Deciphering enzymatic potential in metagenomic reads through DNA language model

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Abstract

Earth's microbial world plays a fundamental role in shaping the biosphere, steering global processes, soil rejuvenation, and ecological fortification. An overwhelming majority of microbial entities, however, remain un(der)studied. Metagenomics stands to elucidate this microbial "dark matter" by directly sequencing the microbial community DNA from environmental samples. Yet, our ability to explore metagenomic sequences is mostly limited to establishing their similarity to curated datasets of organisms or genes/proteins. Aside from the difficulties in establishing such similarity, the reference-based approaches, by definition, forgo discovery of any entities sufficiently unlike the reference collection.

Presenting a paradigm shift, language model-based methods, offer promising avenues for reference-free analysis of metagenomic reads. Here, we introduce two language models, a pretrained foundation model REMME, aimed at understanding the DNA context of metagenomic reads, and the fine-tuned REBEAN model for predicting the enzymatic potential encoded within the read-corresponding genes. By emphasizing function recognition over gene identification, REBEAN is able to label molecular functions carried both by previously explored genes and by new (orphan) sequences. Inherently, REBEAN identifies the functionally relevant parts of a gene even though it is not explicitly trained to do so. It thus expands enzymatic annotation of unassembled metagenomic reads from extreme environments while maintaining consistency with available annotation methods. Here, our comprehensive analysis highlights our models' potential for metagenomic read annotation and unearthing of novel enzymes, thus enriching our understanding of microbial communities.

Key words: Enzymatic activity, Metagenome, Sequence Analysis, Function Prediction, dLM, deep learning

Introduction

The world we live in is estimated to host up to a trillion bacterial species spread wide and deep across the globe (Locey & Lennon, 2016; Louca et al., 2019). Microbes are the foundation of Earth's biosphere and are responsible for much of life as we know it. We learned of the existence of microbes with the discovery of the microscope in the 1600's (Lane, 2015). Centuries later, however, we are only capable of isolating, growing, and studying a tiny fraction of these organisms in the lab (Ruscheweyh et al., 2022; Solden et al., 2016; Staley & Konopka, 1985; Steen et al., 2019).

An alternate to the exploration of individual microbes is studying them directly within their communities via the analysis of metagenomes, i.e. the bulk genetic material of environmental samples. Current applications of metagenomic (and metatranscriptomic) studies are diverse, including but not limited to surveillance of antimicrobial resistance (AMR), exploring microbial adaptation to environmental changes, studying the influence of host microbiome on host health, identification of new microbial species, and discovery of novel genes and gene products (Handelsman, 2004; Ko et al., 2022; Zhu et al., 2018). Significant advances in sequencing techniques of the past two decades have largely overcome the technical challenges associated with metagenomic data extraction, shifting the focus towards downstream analyses, such as gene prediction, taxonomic classification, and functional annotation/profiling (Bharti & Grimm, 2021).

Functional profiling is an important step in deciphering the contents of the microbial sample (Ko et al., 2022; Pushkarev et al., 2018; Zhu et al., 2020). It provides a way to track molecular functionality known to be important and to identify novel genes of known or yet-undescribed functions. Functional labels used in profiling are predefined terms and are part of extant ontologies and domain/family collections, e.g. Enzyme Commission numbers (EC), Gene Ontology (GO) terms, Pfam protein families and InterPro signatures, Clusters of Orthologous Genes (COG), and KEGG genes and pathways (Ashburner et al., 2000; International Union of & Webb, 1992; Kanehisa et al., 2022; Mistry et al., 2021; Paysan-Lafosse et al., 2023; Tatusov et al., 1997). Functional annotation of a metagenomic sample may involve labelling whole genes,

identified from assembled stretches of sequence (transcripts, contigs, or genomes), or inferring putative labels directly from sequencing reads (Bharti & Grimm, 2021).

Most current metagenome annotation methods rely on mapping of genes or reads to related reference, i.e. annotated, gene or product protein sequences. Reference-based function assignments use sequence alignment and k-mer indexing and matching (Aziz et al., 2008; Mistry et al., 2021; Overbeek et al., 2005; Sayers et al., 2024; Tatusov et al., 2000), Hidden Markov Models (HMMs) of families, or structural comparisons. Requiring an existing reference, however, limits the range of discovery possible with these methods (Prabakaran & Bromberg, 2025).

Recent developments in deep learning models, and particularly language models (LMs), have succeeded in predicting protein structure and made significant advances in protein domain and function annotations (Bileschi et al., 2022; John Jumper et al., 2021; Lin et al., 2023; Sanderson et al., 2023). These have been experimented with in the metagenomic world as well (Hoarfrost et al., 2022; Pan et al., 2022), suggesting that they can generalize the information encoded in sequencing reads. In this study, we trained REMME (Read Embedder for Metagenomic exploration), a foundational transformer-based DNA language model (dLM), to learn the “language” of sequencing reads. REMME is adaptable to various downstream tasks, and we describe one such task here: REBEAN (Read Embedding Based Enzyme Annotator) performs reference- and assembly- free annotation of enzymatic activities putatively encoded by microbial genes that give rise to metagenomic sequencing reads. REBEAN demonstrates robust performance, by leveraging understanding on the read context within their “parent” enzymes. Our analyses describe REBEAN’s extensive applicability to metagenome annotation, particularly highlighting its ability to forgo sequence-defined homology in favor of discovering novel enzymes.

We note that much of the recent emphasis in the protein and nucleotide work with dLMs has focused on creating novel sequences that perform desired functions (Madani et al., 2023; Nguyen et al., 2024). This approach, however, discards the well-optimized natural results of billions of years of evolution in favor of synthetic, often questionable, biology. Here we suggest that annotation of the existing rich diversity of sequences,

rather than de novo synthesis, should be the first step in harnessing the power of biological molecules.

RESULTS AND DISCUSSION

Here, we developed a transformer-based language model to annotate enzymatic activity of genes giving rise to sequenced reads. That is, given a particular read, we asked: “What would be the function of the gene that it came from?”

Building a DNA-language model (dLM) to decipher metagenomic reads. Our first (foundation) model was pretrained as a generalized read embedder to understand the DNA “language” using 53.6 million reads from 1,496 prokaryotic genomes (**Methods**). It was then fine-tuned on 19 million non-redundant reads from 19,316 metagenomic samples to annotate enzymatic function in terms of the seven first level EC classes. For clarity of the discussion, we named the pretrained dLM REMME (Read Embedder for Metagenomic exploration) and the fine-tuned model REBEAN (Read Embedding Based Enzyme Annotator).

REMME was trained to embed DNA reads through mask-token prediction, i.e. predicting masked nucleotides in each read. It attained over 98.5% accuracy (**Eq. 1**) in each: training, validation, and testing (**Figure S1**). This performance highlights REMME’s solid understanding of the DNA “spelling”, i.e. the nucleotide order of occurrence. To enforce further understanding of biological context, we gave REMME two additional learning tasks: (1) estimate the fraction of protein-coding nucleotides in each read and (2) if the read is part of a coding DNA sequence (CDS), predict the open reading frame; as part of the latter task, REMME was trained to predict a given read class: 1, 2, or 3 for each of the three-reading frames (with respect to the first position of the read in the 5’ to 3’ direction) and 4 if the read was not transcribed.

REMME learned to identify coding regions within reads well. In both validation and testing, the REMME-predicted fraction of coding nucleotides per read was well correlated with the ground truth fraction of coding nucleotides (**Figure 1A;** Pearson’s r = 0.73, *p-value* < 1E-32; MSE=0.04 and MAE=0.11, **Eqn. 2-3**). Note that the largest number of mispredicted coding fractions was attributed to mostly non-coding reads,

i.e. those containing 0-0.1 known coding residues. This observation is in line with the known difficulty of distinguishing short coding and non-coding stretches (Clamp et al., 2007; Zhang et al., 2016). As the ground truth fraction of coding residues increased, REMME was able to identify the stretches more precisely.

