microbetag: simplifying microbial network interpretation through annotation, enrichment and metabolic complementarity analysis

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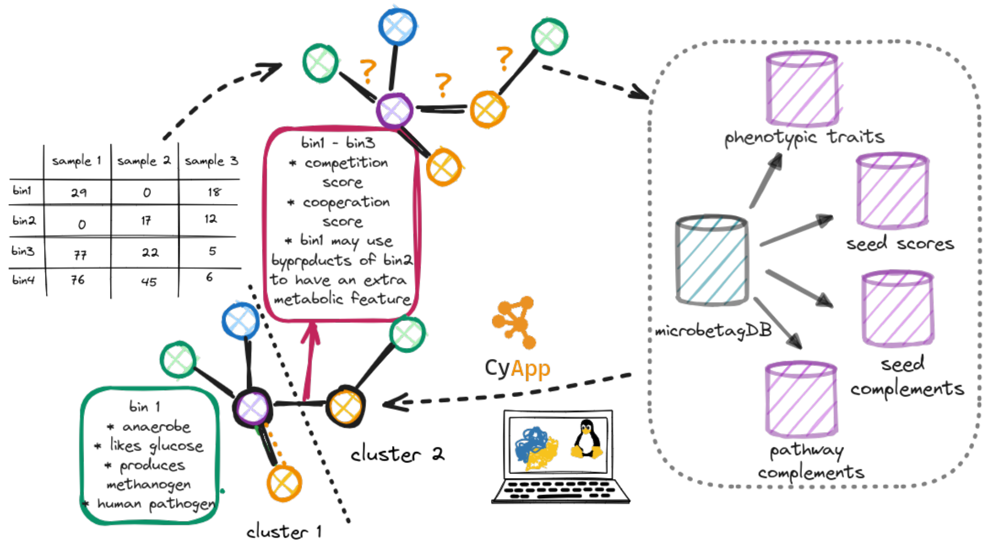
Abstract

Up to 350 words.
The abstract must include the following separate sections:
Background: the context and purpose of the study
Results: the main findings
Conclusions: a brief summary and potential implications



	sample 1	sample 2	sample 3
bin1	29	0	18
bin2	0	17	12
bin3	77	22	5
bin4	76	45	6

Figure abstract.

Keywords: microbial associations, enrichment analysis, data integration, pathway complementarity, seed set

Background

Microbial interactions play a fundamental role in the stability and resilience of ecosystems and their processes; from soils, aquatic environments and biogeochemical cycles [1] to host-associated environments and the human health [2, 3]. Most microbial species live only in communities [4] and most natural microbial communities consist of hundreds or even thousands of species [5]. Each species exhibits a unique repertoire of reactions and adapts to various niches, each with specific nutrient and environmental requirements.

Depending on the net fitness effects that result for the taxa involved, interactions range from cooperation, competition, parasitism, commensalism and amensalism [3]. Metabolic interactions can be established through a range of contact-independent and contact-dependent mechanisms leading to both positive and negative interactions. These interactions can involve either one-way (unidirectional) or two-way (bidirectional) exchanges of metabolites. Depending on the biosynthetic cost borne by the interacting partners, two types of metabolite exchanges occur: by-product cross-feeding, where metabolites result from a selfish act of the producer, and cooperative cross-feeding, where one partner actively invests resources to produce metabolites benefiting the interaction partner [6].

High-throughput sequencing (HTS) has provided insight into the diversity and composition of microbial communities [7]. Uncultivated species can now be detected, and their features can be inferred through their genomic information [8]. Moreover, the composition of thousands of microbiome samples is now accessible allowing for the inference of patterns among sets of samples. A widely used approach to extract such patterns is the creation of co-occurrence networks based on metagenomic read data (amplicon and/or shotgun) [9]. A number of approaches is available for co-occurrence network inference based on a range of statistical concepts such as: correlation (e.g., CoNet [10], SparCC [11]), linear regression (e.g., SpiecEasi [12]) and causal inference (FlashWeave [13]). Nevertheless, microbial co-occurrence networks continue to encounter various challenges [14]. Their inference inherits the challenges of metagenomic data analysis (e.g., compositionality, parameters inference) [15]. As a consequence, the result of network construction is tool-dependent [16, 17]. Moreover, more often than not, the returned network is a "hairball" of densely interconnected taxa. Thus, additional analysis is necessary to generate testable hypotheses [14]. Addressing the question of *What can we learn from the hairballs* posed by Röttjers et *al.* [4] could provide insight into the mechanisms of the interactions.

The assessment of interaction predictions derived from microbial co-occurrence networks has underscored their low accuracy for this task [18]. Theoretical principles derived from network studies might provide indications of emergent biological characteristics [4, 19]. For example, modules (highly interconnected nodes) within microbial co-occurrence networks could serve as indicators of ecological processes that govern community structure, including niche filtering and habitat preference [20]. Data integration and clustering have been suggested to address this challenge [14]. Clusters identified in microbial association networks have demonstrated their ability to mirror key drivers of community composition [21] and several algorithms and implementations are available [22]. However, data integration approaches in microbial co-occurrence networks are so far limited. Here, we present `microbetag`, a microbial co-occurrence network annotator that exploits several channels of information to enhance or reduce the confidence of the associations suggested by the network and generate hypotheses for further investigation both at the taxon pair and the community level.

`microbetag`serves as a comprehensive platform that provides information on taxa along with their potential metabolic interactions from multiple channels (see Implementation 3). The key concept here is the reverse ecology approach [23]. Reverse ecology leverages genomics to explore community ecology with no *a priori* assumptions about the taxa involved. The reverse ecology framework enables the prediction of ecological traits for less-understood microorganisms, their interactions with others, and the overall ecology of microbial communities [24].

A metabolic network's "seed set" is the set of compounds that, based on the network topology, cannot be produced by the organism and need to be acquired exogenously [25] (see Figure 2). Such seeds might be independent, i.e. they cannot be produced by any other biochemical reaction in the metabolic network, or they can be interdependent forming groups of seed compounds. Seeds are a useful proxy for the essential nutrients of an organism [25, 26]. Based on the seed concept, several graph theory-based metrics (indices) have been described to predict species interactions directly from their metabolic networks' topologies [27–30]. Over the last years, the seed approach has been implemented at the Genome-scale metabolic network reconstructions (GENREs) level. GENREs encapsulate mathematical representations capturing the biochemical reactions that could take place within an organism [31–33].

Metabolic complementarity among species reflects the potential for cooperation through cross-feeding or syntrophy. In contrast, metabolic competition refers to the metabolic overlap between two species leading to exploitative competition, e.g. for nutrient resources. Seed and non-seed compound sets can be used to compute complementarity and overlap indices. The examination of such indices can reveal metabolic interactions leading to patterns observed in a co-occurrence network.

However, microbial interactions go beyond metabolite exchange and include for instance, microbial species are recognized to exchange metabolites in order to provide support for other advantageous services, such as detoxifying harmful metabolites or offering protection against predators [34, 35]. They can additionally contribute to the production of metabolites essential for the entire community, even if the species itself does not require them [36]. To explore whether a species may benefit from a partner, it is helpful to check whether their pathways complement each other. We present here a naive approach exporting all possible complements between a pair of species based on their KEGG ORTHOLOGY (KOs) annotations and the KEGG MODULES database [37].

