Explaining microbial phenotypes on a genomic scale: GWAS for microbes

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Abstract

There is an increasing availability of complete or draft genome sequences for microbial organisms. These data form a potentially valuable resource for genotype–phenotype association and gene function prediction, provided that phenotypes are consistently annotated for all the sequenced strains. In this review, we address the requirements for successful gene-trait matching. We outline a basic protocol for microbial functional genomics, including genome assembly, annotation of genotypes (including single nucleotide polymorphisms, orthologous groups and prophages), data pre-processing, genotype–phenotype association, visualization and interpretation of results. The methodologies for association described herein can be applied to other data types, opening up possibilities to analyze transcriptome–phenotype associations, and correlate microbial population structure or activity, as measured by metagenomics, to environmental parameters.

Keywords: *genotype–phenotype association; genome-wide association studies; functional genomics; microbial genomics; random forest*

INTRODUCTION

The 'function' of a gene or protein is a complex concept that consists of several layers, such as molecular function, cellular component and biological process (phenotypic function) [1,2]. Deciphering the function of microbial genes often involves the use of molecular biological techniques to establish function at the molecular level, or knock-out studies to specify cellular or phenotypic annotations. Since (near) complete microbial genomes started becoming available, comparative genomics has been used to prioritize candidate genes for further laboratory testing [1], minimizing the costs of experimental research. The best-known example of functional annotation by comparative analysis is the transfer of functional annotations between orthologous genes, but many other associative methods have been developed [3] including conservation of gene order [4,5] and phylogenetic profiling [6,7]. The recent drop in the cost of genome sequencing has enabled an increase in the scale of comparative genomic analyses. Several thousand bacterial genomes have been sequenced thus far, opening up the potential for microbial genome-wide association studies (GWAS).

In human genomics, GWAS investigate the correlation of genetic variants with phenotypic traits across different individuals [8,9]. These genetic variants mostly consist of simple nucleotide polymorphisms (SNPs) [10], i.e. either point mutations or small insertions or deletions (indels) in the genome sequence. In the first GWAS study to be published, 1133 affected individuals versus 1006 controls were tested for SNPs linked to myocardial infarction [11]. A total of 92 788 SNPs were genotyped, revealing

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one SNP that was strongly associated to the disease. Because such a correlation does not prove causality, further experimental testing confirmed that the SNP induced several cell-adhesion molecules and enhanced the transcription of lymphotoxin-α [11]. Thus, GWAS can be a useful tool for bioinformatic prioritization of candidate SNPs for further research.

A comparable approach is possible for the association of bacterial genes to phenotypes. When the first complete microbial genomes emerged, 'differential genome analysis' of the gene content of related microbes shortlisted gene candidates responsible for certain phenotypes [12,13]. In that study, candidate genes were selected based on a genome-wide comparison of *Helicobacter pylori* to its close relatives *Haemophilus influenzae* and *Escherichia coli*. It was proposed that the genes that were differentially present or absent might be responsible for *H. pylori*'s species-specific features, including the ability to colonize host cells in highly acidic environments. Currently, gene-trait matching or genotype–phenotype association is a more commonly used term for similar analyses.

The concept of correlating phenotypes to the presence or absence of genes in bacterial genomes was scaled up with the introduction of comparative genome hybridization (CGH) microarrays that measured genotypes with high throughput [14,15]. These microarrays were spotted with probes that map to the genes present in a single genome (CGH arrays) or in a number of strains of the same species [16,17], coined pan-genome arrays [18]. The arrays could then be hybridized with fluorescently labeled genomic cDNA to determine the gene content of a query strain [19,20]. For both single genome CGH arrays and the pan-genome arrays, the genes in the query genome can only be determined in terms of the reference sequences, provided that they are sufficiently similar to the probes on the array [20,21]. There are only a few studies describing CGH-based gene-trait matching. In part, this is because consistent trait annotations have been lacking. To correlate the gene content of the selected strains to their phenotypes, consistent phenotyping is required, i.e. the phenotypes should be determined under the same conditions for all the strains that are genotyped.

Today, sequencing has surpassed microarrays for accurately determining the gene content of an organism. Advantages of sequencing include the direct determination of transcripts by RNA sequencing, and the fact that sequencing allows the discovery of new genes, while the genomic diversity that can be determined by microarrays is limited to the reference sequences used for array probe design. Moreover, sequencing increases the resolution of comparative genomic analyses from gene content to individual SNPs. Several high-impact studies have provided draft genome sequences of many strains of the same species [22–25], where genome alignments allow the discovery of single nucleotide differences.

We argue that the greatest bottleneck for applying GWAS to microbial datasets is the availability of experimentally consistent phenotypes or other annotations, such as environmental parameters (metadata). Both types of genotyping data introduced above, CGH arrays and complete genome sequences, are potentially of high value for gene-trait matching, provided that the phenotypes of the strains are also consistently annotated. There are some promising examples, including a publication where 12 phenotypes were determined for 42 *Lactobacillus plantarum* strains [26], however, such datasets are scarce, in part because consistent culturing is crucial to reduce experimental noise. Thus, culturing studies and underpinning microbial physiology are crucial for making best use of the wealth of information that will be available.

In this review, we will focus on genotype–phenotype associations for groups of bacterial strains of the same species. First, it may be more likely that similar traits have been annotated for strains, than for phylogenetically more divergent organisms. Second, the genome sequences of different species are not easily alignable due to the greater differences in gene content and in genome structure. As a result, we would not be able to illustrate the use of SNPs as genotypic characters. Nevertheless, the methods outlined herein are equally suitable for genotype–phenotype associations across different species. We conclude with an outlook of the application of these methods to other data types, including the use of transcriptomic data across different experimental conditions for linking genes to functions within a single species, and the use of functional or taxonomic profiles across metagenomes to link functions or taxa to environmental parameters.

ASSEMBLY AND ANNOTATION

Understanding the functional potential encoded by a given genome starts with an accurate genome sequence and gene annotation. Next-generation sequencing techniques are increasingly being used to sequence the genomes of new microbial isolates [27–30]. As read lengths of most sequencing platforms are in the hundreds of nucleotides, it is imperative to assemble reads into larger contiguous sequences (contigs) and to order and orient contigs into larger scaffolds [31]. These larger DNA fragments allow better prediction of open reading frames (ORFs) and facilitate gene context analyses with comparative genomic approaches. For SNP typing of bacterial strains, the sequence quality of the assembly is very important and there are several strategies to correct the assembly for sequencing errors, including the detection of frameshifts by comparative genomics, and the correction of SNPs in an assembly using Illumina reads [32,33].

