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An environmental perspective on metabolism

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Abstract

In principle the knowledge of an organism's metabolic network allows to infer its biosynthetic capabilities. Handorf et al. [2005. Expanding metabolic networks: scopes of compounds, robustness, and evolution. J. Mol. Evol. 61, 498–512] developed a method of network expansion generating the set of all possible metabolites that can be produced from a set of compounds, given the structure of a metabolic network. Here we investigate the inverse problem: which chemical compounds or sets of compounds must be provided as external resources in order to sustain the growth or maintenance of an organism, given the structure of its metabolic network? Although this problem is highly combinatorial, we show that it is possible to calculate locally minimal nutrient sets that can be interpreted in terms of resource types. Using these types we predict broad nutritional requirements for 447 organisms, providing clues for possible environments from the knowledge of their metabolic networks.

Keywords: Nutrient; Scope; Metabolic network

1. Introduction

Among the numerous approaches developed by Reinhart Heinrich on metabolism, one important focus was the identification of design and optimality principles (see Reinhart Heinrich's annotated bibliography in this issue). He considered such principles as essential prerequisites for understanding evolution of metabolism. It was in this line of thought that Reinhart Heinrich and some of us at the Humboldt University developed the concept of network expansion in the early 2000s (Ebenhöh et al., 2004; Handorf et al., 2005).

The basic principle is that a reaction can only operate if all of its substrates are available as nutrients or can be provided by other reactions of the network. This condition is applied in an iterative manner. Starting from the nutrients, which are called seed compounds, operable reactions and their products are added to an expanding network. This iterative process will end if no further reaction fulfills the above condition. The set of metabolites in the expanded network is called the scope of the seed compounds and represents all metabolites that can in principle be synthesized from the seed by the analyzed metabolic network (Handorf et al., 2005).

This concept has been applied in recent papers, including a discussion on hierarchical structuring of metabolic networks (Handorf et al., 2006), a comparison of metabolic capabilities of organism specific networks (Ebenhöh et al., 2005), a model of metabolic evolution (Ebenhöh et al., 2006) and the analysis of changes of metabolic capacities in response to environmental perturbations (Ebenhöh and Liebermeister, 2006). Further, scopes have been utilized to study the effect of oxygen in metabolic networks (Raymond and Segré, 2006) and to predict the viability of mutant strains (Wunderlich and Mirny, 2006).

In this work we consider the inverse problem of determining sets of seed compounds required for the synthesis of a specific compound or set of compounds. In particular the latter set may comprise metabolic precursors that the cell requires for maintenance or growth. Therefore solving this inverse problem may indicate minimal nutri-

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tional requirements that must be met to sustain maintenance or growth of an organism, based on the knowledge of its metabolic network. We apply this methodology to a number of organisms for which metabolic networks are defined in the KEGG database (Kanehisa et al., 2006) and show that this inverse methodology can indeed provide clues on possible nutritional requirements of organisms and their environment.

2. Methods

2.1. The target set of required metabolites

A key function of metabolism is to chemically convert available nutrients into products which are required by other cellular processes. Precursors for central cellular functions like protein synthesis, DNA replication, energy or cofactor production are ubiquitous. Since the detailed requirements may vary from cell type to cell type, we apply a systematic approach, combined with biological knowledge, to identify a universal set of necessary metabolites, referred to as the target set T.

We construct the target set by determining those metabolites which occur in at least 90% of the analyzed organisms. These include amino acids, nucleotides, many cofactors, organic acids and sugar phosphates. We manually refined this list by including plausible compounds which are missing and removing compounds whose presence in the target set seemed not reasonable. The detailed list of target metabolites as well as the removed compounds and the reasons for their removal can be found in the supplementary material.

