Quantitative assessment of protein function prediction from metagenomics shotgun sequences


*Structural and Computational Biology Unit, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany; †Max Delbrück Centre for Molecular Medicine, D-13092 Berlin, Germany

Edited by Michael S. Waterman, University of Southern California, Los Angeles, CA, and approved July 17, 2007 (received for review March 23, 2007)

To assess the potential of protein function prediction in environmental genomics data, we analyzed shotgun sequences from four diverse and complex habitats. Using homology searches as well as customized gene neighborhood methods that incorporate intergenic and evolutionary distances, we inferred specific functions for 76% of the 1.4 million predicted ORFs in these samples (83% when nonspecific functions are considered). Surprisingly, these fractions are only slightly smaller than the corresponding ones in completely sequenced genomes (83% and 86%, respectively, by using the same methodology) and considerably higher than previously thought. For as many as 75,448 ORFs (5% of the total), only neighborhood methods can assign functions, illustrated here by a previously undescribed gene associated with the well-characterized heme biosynthesis operon and a potential transcription factor that might regulate a coupling between fatty acid biosynthesis and degradation. Our results further suggest that, although functions can be inferred for most proteins on earth, many functions remain to be discovered in numerous small, rare protein families.

Results and Discussion

**An Operational Definition of Protein Function.** Biological function is a fuzzy term summarizing a complex concept applicable to different spatial scales (22, 23). At the molecular and (sub-)cellular level, an operational framework with clearly defined terms and thresholds is therefore required when attempting to quantify protein function. To infer specific function from existing database annotations by using homology, we require similarity to an environmental (partial) ORF >60 bits, corresponding roughly to an e-value of 10^-5 in Uniref90 searches (4). This level of sequence similarity is rather strict in terms of homology identification but without further analysis may be insufficient to distinguish between paralogs and orthologs, thus not capturing all functional features such as enzyme substrate specificity. It is, however, sufficient to capture basic functionality.

We used a hierarchical classification scheme, favoring manual annotation, to divide environmental ORFs and, for comparison, 124 prokaryotic proteomes into four categories based on the level of functional annotation possible: (i) those with strong similarity to, or in the genomic neighborhood of, a gene with specific functional annotation; (ii) those with strong similarity to carcasses on the deep Pacific and Antarctic ocean floor (4), functions have been predicted based on sequence similarity for only 27–48% of the 1.4 million genes in the different samples [see supporting information (SI) Table 1]. This implies that for the majority of proteins in the environment, functions remain unknown, and no attempt has yet been made to discover novel functionality. Furthermore, for each project, different methods, parameters, and even definitions of function were used, which are often not easily accessible to the community, making a comparison of the different samples difficult. To be able to comprehensively predict functions from various metagenomes and to get a consistent overview of function in different environments, we developed a sensitive prediction protocol that complements BLAST- and domain-based function predictions with newly developed and adapted gene neighborhood methods. Applying this protocol to the samples revealed a considerable predictive power, indicating that function can be inferred for most of the genes on earth; yet the majority of functions appear to reside in numerous rare, small protein families that remain largely unexplored.
genes with nonspecific functional information, weak but significant similarity to genes with any functional annotation, or in the genomic neighborhood of either of these; (iii) those with strong similarity to, or in the genomic neighborhood of, a gene of unknown function; (iv) those with neither similarity to sequences in annotated databases nor significant genomic neighborhood (Fig. 1).

We used sequence similarity to infer functional information from the KEGG (24), COG (12), UniRef90 (25), SMART (26), and Pfam (27) databases (see Materials and Methods for parameter choices, benchmarks, and definitions of functional annotation). We used gene neighborhood evidence from the STRING database (21) and adapted existing gene neighborhood function prediction methods, based on intergenic distance and evolutionary conservation, for use in fragmented shotgun metagenomics data. First, we exploited the fact that intergenic distances tend to be shorter between genes of the same operon than between operons (28). Although several operon prediction methods have been introduced that are based solely on intergenic distances (28–31), they are species-specific, trained with experimentally verified transcript information (28), and/or require the context of a complete genome. Here, we calibrated directly on each sample to establish the likelihood of being functionally associated, given a positional distance within a read. Second, we used the fact that neighboring ORFs are more likely to be functionally associated if they are conserved over long evolutionary distances (15, 16, 32). We recorded multiple occurrences of neighboring genes, measured the sequence similarity of the respective neighborhoods to each other, and derived a metric based on evolutionary distance. We then combined these measures for intergenic and evolutionary distance to predict functional relationships between genes in the metagenomic data (see Materials and Methods).