Genome annotations often start with submitting a genome sequence to an online annotation service [34,35]. This results in *ab initio* predicted ORFs consisting of start and stop positions, as well as a predicted function. Start and stop codon prediction is usually performed by ORF calling software implemented in these annotation engines, such as GLIMMER [36], GeneMark [37,38] or Prodigal [39]. It is crucial to use the same ORF prediction method for the different strains of interest, as differences in the ORF predictions could influence downstream analyses, including determining orthologs (see below). It should be mentioned that sequencing of transcripts now enables direct measurement of ORFs, which may be more accurate than automated ORF predictions.

Functional annotation of the predicted ORFs may involve many steps including homology searches to annotated databases, such as RefSeq [40], Genbank [41] and SwissProt [42] using BLAST [43], or hidden Markov model screenings with Pfam [44]. Annotation engines generally provide reasonably accurate automated function annotations for proteins, although they may show deficiencies in genotype–phenotype extrapolation [45–47]. Specifically, they are suited for annotating core metabolic genes, while for genes that are not widely conserved, manual curation remains an important step in identifying function [48]. The time necessary for the curation of gene functions can be reduced by (i) performing the function curation for a representative member of an orthologous group (OG) (see below) instead of for all members; (ii) concentrating curation efforts on the molecular functions of interest and (iii) by examining gene function predictions for targets resulting from the genotype–phenotype matching. The DNA sequences with putative ORFs and their annotations are then ready for comparative genomics and determining structural variations (SVs), single nucleotide polymorphisms (SNPs) and small insertions or deletions (indels).

ORTHOLOGOUS GROUPS OF GENES

Comparing the genes in a selection of genome sequences depends on a reliable annotation of orthologs. Coined by Fitch in 1970, orthology is an evolutionary concept that describes the relationship between genes that diverged following a speciation event [49]. Conversely, paralogy refers to genes that diverged following a gene duplication (Figure 1). A frequent misinterpretation of the concept of orthology is the idea that it signifies functional equivalence. Indeed, orthologs may be likely to represent functional equivalents because of their evolutionary definition, but the original definition *per se* contains no statement about conservation of function [49].

It is relatively straightforward to identify the orthologous genes or proteins for pairs of species by reciprocal homology searches [50]. Comparative genomics of more than two genomes requires an annotation of group orthology [51,52]. OGs of genes can be interpreted as gene families, and consist of all the genes that have descended from a single ancestral gene in the last common ancestor (LCA) of the species considered. Thus, the resolution of a set of OGs depends on which group of species is considered (Figure 1). If the group shares an ancient LCA, say the ancestor of all bacteria (or Bacilli in Figure 1), the single ancestral genes will have had ample time to evolve and diverge, resulting in large and inclusive OGs that may have many representatives in each genome (paralogs). Conversely, a very recent LCA, say for a group of strains of one bacterial species (or the order *Lactobacillales* in Figure 1) leads to smaller, higher resolved OGs with one or a few paralogs in each of the genomes.

There are several methods and tools available that assign genes to OGs for a given set of genomes. These can be divided into methods that construct *de novo* OGs and methods that map proteins to existing OGs by using sequence similarity (both approaches are elaborated in the paragraphs below).

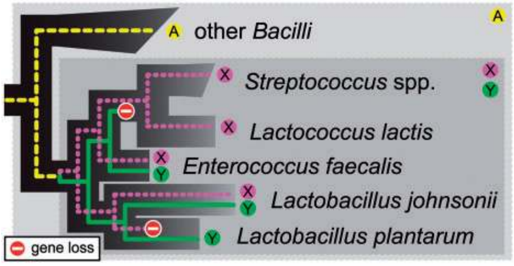


Figure 1: The resolution of an OG depends on the age of the LCA for the studied species. The dark background tree indicates the evolutionary history of the included Bacilli; colored lines indicate the evolutionary history of the genes. Gene family A in the Bacilli duplicated in the LCA of the Lactobacillales to form the paralogs X and Y. When constructing OGs for all Bacilli, all the homologs A, X and Y will be united in one OG, where X and Y are called 'in-paralogs'. If only Lactobacillales are taken into account, X and Y are placed in separate OGs because they had different ancestral genes in the more recent LCA. Note that when species are compared in pairs, paralogs may be mistaken for orthologs due to differential loss of paralogs, e.g. the *Lactococcus lactis* gene X and the *L. plantarum* gene Y. Orthology can be inferred at different levels of resolution by analyzing speciation events and gene duplication events in phylogenetic trees [119].

Methods that map proteins to existing OGs are more practical to use than *de novo* reconstruction of OGs, but have several disadvantages. For example, the resolution of the existing OGs may not be optimal for the selection of species under consideration: the resolution may either be too low (if OGs were reconstructed for a more ancient LCA), or too high, leading to arbitrary subdivision of proteins into distinct OGs, that actually belong in the same OG because they share a single ancestral gene. Moreover, genes that are specific to the newly sequenced genome will not be mapped.

For construction of new OGs, it is imperative that complete genome sequences are available with accurate gene predictions, so that any paralogy relationships can be identified. Given that we are currently within the era of short-read shotgun sequencing, it should be noted that paralogs may accidentally be collapsed by short-read assemblers if they are very similar, e.g. when creating a genome assembly by mapping the sequencing reads to a closely related reference genome [53]. Established tools for *de novo* OG reconstruction include InParanoid/ Multiparanoid [50,54] and OrthoMCL [55]. These programs do not rely on phylogenetic reconstruction, which is computationally expensive and becomes increasingly prohibitive with larger datasets. Finally, orthologs may not necessarily be more similar in sequence [56], and additional information including gene neighborhood (operon structure) can be used to refine their definition.

As sequence databases become more comprehensive, the mapping methods gain in resolution and form a viable alternative to *de novo* OG reconstruction methods. A simple approach is to provide the proteins in the database with OG annotations, and after searching this database with the query genes, assign each to the OG of its highest scoring hit. This works well if the query genome is a close relative of genomes in the database, and the speed of the RAST server (Rapid Annotations using Subsystems Technology [35]) in part depends on this approach. For genomes that do not have reliably sequenced close relatives, the top hit approach might be more likely to spuriously yield distant hits from a different OG. In these cases, Cognitor [57] provides an extension of the simple top-hit rule, by assigning genes to an OG if the majority of the top scoring proteins map to the same OG. Note that proteins belonging to different OGs may occur as fused genes, in which case the Cognitor rule should be applied separately to each of the fused regions. The Signature webserver [58] automatically performs these steps, assigning proteins or protein regions to the OGs from the STRING database [59]. Alternatively, the EggNOG webserver [60] also allows the assignment of protein sequences to OGs.