`microbetag`annotates a user's co-occurrence network by integrating phenotypic traits of the taxa present in the network (nodes) and by mapping potential metabolic interactions onto their associations (edges). `microbetag`includes a Graphical User Interface (GUI) implemented as a CytoscapeApp providing a user-friendly environment to investigate annotations in a straightforward way. All annotations present in microbetagDB are also available through an Application Programming Interface (API). `microbetag`'s source code is distributed under a GNU GPL v3 license and available on GitHub. Documentation and further support on how to use `microbetag`is available at documentation web-site. To the best of our knowledge there is not a software with which `microbetag`could be compared with directly. To validate our annotations, we used a recently published network with partially known interactions between some pairs of species found to be associated [38] (see Results section, paragraph 3). To demonstrate `microbetag`'s potential, we present the main features of its interface, and we discuss a real-world use-case (see Discussion section, paragraph 3).

Implementation [1]

Genomes included

Using the Genome Taxonomy Database (GTDB) v207 metadata files, we retrieved the NCBI genome accessions of the high-quality representative genomes, i.e., completeness $\geq 95\%$ and contamination $\leq 5\%$. A set of $26,778$ genomes was obtained, representing $22,009$ unique NCBI Taxonomy Ids. Using these accession numbers, we were able

[1] This should include a description of the overall architecture of the software implementation, along with details of any critical issues and how they were addressed.

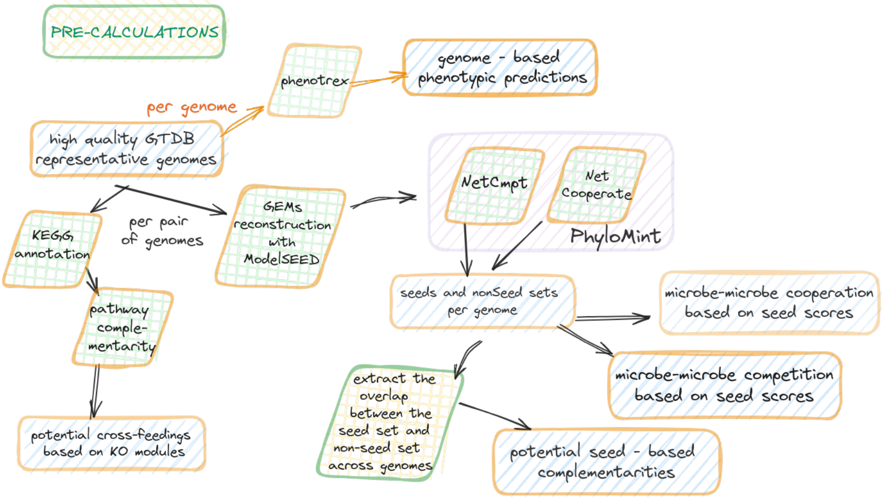

PRE-CALCULATIONS
phenotrex
genome - based phenotypic predictions
per genome
high quality GTDB representative genomes
NetCmpt
Net Cooperate
PhyloMint
per pair of genomes
KEGG annotation
GEMs reconstruction with ModelSEED
seeds and nonSeed sets per genome
microbe-microbe cooperation based on seed scores
pathway comple-mentarity
microbe-microbe competition based on seed scores
extract the overlap between the seed set and non-seed set across genomes
potential cross-feedings based on KO modules
potential seed - based complementarities

Fig. 1: Diagram of the `microbetag`pre-calculations. GTDB v207 representative genomes were filtered and for those of high-quality 33 phenotypic traits were predicted using `phenotrex`. To this end, models were re-trained to sync with the recent version of eggNOG.

to download their corresponding `.faa` files when available leading to a set of 16, 900 amino acid sequence files. The latter were annotated and used to obtain potential pathway complementarities between pairs of genomes (see paragraph 3). Last, when available, their corresponding annotations in the PATRIC database [39] were retrieved to reconstruct GENREs (see paragraph 3).

Taxonomy schemes

`microbetag`maps the taxonomy of each entry in the abundance table to their corresponding NCBI Taxonomy Id and, if available, their closest GTDB representative genome(s), since several GTDB representative genomes may map to the same NCBI Taxonomy Id. Two well established taxonomy schemes are supported: the GTDB [40] that is being broadly used for bins and/or MAGs taxonomic classification and the Silva database [41] that is widely used in amplicon studies. Both taxonomy schemes link their taxonomies to NCBI Taxonomy Ids [42]. In case neither of those two taxonomies was used, and the abundance table contains less than 1,000 taxa, `microbetag`maps the user provided taxonomies to the NCBI Taxonomy. To this end, `microbetag`makes use of the `fuzzywuzzy` library that implements the Levenshtein Distance Metric to get the closest NCBI taxon name and thus its corresponding NCBI Taxonomy Id; a high similarity score is used (90) to avoid false positives. Also, using the nodes dump file of NCBI Taxonomy, `microbetag`may retrieve the child taxa of a taxon in user's data, along with their corresponding NCBI Taxonomy Ids, if requested by the user. If the user provides their abundance table with taxonomies already mapped to the GTDB taxonomy, microbetagwill report the best possible annotations in a time efficient manner.

Network inference

When a co-occurrence network is not provided by the user, microbetagrelies on FlashWeave [13] to build one on the fly. Yet, microbetagsupports the annotation of networks built from any algorithm/software, in any format Cytoscape can load.

microbetagpre-processing

To aid the user to map their sequences to the GTDB taxonomy, DADA2-formatted 16S rRNA gene sequences for both bacteria and archaea [43] were used to train the IDTAXA classifier of the DECIPHER package [44] and are available through the microbetag preprocess Docker image. Likewise, when the abundance table consists of more than $1,000$ taxa, providing a network as an input is mandatory. Again, to help the user, the microbetagpreprocess Docker image supports the inference of a network using FlashWeave.

For a computationally efficient way to annotate large networks, a Docker image is provided, so the user runs a taxonomy assignment using the IDTAXA algorithm [44] of the DECIPHER R package [45]. A co-occurrence network is also built using FlashWeave [13], as microbetagalso does.

Literature based nodes annotation

Using a set of Tara Ocean samples [46] FAPROTAX [47] estimates the functional potential of the bacterial and archaeal communities, by classifying each taxonomic unit into functional group(s) based on the current literature, descriptions of cultured representatives and/or manuals of systematic microbiology. In this manually curated approach, a taxon is associated with a function only if all the cultured species within the taxon have been shown to exhibit that function. In its current version, FAPROTAX includes more than 80 functions based on 7600 functional annotations and covers more than 4600 taxa. Contrary to gene content based approaches, e.g., PICRUSt2 [48], FAPROTAX estimates metabolic phenotypes based on experimental evidence.

microbetaginvokes the accompanying script of FAPROTAX and converts the taxonomic microbial community profile of the samples included in the user's abundance table or of the taxa present in the provided network, into putative functional profiles. Then, it parses FAPROTAX's sub-tables to annotate each taxonomic unit present in the user's data with all the functions for which they had a hit. FAPROTAX annotations are not part of the microbetagDB but are computed on the fly.

Genomic based nodes annotation

phenDB [49] is a publicly available resource that supports the analysis of bacterial (meta)genomes to identify 47 distinct functional traits, e.g. whether a species is producing butanol or has a halophilic lifestyle. It relies on support vector machines (SVM) trained with manually curated datasets based on gene presence/absence patterns for trait prediction. More specifically, the model for a particular trait is trained using a collection of EggNOG annotated genomes where the knowledge of whether that trait is present or absent among its members is available. These models (classifiers) are used to predict presence/absence of their corresponding traits in non-studied species.

