Enhanced Functional Annotation of Protein Sequences via the Use of Structural Descriptors

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In order to circumvent limitations of sequence based methods in the process of making functional predictions for proteins, we have developed a methodology that uses a sequence-to-structure-to-function paradigm. First, an approximate three-dimensional structure is predicted. Then, a three-dimensional descriptor of the functional site, termed a Fuzzy Functional Form, or FFF, is used to screen the structure for the presence of the functional site of interest (Fetrow *et al.*, 1998; Fetrow and Skolnick, 1998). Previously, a disulfide oxidoreductase FFF was developed and applied to predicted structures obtained from a small structural database. Here, using a substantially larger structural database, we expand the analysis of the disulfide oxidoreductase FFF to the *B. subtilis* genome. To ascertain the performance of the FFF, its results are compared to those obtained using both the sequence alignment method BLAST and three local sequence motif databases: PRINTS, Prosite, and Blocks. The FFF method is then compared in detail to Blocks and it is shown that the FFF is more flexible and sensitive in finding a specific function in a set of unknown proteins. In addition, the estimated false positive rate of function prediction is significantly lower using the FFF structural motif, rather than the standard sequence motif methods. We also present a second FFF and describe a specific example of the results of its whole-genome application to *D. melanogaster* using a newer threading algorithm. Our results from all of these studies indicate that the addition of three-dimensional structural information adds significant value in the

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INTRODUCTION

Functional genomics is a field born of necessities. These necessities include both the ability to identify novel biological targets in an environment of highly competitive research and the ability to exploit rapidly evolving volumes of genetic and genomic data. As a matter of practice, the approaches to structural/functional genome analysis have developed as an amalgamation of existing technologies. Pattern recognition algorithms, artificial intelligence methods, computational chemistry and biology techniques, and even linguistic analytical methods have all been pressed into service as approaches to the task of interpreting protein function (Andrade and Valencia, 1998; Attwood *et al.*, 1998; Andrade *et al.*, 1999; Eisenhaber and Bork, 1999; Rindfleisch *et al.*, 1999; Pesole *et al.*, 2000; Thomas *et al.*, 2000; Tomic *et al.*, 2000).

The goal of many functional genomics approaches has been to determine as many protein functions as possible in order to identify novel pharmaceutical targets. In fact, many recent cases document the successful identification of sequence motifs common to a set of related proteins (Henikoff and Henikoff, 1994; Attwood *et al.*, 1998; Sonnhammer *et al.*, 1998; Bateman *et al.*, 1999; Hofmann *et al.*, 1999; Attwood, 2000). However, with the wealth of interest in such pursuits it has become apparent that approaches in which the predictions distinguish themselves in some fashion may be of significantly greater value

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than other competing methods. Distinctive approaches that make accurate predictions of function for greater numbers of proteins, provide accurate annotations of more distantly related proteins, accommodate the multi-functional nature of proteins, or are tailored to annotating protein classes that have garnered little attention are more likely to accomplish the desirable task of identifying a novel function for a previously unannotated protein sequence.

In the practice of making functional predictions for proteins, methods typically follow one of two avenues. The first approach is to identify a suitably homologous, previously annotated protein on the basis of sequence (primary structure) and/or overall structural similarities (secondary or tertiary structure) and transfer that protein's annotation to the query protein. The second means of functional prediction aims to develop or access a database of functional motifs, or templates, derived on the basis of knowledge of known protein sequences or structures and their function. Levels of resolution for both approaches may include comparisons of sequence similarity or sequence-based motifs, analysis of gross structural features (i.e., secondary structure or protein fold similarities), or a detailed three-dimensional feature homology assessment (i.e., specific residues involved in protein function). As might be expected, the level of complexity and expense (computational, temporal, and/or fiscal) generally increases as the level of resolution increases. For example, methods of functional annotation based on sequence-based motifs of known function include Blocks, PRINTS, Pfam, and Prosite (Henikoff and Henikoff, 1994; Attwood *et al.*, 1998; Sonnhammer *et al.*, 1998; Bateman *et al.*, 1999; Hofmann *et al.*, 1999; Attwood, 2000). These methods endeavor to capitalize on the conservation of sequence elements among functionally similar proteins. Generally, sequence-based annotation methods require relatively little in the way of computational time, development of the "rules" for analysis (termed "motifs") is generally facile (and sometimes even automated), and several of the tools are publicly available.

While methods of annotation on the basis of sequence similarity (such as BLAST (Altschul *et al.*, 1997; Altschul and Koonin, 1998; Zhang *et al.*, 1998)) or sequence motifs (such as Blocks, PRINTS, Pfam, and Prosite) have proven successful, they are limited by implicit assumptions underlying their methodology. For instance, some sequence similarity annotation methods rely upon existing database annotations for transfer of functional data. However, the prevalence of such methods and their application to whole genomes has served to inject significant levels of noise into database annotations

(Bork and Koonin, 1998; Karp, 1998). Because the annotation for an entire family might actually be transferred on the basis of sequence similarity, inaccurate annotations may be propagated rapidly.

Recently, development of elaborate automated methods of sequence annotation using overall three-dimensional structural comparison has enabled whole-genome functional analyses. For example, Sanchez and Sali (1998) demonstrated via comparative modeling of the *Saccharomyces cerevisiae* genome that knowing only the fold of a protein is often sufficient to predict its approximate biochemical function, even when sequence-based methods are not capable of an accurate annotation. Another suite of tools has demonstrated the capability of generating functional predictions for 8–17% of the protein sequences of complete genomes (Andrade *et al.*, 1999). However, such methods are also subject to the potential pitfalls of sequence similarity-driven methods of functional annotation. The crux of the sequence similarity issue is at what level and to what extent function may be deduced from structure; even at higher levels of resolution of structural considerations, ambiguities arise from oversimplification of the structure–function relationship. The fact that the number of known folds in protein structure databases is quite tenable from a computational analysis standpoint may seem to enhance the attractiveness of a gross structural feature homology approach. However, biological reality actually indicates just the opposite; the presumption that fold similarities alone are sufficient to identify functional similarity is discredited in numerous cases (Koppensteiner *et al.*, 2000). For example, paralogs (functionally divergent proteins) may share the same fold (such as α/β barrels (Brenner *et al.*, 1997)), and orthologs may exhibit dissimilar fold structures (eukaryotic and prokaryotic serine proteases have differing folds but all function as serine proteases (Fischer *et al.*, 1994)). Hence there is a need to consider a residue-level approach, while retaining three-dimensional information, as a strategy for maximizing the specificity of a structurally based identification of function.