PHAGES

Prophages and mobile elements are the most variable fraction of microbial genomes and often encode functions related to interaction with the changing environment [61], including pathogenicity genes [62]. Moreover, they may contain genes that help bacteria cope with adverse environmental conditions like sub-lethal concentrations of antibiotics or withstanding osmotic, oxidative and acid stresses, as well as increasing growth and influencing biofilm formation [63]. Prophages often contribute the biggest difference between strains and may account for many kilobases of divergent sequence. For example, *Sodalis glossinidius* strain 'morsitans' contains 26 prophages that together cover 956 349 bp of this 5MB genome [64]. In general, prophages encode three types of genes: (i) genes required for the phage cell cycle including phage entry, DNA integration and excision, DNA replication, packaging, and cell lysis, and the expression regulation of those genes; (ii) genes that inhibit the cell cycle of other phages (super-infection exclusion) and (iii) auxiliary metabolic genes that generate energy in the cell and promote viral replication. Thus, we expect prophage and mobile element content to be important for explaining phenotypic differences between closely related strains.

Phage genes can be identified by similarity to known homologs in a relevant database of viral proteins, such as Phantome (http://www.phantome.org/). However, because viruses mutate rapidly [65] and are under-represented in the databases [66], this may not always be sufficient to identify all prophages in a newly sequenced genome. Moreover, it should be noted that prophages often contain homologous sequences inserted at different loci around the genome, hindering sequence assembly efforts. Prophages have specific characteristics, including protein length, transcription strand directionality, AT/GC skews, phage-specific oligonucleotides and phage insertion points (even though different strains can have different prophages inserted at the same chromosomal integration site), that can be used for identification. Recently, the tool PhiSpy [64] has been developed that uses combinations of these characteristics, as well as homology, to identify prophages in bacterial genome sequences. Once phages have been identified, they can be mapped between strains using orthology and gene content.

MUTATIONS

It is possible that the differential presence of OGs, functions, mobile elements or phages cannot explain a given phenotype, if mutations such as SNPs or indels are responsible for gain- or loss-of-function. One famous example is the presence of point mutations in the *E. coli fimH* gene that alters its host specificity [67,68]. Another recent example in fungi is that of very recently diverged *Aspergillus fumigatus* strains that have acquired resistance to toxic azole compounds by a mutation in the transcription factor subunit *HapE* [69], but have an otherwise identical genetic background. Such small mutations can also be included into the list of genotypic features, and associated to phenotypes in the same way as presence/absence patterns of OGs or phages (above). For brevity we will refer to these mutations as SNPs, which could stand for simple (instead of single) nucleotide polymorphisms [10]. In microbial genomics, the state of the art for SNP detection between strain variants consists of mapping the reads to a reference sequence and identifying variants in the conserved regions [23]. Although this will miss larger SV, it will suffice for our purpose, as differences in OG or phage content are already identified with the approaches above.

PHENOTYPES

Phenotypes are here defined as the observable characteristics of an organism. For bacteria, these phenotypes often consist of growth on specific media, for example, containing different carbon sources [26]. Phenotype microarrays [70,71] are a relatively straightforward way of assessing many conditions in parallel, as exemplified by a study of the soil bacterium *Sinorhizobium meliloti* [72]. Another example of a phenotype is the resistance to certain antibiotics, such as measured for the progeny of sexual crossing experiments of *A. fumigatus* [69], or for different bacteria isolated from patients at intensive care units [73]. In the latter study, the observed resistance correlates with geography or other differences in the patient populations. Other phenotypes include: (i) survival/growth under different experimental conditions or presence in ecological niches [74–81]; (ii) the ability to perform a specific molecular function [26,76,82,83] and (iii) metabolomic fingerprints [84].

To perform comparative studies encompassing many strains, it is imperative that phenotypic annotations are consistently performed across the strains. A standard in the description of the environment (metadata including sampling point and habitat) and the actual nucleic acid sequence of individual strains has been launched by the Genomic Standards Consortium, coined the Minimum Information about a Genome Sequence [85]. We recommend that this minimal standard be followed, also when large collections of strains are sequenced simultaneously. Moreover, we imagine that extensions to this standard, that describes the environment and the sequence, may be formulated for the further phenotypic description of specific taxonomic clades or species. An example could be running a standard phenotype microarray for every sequenced strain and one that measures specific properties for the taxonomic group in question. Optimally, the availability of consistent phenotypic information about sequenced microbial strains will allow the application of gene-trait matching approaches, such as those described below, even to bacterial strains sequenced and phenotyped by different research groups. It should be noted that this is not always possible. For example, genome sequences are increasingly being produced from organisms that are not grown in pure culture or even from single uncultured cells.

Phenotype data may be noisy due to several factors, including inaccuracies in the measurements. Moreover, phenotypic measurements assessed on a continuous scale are often converted to categorical values such as 'Yes' or 'No'. However, it might be difficult to categorize some measurements accurately, resulting in ambiguous categories (or phenotypes) such as 'Maybe' or 'Mild'. In association analysis, samples with ambiguous phenotypes are preferably discarded to increase statistical power. For example, some cases that are annotated as 'Maybe' may in fact be a 'No' or a 'Yes', and to prevent noise from obscuring the association signal we recommend excluding such cases from the analysis.

ASSOCIATION ANALYSIS

Association analysis is a multi-step process that links the individual elements of the genotype (e.g. genes, phages, SNPs) to specific phenotypes (e.g. growth on different media, antibiotic resistance; see Figure 2). Depending on the experiment and the association approach, different pre-processing steps will be necessary for the genotype and phenotype data. Below we will describe these steps in more detail.

Large *p* small *n*

Many statistical and classification methods are suited for datasets including few measurements (e.g. genotypic variables) for many samples (e.g. strains). Conversely, the number of variables measured in high-throughput experiments is often much larger than the number of samples (genomes). This is referred to as the large *p* small *n* problem. With few samples, each individual sample has a relatively high importance, and there is a high risk of overtraining the data to individual samples. In such situations, machine learning methods are more suitable than classical statistical methods [86]. Accuracy of a machine learning algorithm can be improved by pre-processing input data, which could also decrease the number of variables significantly. A valid question is how many strains are necessary for a good signal in genotype–phenotype association studies. In general, more samples yield a better statistical power. While we have been able to extract meaningful links with as few as five positive and five negative cases (see also the section on 'Visualization' below), it has been stated that statistical issues can arise when sample sizes drop below 30 subjects. Such a small sample lacks heterogeneity (i.e. diversity) and does not approximate the normal distribution [87]. If possible, we recommend using at least 30 genomes in the association analysis, provided that matching phenotypic data is available.