2.2. Identifying minimal resources

To identify minimal sets of required resources that enable an organism to produce all metabolites contained in the target set, we develop an algorithm that relies on the method of expanding networks which was introduced in Handorf et al. (2005). Starting from a given set of initial metabolites, the seed S, the network expansion algorithm determines all those metabolites which a particular metabolic network is capable to produce when only the seed compounds are available. These metabolites are called the scope of the seed, denoted $\Sigma(S)$. The identification of minimal resources is now described as the problem to identify minimal sets of seed compounds for which the scope contains all target metabolites. A seed S is minimal if its scope contains the target T and no proper subset of S fulfills this condition:

$$S \text{ is minimal seed if } T \subseteq \Sigma(S) \quad \text{and} \quad \forall S' \subset S : T \not\subset \Sigma(S'). \tag{1}$$

For a given network, we determine minimal seeds with the following greedy algorithm: (1) Initially, we define an ordered list containing all metabolites occurring in the network. Clearly, the seed composed of all metabolites from the list must produce a scope containing the target set. (2) Beginning from the top, stepwise each metabolite is removed from the list and the scope is recalculated for the corresponding reduced seed. If now the scope does not contain the full target set, the metabolite is written back to the list, otherwise it remains permanently removed. (3) Step (2) is repeated until the complete list has been traversed. The metabolites contained in the resulting list represent a minimal seed because the further removal of any metabolite would result in a scope that does not contain all target metabolites.

Since the ordering of the list in step (1) determines which metabolites are preferentially removed (those near the top) and which preferentially remain in the seed (those near the end), differently ordered lists will result in different minimal seeds. Clearly, it is impossible to test all possible orderings of the list of metabolites and thus the complete set, denoted $\mathscr{M}$, containing all minimal seeds cannot be calculated. However, significant information about the structure of $\mathscr{M}$ can be obtained by calculating seeds for a sufficient number of random orderings. Further, as not all minimal seeds are equally biologically meaningful, the size of the search space can further be reduced by incorporating biological information on which metabolites can actually be used as nutrients. Such metabolites should reside near the end of the list, which can be achieved in the following way: First, all metabolites are sorted by decreasing molecular weight. This ordering leads to a preferential removal of large metabolites from the list of possible seed compounds and has been introduced to avoid minimal seeds containing only a small number of chemically rich but large metabolites that are unlikely to be transported into a cell. Second, for several metabolites, the transport processes over the membrane are well characterized. For our calculations, we have identified from the KEGG pathways KO02010 and KO02060 all those metabolites which can be translocated by ABC transporters or the phosphotransferase system. This biological knowledge has been considered by symbolically assigning all these compounds a negative "mass" which shifts them towards the end of the list. The resulting list ensures that metabolites that are known to be transported and small molecules are preferentially chosen as seed compounds.

To identify and analyze a large number of possible minimal resource sets, we construct a large number of perturbed lists and apply the algorithm repeatedly for each network. The random perturbation is designed in a way ensuring that large metabolites remain as a tendency near the top of the list. Specifically, we randomly chose two metabolites from the list and exchange their positions with a probability

$$p = \begin{cases} \exp\left(-\frac{1}{\beta}\Delta m\right) & \text{for } \Delta m > 0, \\ 1 & \text{for } \Delta m \leqslant 0, \end{cases} \tag{2}$$

where Δm is the difference of the molecular weights of the molecules at the two positions to be exchanged. It is positive if the heavier compound is the compound situated closer to the beginning of the list. The constant β determines the degree of disorder that is allowed in the resulting list, a value $\beta = 0$ strictly forbids that a heavier compound is placed in a later position than a lighter compound and a choice of $\beta = \infty$ would completely ignore the weights. For our calculation, we chose $\beta = 20\,\mathrm{Da}$ and performed 10,000 exchange operations to generate one randomized list. For each network, 1000 such lists are generated, yielding 1000 minimal, possibly identical seeds.

2.3. Identifying groups of exchangeable resource metabolites

It is typically the case that related substances can be used by a particular organism as alternative resources, for example various sugars may serve as carbon source. We call two metabolites A and B exchangeable if they appear as alternatives in minimal seeds. Formally, A and B are exchangeable if for all seeds $S_A = (A, X_0 \dots X_i) \in \mathscr{M}$, also the seeds $S_B = (B, X_0 \dots X_i)$ are in $\mathscr{M}$. Because the complete set $\mathscr{M}$ is not known, we test this condition on the set of minimal seeds that have been determined by the algorithm described above. First, we determine all metabolites which are found in at least one minimal seed. Then, for every pair A, B of these metabolites we: (1) identify all minimal seeds S_A containing metabolite A; (2) for each S_A construct the corresponding set S_B by replacing A by B (S_B is not necessarily a minimal set); (3) test whether $\Sigma(S_B)$ contains the target set T. If the latter condition is true for all S_A, we assume A to be replaceable by B. Conversely, we test whether B is replaceable by A. The metabolites A and B are considered exchangeable if they can be replaced by each other.