For microbetagDB, classifiers were re-trained using the genomes provided by phenDB for each trait to sync with the latest version of eggNOG [50] and the `phenotrex` [49] software tool. Genomes were downloaded from NCBI using the Batch Entrez program. Then, *genotype* files were produced for all the high quality GTDB representative genomes. Each model was then used against all the GTDB *genotype* files to annotate each with the presence or the absence of the trait. A list of all the phenotypic traits available for the genomes present in microbetagDB is available on `microbetag`'s documentation site. The updated models are also available

Pathway complementarity

To infer potential pathway complementarities, we consider the modules described in KEGG MODULES database [37]. A KEGG module is defined as a functional unit within the KEGG framework that represents a set of enzymes and reactions involved in a specific biological process or pathway [51]. Such a unit consists of several *steps*, each of which may have more than one molecular way to occur (Figure 2). A module's definition is a logical expression and consists of KOs that may be coupled with one another as: a. connected steps of the pathway b. parts of a molecular complex, c. alternatives of the same step, and d. optional entities of a complex. Both (a) and (b) cases should be considered as the `AND` logical operator, while (c) would be the `OR` (Figure 2). Given a module's definition, we will consider as an *alternative* any subset of the KO terms mentioned in the definition, that has exactly one way to perform each step, provided that all the steps of the module are covered. We define a genome as having a *complete* module, only if all the KOs of at least one alternative are present on it. In Appendix A we show an example of a module along with its alternatives.

Within this framework, `kofamscan` [52] was used to annotate with KEGG ORTHOLOGY terms (KOs) the $16,900$ high quality GTDB representative genomes for which a `.faa` was available [53]. The KOs of each genome were then mapped to their corresponding KEGG modules; a KO may map to more than one module ($1 : n$).

All module definitions were retrieved using the KEGG API and parsed to enumerate their alternatives. Each pair of the KEGG annotated genomes was then investigated for potential pathway complementarities, i.e., whether a genome lacking a number of KOs ($genome_A$) to have a complete module ($module_x$) could benefit from another's species genome(s) ($genome_B$). In that case, $genome_B$ does not necessarily have a complete alternative of $module_x$; as long as it has the missing KOs that $genome_A$ needs to complete an alternative of it, $genome_B$ potentially complements $genome_A$ with respect to $module_x$. In total, $341,568$ unique complementarities were exported.

Thanks to the graphical user interface (GUI) of the KEGG pathway map viewer [54, 55], each complementarity can be visualised as part of the closest KEGG metabolic map; where the KOs contributed by the donor are shown in blue-green whereas those coming from the beneficiary genome are coloured in red.

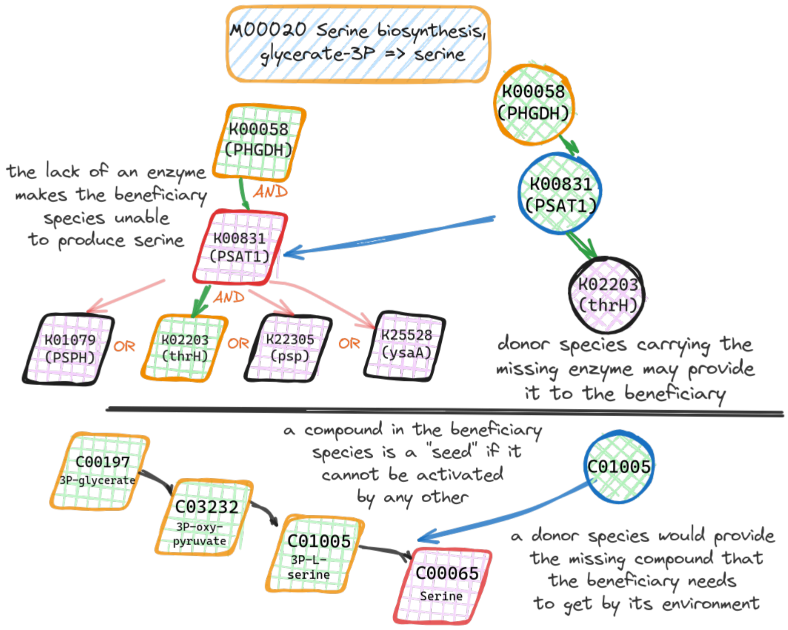

M00020 Serine biosynthesis, glycerate-3P => serine
K00058 (PHGDH)
AND
K00831 (PSAT1)
AND
the lack of an enzyme makes the beneficiary species unable to produce serine
K01079 (PSPH)
OR
K02203 (thrH)
OR
K22305 (psp)
OR
K25528 (ysaA)
K00058 (PHGDH)
K00831 (PSAT1)
K02203 (thrH)
donor species carrying the missing enzyme may provide it to the beneficiary
C00197 3P-glycerate
C03232 3P-oxy-pyruvate
C01005 3P-L-serine
C00065 Serine
a compound in the beneficiary species is a "seed" if it cannot be activated by any other
C01005
a donor species would provide the missing compound that the beneficiary needs to get by its environment

Fig. 2: Pathway complementarity approach. The high-quality GTDB genomes were annotated with KEGG ORTHOLOGY (KO) terms. The various ways of completing a KEGG module were enumerated and all the possible ways a donor species could ”fill” a beneficiary’s non-complete module were calculated. In this case, there are 4 unique ways to complete the serine biosynthesis module; in all of them K00831 is required. However, it is missing from the beneficiary species that supports two out of the three steps of the module’s definition. A donor species having and potentially sharing the corresponding enzyme of K00831 may enable the beneficiary species to produce serine.

`microbetag`annotates the edges of a co-occurrence network by identifying pairs where both taxa map to an annotated genome present in microbetagDB. Since co-occurrence networks are undirected, both nodes of a suggested association are considered as potential donors and beneficiary species. When more than one GTDB representative genome map to the same NCBI Taxonomy Id all the possible genome combinations are considered. Finally, two edges are added in such pairs of taxa in the annotated network: one considering $species_A$ as the potential beneficiary and $species_B$ as the potential donor species, and one vice-versa.

Seed scores and complements using genome scale metabolic reconstructions

The Metabolic Complementarity Index ($MI_{Complementarity}$) measures the degree to which two microbial species can mutually assist each other by complementing each other’s biosynthetic capabilities. As described in [56], it is defined as the proportion of seed compounds of a species that can be synthesized by the metabolic network of another but are not included in the seed set of the latter. $MI_{Complementarity}$ offers an upper bound assessment of the potential for syntrophic interactions between two species. Further, the Metabolic Competition Index ($MI_{Competition}$) represents the similarity in two species' nutritional profiles. This index establishes an upper limit on the level of competition that one species may face from another. Those indices have been described and implemented in the NetCooperate [27] and NetCompt [28] tools respectively. We will be referring to those two indices as "seed scores". Recently, the `PhyloMint` tool [56] was released supporting the calculation of the seed scores of GENREs in SBML format.

In the `microbetag`framework, seed scores were computed using GENREs derived from the high-quality GTDB representative genomes and the `PhyloMint` tool. GENREs were reconstructed using the Model SEED pipeline [57] through its Python interface ModelSEEDpy. The latter requires RAST annotated genomes [58]; if available through the PATRIC database [39], annotations were retrieved. For the rest of the genomes, RAST annotation was performed through RASTtk [59].