### Consistent Functional Characterization of ORFs in Four Environmental Data Sets

By combining homology searches and neighborhood methods, we were able to infer specific functional information for 76% of the 1.4 million predicted environmental ORFs and a more general level of functional information for another 7% (dark and light green segments respectively of the outermost ring in Fig. 2; see also SI Table 2). By using sequence similarity alone, a specific function can be inferred for almost two-thirds (65%) of the ORFs, and a general function for another 13% (inner circle Fig. 2). Neighborhood-based methods provide functional information for 30% of the ORFs (green segments in middle ring; Fig. 2), complementing similarity-based molecular characterizations with functional interactions. They also provide functional information for almost a quarter of the ORFs (75,448), where homology-based methods fail. This 30% of neighborhood-based predictions is considerably lower than the 56% achieved when the same methods are applied to the 124 prokaryotic genomes (SI Table 3). However, only 47% of the ORFs in the metagenomic data sets have a neighbor in the same transcription direction, as compared with 88% in completely sequenced genomes (SI Table 4), which implies that the predictive power of neighborhood methods is comparable in genomes and metagenomes. Indeed, the combined methods perform almost equally well in metagenomes (83% functional characterization) as in fully sequenced genomes (86%). Moreover, the metagenomic ORFs that cannot be characterized by similarity are significantly shorter than those that can (SI Fig. 5). Some of these may be fragmented ORFs that are too short to assign significant similarity; others may have resulted from erroneous ORF predictions. The latter would imply that the true fraction of gene products for which functions can be predicted is even higher. In either case, the quality of predictions should improve in the future because sequence coverage is likely to increase in metagenomics projects, allowing more reads to be assembled into longer contigs.

In the original reports of the metagenomics data sets, specific functions were assigned to 27–48% of the predicted gene products (1, 2, 4), indicating marked differences in the function prediction protocols caused by various technical issues such as the stringency of BLAST cutoffs, the choice of functional databases, and variations in gene calling (a comparison is presented in SI Table 1; for an expanded comparison see ref. 9). Because our benchmarks and manual confirmations of parameter settings show a negligible false-positive rate (see Materials and Methods), we believe that the near doubling in functional assignments is not caused by a looser function definition or more spurious assignments but is due to better utilization of existing functional information. The latter uncovers marked trends such as overrepresentation at the gene, family, or pathway level, in line with earlier studies (4) (SI Table 5). For example, we find that bacterial chemotaxis, flagellar assembly, and type III secretion genes are 3-fold more frequent in the genomes than the metagenomes (dominated by the surface sea water data set), perhaps because of the futility of bacterial motility in strong ocean currents. On the other hand, genes involved in amino acid metabolism as well as in the biosynthesis of nucleotides, carbohydrates, and lipids are significantly underrepresented in the genomes as compared with the metagenomes, perhaps because of the bias toward sequencing obligate pathogens, which tend to acquire these compounds from their hosts.

### Comparison of Environmental Samples

Among the four environments, the fraction of functional assignments differs considerably as it does between organisms (Fig. 2 and SI Figs. 6 and 7). In the surface sea water, specific functions are inferable for 82% of ORFs (dark green sections in Fig. 2); the corresponding fraction in whale fall is 66% and in soil only 53%. These differences can be partially attributed to inherent differences in the sequence data: for example, the average read length of the sea water data is longer than that of soil [818 bp vs. 673 bp after quality filtering (2, 4)] and 60% of the sea water reads can be assembled into longer contigs compared with <1% in soil (33),
Also, environments have been previously characterized to different degrees, and for some environments, complete genome sequences are available that closely resemble those from the environment [e.g., SAR11 as a frequent ocean bacterium (34)]. This means not only more gene content in a certain environment but also more BLAST assignments for short fragmented ORFs and hence more reliable gene predictions. Finally, a major fraction of the acid mine sample is comprised of Archaea, which are generally less functionally characterized than bacteria, thus lowering our functional understanding of the sample. Nevertheless, we believe that most differences between the environments are caused by multiple effects linked to genuine diversity in phylogeny and lifestyle. For example, genomes of species in the sea water samples are smaller than in soil, with a higher fraction of essential, well characterized genes (33), but they also evolve faster (35), which should make homology searches less sensitive. Farm soil might supply the most stressors to microbial life because of its high population density, microhabitats, and physical and systemic perturbations (e.g., temperature, nutrient availability, and pH) (36), leading to a broad repertoire of stress-response phenotypes with hitherto uncharacterized functions. Similarly, the unusual ecological niche created by a deep-sea whale carcass, with its extreme conditions of darkness, cold, and high pressure, lead to highly specialized microbial adaptations such as barotolerance and temperature-induced lipid fluidity (37) that do not resemble those in other environments or genomes.