REMME predicted reading frames with an average accuracy of 48.6% and AUC of 0.789, i.e. not well but much better than a random four-class classifier of 25% accuracy. Note that annotation of CDSs and reading frames used in this study was produced by computational pipelines (Richardson et al., 2023) and, as such, could be incomplete or erroneous. Also note that the reading frame is not well defined for reads, i.e. sequence fragments, possibly shared by multiple CDSs with different reading frames on either forward or reverse strands. Despite these ambiguities, REMME distinguished reads which are part of CDSs (classes 1, 2 & 3) from non-transcribed reads with an accuracy of 88.5% (recall=94.2%, precision=92.7%; **Eqn. 4-5**).

In summary, REMME was pretrained, by enforcing relevant objectives, to understand the biological context of reads and did well in addressing the tasks it was assigned.

Can we annotate enzyme-coding fragments in metagenomic data? To answer this question, we fine-tuned REMME to build REBEAN – a model that predicts the Enzyme Commission (EC)(International Union of & Webb, 1992) first level classes (1: Oxidoreductases, 2: Transferases, 3: Hydrolases, 4: Lyases, 5: Isomerases, 6: Ligases & 7: Translocases) or a non-enzyme class of the proteins encoded by genes that gave rise to each read.

For the purposes of model training, read function annotations were generated by mi-faser (**Methods**), a high precision alignment-based read annotation method (Zhu et al., 2018). Relying on mi-faser annotations for training exposed REBEAN to a broader, real metagenome sequence space, instead of what can be gleaned from simulating reads from experimentally annotated reference databases. This decision also allowed us to train on reads from all genome regions, i.e. coding and non-coding, with no explicit biases.

Note that, somewhat counterintuitively, the task of labelling EC numbers at the first level is more challenging than predicting deeper EC levels, as deeper levels provide a more specific characterization of protein catalytic activity and typically correspond to only a handful of enzymes. For example, it is likely harder to find a unifying, broad, but unique, sequence or structure factor representing the first EC level, e.g. oxidoreductase activity (EC 1.*), than a specific factor explicitly capturing oxalate oxidase activity (EC number 1.2.3.4). Nevertheless, REBEAN attained consistently high accuracy (**Eqn. 1**) in classifying non-redundant reads (<80% sequence identity (Hauser et al., 2016)) across training, validation and test sets (81.7%, 81.0% and 80.6%, respectively; **Table S1** & **Figure 1C**). Furthermore, in testing, REBEAN attained an average multiclass Area Under the Curve (AUC, **Eqn. 9**) of 0.969 and recall of 80.1% with a precision of 83.6% (**Eqn. 4 & 5**). REBEAN's test performance is far better than that of any random classifier (12.5% accuracy). Note that this performance reflects REBEAN's ability to report mi-faser, not experimental, EC annotations.

To test whether pretraining helped training for EC prediction, we used REBEAN's training protocol without REMME weights, instead initiating its base model with random assignments. This model reached a maximum accuracy of only 30% at the end of 200 epochs, illustrating the necessity of pre-training.

Despite the overall strong performance, REBEAN's ability to identify non-enzymatic reads was relatively low (recall=50.6%). That is, REBEAN labelled as enzymatic half (176,254) of the test set reads, which mi-faser had not identified as such; **Figure 1D** illustrates that a portion of non-enzymatic read REBEAN embeddings exists in the same space as enzymatic ones. We note again that mi-faser is a very precise (90% precision at fourth EC level) method of low recall (50%). We thus questioned whether these 176K reads could possibly indeed be enzymatic.

We further found that a third (50,756) of these reads aligned to SwissProt (Hauser et al., 2016; UniProt, 2023) proteins with >30% sequence identity. Three quarters (38,241) of these mapped to enzymes, highlighting alignment-based mi-faser method's low recall and re-affirming reference-free REBEAN's putatively high accuracy levels. Note, that reads aligned to SwissProt often attained higher REBEAN scores than those

that did not align (**Figure 1E**). The remaining (126K) reads, predicted as enzymes by REBEAN, could represent yet-unseen enzymes or simply capture portions of gene sequences re-used in evolution across enzymatic and non-enzymatic sequences alike.

REBEAN performs well in labelling functions on the basis of read sequences. To evaluate REBEAN's performance against experimental, i.e. not mi-faser predicted, annotations, we generated a set of 525,775 fragments (length 200bp) from 4,295 enzyme-coding genes with experimental evidence of enzymatic activity (dataset **SPEnzset1**, **Table 1; Methods**). Each fragment, i.e. synthetic read, was labelled with the first level enzyme class of the parent gene. We evaluated REBEAN's performance in annotating these reads' enzyme classes (**Figure 2A, B**). At a threshold of 0.5, REBEAN attained an average recall=33.4%, precision=71.5%, and accuracy=88.1%; it attained an average Area Under the Receiver Operating Characteristics curve (AUROC, **Eqn. 9**) of 0.79, ranging from 0.73-0.90 across the seven enzyme classes (**Figure 2B** and **Table S1**).

Note that at 90% precision, REBEAN recovered, on average, only 23% (7.6% to 44.5%) of the reads from each enzyme class correctly; i.e., REBEAN attributed high prediction confidence to only a fraction of reads. Since each of the reads in this set is derived from an enzyme, REBEAN's inability to assign high confidence to most reads suggested that it only learned to associate a specific segment in each gene with its molecular activity.

Three possible, non-exclusive, protein biology-driven explanations for this observation come to mind. (1) One is based on the fact that specific functional activities, e.g. catalysis and ligand binding, tend to occupy only *a small portion of the protein*, with the rest of the sequence providing structural support. In homologous enzymes, functional specificity is often tuned within a small protein segment, sometimes as short as a single residue (Allen & Whitman, 2021; Babbitt et al., 1996; Baier et al., 2016). Similarity between independently evolved functionally similar enzymes is also often limited to a subset of residues, i.e. active-site convergence (Davidi et al., 2018). These functionally active protein sections are likely ubiquitous across enzyme classes (Bromberg et al., 2022). (2) Another possibility is that some

proteins may be multifunctional, i.e. evolved to carry out different functions across different segments (Babbitt et al., 1996; Singh & Bhalla, 2020). (3) In the same vein, a read could be *part of multiple genes* encoding different regions of multiple proteins. The prokaryotic genome is densely packed, with an average of 88% of the genome encoding proteins (Chaumeil et al., 2019). In our analysis, as much as 7% of randomly generated reads could be expected to be shared by multiple genes (**Methods**).

REBEAN labels complete genes better than reads. We further tested REBEAN's ability to label enzyme classes of complete genes on the basis of all corresponding read predictions. That is, we assigned each protein in our **SPEnzset1** (**Table 1**) the average score across the corresponding reads. Compared to read-based annotations, our model attained a much higher average AUROC of 0.89 (range: 0.86-0.97) and was able to recover 48% of the enzymes at 90% precision (**Figure 2C & D**).

We observed a strong positive correlation between the number of enzymes correctly classified and the number of reads sampled per gene. For example, the number of genes labelled correctly at 90% precision went up from 30% to 65% when the number of sampled reads increased from one to 50 reads per 1,000 basepairs of a gene. This finding strongly suggests that REBEAN would perform better for samples sequenced at higher depths (**Figure S2**).

We also assessed REBEAN's ability to predict non-enzymes in our **SPset** (**Table 1**) – a problem at read level. Here, a whole protein could only be predicted to be non-enzymatic if all its reads were predicted non-enzymatic. REBEAN attained an AUROC of 0.83, in differentiating enzyme reads vs. putative non-enzyme reads, which is somewhat lower than its performance for labelling protein enzyme classes (~0.89; **Table S1**). There are two potential explanations for this result: (1) some of the putative non-enzymes, as per their lack of annotation in SwissProt, may eventually be labelled as enzymes and (2) unlike enzyme classes, non-enzymatic proteins do not belong to a single class.