Moreover, the computed seed and the non-seed (i.e., metabolic compounds a genome can build on its own) sets of each genome were used to compute their overlap among all the pairwise combinations of those genomes. More specifically, seed and non-seed compounds of each genome were mapped to their corresponding KO terms and those related to any KEGG MODULE were considered further. Focusing on the KEGG MODULE - related KO terms as terms of interest, the overlap of $seed\ set_{species_A}$ with the $non\ seed\ set_{species_B}$ was retrieved. Such *seed complementarities* were calculated for all pairwise GENREs and are now available through microbetagDB. Edges of the co-occurrence network where both taxa have been mapped to at least one GTDB genome can be further annotated mentioning all the KEGG maps for which there is at least one seed compound of the potentially beneficiary species.

Clustering network

`manta` is a heuristic network clustering algorithm that clusters nodes within weighted networks effectively, leveraging the presence of negative edges and discerning between weak and strong cluster assignments. `microbetag`invokes manta [22] to cluster the microbial network. In case `manta` is performed, the annotated network inherits the layout that `manta` returns.

The `microbetag` workflow

As shown in Figure 3, the `microbetag` workflow expects an abundance table representing either amplicon or shotgun data. If a co-occurrence network is already available, the user may provide it too as input. The `microbetag` workflow will first map the taxa present on the abundance table to their corresponding GTDB representative genomes if that is possible, i.e., in case the taxonomy provided does reach the species or the strain level (see paragraph 3). If a network is not provided, `microbetag` will then build one using FlashWeave [13]. Then the abundance table will be used for a literature-based annotation using FAPROTAX [47]. This is the only annotation step that is microbetagDB independent within the web-service workflow. The nodes of the

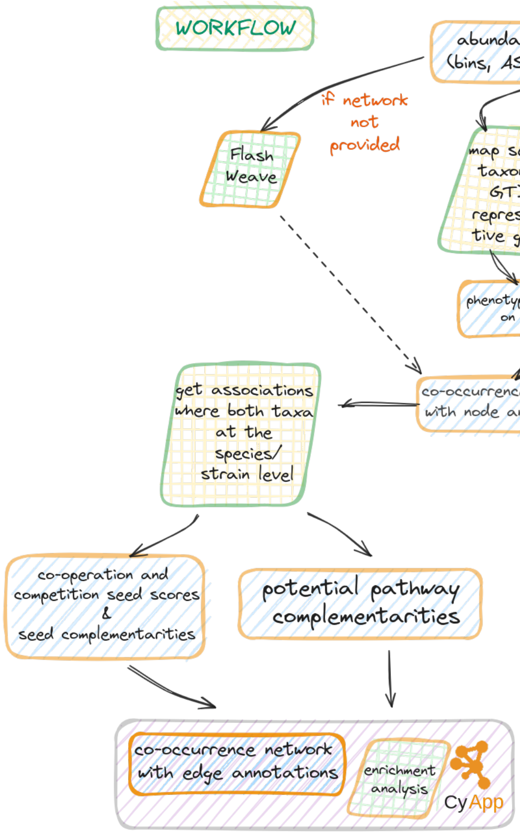

WORKFLOW
abundance table (bins, ASVs, OTUs)
if network not provided
assign
Flash Weave
map sequence/ taxon to GTDB representa-tive genome
FAPROTAX
phenotypical traits based on local phenDB
phenotypical traits based on literature
get associations where both taxa at the species/ strain level
co-occurrence network with node annotations
network clusters
manta
co-operation and competition seed scores & seed complementarities
potential pathway complementarities
co-occurrence network with edge annotations
enrichment analysis
CyApp

Fig. 3: Diagram of `microbetag`'s on-the-fly workflow. `microbetag` expects either an abundance table only as input and infers a co-occurrence network using FlashWeave or an abundance table along with an already inferred co-occurrence network and after mapping taxa present to GTDB reference genomes, for those possible, phenotypic attributes are assigned to the nodes. Literature-based annotations of the nodes are also using FAPROTAX. On the edge level, `microbetag` assigns the pre-calculated potential complements based on the pathway and the seed complementarities approaches. `microbetag` supports optional network clustering with manta. The annotated network can then be parsed into Cytoscape using the MGG app.

network will be further annotated with phenotypic traits based on the model predictions [49]. Edges linking taxa assigned to the species or strain level will be annotated with pathway and seed complementarities and seed scores. Last, a network clustering will be performed assigning each node to a cluster. The annotated network is then returned in a `.cx` format. The user may skip any of these annotation steps if not needed for their analysis.

Groups of annotations

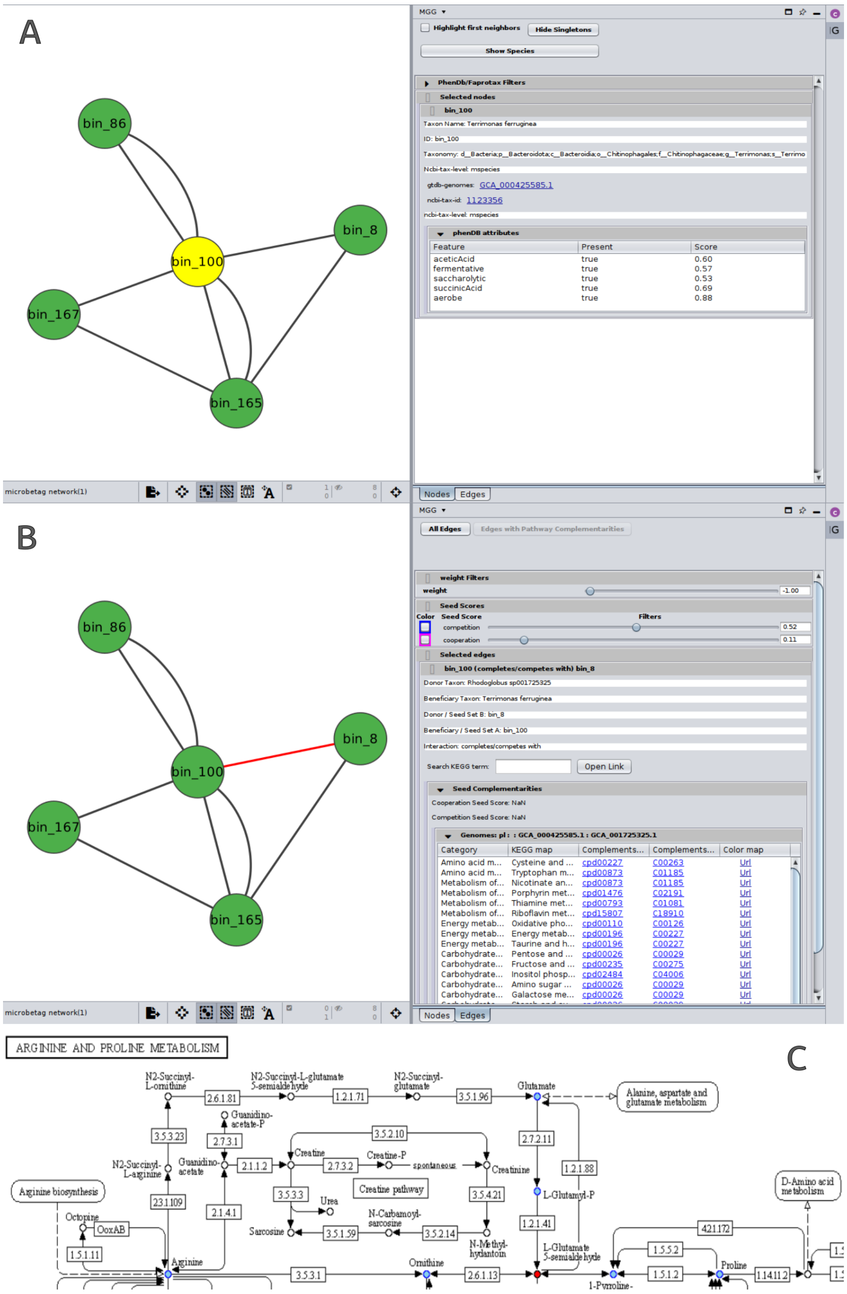
Biologically meaningful groups were described to group phenotypic traits returned from FAPROTAX and phenDB-like annotation steps. The main groups supported are related to: a. the lifestyle of a species, for example being halophilic or thermophyllic etc., b. the biogeochemical processes a species metabolic potential has been found related to, for example Nitrite-oxidizing bacteria (NOB) bacteria and c. important metabolites a species is suggested to produce, e.g. butanol. The aim of these groups are to facilitate filtering of the taxa present. Enrichment analysis for members of such groups (e.g., based on the clusters identified by an algorithm like `manta`) can be performed through the CytoscapeApp.