To this end, the challenges of a manageable approach to devising specific three-dimensional templates of protein function have been addressed by several groups. Artymiuk *et al.* (1994) focused on retrieving structures matching patterns of residue side-chain orientations by use of a graph-theory representation of atoms in both the template and query protein. Wallace *et al.* (1997) and Fischer *et al.* (1994) implemented automated geometric hashing algorithms to derive functional logic from structures in the Brookhaven Protein Data Bank. In addition, Wallace *et al.* (1997) have created a database of enzyme active

site templates called PROCAT (see <http://www.biochem.ucl.ac.uk/bsm/PROCAT/PROCAT.html>). These three-dimensional functional motif databases serve the purpose of *in silico* screening of either existing databases of solved protein structures or novel experimental data regarding three-dimensional structural features. While these approaches have enjoyed success in creating templates for such purposes, their use is curtailed by the rigors and costs associated with the acquisition of high quality protein structures beyond those in publicly available databases. Given the current difficulty and expense of generating high quality structural data for proteins of unknown function, the use of computational methods that predict three-dimensional structural features of protein sequences remains a relatively rapid and increasingly effective means of circumventing such limitations. However, because they rely on detailed knowledge of atom positions, these methods are not directly applicable to the approximate structures generated by today's state-of-the-art structure prediction tools.

The advances in technologies geared toward calculation of protein structures has been well documented via the CASP (Critical Assessment of Techniques for Protein Structure Prediction) series, in which methods are assessed on the basis of their structure predictions for a common set of proteins (Murzin, 1999). Methods of protein three-dimensional structure prediction generally fall into the categories of homology modeling (Skolnick *et al.*, 1997; Karplus *et al.*, 1998; Tramontano, 1998; Fischer, 1999; Kolinski *et al.*, 1999), fold recognition (Rost, 1995; Flockner *et al.*, 1997; Jones *et al.*, 1999; Kelley *et al.*, 2000; Rychlewski *et al.*, 2000; Skolnick, 2001) (for a review of early fold recognition methods, see Friesner and Gunn (1996)), and *ab initio* prediction (Skolnick *et al.*, 1997; Moulton, 1999; Osguthorpe, 2000). Homology modeling and the related method of protein threading rely upon the knowledge of existing structures via the identification of regions in which a template and query protein are structurally similar. Following the identification of a suitable template candidate protein(s), the method aligns the target sequence to the parent structure. It is then another task to build those regions of the query backbone not similar to any parent structure. Fold recognition algorithms aim to exploit knowledge of the relatively small number of folds that proteins utilize. The challenge then is to extract rules for fold assignment even in cases in which there is no obvious sequence homology between structures with related folds. These methods assign a fold, or series of folds, to a target sequence on the basis of these devised conventions. Finally, while *ab initio* is defined as "from the beginning," and although such methods aim to operate with limited knowledge dis-

tilled from existing structures, many utilize data from known structures to calculate scoring functions and assess prediction accuracy.

Our own approach toward identifying function in sequences of unknown structure has been to couple the strength of a library of three-dimensional descriptor-based templates termed FFF (Fuzzy Functional Form (Fetrow *et al.*, 1998; Fetrow and Skolnick, 1998)) with a structure prediction method. Originally we utilized a hybrid threading algorithm (Jaroszewski *et al.*, 1998) and have recently moved to the use of Prospector (Skolnick, 2001) (Protein Structure Predictor Employing Combined Threading to Optimize Results: see http://bioinformatics.danforthcenter.org/services/prospector_doc.html). Prospector improves upon other structure generating methods in that it does not limit prediction considerations to strictly fold recognition or true threading processes. Since "pure" fold recognition methods generally perform best in cases in which significant homology exists between probe and template proteins (Bryant, 1996), and protein threading methods are best suited more distant homology relationships (Murzin, 1999), the Prospector method captures the strength of each approach. The advantage of the marriage of fold recognition and true threading has recently been demonstrated (see http://bioinformatics.danforthcenter.org/services/prospector_doc.html).

The impetus for the combination of the FFF and threading technologies was to implement the optimally suited approach to screening sequences of unknown structure for function. Constructed on the basis of the identities and the geometries between members of a defined subset of active site residues common to functionally similar proteins, FFFs encapsulate the minimum required physical and chemical structural elements for a specific biochemical function. Thus, proteins that exhibit the same function can be automatically identified, even if they are widely divergent in sequence and have dissimilar folds. For example, eukaryotic and bacterial serine proteases have different folds but they all function as serine proteases and share a common active site (Fischer *et al.*, 1994), and they have been successfully identified using the FFF method (Zhang *et al.*, 1998).

Here we describe the value of the use of a three-dimensional template for function in annotating function for protein sequences. We provide specific examples of the new applications of both previously described (disulfide oxidoreductase (Fetrow *et al.*, 1998)) and novel (protein tyrosine phosphatase) FFFs in whole genome screenings to identify novel functional annotations for sequences found therein. We also provide comparisons of these annotations

and those obtained via sequence-based functional annotation methods.