Clearly, since the calculated minimal seeds represent only a fraction of the complete set $\mathscr{M}$, the result is not necessarily correct, rather, pairs of metabolites may wrongly be classified as exchangeable (false positives). The classification becomes more accurate the more seeds are tested. Thus, metabolites occurring only a few times in the set of seeds are especially susceptible for being falsely predicted as exchangeable with other compounds. In order to keep the computing times feasible, a maximum of 50 seeds are tested even if the corresponding metabolites take part in a larger number of seeds. If the compounds A and B are found not to be exchangeable, then there exists a seed S_A where A cannot be replaced by B (or vice versa) in order to obtain the target set T. Hence, the algorithm will not predict false negatives, even though not all seeds in $\mathscr{M}$ are tested.

2.4. Graphical representation of minimal resources

The information on the exchangeability of seed compounds can be illustrated as an undirected graph with nodes representing seed compounds which are connected if they are exchangeable. To increase the reliability of the assembled information, the graph is further reduced by removing all compounds which occur in only one seed. Such a graph decomposes into clusters which may contain one or several compounds. Compounds within one cluster can be alternatively used in the seeds and can never occur together in a minimal seed. Metabolites from different clusters generally cannot be used as alternative seed compounds.

The total number of seeds in which compounds of a cluster occur determines how important it is to include one of the metabolites into a minimal resource. If this number equals the number of distinct minimal seeds, the presence of one of the metabolites from the cluster is essential for the organism. If it is lower, compounds of that cluster can actually be exchanged by metabolites from other clusters. It is also possible that one metabolite is exchangeable by two other metabolites which together provide the same required chemical structures. However, our algorithm is not able to detect such equivalences. For this reason, we call a cluster essential if compounds of this cluster occur in at least 90% of all minimal seeds.

In principle, if no exchangeable metabolites were falsely predicted, all compounds within a cluster should be pairwise exchangeable by transitivity and the cluster should form a complete subgraph (a clique). Therefore, if a cluster is not fully connected, it must contain at least two metabolites which have been falsely predicted as exchangeable.

2.5. Global classification of resource types

To consolidate the information contained in the graphs characterizing the required resources for each single organism, we construct a graph that represents classes of metabolites on a global level. For this, two metabolites are linked if they tend to be exchangeable in most organisms. Specifically, for two metabolites A and B, we determine the numbers of clusters they are found in across all considered organisms, denoted x_A and x_B, respectively, as well as the number of clusters containing both compounds, denoted x_{AB}. We introduce the coefficient

$$C_{AB} = \frac{x_{AB}}{\min(x_A, x_B)} \tag{3}$$

reflecting their co-occurrence as exchangeable metabolites. $C_{AB} = 1$ if these compounds appear exchangeable in all organisms. $C_{AB} = 0$ if they are not found exchangeable in any organism considered. In the present analysis we join two nodes if $C_{AB} \geqslant 0.8$. The resulting graph consists of separate connected components that can be interpreted as global resource types.

3. Results

We retrieved 447 out of 489 organism specific metabolic networks from the KEGG database (Kanehisa et al., 2006)

including information on the reversibility of the reactions (see supplementary material for the detailed procedure). For each of these metabolic networks, we calculated possible resource compounds, determined which of these compounds are exchangeable and represented this exchangeability as a graph. As an example, we present the resource graph for *Escherichia coli* K12 strain MG1655 in Fig. 1. Interestingly, of the 1000 calculated minimal seeds, only 560 are distinct. The fact that many identical seeds were found indicates that our algorithm has searched a considerable part of the biologically relevant minimal seeds. In total, 77 metabolites have been found in at least one of the seeds. All of the separated clusters in the graph are complete subgraphs, indicating that the number of wrongly predicted pairs of exchangeable metabolites is low. The numbers below the metabolite names give the number of distinct seeds in which a compound was found. The sum of these numbers over a cluster characterizes its essentiality.