Software architecture

`microbetag` is a Docker-based application. We deployed the `microbetag` application using Docker containers [60] (v24.0.2) managed by Docker Compose (see Supplementary Figure B1). Docker Compose is a tool for defining and running multi-container Docker applications using a YAML file to configure the services required for the application. Containers of three Docker images are being used simultaneously: a. a MySQL database including the microbetagDB b. a nginx [61] web server and c. the application itself, including the API and the `microbetag` workflow. The latter uses Gunicorn (20.1.0) to build an application server which communicates with the web server using the Web Server Gateway Interface (WSGI) protocol and handles incoming HTTP requests. `microbetag` is implemented as a Flask application (v2.3.2); Flask is a micro web framework for developing Python web applications and RESTful APIs. The API has a route for performing the `microbetag` workflow, either through any Python console or the Cytoscape MGG app, but also several other routes that enable quick and easy access to the microbetagDB content, i.e. the genomes present, their phenotypic traits predicted annotations, pathway and seed complementarities among specific genomes or NCBI Taxonomy Ids and their corresponding seed scores if available. A thorough description of the `microbetag` API is available at the ReadTheDocs web site. The source code of the `microbetag` web service is available on GitHub.

The `MGG` CytoscapeApp

We developed a Cytoscape app to enable a straightforward, user-friendly way to perform the `microbetag` workflow and visualise `microbetag`-annotated networks. The `microbetag` CytoscapeApp (called `MGG`) was built based on the source code of the scVizNet [62]. A visual style was developed to facilitate distinguishing annotated nodes and edges. Nodes are colored based on the level of the taxonomic assignment with those being annotated highlighted in green. Similarly, edges are light green when they carry a positive weight and red when negative. Black edges denote pathway and/or seed complementarities. The latter were not accounted for in the edge weights since edges represent an undirected relationship while complementarity/overlap scores assume a direction, i.e. the complementarity score of species A versus B is not necessarily the same as that of B versus A. `MGG` allows the user to import their data, retrieve an annotated network and investigate the annotations through a series of CyPanels both for node and edge annotations. Figure 4 shows an example of the CyPanels. In the node panel (4.A), the node name, the taxonomy as well as the NCBI Taxonomy Id and the GTDB genome to which the sequence was mapped can be viewed. Depending on the user's settings and the available annotations for a node, genomic based predictions may be present and/or literature-based ones. Further, the annotation groups mentioned in paragraph 3 are on top of this panel allowing for the selection of the nodes carrying either one among several attributes (OR logical relationship) or all of them (AND). Accordingly, in the edges panel (4.B), the beneficiary taxon is specified along with their corresponding GTDB representative sequence identifier. Pathway and seed complementarities are shown each in a table. Potential metabolic interactions are shown in a sub-table entitled with the genome pair under consideration, as several GTDB genomes may have been assigned to a node. In case of pathway complementarities, these tables consist of six columns: a. the KEGG MODULE id of the module to be completed, b. its description, c. a more general metabolic category to which the module belongs, d. the complement itself as a list of KEGG terms, e. the alternative that now represents a complete module in the beneficiary and f. a URL that points to a coloured KEGG map highlighting the complement. If clicked, the user's default browser pops up showing a coloured KEGG map as shown in an example in Figure 4.C. Last, MGG allows to test for enrichment or depletion of the phenotypic traits assigned to the nodes in each of the network clusters. Clusters may have been returned from `manta` [22] while performing the `microbetag` workflow or users may assign them on their own or using any other network clustering algorithm. For thorough instructions on how to use MGG and `microbetag` the reader may visit the ReadTheDocs web site.



MGG ▾

☐ Highlight first neighbors | Hide Singletons

Show Species

PhenDb/Faprotax Filters

Selected nodes

bin_100

Taxon Name: Terrimonas ferruginea

ID: bin_100

Taxonomy: d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagaceae;g__Terrimonas;s__Terrimo

Ncbi-tax-level: mspecies

gtdb-genomes: GCA_000425585.1

ncbi-tax-id: 1123356

ncbi-tax-level: mspecies

phenDB attributes

Feature	Present	Score
aceticAcid	true	0.60
fermentative	true	0.57
saccharolytic	true	0.53
succinicAcid	true	0.69
aerobe	true	0.88

Nodes | Edges

MGG ▾

All Edges | Edges with Pathway Complementarities

weight Filters

weight -1.00

Seed Scores

Color	Seed Score	Filters
🟦	competition	0.52
🟪	cooperation	0.11

Selected edges

bin_100 (completes/competes with) bin_8

Donor Taxon: Rhodoglobus sp001725325

Beneficiary Taxon: Terrimonas ferruginea

Donor / Seed Set B: bin_8

Beneficiary / Seed Set A: bin_100

Interaction: completes/competes with

Search KEGG term: | Open Link

Seed Complementarities

Cooperation Seed Score: NaN

Competition Seed Score: NaN

Genomes: pl : : GCA_000425585.1 : GCA_001725325.1

Category	KEGG map	Complements...	Complements...	Color map
Amino acid m...	Cysteine and ...	cpd00227	C00263	Url
Amino acid m...	Tryptophan m...	cpd00873	C01185	Url
Metabolism of...	Nicotinate an...	cpd00873	C01185	Url
Metabolism of...	Porphyrin met...	cpd01476	C02191	Url
Metabolism of...	Thiamine met...	cpd00793	C01081	Url
Metabolism of...	Riboflavin met...	cpd15807	C18910	Url
Energy metab...	Oxidative pho...	cpd00110	C00126	Url
Energy metab...	Energy metab...	cpd00196	C00227	Url
Energy metab...	Taurine and h...	cpd00196	C00227	Url
Carbohydrate...	Pentose and ...	cpd00026	C00029	Url
Carbohydrate...	Fructose and ...	cpd00235	C00275	Url
Carbohydrate...	Inositol phosp...	cpd02484	C04006	Url
Carbohydrate...	Amino sugar ...	cpd00026	C00029	Url
Carbohydrate...	Galactose me...	cpd00026	C00029	Url