REBEAN captures sequence fragments of functional importance. We investigated the biological relevance of REBEAN read prediction scores by exploring the distribution

of catalytic and binding residues covered by these reads. We extracted the annotated functional site (catalytic and binding) residues from SwissProt proteins covered by our synthetic reads in **SPEnzset2** (UniProt, 2023). We observed an enrichment of functional residues in reads with higher REBEAN scores. Reads predicted with a high prediction score, i.e. achieving 90% per-class precision (**Figure 2**), were 50% more likely to contain catalytic site residues than others (**Figure 3A**).

As function site annotations in databases are often incomplete, we further investigated the spatial distribution of read-encoded amino acid residues in the vicinity of the functional sites in protein structures. We calculated the minimum distance from the read-encoded amino acids to the structurally closest functional site residues. The calculated distance for each read was normalized across all reads from a given gene to account for variable gene lengths. Again, we observed that translations of high scoring reads were significantly closer in 3D space to functional residues (**Figure 3B**).

REBEAN identifies orthologs without relying on sequence similarity. We extended our analysis of REBEAN embeddings to compare reads sampled from OrthoDB (Kuznetsov et al., 2023) genes, representing orthologous and non-orthologous gene pairs across different taxonomy levels, i.e. genus, family, order, class and phylum (**Orthoset1**, **Table1; Methods**). For each gene pair, we calculated the average cosine similarity between read embeddings from each of the two genes (**Eq. 10**). As expected from our earlier work (Hoarfrost et al., 2022), we observed a significant distinction in embedding similarity between reads of orthologous vs. non-orthologous gene pairs at all five taxonomic levels (p-value < 1E-32; **Figure 4**). We also noted a small but significant drop in embedding similarity between reads from orthologous gene pairs in successive taxonomic levels from genus to phylum (p-value < 4E-4 - 1E-102).

We further asked the reverse question: given a particular average read embedding similarity, is the gene pair more likely to be from an orthologous or a non-orthologous set? Embedding similarities were accurate (74.6%) in making this identification (AUROC= 0.84, AUPRC=0.86; **Table 2**) – a performance significantly better than random (50%) or that of MMseqs-derived (Hauser et al., 2016) sequence

identities of gene pairs (50%). As expected, we again observed better performance for lower taxonomic levels; e.g. for gene orthologous pairs at genus level, the accuracy was 79.6% (AUROC=0.88, AUPRC=0.90), while at phylum level the accuracy dropped to 70% (AUROC=0.81, AUPRC=0.82). Note that REMME (instead of REBEAN) embeddings attained a similar performance at this task.

Instead of focusing on whole genes/proteins, we aligned a subset of reads (**Orthoset2**) to find the correlation between read sequence identity (**Methods**) and REMME embedding Euclidean similarity (**Eqn. 11**) of reads was comparably low for read pairs of (1) the same gene (Pearson ρ=0.08), (2) different genes (0.35), (3) genes of the same orthologous group (0.09-0.16 across taxa), or (4) genes of different orthologous groups (0.14-0.30). In comparison, REBEAN read embedding similarity were indifferent to sequence identity regardless of gene pairs ($|\rho| < 0.07$). In other words, REBEAN read embeddings carried substantially more information than sequence identity alone.

That is, fine-tuning our models to predict enzymatic function further reduced the correlation between embedding similarity and sequence identity. That is, REMME (pretrained LM) read embedding similarities were more sequence-driven than REBEAN (fine-tuned for EC prediction) read embedding similarities. REMME is a DNA language model trained to encode information embedded in sequence and hence is expected to capture sequence identity. However, tuning the model to predict function loosens the need for explicit sequence representation.

REMME is thus useful in sequence encoding for, among many applications, identifying coding regions and estimating the likelihood of a given DNA read coming from a specific genome (**Figure S3**). In contrast, REBEAN is useful for exploring enzymatic functions encoded in read collections, e.g. metagenomes.

REBEAN can be used to discover novel enzymes from metagenomes. REBEAN's ability to capture function without requiring sequence similarity can help in identifying proteins that carry out known functions in novel ways. Here, we aimed to evaluate REBEAN's ability to mine potential novel oxidoreductases from a metagenomic set of

reads. Oxidoreductases are broad class of enzymes that catalyze redox reactions by facilitating transfer of electrons. Oxidoreductases are ubiquitous as they are part of every biological energy production mechanism (Hay Mele et al., 2023; Kim et al., 2013) and are likely to have appeared on the scene early in history of life on Earth (Bromberg et al., 2022).

For this analysis, we compiled a synthetic read dataset randomly sampled from the assembled sequences of metagenome-assembled genomes (MAGs) in MGnify (Richardson et al., 2023) (Gurbich et al., 2023) (**MarineMGset**, **Methods**). REBEAN predicted that approximately 10% (12,861,390) of these reads as enzymatic with a high confidence score of >0.9; of these, a quarter (3,019,892 reads) were deemed oxidoreductases (**Figure S4**). Note that almost all (98.7%) of the predicted oxidoreductase reads mapped to MGnify-labelled genes (1,126,995 genes) (Richardson et al., 2023), i.e. this prediction rarely tagged non-coding regions.

In search for truly novel enzymes, we retained only the reads that mapped to genes without any existing MGnify annotations (COG, EC, KEGG, Pfam); due to MGnify filters, for many of the MAG genes the corresponding protein sequences and annotations, were not available. This analysis retained a third of the genes (407,428 genes; 670,637 reads) distributed across all 3,820 MAGs. We further analyzed the available protein sequences encoded by these genes (275,290 of 407,428).

We extracted Pfam domains of these sequences using HMMER (Eddy, 2011). Curiously, although MGnify was missing this annotation for 234,025 proteins, our analysis revealed their 6,883 unique Pfams (E-value < 1E-3, excluding 1,926 DUFs), collectively occurring 740,053 times. To evaluate the significance of Pfam assignments in predicting enzymatic activity — particularly oxidoreductase function — we compared the Pfam distribution within our dataset to that of oxidoreductases in SwissProt (**Table S2**). We observed a significant enrichment of Pfams associated with SwissProt-annotated oxidoreductases in our dataset (Odds ratio = 2.06, Hypergeometric p-value < 1E-32; **Methods**).

We clustered (Hauser et al., 2016) the 275K protein sequences at 30% sequence identity, yielding 51,326 representative sequences. Aligning these against SwissProt (≤30% sequence identity), we identified 39,617 putatively novel proteins. For 32,030 of these proteins (length 100 to 800 residues), we generated structure predictions using ESMFold (Lin et al., 2023). Pfam oxidoreductase analysis, as above, confirmed our predictions (Odds ratio = 2.43, **Table S2**). Note that these proteins were classified based on the prediction of a single-read with oxidoreductase activity. Integrating predictions from all reads corresponding to a protein previously demonstrated higher accuracy (**Figure 2C & D**). Using this refined approach here, we identified 4,901 proteins predicted to function as oxidoreductases at a 90% precision threshold. These proteins exhibited even greater enrichment in oxidoreductase-associated Pfams (Odds ratio = 3.66). These results suggest that REBEAN can, no reference needed, reveal novel proteins that carry out known functions.

To examine the structural similarity of the read-based group of REBEAN-identified proteins (32K) with existing oxidoreductases, we aligned their structures against the PDB database using FoldSeek (van Kempen et al., 2024) to find that 464 (1.4% of the structures) matched 1,698 PDB entries (TM ≥ 0.9). Roughly half (938) of these PDBs lacked an EC annotation. Among the remaining 760 enzymes, half were classified as oxidoreductases (EC 1; 373 structures, 49% of enzymes), confirming REBEAN's ability to label enzyme classes.