Thiamin appears as an isolated compound and was found in 559 minimal seeds, which is consistent with the known auxotrophy of *E. coli* K12. The essential cluster at the top of Fig. 1 consists of sulfur sources including sulfate, thiosulfate and taurine. Dethiobiotin and two other metabolites form a cluster occurring in 546 minimal seeds. It appears in parallel with a compound labelled in KEGG as 'sulfur' (C00087), which is in fact an uncharacterized sulfur source in biotin synthesis. The fact that the biotin cluster appears essential contradicts the known prototrophy of *E. coli* K12 and is due to the lack of annotated pathway for pimeloyl-CoA synthesis in *E. coli*. Other resource clusters are found in significantly fewer minimal seeds, indicating that they are optional resources that can be substituted or synthesized from other types of resources. For example the carbohydrate cluster contains 11 exchangeable compounds that are found altogether in only 135 out of 560 minimal seeds, consistent with the fact that *E. coli* can use alternative carbon sources.

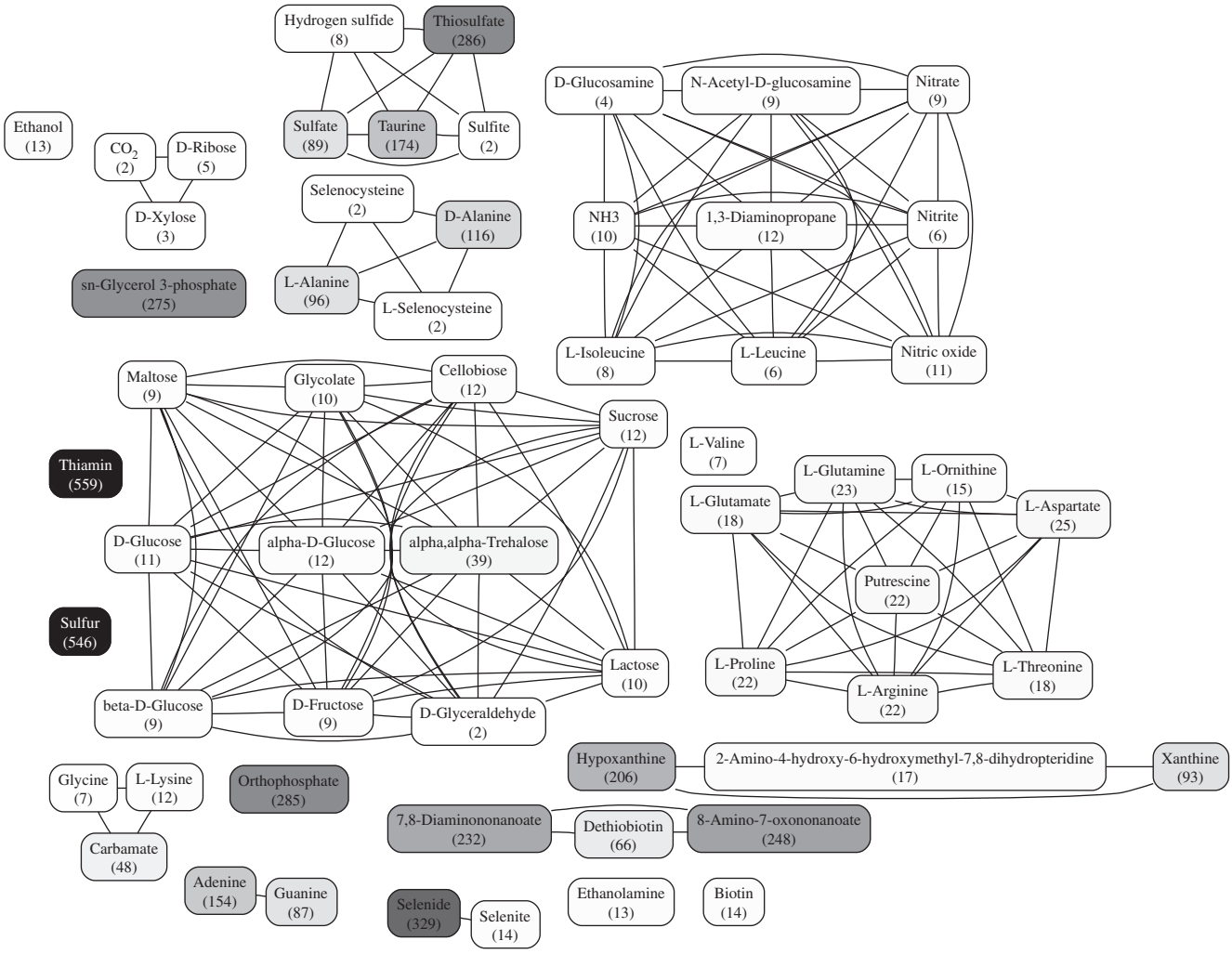

Fig. 1. Graph indicating exchangeable seed compounds for *E. coli* K12 strain MG1655. The numbers in parentheses indicate in how many of the 560 distinct minimal seeds particular compounds occur. The shading supports this information by displaying frequent compounds as dark and rare compounds as light nodes.