Nodes | Edges

Fig. 4: CyPanels of the MGG CytoscapeApp. A. *Nodes* panel display the annotations of each taxon (node) mapped to one or more GTDB genomes. In this example, genomic predicted phenotypic attributes are shown along with their prediction score. B. *Edges* panel displays the list of potential metabolic complementarities between two nodes, specifying which is the potential donor and the potential beneficiary taxon; thus giving a direction to the corresponding edge in the graph. There are two cases of complementarities in `microbetag`. The first are seed complementarities, which are computed based on the species' reconstructed metabolic networks (case shown above). *Seed complementarities* are first exported based on ModelSEED complements (column three) and mapped in KEGG COMPOUNDS (column four). The provided URL points to a colored KEGG map that highlights seeds complementing a beneficiary's metabolic network The same applies for the case of the *Pathway complementarities* only there is no ModelSEED ids as they are computed directly from the KEGG annotated genomes and not from the Genome-Scale Metabolic Reconstructions; that is the case for the *Seed complementarities*. The second are pathway complementarities. These are directly computed from the KEGG annotated genomes and do not involve ModelSEED ids since the metabolic network construction step is not necessary in this case. C. Part of a colored KEGG map returned based on the seed complementarities. Compounds that belong to the beneficiary taxon are colored in cyan while the potential complementing seeds from the donor are highlighted in red.

Results and discussion [2]

Annotating microbial co-occurrence networks with microbetag

The microbetag software ecosystem consists of five main modules: a. microbetagDB including microbetag precalculations, b. the microbetag workflow to annotate the co-occurrence network, c. a web server hosting both the microbetagDB and the microbetag application, d. a CytoscapeApp called MGG that enables users to easily invoke the workflow and investigation of the annotated network, and e. a stand-alone pre-processing workflow provided as Docker image for data sets with more than $1,000$ sequence identifiers (OTUs/ASVs/bins etc.).

Currently, microbetagDB includes more than $34,000$ genomes (Table 1) along with their corresponding annotations. The vast majority of these genomes represent bacterial taxa (364 taxa are Archaea). The presence/absence of more than 30 phenotypic traits have been predicted for those genomes. About 1.4 billion potential metabolic interactions leading to pathway or seed complementarities have been precomputed as well. There are one order of magnitude more seed complements than those corresponding to pathway complementarities as for all GENREs present in microbetagDB all pairwise complements were calculated ($33,755^2$) and stored, even if empty. In the case of pathway complementarities, a genome pair is present in the database and thus counted only if a potential complementarity was found. Yet, in the first case, the number of genomes with no potential seed complements ranges from zero to a few dozen. All annotations can be accessed directly from microbetagDB through the API. Using GENREs for the seed complements and not the genomes per se supports a more realistic simulation of what the corresponding taxa need to get from the environment to grow (seeds) but also, assuming they grow what they may secrete (non-seeds). However, this comes with its own challenges (see paragraph 3).

Running the microbetag workflow is straightforward and can be done using an abundance table with taxonomic assignments as input. When the taxonomy scheme being used is not one among the GTDB, Silva or the GTDB-oriented taxonomy for 16S rRNA amplicon data (see pre-process paragraph 3), the most time-consuming step of the workflow is the one mapping user's taxonomy to a NCBI Taxonomy Id and from that to GTDB representative genomes. Network inference can be a computationally intensive step too, particularly as the number of sequences in the abundance table increases. To enable annotation of large data sets, a stand-alone pre-process workflow is provided with microbetag. The user can either assign their amplicon data to the GTDB-oriented taxonomy and/or reconstruct a network locally. Once a network is available and the taxonomy used is among the standard ones for microbetag, the computational time required for annotation ranges from several seconds to a few minutes based on user's settings. An annotated network in cx2 format is returned,

[2] **Results-related:** Significant advance over previously published software (usually demonstrated by direct comparison with available related software) This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures. This section may be combined with the Discussion section for Software articles. **Discussion-related:** The user interface should be described and a discussion of the intended uses of the software, and the benefits that are envisioned, should be included, together with data on how its performance and functionality compare with, and improve, on functionally similar existing software. A case study of the use of the software may be presented. The planned future development of new features, if any, should be mentioned.

which can be viewed in Cytoscape. A tutorial, frequently asked questions and hints to address the idiosyncrasies of various data sets are available on the ReadTheDocs web site while a Gitter community allows users to exchange experience and ask for more specific help. In the following two sections, we present a validation and use case, highlighting our approach's potential.

Table 1: Summary of the data in microbetagDB

Description	Entries
GTDB representative genomes	34,608
Phen-model-oriented metabolic functions	32
FAPROTAX functions	92
Unique pathway complements	341,568
Pairwise pathway complementarities	184,184,548
GENREs leading	33,755
Seed complements	1,139,400,025
Seed scores	1,105,250,048

A validation case

To validate `microbetag` we used the correlation network of Hessler et *al.* [38] describing mine tailing-derived laboratory microbial consortia. In this study, *Variovorax*, a thiamine producer, and its co-occurrence with a series of thiamine auxotrophs are discussed. The study was selected as a validation case as the authors tested the network's predictions by performing co-culture experiments measuring the thiamine production. Sequence bins corresponding to network nodes and the original network were obtained from the authors. Using GTDB-tk [63], bins were annotated to GTDB taxonomies; those retrieved were added in the original network, which was then annotated with `microbetag`. Supplementary Figure B2 highlights bin_55 that corresponds to *Variovorax* and its first neighbors. The annotated network is available on `microbetag`'s GitHub repository. GTDB-tk returned GCA_001899795.1 as the one closer to bin_55 assigning it to *Variovorax* sp001899795. `microbetag` then suggested that this specific genome corresponds to an aerobe [64], that can grow autotrophically if needed [65] and utilizes D-glucose, while producing ethanol and lactic acid [66]. Last, the Type VI secretion system was suggested to be available on its genome [67].

Hessler et *al.* argue that *Variovorax* is an important thiamine source and can supply neighboring species that cannot produce it (auxotrophs). Indeed, `microbetag` was able to suggest several thiamine-related potential seed complements among the potential metabolic interactions between *Variovorax* as well as its neighbors (Table 2.A). Potential interactions were also found in some cases between the neighbors themselves (Table 2.B). The authors also argue that isolates of that *Variovorax* strain required the addition of pantothenic acid to grow. However, based on the KEGG annotation of the genome that bin_55 was mapped to, it contains both KEGG MODULES related to pantothenate biosynthesis, M00119 (valine/L-aspartate ⇒ pantothenate) and M00913 (2-oxoisovalerate/spermine ⇒ pantothenate). Other genomes though are not capable of either one or any of those (Supplementary Table) B1. This example highlights a challenge of the `microbetag` approach (see Section 3). The complementarities between the nodes would have been different if bin_55 was classified as another *Variovorax* genome, with incomplete modules. For example, if GCF_001577265.1 was picked and its complementarities with the neighboring taxa were retrieved, it would have revealed that all its neighboring species can actually provide it with pantothenate (C00864) as suggested by their seed complementarities (see coloured map).