Predicting Functional Novelty: In-Depth Analysis of Two Neighborhood-Based Findings. Whereas homology-based methods require additional analysis to identify novel functions (e.g., via novel subgroups in a characterized sequence family), neighborhood methods can directly provide novel functional associations. Novelty can be obtained either by (i) seeing unexpected functional coupling of known genes or (ii) assigning unknown genes to known processes. The first is evident in the fact that there are as many as 5,851 pairs of neighboring COGs unique to metagenomes, even though these COGs occur individually in the 124 prokaryotic genomes, implying many novel functional interactions. These frequently include enzymes involved in amino acid biosynthesis with novel links to numerous protein degradation and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6). The second can be seen in the 75,448 ORFs (5% of the total) that are solely characterized by neighborhood in the 124 prokaryotic genomes, implying many novel functional interactions. These frequently include enzymes involved in amino acid biosynthesis with novel links to numerous protein degradation and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6). The second can be seen in the 75,448 ORFs (5% of the total) that are solely characterized by neighborhood in the 124 prokaryotic genomes, implying many novel functional interactions. These frequently include enzymes involved in amino acid biosynthesis with novel links to numerous protein degradation and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6). The second can be seen in the 75,448 ORFs (5% of the total) that are solely characterized by neighborhood in the 124 prokaryotic genomes, implying many novel functional interactions. These frequently include enzymes involved in amino acid biosynthesis with novel links to numerous protein degradation and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6). The second can be seen in the 75,448 ORFs (5% of the total) that are solely characterized by neighborhood in the 124 prokaryotic genomes, implying many novel functional interactions. These frequently include enzymes involved in amino acid biosynthesis with novel links to numerous protein degradation and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6). The second can be seen in the 75,448 ORFs (5% of the total) that are solely characterized by neighborhood in the 124 prokaryotic genomes, implying many novel functional interactions. These frequently include enzymes involved in amino acid biosynthesis with novel links to numerous protein degradation and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6).

Neighborhood information can help characterize a gene family if members of that gene family occur next to different genes belonging to the same pathway in different species. By using such a query, we discovered members of a large uncharacterized gene family (COG1981) with several hundred ORFs in the surface sea water and whale fall samples, adjacent to various enzymes from the COG1981 and regulatory proteins, probably reflecting the different nutritional constraints (SI Table 6).
regulator appears on 20 distinct contigs in the sea water, of which there are at least five unique operon arrangements. The putative transmembrane protein belonging to an uncharacterized COG (COG1981 shown in red) that consistently cooccurs with members of the well characterized heme biosynthesis pathway (colored blue). The putative membrane-associated protein occurs on 174 distinct contigs in the surface sea water and whale fall data sets that can be grouped into at least 15 unique operon arrangements, strongly suggesting a role in this process. (a) A predicted putative regulator, shown in red, that links fatty acid biosynthesis (upstream, colored green) with fatty acid degradation (downstream, colored blue), a functional link not seen in fully sequenced genomes. (b) A predicted putative regulator, shown in red, that links fatty acid biosynthesis (upstream, colored green) with fatty acid degradation (downstream, colored blue), a functional link not seen in fully sequenced genomes. The regulator appears on 20 distinct contigs in the sea water, of which there are at least five unique operon arrangements.

and metabolic regulation (38). In addition, it functions as a prosthetic group to proteins involved in bacterial stress response, oxidative damage, and virulence (39). Sequence analysis of the uncharacterized family reveals that it comprises hydrophobic, putative membrane-associated proteins that are unlikely to have enzymatic functions. They might thus be implicated as scaffolding proteins in tethering the pathway to the membrane and/or enabling sufficient substrate fluxes.