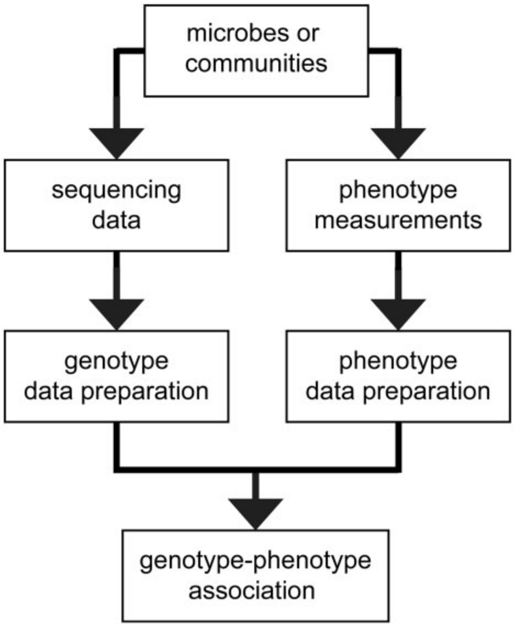

microbes or communities
sequencing data
phenotype measurements
genotype data preparation
phenotype data preparation
genotype-phenotype association

Figure 2: Flow diagram for genotype–phenotype association analysis. Genomic and phenotypic data are collected for microbial strains. Phenotypes can be determined by, e.g. phenotype microarrays or analytical profile indices. Both the genotypic and phenotypic data are then preprocessed before genotype–phenotype association analysis. In the association analysis, correlations between genotype and phenotype are determined and visualized.

Data filtering

When using data from high-throughput platforms that simultaneously assess many variables for many samples (e.g. next-generation sequencing technologies or phenotype microarrays), it is often necessary to apply some kind of pre-processing to filter for intrinsic noise [70,88]. Moreover, separately observed variables may contain redundant information for association analysis, for example, if two genes are in the same operon they will likely show the same presence/absence pattern across strains, and it might be better to collapse them. The most straightforward way to remove redundant variables is by using a correlation metric and combining strongly correlated features [26]. Finally, variables could be non-informative: i.e. a variable has the same or similar values across all samples, for example, a housekeeping gene that is present in every genome. Because these noisy, redundant or non-informative observations will not add relevant information about the different phenotypes, they need to be excluded from the association analysis [89,90].

Biased sampling of phenotypes

In addition to noise, the number of samples for each phenotype also affects the association analysis. In particular, an imbalanced distribution of samples across phenotypes decreases the accuracy of classification algorithms [91]. Due to overtraining of the algorithm toward the largest phenotypic group, strains from relatively rare phenotypic groups will often be classified into the more frequent phenotypic groups, as this is a 'safe bet' for the classifier. The effect of this phenotype imbalance can be decreased by bagging, where each bag contains an equal number of randomly selected samples, or by assigning reversely proportional weights to phenotypes [91,92].

Genotype–phenotype association

Here, we describe three approaches for genotype–phenotype association (Figure 3): statistical tests, correlation analysis and machine learning. Note that although these methods are often used, they are merely examples that illustrate a range of possibilities.

Comparison of means

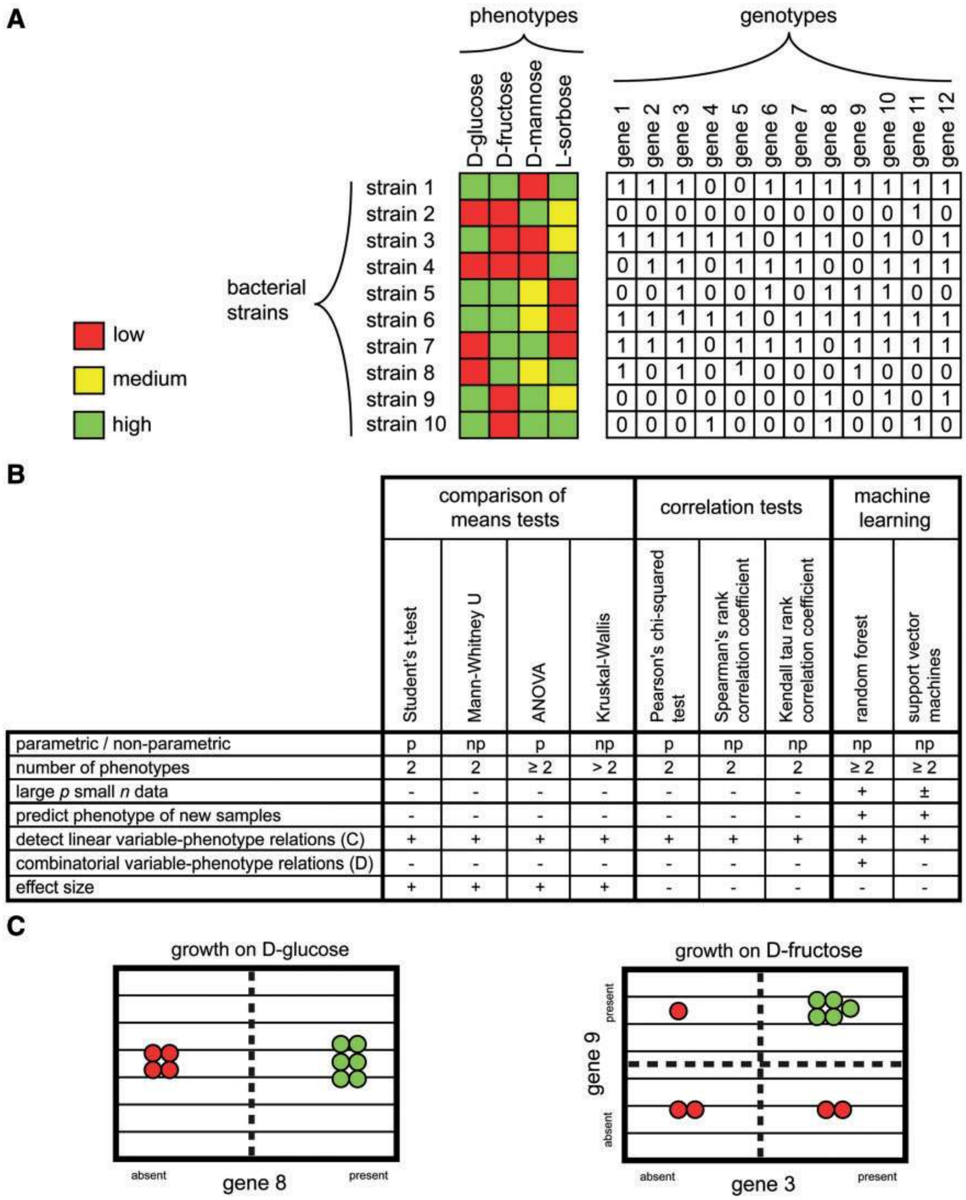
One of the most straightforward ways of detecting genotype–phenotype associations is by using a statistical test to compare the mean values of the genotypic variables between different phenotypes. It is important to know the distribution of the data, because it determines which tests can be used. For normally distributed data, (parametric) the Student's *t*-test (two phenotypes) [93] and ANOVA (two or more phenotypes) [94] are used, for other data the respective non-parametric (rank based) counterparts can be used, i.e. the Mann–Whitney U (two phenotypes) [93] and Kruskal–Wallis tests (two or more phenotypes) [95]. After multiple testing correction, these statistical tests supply the user with a *P*-value for each variable, which indicates the probability that the distributions of the different phenotypes do not differ significantly. As each genotypic variable is tested individually, no combinatorial dependencies between variables can be detected.