A. *Variovorax* thiamine-related benefits to its neighbors

Neighboring taxon	**node id**	**KEGG compounds**	**url**
Kapabacteria thiocyanatum	bin_59	C15809	url
Terrimonas ferruginea	bin_100	C15809;C01081	url
Tahibacter sp001725155	bin_167	C15809	url
Microbacterium sp900156455	bin_28	C15809; C20246	url
Sphingobium sp001899715	bin_155	Iminoglycine C15809;	url
Nitrosospira sp001899235	bin_176	None	None
62-47 sp001899255*	bin_233	None	None
Bosea sp001898115	bin_273	C04327;C01279	url
54-19 sp001898225**	bin_41	C15809	url
Rhodoglobus sp001725325	bin_8	C15809	url

B. Potential thiamine-related complements among *Variovorax* neighbors

Beneficiary	**Donor**	**potential complement**
T. ferruginea	*Tahibacter* sp001725155	C01081
T. ferruginea	*Rhodoglobus* sp001725325	C01081
Nitrosospira sp001899235	*Bosea* sp001898115	C04327;C01279
Chloroflexi	*Bosea* sp001898115	C15809
Chloroflexi	Xanthobacteraceae	C15809
Chloroflexi	*Nitrosospira* sp001899235	C15809

Table 2: Thiamine biosynthesis related seed complements between *Variovorax* and its first neighbors in the network of Hessler *et al.*[38] (A), and between pairs of the neighbors (B). Bin sequence files were mapped against GTDB using GTDB-tk. Chloroflexi refers to the GTDB taxonomy of: 54-19 sp001898225

Interpreting a real-world network with `microbetag`

Annelies' dataset.

One last visual component from the use case would be nice to have.

Potential and limitations

The previous paragraph shows the potential of `microbetag`in the interpretation of co-occurrence networks and how it can be used to generate new hypotheses derived from those. However, `microbetag`benefits the microbiome community in several other ways. The microbetagDB provides a vast number of annotations; 31 predicted traits for more than $30,000$ genomes, their GENREs along with their corresponding seed sets, potential metabolic complementarities and cooperation/competition scores. Such a resource may support a range of studies; from a more theoretical perspective regarding the distribution of the complements among taxonomic groups or how often a complement potentially appears, to applications such as eco-evolutionary studies and the investigation of interactions.

Yet, there are several challenges involved in our approach. First, `microbetag`inherits all the biases and drawbacks of both the data and the software it is based on. Functional annotation comes with its own limitations. Some domains boast richer annotations and more comprehensive descriptions compared to others, thus exhibiting a wealth of detail and employing more precise terminology, particularly for widely recognized processes.

In the validation case, the bin representing the *Variovorax* strain was mapped to a genome that is supposed to contain the pantothenate KEGG modules. Thus, the fact that it requires to receive pantothenate from its environment to grow, as the authors mention, would not have been predicted in the `microbetag`framework. Beyond the sequencing and the annotation challenges, we also need to consider the fact that a pathway may not be fully represented in a KEGG module. Of course, various factors can prevent the actual production of an enzyme even if its genetic information is included in a species' genome.

Pathway complementarity can only be as accurate as the KEGG MODULE database and as precise as the software annotating genomes with KO terms. It is well known that automated Genome-Scale Metabolic Reconstruction comes with a great number of challenges and different software for this task come with their intrinsic limitations [68]. Using ModelSEED with a complete medium may limit potential metabolic interactions but, on the other hand, the retrieved ones will be of higher confidence.

It is also well known that higher-order interactions, i.e. interactions involving more than two species [29], should also be considered. Pairwise relationships do not capture the more complex forms of ecological interactions, in which species depend on (or are influenced by) multiple other species [3]. However, since `microbetag`is decoupled from network inference, it could annotate a network with hyperedges (i.e. edges connecting more than two taxa) produced by a future tool capable of inferring higher-order interactions.

Last, the limited number of Archaea in microbetagDB is also the result of a software limitation. As shown in [69] (Figure 6b), the original version of CheckM [70] that is still being used by GTDB returns lower completeness scores for genomes that correspond to phyla known for having smaller genomes in general, e.g. Patescibacteria representative genomes on GTDB have an average completeness of $\sim 65\%$. Thus, only few representatives from these taxonomic groups passed our filters leading to an important under-representation of Archaea.

Future work

In the near future, we plan to develop two main features: a. the integration of transcriptomics data provided by the user, which would enhance or lower the probability for a potential metabolic interaction to occur based on whether the KO terms involved are present or not, and b. the integration of spatial data; it is well-known that the distance between cells determines whether an interaction occurs [71]. For this, we intend to support data with spatial dimensions. Thus, potential metabolic interactions between taxa that are closer to one another would be more probable to occur.

Last, we already work on a *"for advanced users"* version, a server-independent version of `microbetag`is about to be released, so the user can provide bins/MAGs of theirs and annotations will be made not by mapping taxonomies to reference genomes but using their sequencing data directly. This would require important computing resources and time and cannot be supported in an app-framework like the one presented here. In this case, one will be again able to investigate the annotated network returned through Cytoscape and the MGG app. [3]

[3] Could be part of this release; time will tell.

Conclusions [4]

Co-occurrence networks are widely used in microbiome studies to explore associations [4]. However, their inference and their interpretation come with a range of challenges [14]. Metabolic exchanges among microbial taxa are considered ubiquitous [72] n a large number of environments. In our study, we exploit reverse-ecology approaches and publicly available genomic data and software to predict phenotypic traits and construct metabolic networks to annotate co-occurrence networks derived from amplicon or shotgun data. Our annotation was in-line with the study of Hessler et *al.* [38] predicting thiamine-related metabolic interactions among *Variovorax* and its closest neighbors, suggesting several ways to achieve them. Using ..... Enrichment analysis using them combined with network clustering algorithms can further benefit their interpretation. Both microbetagDB and `microbetag`will benefit microbiome studies, as a resource and as a hypothesis generation tool.

Use case

Supplementary information. List of supplementary figures and tables.

Supplementary Figure 1: `microbetag`software ecosystem architecture.

Supplementary Figure 2: *Variovorax* (node_55) and its closest neighbors. *Variovorax* annotations are shown in the node CyPanel.

Supplementary Table 1: *Variovorax* genomes present on microbetagDB and their corresponding complete/incomplete presence of the pantothenate - related KEGG modules

Supplementary Table 2: Computing times per step of the `microbetag`workflow using four different data sets.

Declarations

- **Availability of data and materials**
  - Raw sequences for the use case:
  - Raw data for the validations case:

- **Funding**
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- **Conflict of interest/Competing interests**
  The authors declare that they have no other competing interests.
- **Authors' contributions** [5]
  Conceptualization: K.F. Methodology: K.F. and H.Z. Software: H.Z., E.I.M.D. and J.M. Validation: H.Z. and K.F. Formal analysis: H.Z. and K.F. Investigation: H.Z. Resources: K.F., A.E. and A.G. Data Curation: H.Z. Writing - Original Draft: H.Z.

[4] This should state clearly the main conclusions and provide an explanation of the importance and relevance of the case, data, opinion, database or software reported.

[5] Based on the CRediT system.

and K.F. Writing - Review & Editing: all Visualization: H.Z. Supervision: K.F., H.Z. and S.M. Project administration: K.F. Funding acquisition: K.F., H.Z.