The remaining enzyme matches comprised 143 transferases (19%; EC 2), 128 hydrolases (17%; EC 3), 28 lyases (4%; EC4), 86 isomerases (11%; EC 5) and 2 ligases (EC 6). While these matches suggest REBEAN's error in function labelling, additional considerations may be at play. For example, the top two non-oxidoreductase EC classes matched by this alignment were 2.7.7.7 (DNA polymerase, 42 structures) and 5.3.1.5 (Xylose Isomerase, 31 structures). DNA polymerase serves a critical but a non-redox function. However, these enzymes, like oxidoreductases, bind cations and nucleotides – a functionally crucial activity (Hay Mele et al., 2023; Selles Vidal et al., 2018; Steitz, 1999). EC class 5.3.1.5 is also like EC 1's; it is an isomerase subclass of Intramolecular oxidoreductases (EC 5.3) that carries out oxidation and reduction within

a molecule. We thus suggest that, at least in this case 19% (72 of 387) of the REBEAN errors may be driven by functional similarity of enzyme regions due to evolutionary and/or molecular activity constraints. For other putative false positive labels while some overlap with other enzyme classes may indeed be an error, it is also possibly a consequence of the evolutionary promiscuity and functional crossover observed among enzymes (Martinez Cuesta et al., 2015).

Interpreting REBEAN predictions through Embeddings. What did the model learn? To answer this question, we computed read-pair Euclidean similarity (**Eqn. 10**) and cosine similarity (**Eqn. 11**) between confidently predicted reads with REBEAN score above 0.90. For both metrics, we then computed the average similarity (**Eqn. 12-13**) of each read with other reads in our dataset of 54,115 reads generated from 4,295 enzyme genes (**SPEnzset2**, **Table 1**). Note that both cosine and Euclidean similarity metrics maintained similar trends, so from here on we will only describe the Euclidean similarity results.

The average similarity of a read with other reads sampled from the same gene was 0.64±0.06 and varied widely from 0.37 to 1.0 (**Figure 5A**) indicating contextual differences of various gene regions. On the other hand, similarities of read embeddings from different genes were only slightly lower (0.59±0.03), irrespective of their EC class (range: 0.30 to 0.64). These observations reinforce the conclusion that much of any given gene sequence is likely shared between enzymes for e.g. structural or evolutionary reasons, with only a small fraction being specific to a particular function.

Similarity between overlapping reads within a gene was higher than that of non-overlapping reads (**Figure 5A**), i.e. as expected, reads that share a portion of the same exact sequence within a gene tend to have higher embedding similarity. Note that, as we had demonstrated with the ortholog dataset, we did not observe any correlation between embedding similarity and sequence identity of read pairs from different genes (Hauser et al., 2016). Together, these observations suggest that the model indeed learned functional, rather than solely sequence-based, signatures encoded in genomic data. We thus probed deeper into the question: how much function did the model learn?

We computed the embedding similarity between all pairs of reads within same EC class at first, second, and third EC levels (e.g. EC 1.*, 1.1.*, and 1.1.9.*; **Figure 5B**). At all three levels, reads from same enzyme classes were more similar than reads from different classes, although the difference was more pronounced for higher EC levels (first>second>third level). Note, however, that the embedding similarities were higher within lower EC levels (first<second<third). The average similarity varied across enzyme classes (EC1-7), with translocase (EC 7.*) reads tended being least similar. This observation is in line with the fact that this class comprises subgroups that were historically part of EC designations of oxidoreductases, hydrolases, and lyases (McDonald & Tipton, 2023).

Comparing REBEAN predictions with other annotators. We compared REBEAN predictions for 8 million reads from 19 metagenomic samples (**Table 1: ExtremeMGset**) from three aquatic environments (hydrothermal vents, hypersaline and salt crystalizer ponds) against annotations from existing methods, i.e. mi-faser, HUMAnN, and MGnify (pipeline v5 and v4.1) (**Methods**) (Beghini et al., 2021; Zhu et al., 2018). The selection of extreme environments for this evaluation is in line with the desire for novel function discovery. Furthermore, these environments provide a microbial diversity profile distinct from those typically represented in reference databases, which are typically used by alignment-based annotation tools. While very precise (~90% precision), mi-faser was only able to label 1.9% of the reads as stemming from enzyme-coding genes (in line with its reported low recall, **Figure 6A & B**). On the other hand, REBEAN annotated all reads but only a fraction of these confidently. For example, at a very high threshold of confidence (0.90), REBEAN annotated 10.4% of the reads; note that this loss in coverage is expected to be accompanied by a gain precision. Among the three environments from which the metagenomes were sampled, salt crystallizer ponds microbiome was the least well annotated by both methods, with 0.27% and 8.8% of reads captured by mi-faser and REBEAN, respectively.

Devoid of a ground truth for comparisons, we computed Pearson's correlation and L1 norm distances between fractions of reads from each EC class to assess the

method agreement. All annotation methods assigned somewhat similar (correlation>0.518) fractions of EC classes to each of the metagenomes. REBEAN predictions, however, were much better correlated with MGnify annotations, than mi-faser and HUMAnN predictions (0.698 vs. 0.637 and 0.518, respectively; **Figure 6C**). REBEAN annotations also agreed with mi-faser (0.844) better than with HUMAnN (0.561). This is not unexpected as REBEAN was fine-tuned on mi-faser annotations but may also indicate the difference between per read and RPK prediction units. In addition to the correlation scores, we computed the L1 norm (Manhattan distance) between tools across all the samples (**Figure 6C**). In this comparison, REBEAN had the lowest distance from all other tools.

Addressing the need to step up tool development. Accurate functional annotation of metagenomes is an important step in understanding microbial communities. However, discovery and identification of novel functions in metagenomic data requires innovative approaches that transcend traditional homology-based methods (Prabakaran & Bromberg, 2025). For metagenomic tools to effectively aid in discovery, they must be both robust and highly accurate.

Recent advances in language model development have accelerated the field of protein structure and function prediction (J. Jumper et al., 2021; Lin et al., 2023) and we believe that a similar leap in metagenomic functional annotation is essential.

We believe that tools like REMME and REBEAN can pave the way for this transformation. By capturing biologically relevant insights, REBEAN has shown considerable potential for the discovery of truly novel, i.e. sequence and structure dissimilar, means of carrying out enzymatic activity. This capability is crucial as the field moves toward exploring the uncharted territory of novel enzymes.

Conclusion

The increasing accumulation of metagenomic samples presents both new challenges and opportunities for tool development. To address this, we have developed REMME, a robust foundational DNA-language model tailored to capture and interpret biological context encoded within metagenomic reads. REMME's

versatility makes it valuable for diverse research applications including generating read embeddings for machine learning applications, clustering reads for comprehensive ecological and evolutionary studies and, also, building fine-tuned models like REBEAN for specific downstream tasks.

REBEAN, a method for predicting enzymatic functionality from read information alone, was trained using millions of diverse metagenomic reads. Unlike alignment-based methods, REBEAN can actually discover novel enzymatic sequences. Moreover, though never explicitly trained to do so, its predictions highlight functionally significant residues within a given read. Our extensive analysis highlights REMME's and REBEAN's potential for metagenomic read annotation and the discovery of novel enzymes – a route in metagenomic exploration that has not yet been attempted by other computational techniques.

MATERIALS AND METHODS

Model Development: REMME - Read Embedder for Metagenomic exploration.

Data. We obtained 1,496 genomic assemblies representing 1,496 prokaryotic species from the marine microbiome samples in MGnify (Richardson et al., 2023). From these assemblies, we extracted 72.9 million reads (72,966,774) by randomly sampling 20 reads per 1Kbp with an average length of 136 bp (60-300 bps), as described in (Hoarfrost et al., 2022). The fraction of coding region residues in each read, along with the read position relative to the start codon were noted. Of these ~73 million reads, 65.1M and 7.8M reads were from coding and non-coding region, respectively. We randomly selected 20% (14.6M) of these reads for testing (13M and 1.6M of coding and non-coding reads, respectively) and used the remaining 58 million for training.