Correlation tests

Thus far, we have only discussed discrete genotypic variables (e.g. presence/absence of OGs, SNPs or phages). However, in some cases, it may be possible to assign continuous values (e.g. gene copy number or percent sequence identity). Similarly, some phenotypes may also be better represented as continuous values rather than dividing them into groups. In such cases, it is more appropriate to correlate the genotypic and phenotypic values. Similarly as for the comparison of means tests, the distribution of the data is an important factor in determining which method to use. For normally distributed data, the Pearson's chi-squared test [96] can be applied. The other two methods listed in Figure 3, the Kendall tau rank correlation coefficient (tau) [97] and Spearman's rank correlation coefficient (rho) [98] are both non-parametric, which means that they do not rely on a specific distribution in the data. On average, rho gives a higher correlation value than tau, while more severely penalizing individual samples that correlate badly [99]. As for the comparison of means tests, each genotypic variable is tested individually, and no combinatorial dependencies can be detected.

Machine learning

Machine learning methods, including random forest (RF) [100] and support vector machines (SVMs) [101] use training data to create a classifier (predictor) to classify phenotypes of new samples. These methods can generate an importance score for each of the genotypic variables for distinguishing the phenotypes (this is a native feature of RF; SVM requires an additional module such as the R module caret). Using this importance, variables can be removed that do not significantly contribute to the classification or improve the prediction accuracy [26,102,103]. The advantage of machine learning methods is that they build decision models that encompass multiple variables at once, allowing the prediction of phenotypes based on combinations of genotypic variables, i.e. two variables that contain no information on their own, but are predictive when assessed together (see Figure 3D) [104–106]. These interactions are



	D-glucose	D-fructose	D-mannose	L-sorbose
strain 1	🟩	🟩	🟥	🟩
strain 2	🟥	🟥	🟩	🟨
strain 3	🟩	🟥	🟥	🟨
strain 4	🟥	🟥	🟥	🟩
strain 5	🟩	🟩	🟨	🟥
strain 6	🟩	🟩	🟨	🟥
strain 7	🟥	🟩	🟩	🟥
strain 8	🟥	🟩	🟨	🟩
strain 9	🟩	🟥	🟩	🟨
strain 10	🟩	🟥	🟩	🟩

	gene 1	gene 2	gene 3	gene 4	gene 5	gene 6	gene 7	gene 8	gene 9	gene 10	gene 11	gene 12
strain 1	1	1	1	0	0	1	1	1	1	1	1	1
strain 2	0	0	0	0	0	0	0	0	0	0	1	0
strain 3	1	1	1	1	1	0	1	1	0	1	0	1
strain 4	0	1	1	0	1	1	1	0	0	1	1	1
strain 5	0	0	1	0	0	1	0	1	1	1	0	0
strain 6	1	1	1	1	1	0	1	1	1	1	1	1
strain 7	1	1	1	0	1	1	1	0	1	1	1	1
strain 8	1	0	1	0	1	0	0	0	1	0	0	0
strain 9	0	0	0	0	0	0	0	1	0	1	0	1
strain 10	0	0	0	1	0	0	0	1	0	0	1	0

	comparison of means tests				correlation tests			machine learning	
	Student's t-test	Mann-Whitney U	ANOVA	Kruskal-Wallis	Pearson's chi-squared test	Spearman's rank correlation coefficient	Kendall tau rank correlation coefficient	random forest	support vector machines
parametric / non-parametric	p	np	p	np	p	np	np	np	np
number of phenotypes	2	2	≥ 2	> 2	2	2	2	≥ 2	≥ 2
large *p* small *n* data	-	-	-	-	-	-	-	+	±
predict phenotype of new samples	-	-	-	-	-	-	-	+	+
detect linear variable-phenotype relations (C)	+	+	+	+	+	+	+	+	+
combinatorial variable-phenotype relations (D)	-	-	-	-	-	-	-	+	-
effect size	+	+	+	+	-	-	-	-	-

Figure 3: Choosing an approach for genotype–phenotype association. (**A**) Dataset consisting of phenotypes (e.g. growth rates on different carbon sources) and genotypes (e.g. gene content) for 10 bacterial strains (rows). (**B**) Nine possible methods (four comparison of means statistical tests, three correlation analyses and two machine learning methods) for detecting genotype–phenotype associations. The compatibility with specific data types and applicability in microbial GWAS is shown. (**C**) Hypothetical example of a linear genotype–phenotype relation. Green strains grow on D-glucose; red strains do not. The presence of gene 8 is predictive of the growth on D-glucose. (**D**) Hypothetical example of a combinatorial genotype–phenotype relation. All six strains that grow on D-fructose contain gene 9 and gene 3. In other words: the interaction between gene 9 and gene 3 is predictive of the growth on D-fructose.

implicitly modeled in current implementations of RF and SVM. However, there is no explicit importance measure for interactions between variables. Using machine learning methods allows the selection of the most important variables for further visualization (see below), interpretation and follow-up experiments in the laboratory.

We note that the steps outlined earlier can be applied to answer many different types of research questions that aim to find relations between large-scale datasets. Advanced users may choose to perform all the data processing steps using R and a set of specific modules. As an alternative, web tools are available such as PhenoLink [26] that uses RFs, and automatically performs many of the steps outlined above. Such automated pipelines allow for rapid, in-depth analysis of large amounts of data.