MATERIALS AND METHODS

The disulfide oxidoreductase and protein tyrosine phosphatase FFF. As described in previous work (Fetrow and Skolnick, 1998), the FFF for the disulfide oxidoreductase function was constructed of three active site residues: two cysteines, which are two residues apart in the sequence, forming a CXXC motif, and a proline distant in sequence but close in three-dimensional space. The second cysteine can be replaced by a serine and the same geometric constraints for a functional template are used. We utilized physicochemical data from three entries in the PDB in constructing the disulfide oxidoreductase FFF: 1aaz (a bacteriophage T4 thioredoxin) (Eklund *et al.*, 1992), 1ego (an *Escherichia coli* glutaredoxin) (Xia *et al.*, 1992), and 1dsb (an *E. coli* disulfide bond A protein) (Martin *et al.*, 1993). With only the data from these PDB entries, our resulting functional template was sensitive enough to discriminately identify all 8 disulfide oxidoreductases in a test database of 1500 nonredundant PDB structures.

We compared the disulfide oxidoreductase FFF to two Blocks, the thioredoxin and glutaredoxin Blocks. The disulfide oxidoreductase FFF is designed to identify both thioredoxins and glutaredoxins, as well as disulfide isomerases, as all of these perform a similar chemical reaction at their active site. Given that, by definition, an FFF serves as a template of the underlying chemical functionality of a protein, the use of a single FFF corresponding to separate thioredoxin and glutaredoxin Blocks is appropriate.

The protein tyrosine phosphatase (PTP) FFF was constructed in the same manner described for the disulfide oxidoreductase FFF utilizing the following amino acids: a Cys that initiates catalysis via nucleophilic attack; an Arg that stabilizes the reaction intermediate; and an Asp that acts as a general acid (Sarmiento *et al.*, 1998). As with the disulfide oxidoreductase FFF, our use of 10 PDB structures in defining the FFF yielded a template sensitive enough to detect all 23 PTPs in our test database of 4042 nonredundant PDB structures (this database is an expanded version of the one utilized in validating our disulfide oxidoreductase FFF).

Threading algorithm. The disulfide oxidoreductase FFF was applied to the *Bacillus subtilis* genome (<http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/eframik?db=Genome&gi=27>). First, a threading algorithm (Jaroszewski *et al.*, 1998) was applied to the *B. subtilis* genome, as described previously (Fetrow and Skolnick, 1998). The algorithm evaluated the threading results using three scoring functions, sq (sequence to sequence alignment), br (sequence to structure alignment), and tt (structure to structure alignment) (Jaroszewski *et al.*, 1998). Threading was performed against an extended database of 1501 known nonredundant structures, 8 of which are known disulfide oxidoreductases: 1bed (Hu *et al.*, 1997), 1ego (Xia *et al.*, 1992), 1erv (Weichsel *et al.*, 1996), 1fvk chain A (Guddad *et al.*, 1997), 1kte (Katti *et al.*, 1995), 1thx (Saarinen *et al.*, 1995), 1tof (Mittard *et al.*, 1997), and 2trx chain A (Katti *et al.*, 1990). Next, the disulfide oxidoreductase FFF was applied to the threading results. The genome sequences that aligned correctly to these 8 structures, in accordance with the FFF, were further analyzed as described under Results.

The PTP FFF was applied to the open reading frames encoded in the left arm of chromosome 2 in the *D. melanogaster* genome (2486 sequences). In this experiment a more recent threading algorithm, Prospector (Skolnick, 2001), was used. Prospector uses two sequence-to-sequence alignment and two sequence-to-structure alignment scoring functions. For each type of scoring function, a closely related alignment and a distantly related alignment are calculated. Thus, a total of four scoring functions are

employed in a Prospector analysis: close or distant sequence profiles, followed by application of a scoring function based on either sequence alone or sequence plus secondary structure plus pair interaction profiles. The closely related alignment is defined by the query sequence and the template sequences possessing 35–90% sequence identity (as calculated by BLAST). The distantly related alignments are defined by the query sequence and the template sequences possessing a BLAST *E* value ≤ 10 . Threading via Prospector was performed against a nonredundant structure database of 3332 proteins assembled by clustering the sequences of the protein data bank into groups with $\geq 40\%$ sequence identity and selecting a representative from each cluster to populate the threading database. A *Z* score is calculated for each *Drosophila* sequence aligned to a structure by comparing normalized raw scores from each scoring function for each sequence. The PTP FFF was applied to the results of these threading calculations.

Conservation profile. To check the conservation of active site residues in a given sequence, we calculated a conservation profile (Zhang *et al.*, 1998). First, a multiple sequence alignment was generated for the query sequence using BLAST (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast, (Altschul *et al.*, 1997)), with an *E* value cutoff of 0.01. Next, gaps were ignored, and for each column in the multiple sequence alignment the number of appearances of each residue was tallied. The resulting conservation profile depicts the degree of conservation among the set of related sequences in the multiple sequence alignment.

Searching for one sequence motif of the Blocks database against a whole genome. In order to find all the sequences in each genome that Blocks (http://blocks.fhcr.org/blocks_search.html, Blocks Database Version 11.0, July 1998 (Henikoff and Henikoff, 1994)) identifies as a thioredoxin, a screening of the *B. subtilis* was performed. One sequence motif ("block") in the Blocks database correlated with thioredoxin activity (Block BL00194). It contains the active site cysteines motif, CXXC, and does not have a second sequence motif corresponding to the proline.

The *B. subtilis* genome was downloaded from the the NCBI web site (<http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/eframik?db=Genome&gi=27>). A search for the query Block was conducted on all sequences of the genome. Blocks aligns the query block to each sequence in the genome and looks for matching alignments. The result of each screening was a list of sequences, ranked by the Blocks score, that was found by Blocks to exhibit the thioredoxin motif.

