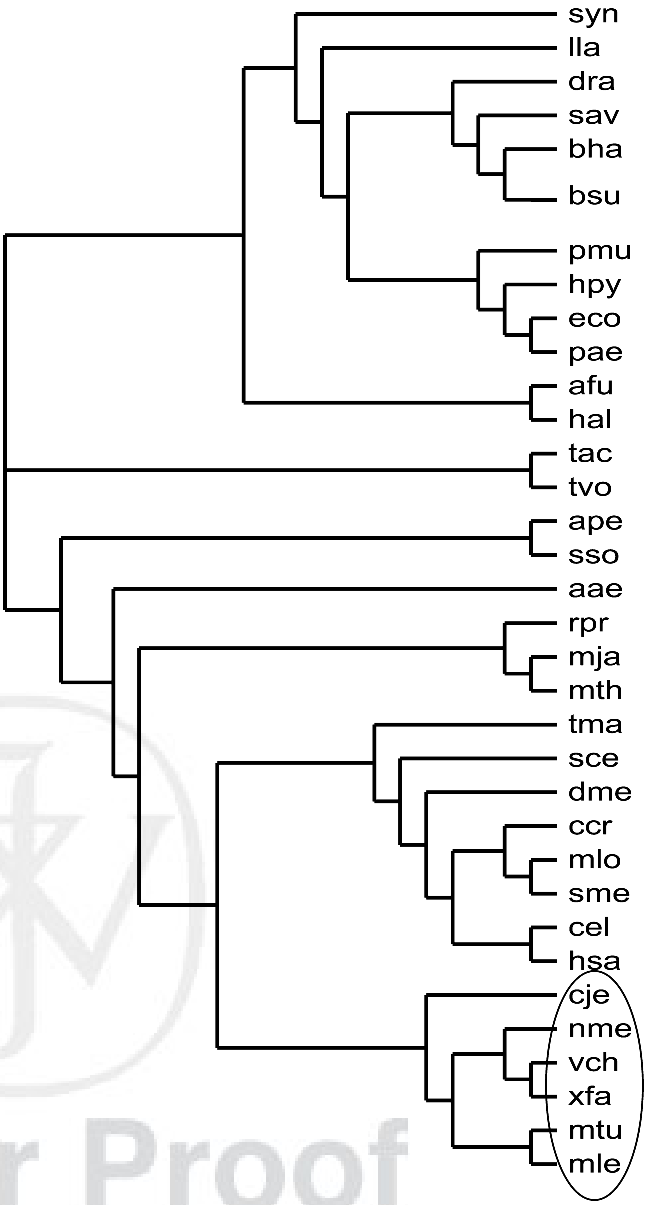

Fig. 5. Phylogenetic clustering based on isocitrate dehydrogenase sequences of the organisms. Although organisms cluster differently in the ribosomal tree, the 6 most diverged isocitrate dehydrogenase sequences cluster together.

For both enzymes, isocitrate dehydrogenase and fumarate reductase, we conclude that the active-site structures of the enzymes in the suspect organisms are most likely to be similar to the known structure. Thus, the sequence variability observed near the functional site probably has a role in altering the binding affinity of the substrate in the active site and is possibly one of the mechanisms for controlling of the enzymatic function in the organisms. The number of sequences in fumarate reductase was 10. The probability of a sample of sequences of this size having a mean ASC ratio of 0.94 (mean ratio for the modified organisms) was tested using normal statistics and is a marginal 0.065. This can be a result of small size of the data set.

In addition, we have also observed a few glycolytic enzymes for which there are a group of organisms with modified active sites. Fructose-1,6-biphosphatase is one such enzyme that catalyzes conversion of fructose-1,6-biphosphatase to fructose-6-phosphate. We observed that four organisms—*Lactobacillus lactis*, *Clostridium acetobutylicum*, *Bacillus subtilis*, and *Staphylococcus aureus*—have very diverged sequences around the active site. Similarly, archaeal glyceralde-3-phosphate dehydrogenases have very modified sequences in the active site. However, we were not able to characterize these enzymes further, either structure- or sequencewise.

Statistical Test to Verify the Possibility of Bad Alignment

It should be pointed out that the average pairwise sequence similarity is quite low for some of the enzymes we studied, as shown in Figure 1(B). In particular, the two enzymes (EC Nos.: 11142 and 11137) for which we observed significant sequence variation around the active site have low-average pairwise sequence similarity. It is possible that the observed sequence variation in the active site for these two enzymes can be a result of a bad local sequence alignment in that particular region. This will lead to low pairwise sequence similarity for the active-site sequence. We therefore performed a statistical test to verify this possibility. We calculated the average pairwise sequence similarity for a large number of random clusters, each comprising ~90 residues. To generate a random cluster, we selected a random residue in the representative sequence with known structure and then determined all the residues that are present within the sphere of a 10 Å radius. Using this procedure, we have generated large number of random clusters. If the alignments in the active-site sequences were particularly bad, active-site sequence similarity would be lower than the average sequence similarity values for these random clusters. For example, in *Vibrio cholerae,* an organism with modified isocitrate dehydrogenase, the average pairwise sequence identity is 21% for random clusters, and that of active-site cluster is 27%. Although the values are low, calculation shows that active-site identity of 27% has a *P*-value of 1.2×10^{-12}. Similarly for malate dehydrogenase, results show that the *P*-values are less than 0.01 for the organisms with modified active site. Therefore, we conclude that the residues in the active-site sphere most likely represent the active site in the enzyme and do not represent random residues, as would be expected in case of bad alignment. However, in the case of fumarate reductase, *H. pylori* and *C. jejuni* have average active-site similarity of 36% and less that average pairwise identity of random clusters (40%), and have a *P*-value of less than 2.87×10^{-7} to occur
AQ: 11 by chance. Clearly, the smaller number of organisms (i.e., 10) in our analyses biases the results in fumarate reductase. However, the biological significance of this result is not clear.

CONCLUSIONS

In this article, we have introduced a method to identify functional shifts in protein families based on the calculation of an active-site conservation (ASC) ratio. For a structurally based alignment of a protein family, this ratio is the average sequence similarity for the active-site region compared to the protein's full-length. We have analyzed AQ: 12
the sequence variation of enzymes around active sites in a large number of organisms. For most of the organisms, the results showed that sequences are highly conserved around the active site. However, there are 3 enzymes in the TCA cycle for which we have observed that sequences are extremely divergent. Homology modeling showed that diverged sequences might have an active-site structure similar to the known structure. Most interestingly, 6 pathogenic organisms have unique isocitrate dehydrogenase that has sequence similarity to a cytotoxin in *Y. pestis* that is linked to bacterial pathogenicity.

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AQ1: If running head is not OK, please provide one that is 45 or fewer characters.

AQ2: Sentence beginning "We found that. . ." as meant?

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AQ5: Sentence beginning "In this article. . ." as meant?

AQ6: Sentence beginning "In some cases. . ." as meant?

AQ7: In sentence beginning "We developed. . .", is wording change OK, so that the term matches the acronym?

AQ8: Journal style requires that references be cited in numerical order. Ref. 26 has been changed to ref. 16, and succeeding references have been renumbered.

AQ9: CO2 as meant?

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