VISUALIZATION

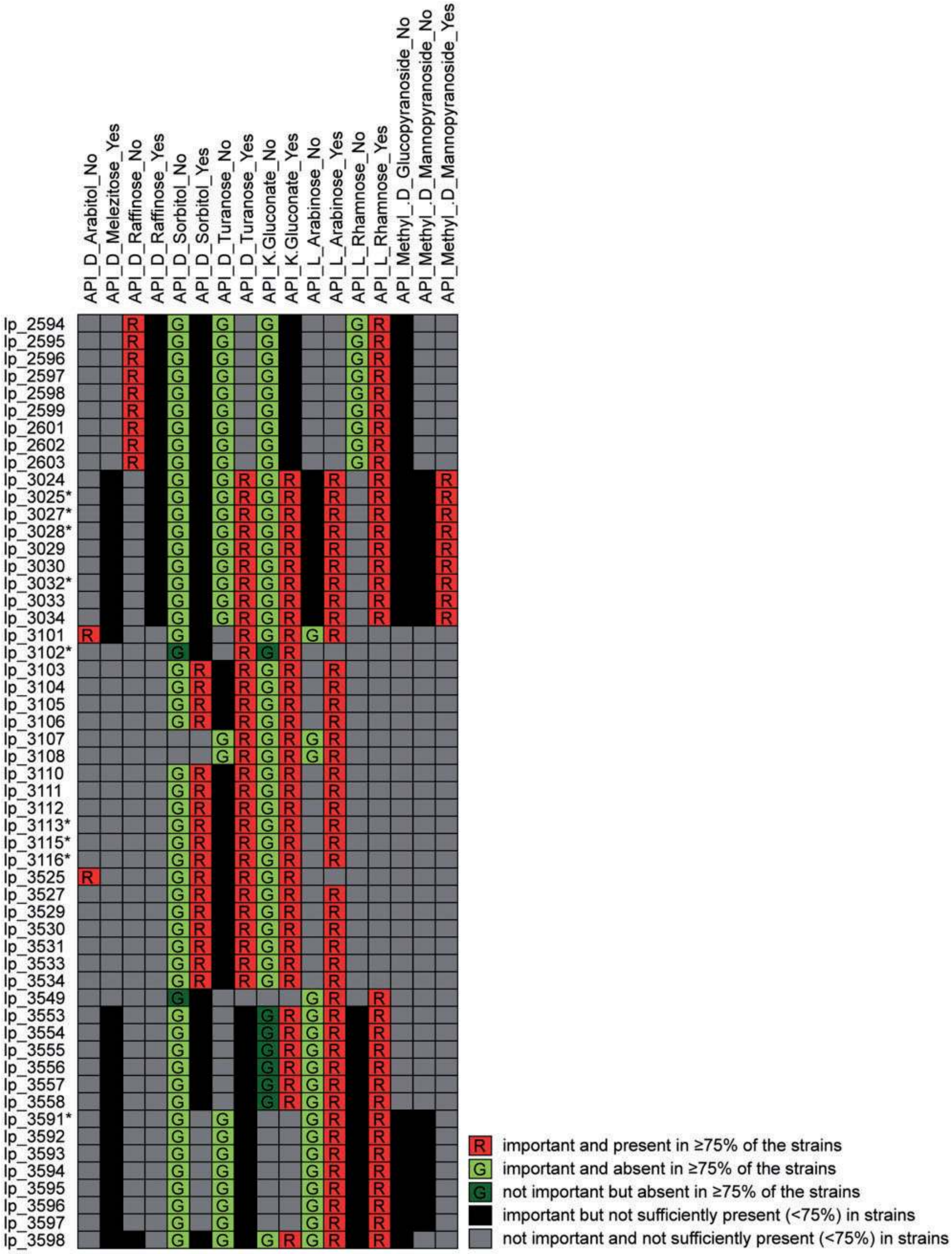
Mining very large datasets, like the collections of genotypic variables described above, may yield many significant associations. Although these genotype–phenotype associations are selected on the basis of their statistical significance, some associations might make more biological sense than others. For instance, the absence of a gene could have a high correlation with growth on a given carbon source, but if there is no function known for that gene (e.g. the gene could be a regulator repressing an operon required for growth on that sugar) a meaningful explanation for the statistical association might be difficult to predict. The integration of additional biological descriptors can help to formulate a meaningful interpretation of the results and to select the most promising associations for follow-up experiments.

Visualization allows us to integrate multiple information sources, including biological descriptors, and facilitate distinguishing relevant from irrelevant links. An optimal visualization would allow a general overview of the data, while simultaneously providing the possibility for in-depth examination of specific associations. To allow the inclusion of different sources of information, the visualization may require using multiple dimensions, each dimension representing a different source of information. Network graphs are an often used visualization that allows the incorporation of data from different sources [107]. However, such graphs can quickly become complex and often very large for many associations. Moreover, incorporating information from different sources is cumbersome when they are inter-dependent. For instance, visualizing a link between a gene and a phenotype based on their co-occurrence across a selection of strains could illustrate why this specific gene is predicted to be important. While visualization of such a three-way relationship (gene-strain-phenotype) is straightforward for a single gene, it is not easy for hundreds of genes and multiple phenotypes. Thus, color-coded tables (Figure 4) can be used to represent information using two different views: (i) a general figure that shows the relationships between all selected variables and their related classes and (ii) a detailed figure that shows the relationships between variables and samples for only a few classes. Similarly, multiple scales can be achieved in graph-based visualizations by interactive visualization of associations, initially showing a general view and also allowing interactive browsing of specific relations [108].

To illustrate the different possible visualizations, we show the results of a genotype–phenotype association analysis of 42 *L. plantarum* strains performed using PhenoLink [26]. All three visualizations show the same genes (genotype) selected for their importance in predicting one of the phenotypes (growth or non-growth on several sugars). The first visualization of these data comes from PhenoLink, using color-coding as an additional dimension of information to visualize the relations between 54 genes and 17 phenotypes (Figure 4). This figure highlights: (i) relations between genes that are relevant to similar phenotypes; (ii) relations between phenotypes that share a similar set of related genes and (iii) relations between individual genes and phenotypes. The second visualization, made with the graph-based visualization tool STRING [3], shows proteins as nodes and predicted pairwise interactions as edges (Figure 5). This graph shows additional links that have been mined from publicly available literature and experimental datasets. The third visualization (Figure 6) was made with iPath [109], and highlights the proteins in a global map of metabolic pathways, placing the selected genes in the context of their biological system.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recent technological advances, combined with large-scale genotype–phenotype association studies, hold a great promise for microbial functional genomics. Specifically, next-generation sequencing technologies have made DNA sequencing orders of magnitude faster and cheaper [27], and advances including the phenotype microarray [70,71] allow high-throughput measurement of microbial phenotypes. However, while bacterial genome sequences are appearing faster than they can be analyzed, the consistent measurement of phenotypes across the sequenced strains is often still lacking. Some examples of available datasets involving lactic acid bacteria include growth on different carbon sources [26,74,78]] organic acid production (measured in a single *L. plantarum* strain grown under different fermentation conditions [110]), adhesion to eukaryotic cells (*Saccharomyces cerevisiae* [111]) and IL-10 and