We clustered training and test datasets at 80% sequence identity using MMseqs (Hauser et al., 2016) to obtain 45.2M and 8.4M sequence-dissimilar representatives, respectively. Further, the training representatives were split into training (70%) and validation (30%) sets for pre-training.

Training. REMME is an encoder-only transformer model, holding six transform layers with eight multi-attention heads. Each input DNA sequence was transformed into a

sequence of overlapping nucleotide triplets (tokens) with a stride of one nucleotide. The tokens were fed into a token embedder that encodes each token in the sequence as a numeric vector of length 128 and a position embedder that encodes the sequence position of each token as a vector of length 128. The combined encoding from token embedder and position embedder was fed into the encoder module containing six encoder layers with eight multi-head attention heads. We had used GELU activation along with a drop out of 0.10 throughout the model everywhere, unless specified otherwise. The total number of trainable parameters was 3,520,561.

The encoder model was trained to perform masked-token prediction. Here, 15% of the nucleotides to the encoder input were perturbed, similar to BERT (Devlin, 2018) model training (80% masked and 10% random). The transformed read embeddings from the encoder module were then fed into three modules: 1) a *decoder* module, of three linear layers reconstructing the original nucleotide triplet sequence, 2) a *regression* module, comprising a 2d Fractional Max pooling layer, combined with two dense layers to predict the fraction of coding/non-coding residues in the read, and 3) a four-class *classifier* module, predicting the reading frame of the coding DNA sequence (CDS) that overlaps with a given read. The four classes represent three reading frames (classes 1, 2 & 3) and the non-transcribed reads (class 4). The losses from all three modules were summed and back propagated during training. The model was trained for 53 epochs until no significant decrease in total loss was observed.

Model Development: REBEAN - Read Embedding Based Enzyme Annotation.

Data. We collected 306 studies containing 19,316 diverse metagenomic samples of non-viral origin from the recent update to the SRA database (20230627) (Katz et al., 2022). Among these, 3,136 metagenomic samples belonging to microbiomes associated with soil, water, coastal water, sea water, oligotrophic water, deep marine sediment, phyllosphere, algal, and anthropogenic material, we randomly selected 40 SRA experiment runs from each of the nine environments, yielding 360 runs representing 332 samples and containing 267.3M reads (**Appendix A**).

The mi-faser method (Zhu et al., 2018), using 41,640 enzyme sequences as reference, was used to annotate these metagenomic reads with an enzymatic activity assigned to their putative 'parent' genes, i.e. gene sequences from which the read is taken. The enzyme reference set comprised genes (extracted from NCBI RefSeq (O'Leary et al., 2016)) encoding Swiss-Prot proteins with Enzyme Commission (EC) number annotations and experimental evidence of protein existence (Sayers et al., 2024; UniProt, 2023). Note that only a third (14,229) of these enzymes had experimental evidence of their enzymatic activity. Mi-faser annotated 59.5M of 267.3M reads (22%) as belonging to enzymes. We extracted reads of length 60 to 300 bps and clustered them, at 80% sequence identity using MMseqs, to retain 16.6M (16,624,341) and 112.3M (112,334,039) sequence-dissimilar reads of enzymatic and non-enzymatic origin, respectively. To create a balanced class distribution for training, we randomly sampled 2.4 million non-enzymatic reads – a number representative of the average number of reads in each EC class. Final composition of the 19 million read dataset was 17.7% of oxidoreductase (EC 1), 25.9% of transferases (EC 2), 11.3% of hydrolases (EC 3), 8.8% of lyases (EC 4), 5.8% of isomerases (EC 5), 10.5% of ligases (EC 6), 7.6% of translocases (EC 7), 12.5% of non-enzymes. We held out 3.8M (20%) of the reads as a test dataset and split the rest of the data (15.2M) into training (13.7M, 90%) and validation (1.5M, 10%) without altering the proportion of each enzyme class.

Training: REBEAN is a version of REMME that was fine-tuned to label reads that come from 'parent' enzyme-coding genes. REBEAN consists of the six encoder layers from REMME coupled with one classifier module comprising three dense layers. The classifier annotates a given read as being class one through eight, representing seven first level EC classes and non-enzymes, annotated by mi-faser as described above. We trained REBEAN using an ADAMW optimizer and cosine restarts scheduler. The model was trained for 188 epochs until no significant loss decrease was observed.

Model Evaluation

Performance measures. We used multiple standard metrics such as accuracy, recall, precision, specificity, F_1 score, balanced accuracy, mean squared error (MSE) and mean absolute error (MAE) (**Eqn. 1-8**) to evaluate predictive performance of REMME

and REBEAN in training and several independent analyses. We calculated the MSE (**Eqn. 2**) and the MAE (**Eqn. 3**) to quantify the difference between "ground truth" (f) and predicted coding fraction (f^p) of reads. We also computed the Area Under the Curve (AUC, **Eqn. 9**) for Precision-recall (PR-AUC) and Receiver operating characteristic (ROC-AUC) curves (Fabian et al., 2011).

$$Accuracy = \frac{Correctly\ predicted\ positives\ and\ negatives}{Total\ number\ of\ positives\ and\ negatives} \quad \textbf{(Eqn. 1)}$$

$$MSE\left(f_i, f^p{}_i\right) = \frac{1}{no.\ of\ reads}\sum_{i=1}^{no.\ of\ reads}\left(f_i - f^p{}_i\right)^2 \quad \textbf{(Eqn. 2)}$$

$$MAE\left(f_i, f^p{}_i\right) = \frac{1}{no.\ of\ reads}\sum_{i=1}^{no.\ of\ reads}\left|f_i - f^p{}_i\right| \quad \textbf{(Eqn. 3)}$$

$$Recall = \frac{Correctly\ predicted\ positives}{Total\ number\ of\ positives} \quad \textbf{(Eqn. 4)}$$

$$Precision = \frac{Correctly\ predicted\ positives}{Total\ number\ of\ predicted\ positives} \quad \textbf{(Eqn. 5)}$$

$$Specificity = \frac{Correctly\ predicted\ negatives}{Total\ number\ of\ negatives} \quad \textbf{(Eqn. 6)}$$

$$F_1\ score\ = \frac{2\ (\ Precision \times\ Recall\ )}{Precision + Recall} \quad \textbf{(Eqn. 7)}$$

$$Balanced\ accuracy = \frac{Recall + Specificity}{2} \quad \textbf{(Eqn. 8)}$$

$$AUC_{macro} = \frac{1}{C}\sum_{i=1}^{C} AUC(i) \quad (\textbf{Eqn. 9})$$

Embeddings similarity between reads. We used cosine similarity and Euclidean similarity for comparing read embeddings (**Eqn. 10-11**). Apart from the inherent differences in similarity distributions, our inferences were consistent across these metrics. For clarity, we only show the results of Euclidean similarity unless specified otherwise.

$$S_{Euclidean}(E1,\ E2) = \frac{0.5}{0.5 + \sqrt[2]{\sum_{i=1}^{l}\left(E_1{}^i - E_2{}^i\right)^2}} \quad (\textbf{Eqn. 10})$$

$$S_{Cosine}(E1,\ E2) = \frac{E_1.E_2 + 1}{2} \quad (\textbf{Eqn. 11})$$

In several comparison, we computed embedding similarity of a read against a set of reads representing a gene, gene set, genome or metagenome. In these cases, we represent the multiple read-pair similarities as an aggregate score using mean and maximum statistics. For a read *k* compared against a set of *n* reads, the aggregate score is defined using **Eqn. 12 and 13**. **Eqn. 12** depicts the average embedding similarity of the read *k* against the read set representing a gene or genome. **Eqn. 13** represents the embedding similarity with the best-aligned read in the read set.

$$\langle CS\ (R_k)\rangle = \frac{1}{n}\sum_{i=1}^{n} S_{Cosine}(E_k,\ E_i) \qquad \textbf{(Eqn. 12)}$$

$$CS_{max}\ (R_k) = \ max\ \{S_{Cosine}(E_k,\ E_i)\ : i = 1, \dots\ , n\ )\} \qquad \textbf{(Eqn. 13)}$$

Genes-to-synthetic reads. For multiple analysis, we generated synthetic reads from gene sequences to simulate metagenomic samples (**Table 1**). Unless otherwise specified, we randomly sampled, with no restrictions on overlap or coverage, ten gene fragments of length 200 nucleotides per 1Kbp of a gene. For example, for a gene of length 2Kbp, the number of reads sampled would be 20. This process of reads generation was also employed to generate reads from genomes.