In order to assess our predictions, we also compared the resource graphs found for *Rickettsia prowazekii* and *Tropheryma whipplei* with the information provided in the Metagrowth database (Ogata and Claverie, 2005). The Metagrowth database provides evidence and hypotheses on culture conditions of selected obligate parasitic bacteria. Our calculations are in good agreement with most deficiencies of biosynthetic pathways reported in Metagrowth. Most compounds lacking a biosynthetic pathway according to Metagrowth could be predicted by our algorithm. The others were not found because they were not necessary for the synthesis of any compound in the target set. Details can be found in the supplementary material.

Resource graphs were identified for all 447 analyzed organisms. In most cases these clusters can also be categorized, for example as carbohydrates, amino acids or vitamins. However, they usually differ in their exact compositions, which is not surprising because of the structural differences of the underlying networks. As described in the methods section, we merged the information contained in the organism specific resource graphs into a graph representing global resource type clusters (see supplementary material). Due to the variety of the organisms and the fact that the algorithm is based on a statistical approach, different clusters in this graph may actually represent the same resource types. Therefore we merged manually some of the clusters containing closely related biochemical compounds. Also some compounds not normally used as nutrients, such as sugar phosphates, have not been considered further in this analysis. Table 1 provides a representative for each of the 45 resource types, with the full lists of the corresponding compounds given in the supplementary material.

The global resource types have been used for a comparative analysis of the nutritional requirements of all organisms. For each organism, all resource types have been matched with seed compound clusters of that organism if they have at least one compound in common. A specific resource type has been characterized as essential for a particular organism if at least one of the corresponding clusters is present in at least 90% of all its distinct minimal seeds. Consequently, it is characterized as optional if it is present in fewer than 90% of the seeds and it is unused if no compound associated with the resource type is present in any seed. The results for the Proteobacteria phylum are presented as a matrix in Fig. 2, where each row corresponds to an organism and each column to a global resource type. The corresponding entries in the matrix indicate the category of the resource type, red denoting essential, green optional and black unused resources. To visualize the relatedness of the considered organisms, they are ordered according to the topology of the phylogenetic tree as defined in KEGG (Kanehisa et al., 2006). The global resource types were sorted, maximizing the similarity of neighboring columns using a seriation method as introduced in Gelfand (1971) and an Euclidian distance between the nutrient profiles. The complete matrix comprising all considered species can be found in the supplementary material.

Table 1
List of resource types used in the comparative analysis of the nutritional profiles of the analyzed organisms

Acetate	Bases	beta-Alanine	Biotin	CO_2
D-Fructose	D-Glucose	D-Mannose	D-Ribose	Folate
Glycerol	Glycine	Isopentenyl	L-Alanine	L-Arginine
L-Asparagine	L-Aspartate	L-Cysteine	L-Glutamate	L-Histidine
L-Isoleucine	L-Leucine	L-Lysine	L-Methionine	L-Ornithine
L-Phenylalanine	L-Proline	L-Serine	L-Threonine	L-Tryptophan
L-Tyrosine	L-Valine	Maltose	Nicotinate	Nucleoside
Organic acids	Orthophosphate	Pantetheine	Propanoate	Riboflavin
Shikimate	Succinate	Sucrose	Sulfate	Thiamin

4. Discussion

In this work we presented a novel method for the analysis of nutritional requirements of organisms based on the structural analysis of their metabolic networks. The algorithm is effective in predicting precursors of essential compounds that cannot be synthesized by the organism itself. It also allows to determine sets of alternative non-essential nutrients representing metabolic options for potential different environments. Predicted nutrient sets differ among organisms, depending on their metabolic networks. In order to perform a comparative analysis, we defined general resource types containing well known resources, such as sugars, aminoacids or vitamins or their direct precursors. Organisms could thus be compared in terms of their usage of nutritional resource types in an essential or non-essential way.