Comparison of the FFF results with sequence motif databases. The threading/FFF results were compared to three known sequence motif databases: PRINTS (<http://www.biochem.ucl.ac.uk/bsm/dbbrowser/PRINTS/PRINTS.html> (Attwood *et al.*, 1998), PRINTS20.0, released October 25, 1998); Prosite (<http://www.expasy.ch/tools/scnpsit1.html> (Hofmann *et al.*, 1999), release 15, July 1998); and Blocks. These databases receive a sequence as input and output a list of sequence motifs, ranked by score, that may match the function of the query sequence. The thioredoxin and glutaredoxin families represent the disulfide oxidoreductase function in these local sequence motif databases. PRINTS uses a three-element fingerprint for thioredoxin (PS00160) and glutaredoxin (PS00421): one for the active site cysteines motif, CXXC; one for the proline motif; and a third one. Prosite uses only one signature containing the active site cysteines for thioredoxin (pattern PS00194) and glutaredoxin (pattern PS00195). Blocks uses one block for thioredoxin, BL00194, containing the active site cysteines motif, CXXC, and two blocks for glutaredoxin: BL00195A, encompassing the active site cysteines motif, and BL00195B, including the proline. Each of the *B. subtilis* protein sequences found by the FFF to contain the disulfide oxidoreduc-

TABLE I
Proteins in the *B. subtilis* Genome Predicted to Have the Thiol-Disulfide Oxidoreductase Active Site

Sequence	Thrd/FFF ^a	CP	Functional motif			Active site			Database description
			Pr	PS	Bl	C	C/S	P	
trxA	X	X	X	X2	X	29	32	73	Thioredoxin
yusE	X	X	X2	X3	X	26	29	69	Possible thioredoxin
ykvV	X	X	X ^b	X4	X	65	68	135	Possible thioredoxin
resA	X	X	X	X5	X	76	79	143	Possible cyt- <i>c</i> biogen protein
ytpP	X	X	X	—	X	28	31	71	Possible thioredoxin
yvgV	X	X	X	—	X	69	72	193	Unknown
yneN	X	X	X	—	X	71	74	139	Possible dsb
ydbP	X	X	X	—	X	29	32	72	Possible thioredoxin
yolI	X	X	X	—	X3	58	61	104	Possible thioredoxin
ytnI	X	X	X	—	X3	15	18	57	Unknown
yosR	X	X	X	—	X4	11	14	49	Possible thioredoxin
ydbE	X	X	—	—	X	41	44	110	Unknown
ykuV	X	X	—	—	X	41	44	112	Unknown
yuzD	X	X	—	—	—	16	19	76	Unknown
ydfQ	X	X	—	—	—	32	35	75	Possible thioredoxin
ywbO	X	?	—	—	X4	13	16	171	Unknown
yqiW	X	?	—	—	—	52	54	103	Unknown
yqkB	X	?	—	—	—	34	36	63	Unknown
yczA	X	?	—	—	—	12	15	25	Unknown
ytxJ	X	?	—	—	—	31	34	79	Unknown

^a Abbreviations used: Thrd/FFF, top six alignments of sequences from the *B. subtilis* genomes to the sequences of 1bed, 1ego, 1erv, 1fvk (chain A), 1kte, 1thx, 1tof or 2trx (chain A), using an older threading algorithm (Jaroszewski *et al.*, 1998), followed by analysis of the resulting sequence–sequence alignment for the active site residues specified by the FFF for the thiol-disulfide oxidoreductase activity of the glutaredoxin/thioredoxin family (Fetrow *et al.*, 1998); CP, conservation profile of homologous sequences shows if conservation of all three active site residues is greater than 50%. The *E* value cutoff is 0.01; a question mark (?) means that too few homologous sequences were found, so calculation of a conservation profile is not possible; Functional motif, search of each sequence found by Thread/FFF protocols against the local signature databases PRINTS (Pr; <http://www.biochem.ucl.ac.uk/cgi-bin/attwood/SearchPrintsForm2.pl>), Prosite (PS; <http://expasy.hcuge.ch/sprot/scnpsit1.html>), or Blocks (Bl; http://www.blocks.fhcrc.org/blocks_search.html). The number following the X indicates the rank of the sequence in the search.

^b Of the three glutaredoxin/thioredoxin motifs found in PRINTS, only the C-C motif and a third one were found.

tase active site was used as input in the web version of these three motif databases. We then determined whether the sequence motif database identified the query sequence as either a thioredoxin or glutaredoxin, and, if so, at what position the motif database ranked the disulfide oxidoreductase for this sequence.

RESULTS

Enhanced data mining using FFFs: Mining the B. subtilis genome with the disulfide oxidoreductase FFF. To evaluate the effectiveness of using FFFs for biochemical function annotation across whole sets of genome sequences, the disulfide oxidoreductase FFF was used to screen models created from a threading alignment. Table I shows all sequences in the *B. subtilis* genome that were identified by the FFF as having the disulfide oxidoreductase structural motif. To validate these results, a conservation profile was calculated for each sequence (Zhang *et al.*, 1998—see Methods) and the conservation of the identified active site residues in each sequence was determined (Table I, column labeled “CP”). Our emphasis in calculating the conservation profile was on the conservation of the three active site residues

identified by the FFF—the two cysteines and the proline. In most cases, the active site residues of the sequences that were identified by threading/FFF as disulfide oxidoreductases were conserved. Overall, in eight genomes analyzed (data not shown; *B. subtilis* data in Table I shown as example), 72% of the sequences identified by this FFF as disulfide oxidoreductases had conserved active site residues, while 14% of the sequences did not have enough homologous sequences to calculate a conservation profile, and another 14% of the sequences did not have conserved active site residues. Thus, for nearly three quarters of the identified sequences, there was substantiating evidence that the prediction of the exact functional residues might indeed be correct. We wanted, however, to delineate in more detail which of the FFF hits were true positives and which were false positives. One way to approach this question was to employ a number of function prediction tools and simply stipulate those sequences that were identified by a majority of methods as “true positives.” The term “consensus positives” more accu-

rately describes these sequences, as many lack experimental evidence regarding function, and therefore their classification as true positives relies on the consensus between various methods. Thus, the sequences identified as disulfide oxidoreductases by subsequently applying the FFF to the threading models were analyzed using the local sequence signature databases PRINTS (Attwood *et al.*, 1998), Prosite (Hofmann *et al.*, 1999), and Blocks (Henikoff and Henikoff, 1994). To obtain the data in Table I, each motif database received a protein sequence as input and, after comparing this sequence to all the motifs in the database, each annotation tool provided a list of the top scoring motifs for that sequence. A BLAST analysis was also used to classify sequences as consensus positives, though these data are not shown in Table I.

