

'Load Points' and 'Choke Points' as Nodes for Prioritizing Drug Targets in *Pseudomonas aeruginosa*

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Abstract: Biological pathways information has accumulated along with Genomic sequence data. These metabolic pathways help us in understanding network robustness and complex reaction networks. They also provide a framework for improved understanding of microbial physiology and for antimicrobial drug discovery. This article is an attempt to understand the local and global properties of metabolic networks in *P. aeruginosa* and to identify potential drug targets through 'load point' and 'choke point' analyses. In this study, we identify 25 choke point enzymes in pathways unique to *P. aeruginosa* and 202 choke point enzymes in the common pathways between the pathogen and the host human. We also list top 10 choke point enzymes based on the load point values and number of shortest paths and propose them as putative targets. These data underscore the utility of systems analyses methods for understanding human metabolic network in drug discovery process and in-depth understanding of the mechanism of diseases.

Keywords: *P. aeruginosa*, choke points, load points, Pathway Hunter Tool (PHT).

INTRODUCTION

The advent of the microbial genomics era has provided a wealth of information on a variety of microorganisms. At the same time, genomics has facilitated the identification and validation of a number of genes that could serve as targets for the discovery of novel antibacterial agents. The overall process of drug discovery and development seems poised to cure disease by identifying potential antibiotic drugs. However, this goal has been difficult to fulfill in recent years. Despite the advent of the high-throughput techniques sparked by the genomics revolution, discovery and development of new antibiotics has lagged in recent years due to the serious problem of evolution of antibiotic resistance [1].

Comparative genomics has provided a gradual increase in targets and the number of attractive targets available are no longer rate limiting. The problem of searching for potential drug targets is now becoming increasingly shifted towards the question: which are good target among the selected ones? [2]. Thus, the current challenge lies in short listing and prioritizing the targets [3].

One of the most useful applications of computational analysis of microbial genomes is to extract as much information as possible *in silico*, in order to simplify target selection for antimicrobial drug discovery. Putative targets must be essential for the survival of the pathogen and must be selectively present in the pathogen [4, 5]. Furthermore, genes that are conserved across different pathogens and have no human homologs represent attractive target candidates for new broad-spectrum antibiotics [6-8]. It is also possible to examine the complement of genes within a certain pathway or

discrete cellular function to help judge the suitability of a target [9]. These data could be supplemented by knowledge on metabolic modeling from genome revolution [10].

Concurrently, comparing genomes from related pathogenic and non-pathogenic species (differential genome analysis) can help in identifying pathogen-specific genes that could be tested for their pathogenicity. The many successes of this metabolic genome analysis include the complete reconstruction of the biosynthetic pathways for all 20 amino acids in *Escherichia coli*, *Haemophilus influenzae*, and *Bacillus subtilis* [11]. Thus, specific lists of target candidates can be generated and validated rapidly using bacterial genetics [12]. Alongside, graph theory based pathway analyses methods are useful in analyzing metabolic networks consisting of reactions, metabolites and enzymes [13-15]. Here, metabolic networks are represented as a metabolite graphs consisting of nodes (metabolites) and edges (reactions) with large number of connecting links. Such representation of network allows the characterization of the metabolic pathways with respect to degree of metabolite (nodes) connectivity defined as possible number of reactions by a metabolite; and the degree of interconnectivity or average network diameter defined as the average shortest path length [16].

Moreover, essentiality is almost perfectly predicted by the lack of an alternative pathway. Most crucial points in metabolic networks correspond to the enzymes at the periphery of the biological system [17]. Palumbo *et al.* [18] showed that the lethality corresponds to the lack of alternate paths in the perturbed network linking the nodes affected by the removal of the enzyme. Cellular metabolism is described and interpreted in terms of the biochemical reactions that make up the metabolic network. It is also crucial to define metabolic pathways in the context of network framework to determine the importance of enzymes. Defining the metabolites of the metabolic network as connections between biochemical reactions via substrate and product metabolites develops

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complex metabolic networks that may be analyzed to gather information on protein structure/function and metabolite properties.

These network frameworks take into consideration different aspects of metabolic chemistry and demonstrate the importance of metabolic biochemistry on the local and global properties of cellular metabolism. The integration of these metabolic pathway approaches along with systematic classification of the chemical structure of metabolites will not only enhance our understanding of metabolic pathways, but will also improve our ability to predict enzyme function and novel potential drug targets.

Pseudomonas aeruginosa is an opportunistic respiratory tract infection causing ubiquitous bacterium. The organism is inherently resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs and has the ability to adapt and thrive in many ecological niches, including humans [20]. Besides *P. aeruginosa* continues to be a major pathogen among patients with immunosuppression, cystic fibrosis, malignancy, and trauma [21]. Recently, we performed a differential genome analysis of metabolic enzymes in *Pseudomonas aeruginosa* for drug target identification and identified potential drug targets in unique and common pathways [19]. In this manuscript, we extend our analyses to the identification of potential drug targets based on the concept of 'load points' and 'choke points'. We propose top 10 choke points in *P.aeruginosa* based on the number of shortest paths in the bacterial network. A comparative analysis between the identified top 10 choke point enzymes and the human metabolic network is also performed. This approach allows for understanding of the local and global properties of the metabolic network thereby helping to chaff and prioritize the potential drug targets for this pathogen.