UniProt annotated enzyme read dataset. From UniProt, we extracted 12,277 manually curated enzymes annotated with one unique EC number and experimental evidence of enzymatic activity (ECO:0000269) and mapped these to gene sequences using NCBI Entrez (Sayers et al., 2024; UniProt, 2023). Of these, we selected the 4,295 enzymes belonging to prokaryotes and archaea. These included 1,096 (EC 1), 1,300 (EC 2), 785 (EC 3), 509 (EC 4), 315 (EC 5), 216 (EC 6), and 74 (EC 7) enzyme sequences. We further generated a dataset of 525,775 reads by randomly sampling 100 reads of length 200 bp per 1Kbp of a gene (as described in section **Genes-to-synthetic reads).** Each read was labelled with the 1st level enzyme class of the parent gene. Among the half million reads, 129,111 (24.6%), 160,065 (30.5%), 95,638 (18.2%), 60,325 (11.5%), 35,476 (6.7%), 34,365 (6.5%) and 10,795 (2.1%) were annotated as EC class 1 to 7, respectively, based on the parent gene. This dataset of 545,775 reads (**SPEnzset1**) was used to access REBEAN's prediction performance. The performance statistics were computed by sampling 10% of the reads over 100

independent iterations to simulate a read sample of 10 reads per 1Kbp. We also created additional read datasets by varying the sampling rate (1, 2, 5, 10, 20, 50 and 100 reads per 1Kbp) and read length (50, 100, 150, 200, 250, 30 and 400 bp) to assess the influence of sequencing depth and other parameters.

SwissProt annotated enzyme versus unannotated proteins. To evaluate REBEAN's ability to differentiate enzymes and non-enzymes, we collected gene sequences of 473,854 SwissProt proteins, as described above. Among these proteins, 231,203 were annotated as enzymes based on *in silico* or experimental evidence, while the remaining 242,651 were unannotated. From these 473 genes, we generated 5,305,894 synthetic reads of 200 bp in length (**SPset)**, including 2,965,689 reads derived from the 231,203 enzyme-annotated genes.

Ortholog dataset. We randomly selected 1,000 different ortholog groups (OGs) from OrthoDB (Kuznetsov et al., 2023) at each of the five taxonomic levels: genus, family, order, class and phylum. For each OG, we randomly chose five genes and identified ten random ortholog gene pairs between them. We then formed ten non-ortholog pairs by pairing genes across different OGs of the same taxon. The final dataset comprised 65,169 unique genes which includes 24,938 genes in 50,000 orthologous gene pairs and 65,169 genes in 50,000 non-orthologous gene pairs. We mapped these 65,129 genes to EMBL CDS to obtain respective gene sequences. 707,947 reads (**Orthoset1)** were sampled from these 65,129 genes as described above (as described in section **Genes-to-synthetic reads)**.

Further, we selected a subset of 7,311 genes (79,783 reads, **Orthoset2**) from 100 ortholog groups for in-depth analysis of read pair embedding similarity. For the ~6.4B corresponding read pairs, we computed sequence identity using Smith-Waterman (Cock et al., 2009; Smith & Waterman, 1981) with match, mismatch, gap scores 1, -2 and -1, respectively.

Synthetic Metagenome Read dataset. We collected 3,820 marine metagenome-assembled genomes (MAGs) from MGnify (Gurbich et al., 2023) and generated 124 million genome fragments of 200bp length resembling metagenome reads

(**MarineMGset)**. We sampled ten reads per 1Kbp of a genome without restrictions on overlap. In addition to the genomes, we also retrieved corresponding coded protein sequences and annotations of EggNOG-mapper, HUMANN, HMMER, and KEGG mappings from MGnify (Eddy, 2011; Gurbich et al., 2023; Huerta-Cepas et al., 2019; Richardson et al., 2023; Seemann, 2014).

Additionally, we ran HMMER (Eddy, 2011) to assign Pfams to the putative oxidoreductases identified by REBEAN, with E-value threshold set at 1E-3. To assess the enrichment of oxidoreductase-associated Pfams in REBEAN-filtered proteins, we compared them to Pfams assigned to oxidoreductases in SwissProt (**Table S2**). Note Pfams of unknown function (DUFs) were excluded from this analysis. Enrichment was quantified using the odds ratio, and statistical significance was determined using the Hypergeometric test (*Categorical Data Analysis*, 2002; Virtanen et al., 2020).

Metagenomes from extreme environments. We collected 803 million metagenomic reads from 19 samples (27 Runs) belonging to three extreme aquatic environments: hydrothermal vents, hypersaline and Salt crystallizer ponds from MGnify to compare REBEAN predictions against other annotation tools. We randomly selected 1% of the reads (8,030,102, **ExtremeMGset**) for our analysis and ran annotation tools: Humann and Mi-Faser (Beghini et al., 2021; Zhu et al., 2018). In addition, we also extracted existing GO annotations for CDSs from MGnify and mapped to EC numbers [GO2EC (Gene Ontology et al., 2023)].

Embedding visualization and statistical tests. We used TSNE for dimensionality reduction of the embedding space. TSNE 2D-projections were computed using (Policar et al., 2024) with following parameters: n_neighbors = 15, min_dist = 0.1 using *cosine* metric. Statistical significances were assessed through the Wilcoxon rank-sum test and Student's t-test using SciPy (Virtanen et al., 2020).

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Coda and Data availability

The models and supplementary scripts will be made publicly available on GitHub after acceptance. In addition, REBEAN is accessible as a free web service at https://services.bromberglab.org/rebean/.

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Figure captions

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Figure 2: REBEAN synthetic read annotation. We extracted read-sized gene fragments from 4,295 prokaryotic enzymes and labelled them using REBEAN. **(A)** ROC and **(B)** PR curves demonstrate the performance of REBEAN in annotating reads with level 1 EC classes. **(C)** ROC and **(D)** PR curves represent performance of REBEAN in annotating complete proteins as an as average score of multiple reads with level 1 EC classes. The performance at chosen threshold corresponding to 90% precision is marked with '*' on the curve.