Whereas the heme-associated gene family had previously been observed in fully sequenced genomes, another family of 20 members was found exclusively in the surface sea water samples by using our clustering procedure (see Materials and Methods). Even though no homology could be found by using our automated methods, detailed analysis revealed weak but significant similarity to a family of helix–turn–helix (HTH) transcription factors. An examination of its neighboring genes imply that this family is found in a variety of species, the most closely related being Actinobacteria. As the genes are on various contigs with differing gene orders, we could assign it to an entire operon that additionally contains three downstream genes consistently occurring in the same orientation. The first downstream gene of unknown function (NOG05011) has been observed in completely sequenced genomes; in-depth sequence and secondary structure analyses suggest an enzymatic function (data not shown). The second and third genes of this potential operon (COG1961 and COG1960) catalyze successive steps of the β-oxidation of fatty acids (usually involved in degradation) (38, 40). Interestingly, this invariant operon, apparently controlled by the newly predicted transcriptional regulator, frequently occurs downstream of various genes involved in fatty acids biosynthesis (Fig. 3b). Thus, context-based methods predict a coupling between fatty acid degradation and biosynthesis, whereby the previously undescribed gene might provide the regulation of this link. It is intriguing to speculate that this coupling of two antagonistic processes is an adaptation to repeatedly changing environmental conditions. For instance, strongly regulated circadian rhythms are followed by several marine bacteria (41). These bacteria actively migrate to different depths in a periodic fashion to balance the efficient usage of light for energy against the danger of DNA damage (42, 43). Energy storage during the light-dependent phase by biosynthesis of fatty acid and energy release in the light-independent phase could thus be a regulated switch during locomotion from light to dark and vice versa.

**Functional Prediction vs. Functional Diversity.** As more environments are explored, we expect that core protein functions (for example, translational machinery) will be seen repeatedly and will dominate every sample. Novel, rare, and perhaps environment-specific functions, on the other hand, might not be classifiable because they are not yet captured by the experimental studies that underlie most current knowledge about biological function. To reconcile our gene-centric view of the data with a function-based one, we performed an all-against-all similarity search of all predicted ORFs in all four environments, clustered the results into gene families, and recorded their functional status according to our operational definition (see Fig. 4 and Materials and Methods). We find that specific functional knowledge is indeed heavily skewed toward large families: functionally characterized families make up 89% of the largest families (200 or more members), whereas uncharacterized ones make up 72% of the smallest families (three or fewer members). Thus, although most of the proteins in the environmental samples can be functionally characterized because they belong to well studied large gene families, numerous distinct, rare functions remain to be identified. Because these are likely to be adaptations to specific environmental constraints, they should have the poten-
Most large gene families have a known function, whereas small and rare families of unknown functions, analogous to homologous neighborhoods. An edge was placed between two overlapping genes, see only codirectionally transcribed genes (for the treatment of uncharacterized clusters, nonspecific functional annotation if they contain a domain from the SMART and Pfam databases. These annotations were combined in a hierarchical manner, favoring manually annotated databases, placing each ORF into one of the above categories. By definition, any ORF that mapped to KEGG was considered to have a specific function assigned. Of the remaining ORFs, those that mapped to a COG were considered to have a specific function assigned, with the exception of those in functional classes “R” and “S,” which were considered to have nonspecific and no function assigned, respectively. The remaining ORFs were considered to have specific functional annotation if they had strong similarity (≥60 bits) to functionally characterized UniRef90 clusters, nonspecific functional annotation if they contain a domain from the SMART or Pfam A database or have remote similarity (≥40 bits) to functionally characterized UniRef90 clusters. All other ORFs were considered to have no function assigned, those with similarity to uncharacterized UniRef90 clusters were considered to be part of a family, and the rest singletons.

Gene Family Analysis. We grouped genes from all four environmental data sets into 206,217 gene families by first constructing a single-linkage graph of an all-against-all BLAST (60-bit cutoff), with nodes representing proteins, and edges representing BLAST hits between proteins weighted by BLAST bit scores. This graph was then clustered by using Markov chain linkage clustering with an inflation value of 1.1 (44, 45) (SI Table 8).