	API_D_Arabitol_No	API_D_Melezitose_Yes	API_D_Raffinose_No	API_D_Raffinose_Yes	API_D_Sorbitol_No	API_D_Sorbitol_Yes	API_D_Turanose_No	API_D_Turanose_Yes	API_K.Gluconate_No	API_K.Gluconate_Yes	API_L_Arabinose_No	API_L_Arabinose_Yes	API_L_Rhamnose_No	API_L_Rhamnose_Yes	API_Methyl_.D_Glucopyranoside_No	API_Methyl_.D_Mannopyranoside_No	API_Methyl_.D_Mannopyranoside_Yes
lp_2594	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2595	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2596	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2597	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2598	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2599	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2601	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2602	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_2603	▨	▨	R	■	G	■	G	▨	G	■	▨	▨	G	R	■	▨	▨
lp_3024	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3025*	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3027*	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3028*	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3029	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3030	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3032*	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3033	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3034	▨	■	▨	■	G	■	G	R	G	R	■	R	▨	R	■	■	R
lp_3101	R	■	▨	▨	G	■	▨	R	G	R	G	R	▨	▨	▨	▨	▨
lp_3102*	▨	▨	▨	▨	G	■	▨	R	G	R	▨	▨	▨	▨	▨	▨	▨
lp_3103	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3104	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3105	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3106	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3107	▨	▨	▨	▨	▨	▨	G	R	G	R	G	R	▨	▨	▨	▨	▨
lp_3108	▨	▨	▨	▨	▨	▨	G	R	G	R	G	R	▨	▨	▨	▨	▨
lp_3110	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3111	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3112	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3113*	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3115*	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3116*	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3525	R	▨	▨	▨	G	R	■	R	G	R	▨	▨	▨	▨	▨	▨	▨
lp_3527	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3529	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3530	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3531	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3533	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3534	▨	▨	▨	▨	G	R	■	R	G	R	▨	R	▨	▨	▨	▨	▨
lp_3549	▨	▨	▨	▨	G	■	▨	▨	▨	▨	G	R	▨	R	▨	▨	▨
lp_3553	▨	■	▨	▨	G	■	▨	■	G	R	G	R	■	R	▨	▨	▨
lp_3554	▨	■	▨	▨	G	■	▨	■	G	R	G	R	■	R	▨	▨	▨
lp_3555	▨	■	▨	▨	G	■	▨	■	G	R	G	R	■	R	▨	▨	▨
lp_3556	▨	■	▨	▨	G	■	▨	■	G	R	G	R	■	R	▨	▨	▨
lp_3557	▨	■	▨	▨	G	■	▨	■	G	R	G	R	■	R	▨	▨	▨
lp_3558	▨	■	▨	▨	G	■	▨	■	G	R	G	R	■	R	▨	▨	▨
lp_3591*	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3592	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3593	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3594	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3595	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3596	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3597	▨	■	▨	▨	G	▨	G	■	▨	▨	G	R	■	R	■	■	▨
lp_3598	▨	■	■	▨	G	■	G	■	G	R	G	R	■	R	■	▨	▨

R important and present in ≥75% of the strains
G important and absent in ≥75% of the strains
G not important but absent in ≥75% of the strains
■ important but not sufficiently present (<75%) in strains
▨ not important and not sufficiently present (<75%) in strains

Figure 4: Different ways to visualize *L. plantarum* genes that were found to be important to predict growth or non-growth on multiple sugars using PhenoLink [26]. Color-coded table of links between the 54 selected genes and growth on different sugars using from PhenoLink. 'Yes' or 'No' suffixes in column names indicate growth and non-growth, respectively. Asterisks (*) besides gene names (rows) indicate that the gene could not be mapped to COGs (see Figure 6). The color scheme integrates the importance of genes to predict phenotypes, and their occurrence in strains with that phenotype: bright red/green indicates genes that are important to a phenotype and present/absent in ≥75% of the strains with this phenotype; dim red/green indicates genes that are not important to a phenotype but are present/absent in ≥75% of the strains with this phenotype; black indicates genes that are important to a phenotype but are not sufficiently present/absent (<75%) in strains with this phenotype; gray indicates genes that are not important to a phenotype and are not sufficiently present/absent (<75%) in strains with this phenotype.