We focused on Proteobacteria as an example for this inverse metabolic analysis. This group of bacteria is quite diverse in terms of metabolic requirements, with organisms ranging from prototrophs to multiple auxotrophs. The analysis of the nutritional requirements of different species generally indicates similarities in the requirements of closely related species (Fig. 2), which is consistent with classical approaches of microbial identification based on spectra of growth substrates or fermentable products. For example among Enterobacteriaceae, genera like *Escherichia*, *Yersinia*, *Salmonella* or *Shigella* have similar nutritional profiles and can utilize many nutrient types in a non-essential way. On the other hand and in the same family, the *Buchnera* genus possesses a completely different profile, requiring many nutrient types including amino acids in an essential way. This is consistent with the endosymbiotic nature of these organisms whose metabolism relies heavily on their hosts (aphids). Other predicted multiple auxotrophs include many obligate symbionts or parasites. For instance all Rickettsiales including the symbiotic *Wolbachia* belong to this group, as well as mollicutes and chlamydiales outside the Proteobacteria. Thus our results clearly distinguish between organisms having a relatively

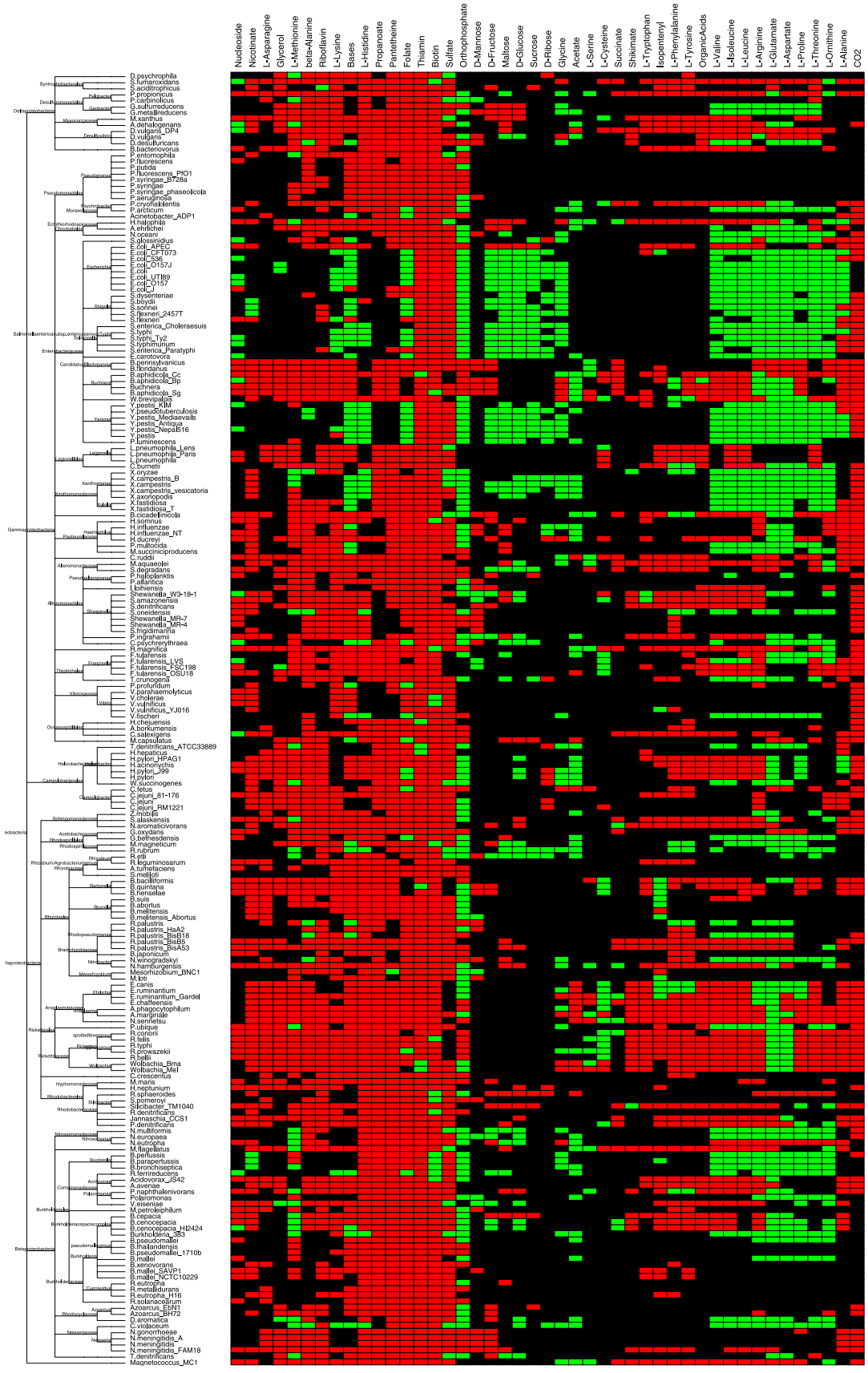

Fig. 2. Predicted nutritional profiles of Proteobacteria. For each organism it is indicated whether it uses the resource types of Table 1 in an essential (red) or optional way (green).

low number of essential nutrients and those exhibiting numerous predicted auxotrophies, allowing for a broad prediction of possible life-sustaining environments.

We compared the amino acid nutrition profile obtained for the *Buchnera* strain APS with the analysis of amino acid essentiality of Shigenobu et al. (2000). We found that Ala, Asn, Tyr and Pro are correctly predicted as required for *Buchnera* as they are provided by the host. Gly can be viewed as required instead of Ser since they are interchangeable. His, Trp, Thr, Phe and Arg are correctly predicted as not required, even though Phe and Arg are found to be utilizable. Asp and Glu on the other hand should be required according to Shigenobu et al. (2000). However, they are only partially used in our calculations. Pathways for Ile, Leu, Val and Met are incompletely annotated according to Shigenobu et al. (2000), which explains our prediction that they are essential. While the correspondence with our results is quite good, this example also shows some limitations of our algorithm. In particular, the discrimination between essential and non-essential nutrients is dependent on a tunable threshold value. With the current value, Asp and Glu were not predicted as exchangeable. When looking at the resource graph of *Buchnera* (supplementary material) it can, however, be seen that their occurrences are significantly higher that those of Phe and Arg that are not required according to Shigenobu et al. (2000).