Function Prediction Using Sequence Similarity. ORFs were assigned to KEGG pathways and COGs by using the method described by Tugule et al. (4) using a 60 bit cutoff. For the 124 prokaryotic genomes, the KEGG and COG assignments from the STRING database were used. ORFs were also compared against the UniRef90 database, divided into functionally characterized and uncharacterized clusters (see SI Text) and annotated with domains from the SMART and Pfam databases. These annotations were combined in a hierarchical manner, favoring manually annotated databases, placing each ORF into one of the above categories. By definition, any ORF that mapped to KEGG was considered to have a specific function assigned. Of the remaining ORFs, those that mapped to a COG were considered to have a specific function assigned, with the exception of those in functional classes “R” and “S,” which were considered to have nonspecific and no function assigned, respectively. The remaining ORFs were considered to have specific functional annotation if they had strong similarity (≥60 bits) to functionally characterized UniRef90 clusters, nonspecific functional annotation if they contain a domain from the SMART or Pfam A database or have remote similarity (≥40 bits) to functionally characterized UniRef90 clusters. All other ORFs were considered to have no function assigned, those with similarity to uncharacterized UniRef90 clusters were considered to be part of a family, and the rest singletons.

Function Prediction Using Genomic Neighborhood. Using the contig positions of the ORFs in each data set, we constructed a list of pair-wise neighborhoods. For this analysis, we considered only codirectionally transcribed genes (for the treatment of overlapping genes, see SI Text). To investigate the conservation of neighborhoods, we constructed a graph for each set of homologous neighborhoods. An edge was placed between two neighborhoods if there were BLAST hits >60 bits between both pairs of genes, except in cases where a gene from one neighborhood hit both genes in the other. This graph was then used to construct clusters of neighborhoods representing a conserved gene pair. To estimate the evolutionary distance over which a neighborhood is conserved, we adapted a weighting scheme used for multiple sequence alignment (46) to derive a score with the property that it will be low for small clusters of closely related sequences and large for clusters with distantly related sequences. For each metagenomic data set, we then constructed a benchmark set of pair-wise neighborhoods where both genes have a KEGG mapping. At each intergenic and evolutionary distance within the benchmark set, we determined the proportion of neighborhoods that map to the same KEGG pathway. This relationship was then interpolated.

Materials and Methods

Sequence Data and Similarity Searches. We analyzed published microbial shotgun sequence data from four environmental samples, totaling 1,438,944 genes: 1,086,400 genes from tropical surface water from the Sargasso Sea (2), 183,586 genes from farm soil from Minnesota (4), 122,146 genes from isolated whale fall carcasses (4), and 46,862 genes from an acidophilic biofilm from an iron ore mine (1). In parallel, we analyzed 344,619 genes from 124 prokaryotic genomes from the STRING database (21) (SI Table 7). Analyses were carried out at three different levels of stringency, the figures reported here use a bit score cutoff of 60 bits for orthology assignment (a prerequisite to predict specific functions) and 40 bits for homology assignment (for details of parameter exploration see SI Text). To map functionally characterized domains to metagenomic ORFs, we scanned the HMM profile signatures from Pfam (27) and SMART (26) against the metagenomic sequences by using HMMER (http://hmer.wustl.edu/) software and applied the corresponding family-specific cutoffs.
and used to derive a value \( P \) for each neighborhood in the data set, corresponding to the probability that a pair of genes in a neighborhood is functionally related (SI Figs. 6 and 8–11 and SI Table 2). We also applied this method to individual organisms (SI Figs. 7 and 12 and SI Table 3) to assess the effect of species-specific genome architectures on the method. It is clear that the relationship between intergenic and evolutionary distance and \( P \) is highly species-specific. The vast majority of \( P \) values exceed the random expectation (16%, the probability that a random pair of genes map to the same KEGG pathway). To ensure that we were dealing with high quality predictions, we considered a pair of genes to be functionally linked only if the \( P \) value was \( >0.4 \) [found to have an accuracy approaching 70% at the level of functional modules (47)]. For the ORFs that map to COGs, additional neighborhood information was taken from the STRING database (see SI Text).

We thank the P.B. group for helpful discussions. E.D.H. was supported by the European Community’s FP6 Marie Curie Fellowship for Early Stage Training (E-STAR) under contract number MEST-CT-2004-504640. This work was supported by the European Union 6th Framework Program (Contract No. LSHG-CT-2004-503567).