In this study, we identified 13 consensus positive sequences in the *B. subtilis* genome. Of these 13 sequences, 1 is known to be a thioredoxin (trxA). Of the remaining 12 sequences, 8 are annotated in the genome database as having disulfide oxidoreductase function, i.e., possible thioredoxin, possible cytochrome c biogenesis protein, or possible disulfide bond formation protein. All 13 consensus positive sequences are identified by the threading/FFF method.

The FFF identified two additional sequences (yuzD and ydfQ) as disulfide oxidoreductases. Neither of these sequences is identified by any publicly available tool, but the conservation profile analyses of these sequences supported the FFF function prediction. While these sequences were not identified by any of the sequence-based methods, and therefore may not be considered as consensus positives, their conservation profiles suggest that they may in fact be novel hits. Additionally, ydfQ has been shown to have properties similar to those of thioredoxin (Beloïn *et al.*, 1997); thus a literature report suggests that this function prediction made only by the FFF and not by other methods is indeed correct.

There were four sequences (yqiW, yqkB, yczA, ytxJ) that did not have corroborating conservation profiles or supporting annotations from other annotation methods. In the same vein, ywbO does not fall in the consensus positive database and there is no supporting experimental evidence; therefore, it should be counted as a false positive in using the consensus method. In total, using a consensus model, the disulfide oxidoreductase FFF identified six false positives (yuzD, yqiW, yqkB, yczA, ytxJ, and ywbO) in the *B. subtilis* genome. However, of these six proteins, our annotation of disulfide oxidoreductase is supported in the case of ywbO by Blocks analysis and in the case of yuzD by conservation profile analysis.

FIG. 1. The thioredoxin Blocks (Henikoff and Henikoff, 1994) score distributions for the *B. subtilis* genome with a comparison to the FFF results reported in Table I. The thioredoxin block (BL00194) was used to search each genome, as described under Materials and Methods. White bars are those sequences identified only by the Blocks sequence motif; gray bars are those sequences identified by the Blocks sequence motif, the FFF structural motif, and validated by the conservation profile (see Table I for a list of these sequences). In the analysis done here, these are counted as “true positives” or “consensus positives.” The hashed bar represents one of the two sequences that were found by the FFF, validated by the conservation profile, and does not fall in the consensus positive group of sequences.

Whole genome mining: Structural motifs vs sequence motifs. In order to accurately assess the effectiveness of a specific example of an FFF, we have undertaken studies to generate comparisons of FFF results and those of other functional annotation tools. We estimated the ability of the FFF to distinguish between true and false positives via a direct comparison with Blocks (Henikoff and Henikoff, 1994), which, according to the results presented in Table I and other unpublished results, provides more predictions than either Prosite or PRINTS. In making this direct comparison to the FFF, the sequences from the *B. subtilis* genome were screened using one Blocks motif at a time. Our analyses employed three Blocks motifs: the thioredoxin block (BL00194), which includes the active site cysteines, and the two glutaredoxin blocks (BL00195A and BL00195B), which encompass the cysteine and the proline motifs, respectively. As described under Materials and Methods, our use of a single FFF corresponding to both thioredoxin and glutaredoxin Blocks is appropriate given that the disulfide oxidoreductase encompasses both of these related biochemical functions. It should be emphasized that in this analysis each Block was screened against all sequences in the genome. Thus, three screenings were performed (one for each Block motif: BL00194, BL00195A, and BL00195B), producing three lists of sequences ranked by their calculated significance score against each Block. The distribution of the top scoring sequences for this genome is shown in Fig. 1,

which depicts the number of sequences identified by Blocks alone; by conservation profile, FFF, and Blocks; and by FFF and conservation profile, but not Blocks. The Blocks predictions were compared to the FFF predictions and to the conservation profile results from Table I.

To summarize the thioredoxin block search results, there was a general overlap of the FFF and Blocks results for the very high scoring sequences, but the results diverged for sequences that received lower scores. A predominant problem in interpreting Blocks results is a lack of guidance for a specific cutoff value that distinguishes clearly between true and false positives. For example, in the thioredoxin Blocks search there was neither a clustering of scores nor some threshold scoring gap clearly distinguishing a true ("consensus") positive from a false positive among sequences in this genome (scores ranging from 1021 to 735). The FFF identified sequences that received Blocks scores of 1021 to 808 as well as two additional FFF hits that received Blocks scores of 780 and 749. Both of the latter sequences have been previously annotated as possible thioredoxins, and BLAST predicts both of them to be thioredoxins. However, they fell within a Blocks score range with many other sequences of alternate functional annotations. Blocks identifies 7 additional sequences at scores of 770–780 and 12 additional sequences at scores of 744–750 that the FFF did not identify, all of which are of unknown function or are known false positives.

As seen in Fig. 1, the analysis of thioredoxin Blocks figure showed that 25 false positives were found before all consensus positives were found. Since higher scores listed on the *X* axis indicate higher confidence in the Blocks prediction, in order to identify all of the consensus hits (denoted FFF + CP + Blocks in Fig. 1), a consideration of sequences scoring 750 and above would be required. However, 25 sequences that were not consensus hits were also identified in this scoring range—in these analyses, these are considered the false positives. Similar FFF analyses shown in Table I yielded only 6 false positives, a significant reduction in false positive data for this genome.

FFFs extend structural information derived from threading algorithms. An important issue in structural genomics is whether threading scores alone are sufficient to accurately predict the function of a sequence. To address this issue, the threading results for the *B. subtilis* genome were evaluated by three scoring functions, abbreviated sq, br, and tt. From each raw score, a significance score was calculated (Jaroszewski *et al.*, 1998). The significance score distribution for the whole genome was plotted

FIG. 2. The distribution of significance scores derived from the threading algorithm applied to the *B. subtilis* genome. In each figure, the distribution of the three scoring functions, sq (sequence to sequence alignment), br (sequence to structure alignment), and tt (structure to structure alignment) are shown in A, B, and C, respectively. The more significant scores are more negative. The black bars represent sequences in the genome identified by the disulfide oxidoreductase FFF.

and alignments that were identified by the FFF as having the disulfide oxidoreductase active site were identified (Fig. 2). For each scoring function, there was a consensus of annotation between threading and the FFF analysis among sequences receiving a very significant score. But interpretation of the less significant scores indicates there is no obvious cutoff value that distinguishes between sequences identified by the FFF as potential disulfide oxidoreductases and those that are not. These results demonstrate that the raw threading score alone is not sufficient to predict the function of a given sequence.