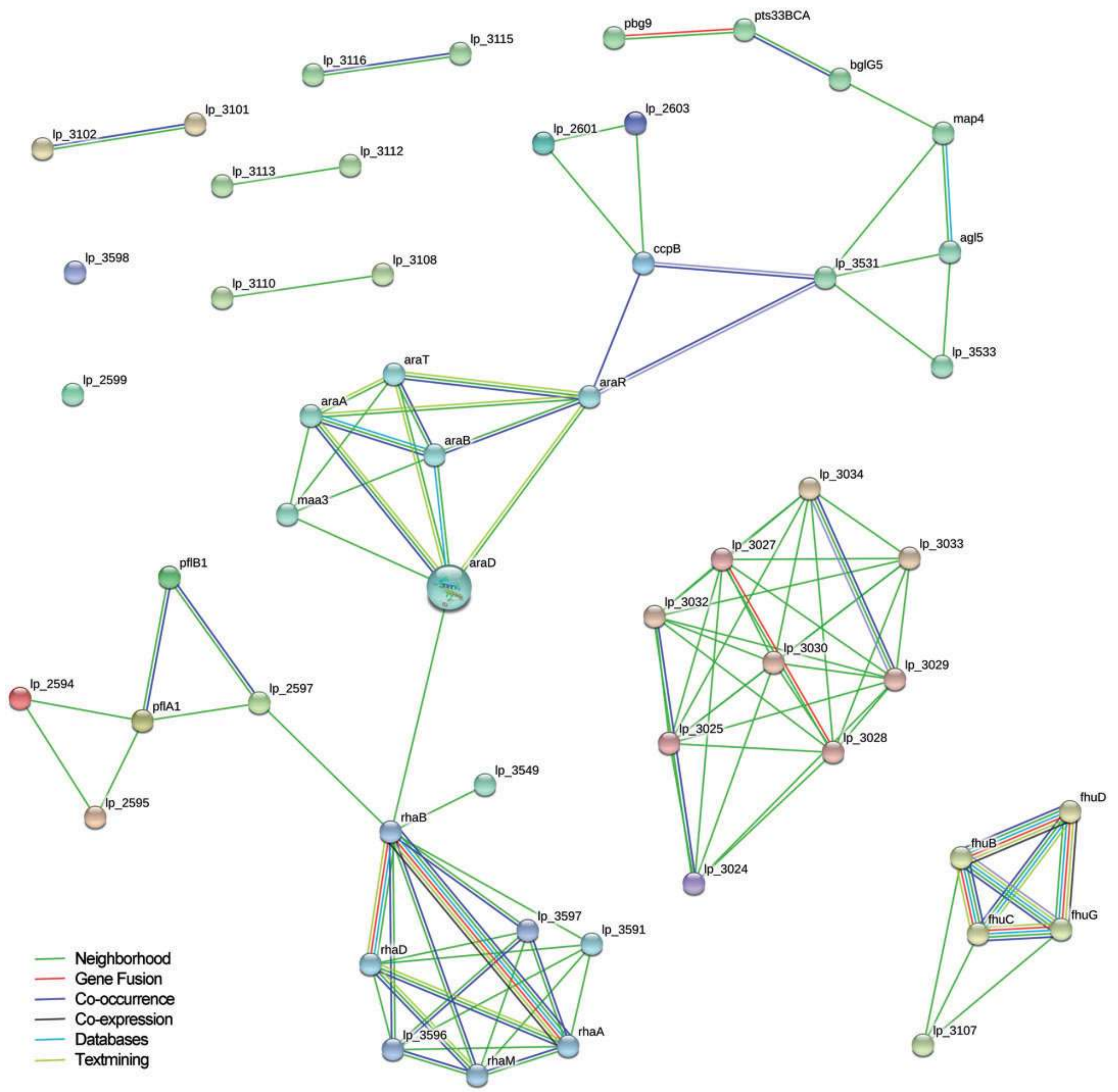


Figure 5: Different ways to visualize *L. plantarum* genes that were found to be important to predict growth or non-growth on multiple sugars using PhenoLink [26]. STRING evidence graph [3] of all 53 genes important for growth or non-growth on multiple sugars (all phenotypes combined). The gene *lp3111* did not encode a protein and was omitted from this figure.

IL-12 response in human cell lines (measured for 42 *L. plantarum* strains [112,113]).

The association methods described herein for discovering genotype–phenotype associations can also be applied to other types of data. For example, it may be valuable to link metagenomic entities including functions or taxa (operational taxonomic units) observed across metagenomic samples to clinical [114] or environmental [115] metadata. Indeed, discovery approaches have been published for such 'metagenome-wide' association of environmental parameters to metagenomic entities [115–117].

One of the disadvantages of the static measures of genotype described in this review (e.g. presence of OGs, phages and SNPs on the genome) is that they do not take into account other levels of cellular regulation, such as gene expression and protein abundance. Although the presence of OGs, phages or SNPs on a genome may have important consequences for the functioning of an organism, the question remains whether the gene product is actually present and functional. Addressing this issue, the first transcriptome-trait matching studies have recently been published [110,118], which are a

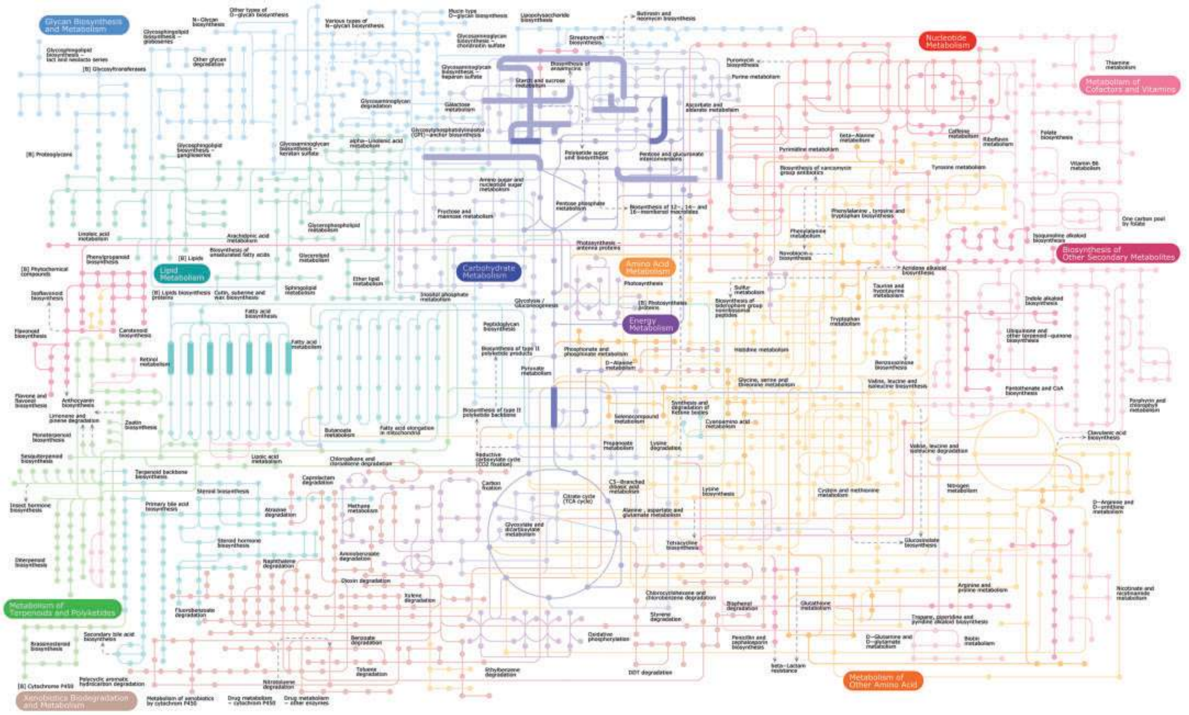

Glycan Biosynthesis and Metabolism
Nucleotide Metabolism
Metabolism of Cofactors and Vitamins
Biosynthesis of Other Secondary Metabolites
Lipid Metabolism
Carbohydrate Metabolism
Amino Acid Metabolism
Energy Metabolism
Metabolism of Terpenoids and Polyketides
Xenobiotics Biodegradation and Metabolism
Metabolism of Other Amino Acids

Figure 6: Different ways to visualize *L. plantarum* genes that were found to be important to predict growth or non-growth on multiple sugars using PhenoLink [26]. iPath global metabolic map [109] of the same genes mapped to COGs (47 unique COGs), where reactions with at least one mapped COG are indicated with a thick line.

particularly attractive way of comparing genotype and phenotype, and designing testable hypotheses. These studies determined the transcriptomes of a single strain grown under different conditions. Moreover, several phenotypes were measured in each of these cultures, including the production of organic acids, and the survival in the gastrointestinal tract. These phenotypes were then linked to the genes identified in the transcriptomes to discover which gene products correlated best with each specific phenotype.

Future microbial genotype–phenotype association studies will require the integration of consistent genome annotations with consistent phenotypic datasets, available in a computer readable format. The challenge does not lie in the generation of sequence data or in the development of novel statistical techniques, but rather in the generation, annotation and databasing of phenotypic data about the genomes and metagenomes that are being studied. Novel computational techniques, see for example [102], applied to these large datasets will allow determining interacting genes or SNPs that govern currently not understood complex phenotypes. These findings in turn will fuel the prediction of novel microbial properties for newly sequenced strains.

Key points

- The growing number of (draft) genome sequences constitutes a rich resource for microbial functional genomics.
- Phenotypes should be consistently measured and documented for the sequenced strains, so that computational tools can be readily applied.
- Datasets of consistently measured phenotypes across a collection of sequenced strains are still rare.
- Visualization can turn the sometimes abundant statistically significant genotype−phenotype associations into biological interpretations.

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