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- **Ethics approval**
  Not applicable
- **Consent to participate**
  Not applicable.
- **Code availability:**
  - microbetagDB related scripts: https://github.com/hariszaf/microbetag
  - `microbetag`application: https://github.com/msysbio/microbetagApp.
  - MGG CytoscapeApp: https://github.com/ermismd/MGG/
  - Validation and use case: [6]
  - Documentation web-site: https://hariszaf.github.io/microbetag/

Appendix A Background on pathway and seed complementarity

For a genome to have a KEGG module *complete* means it provides at least one complete *alternative*. Based on the module's definition, alternatives are considered as the unique combinations of KOs that will enable the module. For example, the definition of the D-Galacturonate degradation in Bacteria (M00631) is:

```
K01812 K00041 (K01685,K16849+K16850) K00874 (K01625,K17463)
```

Once breaking down, it leads to 4 alternative sets of KOs (pathways):

```
K01812 K00041 K01685 K00874 K01625
K01812 K00041 K16849+K16850 K00874 K01625
K01812 K00041 K01685 K00874 K17463
K01812 K00041 K16849+K16850 K00874 K17463
```

In alternatives two and four, the K16849+K16850 is a *complex*, meaning both KO terms are required for the step to be available.

In case of seed complementarity, in `microbetag`we focus on the effect that a metabolic exchange between two taxa might have if the seed of the beneficiary taxon is linked to a KEGG MODULE. Therefore, the KOs that were found linked to modules were mapped to ModelSEED ids. The initial seed and non-seed sets that were exported as sets of ModelSEED ids were then mapped to KOs too. When the non-seed set of a genome (donor) provided a seed related to a KEGG module to another genome (beneficiary), this is considered a potential metabolic interaction.

[6] Consider moving that under the 3D'omics organization

Appendix B Validation

Table B1: *Variovorax* genomes present in microbetagDB and their corresponding complete/incomplete presence of the pantothenate-related KEGG modules. The genome that bin_55 was mapped to is shown in bold.

Genome	md:M00119	md:M00913
GCA_004210915.1	incomplete	complete
GCA_902506565.1	incomplete	incomplete
GCF_000184745.1	complete	complete
GCF_000282635.1	complete	complete
GCF_000463015.1	complete	complete
GCF_000834655.1	complete	complete
GCF_001424835.1	complete	complete
GCF_001425205.1	complete	complete
GCF_001426505.1	complete	complete
GCF_001577265.1	incomplete	incomplete
GCF_002157355.1	complete	complete
GCF_002754375.1	complete	complete
GCF_003019815.1	incomplete	complete
GCA_001899795.1	**complete**	**complete**
GCF_003852515.1	complete	complete
GCF_003951285.1	complete	complete
GCF_003952165.1	complete	complete
GCF_003952185.1	complete	complete
GCF_003984625.1	complete	complete
GCF_003984645.1	complete	complete
GCF_006438845.1	complete	complete
GCF_007828835.1	complete	complete
GCF_009498455.1	complete	complete
GCF_009755665.1	complete	complete
GCF_010499245.1	complete	complete
GCF_013376045.1	complete	complete
GCF_014170375.1	complete	complete
GCF_014302995.1	complete	complete
GCF_014303735.1	incomplete	incomplete
GCF_901827175.1	complete	complete
GCF_901827205.1	complete	complete

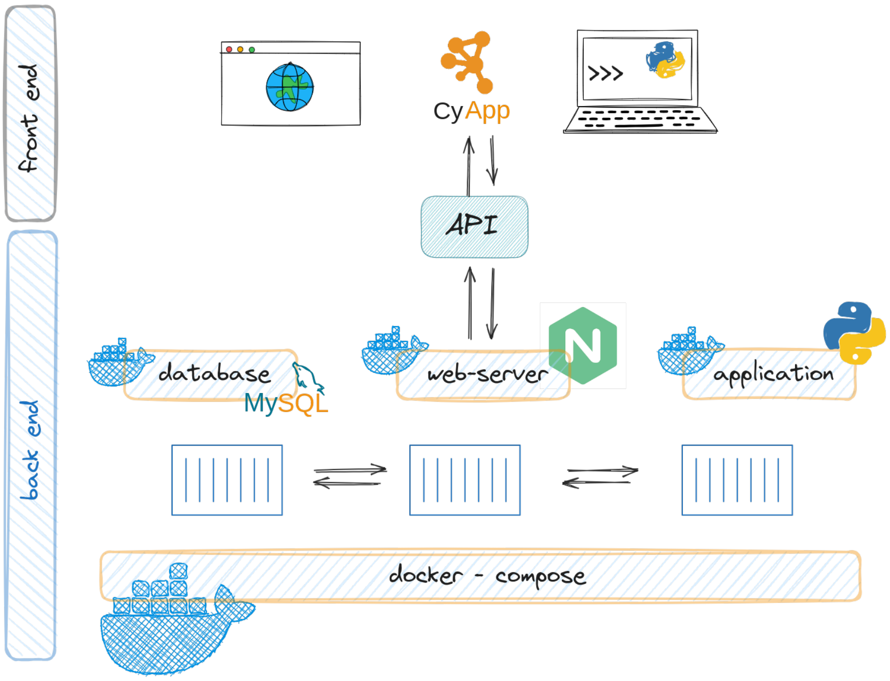
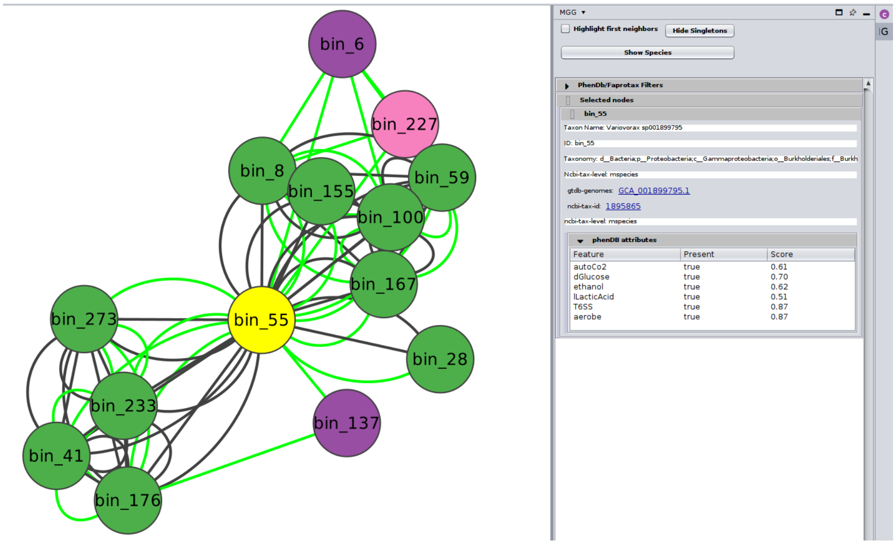


Fig. B1: `microbetag`software ecosystem.



Taxon Name: Variovorax sp001899795

ID: bin_55

Taxonomy: d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkh

Ncbi-tax-level: mspecies

gtdb-genomes: GCA_001899795.1

ncbi-tax-id: 1895865

ncbi-tax-level: mspecies

Feature	Present	Score
autoCo2	true	0.61
dGlucose	true	0.70
ethanol	true	0.62
lLacticAcid	true	0.51
T6SS	true	0.87
aerobe	true	0.87

Fig. B2: *Variovorax* node (bin_55) and its neighbors annotated by `microbetag`. All but three of them were not mapped to a GTDB representative genome. Green edges represent the positive association weights. The black edges represent pairwise seed complementarities and scores.

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