When the threading alignments and models are augmented by the application of a three-dimensional descriptor template (such as an FFF), sequences with a specific structure and function can be distinguished easily among all the sequences in a genome.

Structural descriptors enable accurate annotation of biological function in sequentially dissimilar proteins. Here we describe the development of an FFF for the function PTP. We have applied this FFF to structural predictions from Prospector, our latest threading algorithm (Skolnick, 2001). The PTP family of enzymes includes low-molecular-weight PTP, receptor-like PTP (RPTP), and dual-specificity Tyr-Ser/Thr phosphatases. Members of this family catalyze the removal of a phosphate moiety from tyrosine in phosphoproteins. Given the low sequence and structural similarity among the diverse members of the protein tyrosine phosphatase family, development of an accurate set of three-dimensional descriptors of protein function would appear to be a daunting task. However, by identifying three amino acids essential for catalysis (a Cys that initiates catalysis via nucleophilic attack, an Arg that stabilizes the reaction intermediate, and an Asp that acts as a general acid (Sarmiento *et al.*, 1998)) and their characteristic ranges in three-dimensional coordinates common to known PTPs, we successfully developed and validated a three-dimensional template of PTP function. The three key amino acids chosen as the basis of this template are structurally conserved within the PTP functional family, despite differing in their sequential positions (Fig. 3).

Figure 3 depicts the relative primary and tertiary structural positions of the PTP catalytic amino acids for the sequences of the human PTP 1B (PDB entry 2hnp (Barford *et al.*, 1994)) and human low-molecular-weight PTP (PDB entry 5pnt (Zhang *et al.*, 1998)). These two proteins exhibit only 2% sequence identity when aligned by clustalW (http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html), yet perform the same biological function. In addition, the sequence position of the amino acids important for the phosphatase function differs between the two proteins (Fig. 3A). The Cys residue is located 6 amino acids from the Arg in both PTP sequences. In the sequence of 2hnp, the Cys and Arg residues are located in the C-terminal end of the protein, but are alternatively located in the N-terminal end of the PTP protein 5pnt. Likewise, these discrepancies in sequential positions are augmented by the fact that the position of the catalytic Asp is inverted between 2hnp and 5pnt with respect to the C- and N-termini of the protein (Fig. 3). Thus, the amino acids that make up the catalytic site fail to align in the primary sequence. The difference in the

positions of Cys and Arg in the overall sequence of 2hnp and 5pnt would preclude use of the sequence of one to identify the chemical function of the other. While the potential exists for identifying the PTP function in both proteins using the CXXXXXR sequence motif common to both forms of PTP, doing so ignores the critical contribution of Asp to the chemistry of the catalytic mechanism.

A comparison of the overall structures of the two PTP proteins (Fig. 3B), and more specifically the active site (Fig. 3C), reveals that the relative positions of these amino acids in three-dimensional space are conserved. In both 5pnt and 2hnp, the Cys is at the C-terminus of a β -strand, and Arg is at the N-terminal end of an α -helix. The Asp in 5pnt is at the C-terminal end of a β -strand and at the N-terminal end of a β -strand in 2hnp. Together, this conserved structure positions these key catalytic amino acids in a cleft on the surface of the proteins (Fig. 3B) and affords the basis for our structural motif that is designed to identify similarities in chemical function.

Mining the D. melanogaster genome using the protein tyrosine phosphatase FFF. In a whole genome comparative analysis of *D. melanogaster*, as deposited into the publicly available database GenBank (see <http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/7227.html>), we have noted that the protein tyrosine phosphatase FFF has identified sequences for function that BLAST, Blocks, PRINTS, and Pfam have failed to identify. Here we list the results for one such sequence of unknown structure—a product of the chromosome referred to as 2L—identified by our PTP FFF as having protein tyrosine phosphatase activity. This sequence, listed in Fig. 4A, is 785 amino acids in length, and we hereafter refer to it by the Accession number gi|7295991. The entry for gi|7295991 in the Gene Ontology Consortium Gene Associations database (see http://www.geneontology.org/gene_association.fb) indicates that it has previously been associated with the cellular functions of “chaperone,” as a component of the nucleus, and/or associated with the “DNA packaging” apparatus. These assessments were made on the basis of sequence similarities to other annotated proteins.

Results from threading via Prospector (Fig. 4B) indicate a Z score of 1.25 via the 3590 structural scoring method for threading of a fragment of gi|7295991 to the PDB entry 1vhr (chain A), a score which is too insignificant to allow structure or function identification from threading alone. Subsequent analysis of the Prospector-predicted structure of sequence gi|7295991 via our PTP FFF indicated that this protein possesses not only the requisite Cys,

FIG. 3. Protein tyrosine phosphatase active site. (A) Sequences from the human protein tyrosine phosphatase 1B (PDB entry 2hnp) and human low-molecular-weight protein tyrosine phosphatase (PDB entry 5hnp). The Cys (blue), Arg (red), and Asp (purple) identified as important to the phosphatase activity are colored as indicated. (B) Structure of low-molecular-weight PTP and PTP 1B from human. The Cys, Arg, and Asp in both structures are colored as in A. (C) Magnified view of superimposed PTP active sites. The low-molecular-weight PTP appears in red, and the PTP 1B appears in blue, with Cys, Arg and Asp depicted as ball and stick models.