Additional information will be required in order to reach a more precise and detailed prediction of life-sustaining environments. First, the list of preferred seed compounds and its sorting are critical. Different organisms may use compounds with different priority and the set of metabolites that they can transport will vary. Therefore our analysis will miss some specific requirements of individual organisms. Second, our analysis is sensitive to errors or gaps in the described metabolic networks. Here we used metabolic networks from the KEGG database as derived from KEGG orthology groups (Kanehisa et al., 2006). These networks are constructed from a systematic analysis of putative enzyme coding genes on the basis of homology, which inevitably leads to a small rate of false or missing predictions (see for instance Claudel-Renard et al., 2003). Missing enzyme predictions may cause false inference of auxotrophies, as seen for instance for biotin auxotrophy with *E. coli*. Third, information on reaction directionality is not yet systematically available for all enzymes, which may cause some unrealistic predictions. In addition our approach could be extended by adding thermodynamic constraints or flux balance constraints to the structural constraints considered here, which would require a different set of methods (see for instance Imielinski et al., 2006 for a recent approach based on convex cone analysis).

The inverse metabolic analysis presented here could prove useful in several respects. For instance there are numerous fastidious bacteria that are not readily cultivatable. They can be found among pathogenic species, in symbiotic associations or in various environmental samples. For these bacteria it has paradoxically become more accessible to obtain their complete genome sequence than to manipulate them experimentally, because of the lack of a culture medium. The method proposed here could be used to infer essential additions to culture media, which would complement manually curated metabolic knowledge bases such as Metagrowth (Ogata and Claverie, 2005). Inverse metabolic analysis could also provide clues on possible environments for poorly characterized organisms for which genome data are available. On the other hand, for well known organisms, the method can be used to uncover shortcomings in metabolic network annotation. In the longer term we envision to combine this type of inverse metabolic approach with the reconstitution of ancestral metabolic networks (Ebenhöh et al., 2006), which could provide clues on environments associated with the emergence and evolution of ancestral phyla.

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Appendix A. The supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtbi.2007.10.036.

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