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Tables

Table 1: List of sequence datasets used in analysis

Dataset name	Description	Type	Number of entries	Number of reads	Synthetic reads[1]	Read sampling rate (reads/kbp)
SPEnzset1	Reads from SwissProt Exp. Enzymes I	Gene	4,295	525,775	Yes	100
SPEnzset2	Reads from SwissProt Exp. Enzymes II	Gene	4,295	54,115	Yes	10
SPset	Reads from SwissProt Enzymes and Non-enzymes	Gene	473,854	5,305,894	Yes	10
Orthoset1	Reads from Ortholog genes from OrthoDB	Gene	24,938	707,947	Yes	10
Orthoset2	A subset of Orthoset1	Gene	7,311	79,738	Yes	10
MarineMGset	Synthetic Metagenome from MGnify MAGs	Genomes	3,820	124,269,382	Yes	10
ExtremeMGset	Extremophile metagenomes	Metagenome	27	8,030,102	No	-

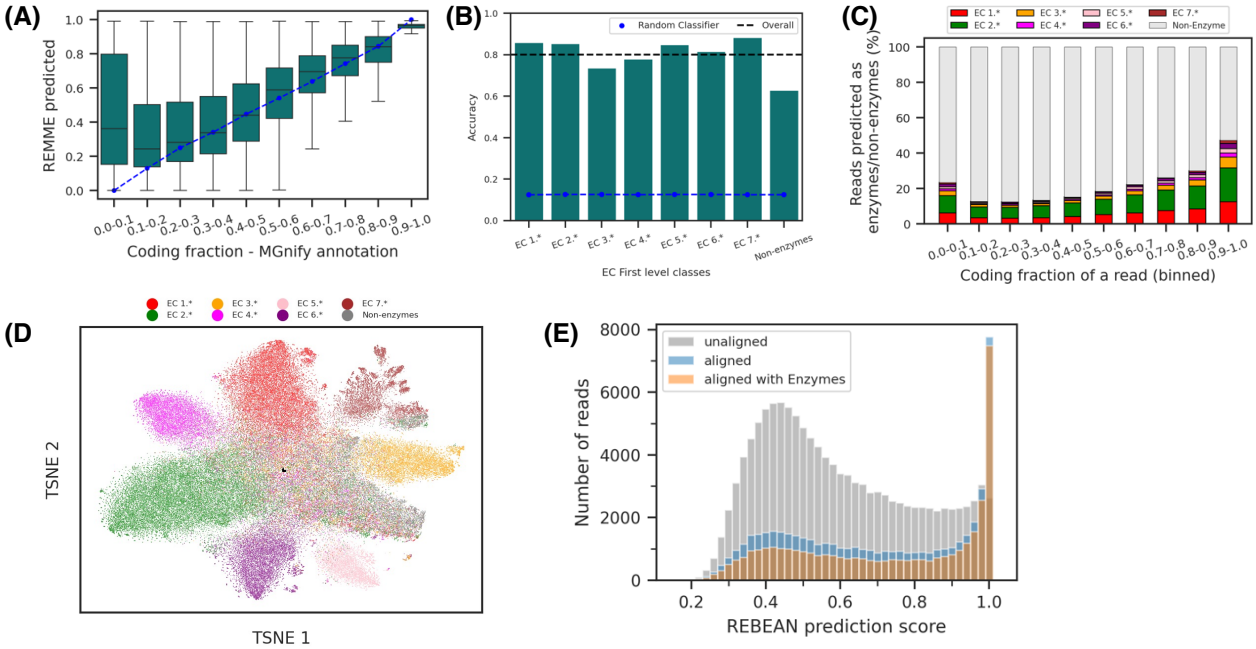
[1] Synthetic reads were sampled from gene and genomes sequences as described in the section “Genes-to-synthetic reads”.

Table 2: Predicting orthologs gene pairs across taxa

Taxa	Method	AUC ROC	AUC PR	F1 max	threshold[2]	Recall	Precision	Accuracy
Genus	REBEAN	0.880	0.895	0.799	0.707	0.830	0.770	0.791
Family	REBEAN	0.851	0.866	0.772	0.703	0.846	0.709	0.750
Order	REBEAN	0.854	0.869	0.773	0.706	0.810	0.739	0.762
Class	REBEAN	0.824	0.835	0.753	0.699	0.820	0.697	0.732
Phylum	REBEAN	0.808	0.82	0.742	0.693	0.861	0.651	0.700
All	REBEAN	0.843	0.858	0.766	0.701	0.837	0.705	0.744
All	Seq. Similarity	0.319	0.526	0.667	0	1	0.5	0.5

[2] The threshold corresponds to the maximum F1 score.

Figures



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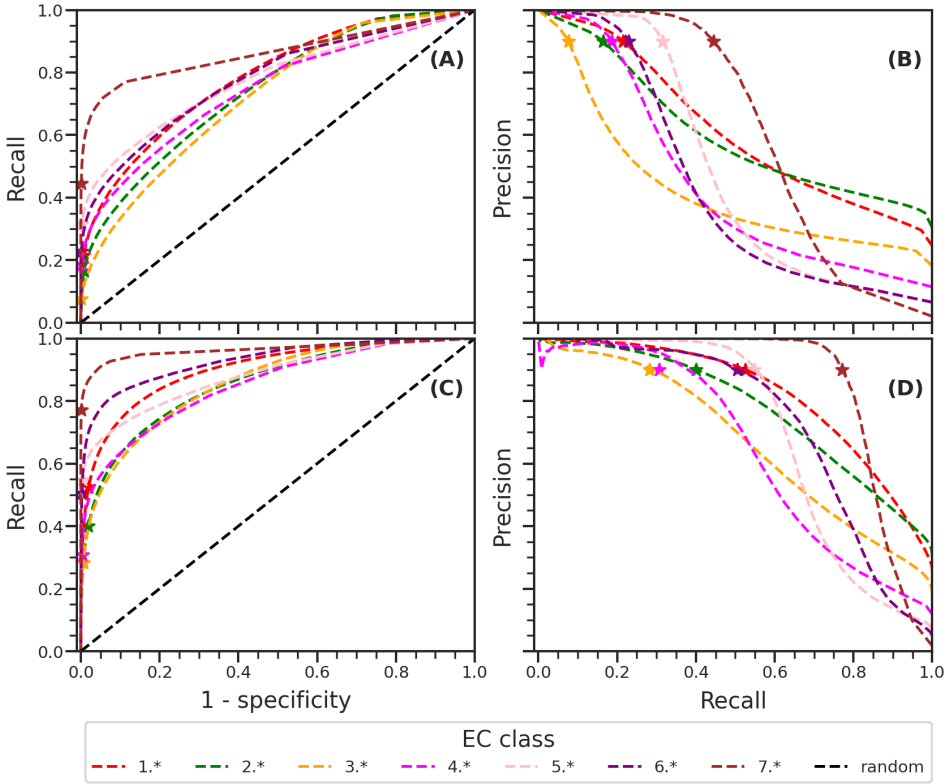

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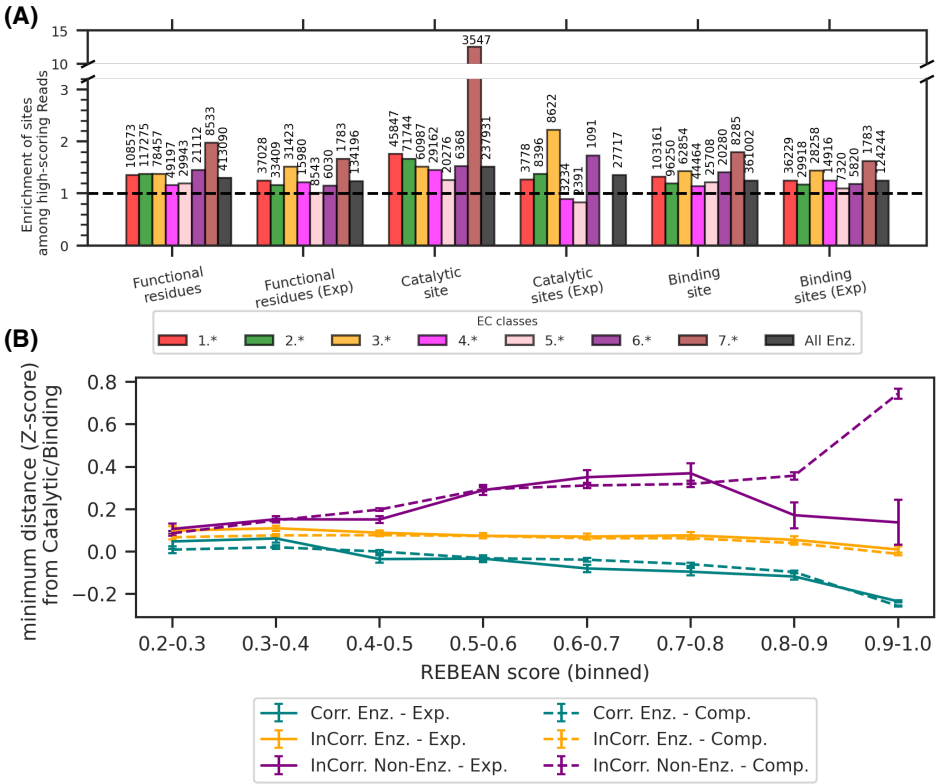