A

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>gi|7295991|gb|AAF51288.1| CG7074 gene product [Drosophila melanogaster]
MSGNTHGLSWFFPHFDPKFSVWGQEIHLVEVRRKDDHSQKSRLPYISVNYLANESRYQYARCVAAASYHSDQ
PIIAVGLADGKVGICNFRDITYDSSWEYTPROQRMCTCLAWNELDANILAIGHDRHRNDTCITIWDERGV
PKETANFFGVGESANSICWDRNHRTVIAGMSQKMIKFLDLRQSNATCQSIQTKTVQGLSVSPNGNYLCSY
VDSVITLWDPNRIKSPRLRQIQSSKNHLQIAWCPTRTSLSSLLQDSSSYITLYDIRSVDTNDSGEIYHVKR
QISPPARYQHSGKFSFVNCLSWHSRDFERALLADALNILDRLPATLHTAHSNRRKPLLLMQRPLYTP
ASPTSTAATPTQQOPTSSCSTNSGSSLDFTSTPGGSPFNVDLLKPELFELDLVDETRQRALEDYGIKPDNK
RFGELHLPYLRNVWSTLNVVYSEDRLTGLKATLGINLGTSEALMASSRIESQVLQWPEGINNSNKLIC
YRSEQRDLALQLCGWAFEQELDRFIDQLYANKEYSRAAMICVFHLKIFHACNILSSAADNMRDPSMYRIT
VIALSSFNADRCSSSTWRNQRSSANMQIHDPHLRAVFSFLTMEKDNFDAVLKEEGVSLSDRMAFACKYLSE
TKLADYVAQQIQAAIDGGDLNGLLLTGESQDGIDILQSYMDTSFQVQTVLVAINYFRQELFEDKRIQYW
IASYLDHLNSWGLWEKRAELDIKIESIRPSSRSSRTVFLSCNFCGKSVSNALLDEPRPRSTTTSTNRLSS
CPSCRKPLPRCSLCL
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B

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gi|7295991      ..... VSPNGNY... .L.CSYVDSV ITLWDPNRIK SPLRQIQSSK
1vhrA          SVQDLNDLLS .DGGSCYSLP SQPCNEVTPR IYVGNA.SVA QDIPKLQKLG

gi|7295991      NHLQIAWCPT RTSL...LSS LQRDSSYITL YDIRSVDTN SGEIYHVKRQ
1vhrA          ITHVLNAAEG RSFMHVNTNA NFYKDSGITY LGIKANDTQE FNLSAYFERA

gi|7295991      ISPPARYQH SGKFSFVNCL SWHSRDFERA LLLADALNIL DFLRPATLHT
1vhrA          ADFIDQALAQ KNGRVLVHCR EGYSRSPTLV IAYLMMRQKM DVK..SALSI