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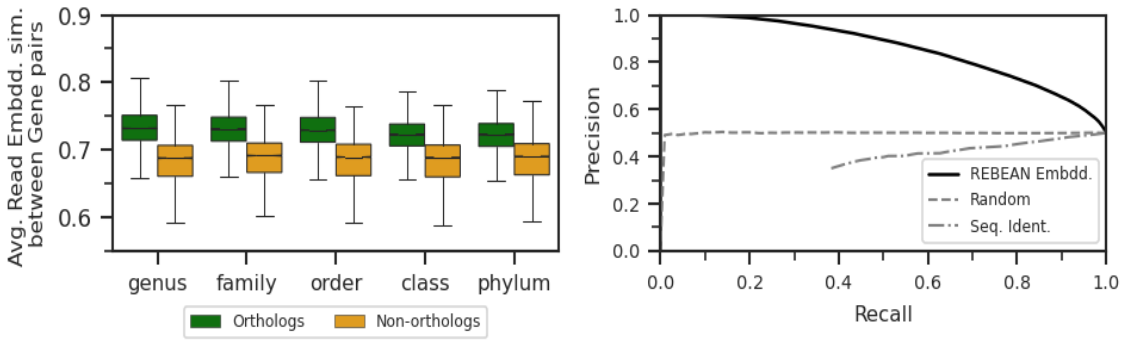

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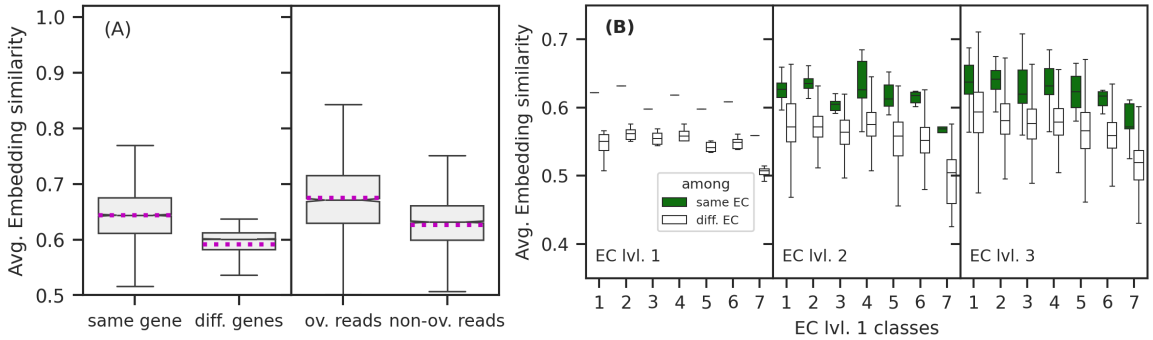

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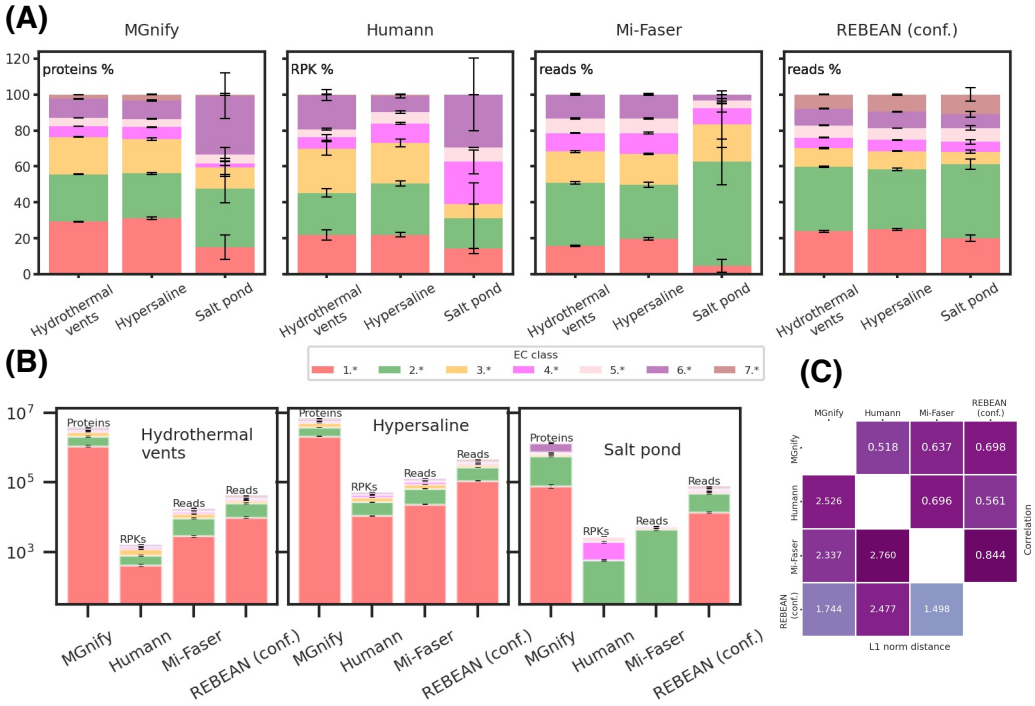

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Appendix A

ERR058011	ERR058011	ERR058014	ERR058017	ERR058017	ERR058019	ERR058021
ERR058022	ERR058023	ERR058023	ERR058024	ERR058024	ERR058025	ERR058026
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ERR058036	ERR058036	ERR058037	ERR058038	ERR058039	ERR058040	ERR058042
ERR058043	ERR058045	ERR058046	ERR058046	ERR058047	ERR058047	ERR058051
ERR058053	ERR058054	ERR058055	ERR058057	ERR058058	ERR205703	ERR205705
ERR205717	ERR205719	ERR205720	ERR2102868	ERR2102871	ERR2102875	ERR2102877
ERR2102883	ERR2102890	ERR2102891	ERR2102894	ERR2102900	ERR2102910	ERR2102916
ERR2102917	ERR2102919	ERR2102922	ERR2102931	ERR2102946	ERR2102946	ERR2102949
ERR2102951	ERR2102951	ERR2102956	ERR2102956	ERR2102964	ERR2102970	ERR2102971
ERR2102975	ERR2102977	ERR2102986	ERR2102987	ERR2102988	ERR2102989	ERR2102995
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ERR2103032	ERR2172154	ERR2172155	ERR2172165	ERR2172173	ERR2172173	ERR2172174
ERR2172174	ERR2172175	ERR2172185	ERR2172186	ERR2172206	ERR2172209	ERR2172215
ERR2172230	ERR2172243	ERR2172245	ERR2172245	ERR2172248	ERR2172248	ERR2172249
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ERR3415785	ERR3415786	ERR3415791	ERR3435075	ERR3435077	ERR3435081	ERR3435082
ERR3435091	ERR3435093	ERR3435094	ERR3435104	ERR3435105	ERR3435111	ERR3435112
ERR3435119	ERR3435128	ERR3525229	ERR3525233	ERR3525236	ERR3525238	ERR413154
ERR413168	ERR413176	ERR413179	ERR413196	ERR413201	ERR413203	ERR413216
ERR413218	ERR413224	ERR413227	ERR413254	ERR413254	ERR413261	ERR413265
ERR413277	ERR413286	ERR413291				