gi|7295991      AHSNRRKLP. .... .LLMQRPLY TP
1vhrA          VRQNREIGPN DGFLAQLCQL NDRLAKEGKL KP
```

FIG. 4. (A) FASTA format and Accession information for the *D. melanogaster* sequence identified as a protein tyrosine phosphatase by the FFF template. (B) Resulting alignment from threading of fragments of the gi|7295991 sequence to the PDB entry 1vhr (chain A). Key catalytic residues are shown in bold type and marked by asterisks.

Arg. and Asp residues for PTP activity, but also that they are positioned appropriately in three-dimensional space to enable their roles in the PTP biochemical function. Although Prospector aligned gi|7295991 to 1vhr with a low *Z* score (*Z* scores of >4.5 are typically considered biologically relevant), we again suggest that FFFs extend structural information derived from folding algorithms (Fig. 2). 1vhr is annotated in the PDB as “Human-Vh1-Related Dual-Specificity Phosphatase” (Yuvaniyama *et al.*, 1996). The function of dual specificity protein phosphatases (DSPs) has been reported to be the regulation of mitogenic signal transduction and the control of the cell cycle. 1vhr is less substrate-specific than PTPs, as it allows for the hydrolysis of phosphorylated serine, threonine, or tyrosine protein residues, whereas PTPs are restricted to only phosphotyrosine. However, given the variety of substrates acted upon by 1vhr, the 1vhr structure defines a conserved structural scaffold for both DSPs and PTPs that defines an overlap of biological function (Yuvaniyama *et al.*, 1996). Hence, Prospector aligned gi|7295991 to a protein with known PTP activity, and our PTP FFF subsequently identified

gi|7295991 as possessing the requisite three-dimensional structural motifs to infer that it is, in fact, a PTP.

We have also performed analyses by BLAST, Blocks, Pfam, and PRINTS (using default values) for gi|7295991. None of these methods identified gi|7295991 as a protein tyrosine phosphatase. Moreover, none of these methods identify gi|7295991 as a phosphatase of any type. Even BLAST, which typically identifies protein sequences matching a particular function at some level (i.e., the *E* value may be quite high for a particular BLAST function assignment), does not identify any similar sequences that are annotated as protein tyrosine phosphatases, up to the cutoff *E* value of 10. The sequence-based tools associated the following functions most strongly with gi|7295991: BLAST, “similar to yeast hypothetical protein ybk4” (*E* value = $1e-51$); Blocks, “adhesin family signature” (combined *E* value = 0.093); Pfam, “Peptidase_S15 X-Pro dipeptidyl-peptidase” (*E* value = 5.0); PRINTS, “thyrotropin receptor precursor signature” (*E* value = 58). These results strongly indicate that identification of gi|7295991 as a protein tyrosine

phosphatase is not readily accomplished utilizing existing annotation technologies. Given our confidence in the PTP FFF based upon our choice of structural motif components, and the fact that it identifies sequences in *D. melanogaster* as PTPs that sequence-based annotation methods fail to similarly annotate, we conclude that the FFF method of functional annotation holds a potential competitive advantage. Our method provides a novel hypothesis about biochemical function—one consistent with current annotations of cellular function via the Gene Ontology Consortium Gene Associations database—that may now be experimentally validated.

DISCUSSION

While the use of overall three-dimensional structural information to extend functional annotation beyond the realm of sequence-based methods has been demonstrated to be effective (e.g., see Xu *et al.* (1999), the subsequent application of a protein function template clearly enhances the annotation process even further. Although results from threading provide insight into protein function on a fold-comparison basis, it is only with consideration of the geometries and constituents of the active sites of members of a related set of proteins that the most specific and robust predictions are possible. Using examples of two separate functions and proteins from two genomes, we have documented the abilities of this combination of structure and functional template and compared it with sequence-based analyses. Moreover, we have established novel functional predictions that provide hypotheses for future experimental exploration.

The *B. subtilis* genome was screened with the disulfide oxidoreductase FFF in search of sequences that exhibit this functionality. Sequences identified by the disulfide oxidoreductase FFF were also assessed for the conservation of their active site residues. In addition, these sequences were analyzed for function via the web versions of the three sequence motif databases, PRINTS, Prosite, and Blocks. Not surprisingly, sequences identified by the FFF and annotated as disulfide oxidoreductases or as possible disulfide oxidoreductases had conserved active site residues and were also identified by at least two of the sequence motif databases.

In the comparison of the screenings by the FFF and by Blocks, a drawback to the Blocks method is the lack of a clear cutoff score that distinguishes between true and false positives. A possible reason for this difficulty in defining a clear scoring cutoff is that the Blocks motifs are specific to a certain family of proteins, i.e., thioredoxins or glutaredoxins, and therefore their sensitivity in identifying other disul-

fide oxidoreductases is lower, thus producing scores that are not significant when compared to the many sequences found in a given genome. The advantage of the FFF is that it treats various protein families with equal sensitivity when searching for a specific function that is common to a few families and folds.

There are six sequences identified by the disulfide oxidoreductase FFF that do not fall in the consensus positive data set (Table I). Thus, at worst (since some evidence supports our annotation for two of these sequences), the FFF gives 6 false positives in screening the *B. subtilis* genome. Our comparison of the thioredoxin Block and the disulfide oxidoreductase FFF demonstrates that Blocks identifies 25 false positives before finding all consensus positives. Therefore, the FFF gave a substantially lower false positive rate in whole genome screening, a competitive advantage when considering the time and expense of the biological verification of function for unknown sequences.

The noted discrepancy between the number of disulfide oxidoreductase sequences identified by the disulfide oxidoreductase FFF (6 potential false positives) and the thioredoxin Block (25 potential false positives) could be attributed to the fact that the thioredoxin Blocks includes only the active site cysteines CXXC motif. Based on these results, it might be expected that the glutaredoxin Block search would yield a better accord between Blocks and the FFF, since two Blocks are available: the cysteines block sequence motif (BL00195A) and the proline block (BL00195B). But our results indicated that there was still a large discrepancy in the performance of the glutaredoxin blocks and the FFF, even among the high scoring sequences (data not shown).

The use of threading scores without the subsequent application of a three-dimensional descriptor template is insufficient to optimally predict the function of a sequence. As the threading results for the *B. subtilis* genome indicate (Fig. 2), threading results, when considered alone, appear to fall prey to several sequence-based motif methods of functional annotation; interpretation of the less significant scores fails to delineate an obvious cutoff value that distinguishes between those sequences that are identified by the FFF as potential disulfide oxidoreductases and those that are not. These results demonstrate the added value of the use of structural descriptors for function when utilized in combination with threading results.

Using the particular case of the FFF for protein tyrosine phosphatase and our updated threading algorithm, we have demonstrated the circumvention of certain limitations inherent to sequence-based similarity or motif methods of functional annotation. Of the sequence-based methods, only BLAST annotated

gi|7295991 with a score of any confidence, and even in that instance, it was as having similarity to a yeast hypothetical protein. The failure of these sequence-based methods in identifying gi|7295991 as a PTP is indicative of the exceptional strength of the FFF approach; while the sequence-based methods may be suitable at identifying PTPs that are similar to currently known PTP sequences, the PTP FFF is capable of identifying novel PTP sequences regardless of sequence similarity. Although the PTPs 2hnp and 5pnt are listed as members of separate superfamilies in the Structural Classification of Proteins database—"(phosphotyrosine protein) phosphatases II" (<http://scop.mrc-lmb.cam.ac.uk/scop/data/scop.1.003.040.001.002.001.html>) and "phosphotyrosine protein phosphatases I" (<http://scop.mrc-lmb.cam.ac.uk/scop/data/scop.1.003.039.001.001.002.html>), respectively, they are both accommodated by our PTP functional template. Since the FFF template is defined on the basis of the minimum required chemicostructural features common to all PTPs, *any* PTP, whether currently known or not, is likely to be accurately identified by the FFF.

The entry for gi|7295991 in the Gene Ontology Consortium Gene Associations database indicates that it has previously been associated with the functions of "chaperone," as a component of the nucleus, and/or associated with "DNA packaging" apparatus. Certainly, these annotations are not contrary to our prediction of PTP activity for gi|7295991. PTPs have been implicated in interactions with chaperone proteins (Fu and Galan, 1998; Montagna *et al.*, 2000) and have been confirmed to be nuclear components (Misawa and Yamaguchi, 2000; Tsurusaki *et al.*, 2000). Moreover, PTPs have been demonstrated to be participants in mitotic events, including an important role at the G1/S-phase transition (Nilsson and Hoffmann, 2000). Thus, our predicted biochemical function for gi|7295991 is consistent with its previously annotated cellular function. The protein does appear to have a WD-repeat domain, but this is a separate domain from the putative phosphatase. In addition, WD repeats are found in proteins with a wide range of functions, including those involved in signal transduction, and specifically in subunits of phosphatases (Smith *et al.*, 1999).

CONCLUSION

As with any alternative method, the use of FFFs in functional annotation is not failsafe. However, we have demonstrated that their use has proven to be highly successful in distinguishing between true ("consensus") and false positives when screening whole genomes. Combined with an accurate struc-

tural prediction algorithm, such as Prospector, this method of the use of three-dimensional descriptors of function allows new insight into the subset of proteins for which sequence-based methods alone are not sufficient for functional annotation.

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