METHODOLOGY

A prerequisite for mapping metabolic pathways on genomic data is the annotation of proteins with metabolic information. The commonly known Enzyme Commission (EC) numbers are used to characterize proteins in order to classify the enzymatic chemical reactions of proteins. We illustrate the approach of load point and choke point analyses of metabolic pathways in *Pseudomonas aeruginosa* to identify potential drug targets. The analyses are carried out using the Pathway Hunter Tool (PHT) [22] to identify enzymes that are essential for the bacterial network.

Load Point and Choke Point Analyses

'Load point' of a metabolite in a metabolic network is defined as the ratio of number of k-shortest paths passing through the metabolite and its nearest neighbour links [23]. These load point values give a global view of the metabolic network and help in analysis of the metabolic pathway reactions. Pathways that are highly connected in the metabolism of the cell tend to have high load values. Moreover, the lethality of an enzyme depends on the number of connections it has in the whole metabolic network [24]. Enzymes with large number of connections are found to be highly essential and hence targeting them would result in disruption of the entire metabolic network. Jeong *et al.* [24] further concluded that the highly interconnected proteins would play an impor-

tant part in the central metabolism of the bacterial cell and are found to be three times more essential than proteins that interact with only a few other neighbors.

On the other hand, 'choke point' enzymes are those that take part in a reaction that consumes a unique specific metabolite (substrate) or uniquely produces a specific metabolite (product) in the metabolic network [25]. These choke point enzymes are crucial points in the metabolic pathway and inactivation of these important enzymes may lead to the disruption of the metabolic network of the bacterium. Thus, choke point enzymes are potential drug targets. Further, these load points and choke points help in determining the importance of metabolites in the metabolic network of the pathogen.

Step-1: Identification of Potential Drug Targets

The complete genome sequence of *P.aeruginosa* PAO1 and *Homo sapiens* was downloaded [20, 26]. Metabolic pathway information was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) a knowledge base for systematic analysis of gene functions [27].

Using the PHT tool we calculated shortest path distribution, the average path length and average alternate paths in the *P.aeruginosa* metabolic network. It is earlier suggested that the important aspects of global similarity (structural similarity between a metabolite over a series of other metabolites in a pathway) and local similarity (similarity between two consecutive metabolites) need to be considered while performing choke point analysis since higher the similarities smaller will be the network diameter and the average degree of nodes [22]. In this study we chose the local similarity score as 15% and the global similarity score as 5%. We then identified top 10 choke point enzymes based on the number of shortest paths. The obtained list of choke point enzymes was subject to a comparative analysis with our previously reported potential drug targets (361) in *P.aeruginosa* to list out top candidate essential targets [19]. A comparative study was performed between the pathogen choke point enzymes and the human metabolic network enzymes to differentiate human choke points from those of the bacterial choke points. Finally, for the predicted list of choke points in the pathogen, we performed a homology search against the human genome using BLAST [28].

RESULTS AND DISCUSSION

In this study, we present an analysis of metabolic networks in *P.aeruginosa* by ranking the metabolites on the basis of their load point and choke point properties. The results presented help to identify potential drug targets in the bacterial metabolic network.

Choke Point Analyses of the Metabolic Network of *P. aeruginosa*

Network models are crucial for shaping our understanding of complex networks and help to explain the origin of observed network characteristics. One of the most elementary characteristic of a node (metabolite) is its degree (or connectivity), k , which tells us how many links the node has to other nodes. The edges (reactions) explain the number of interactions each node has with other neighboring nodes. Based on these network characteristics, metabolite informa-

tion can be used to calculate the k-shortest paths between metabolites (substrate and product). Distance in metabolic networks can be measured in terms of path length, which represents the number of links passing between two nodes. As there are many alternative paths between two nodes, the shortest path with the smallest number of links between the selected nodes has a special property. The average path length represents the mean over the shortest paths between all pairs of nodes in the entire network of the bacterium [16].

PHT tool reveals that the metabolic network for *P.aeruginosa* PAO1 used in this study contained 996 reactions and 1063 metabolites with a network diameter of 34 and the average degree distribution (Connectivity) of 3.16 (Table 1). Comparison of the 361 enzymes (50 enzymes in unique pathways and 311 enzymes in shared pathways) [19] to that of the choke point enzymes obtained above shows that 227 targets match the previously reported 361 targets of which 25 targets belong to the unique pathways of the pathogen (Table 2). The remaining 202 targets match with that of the enzymes in the shared pathways between the pathogen and human. The list is provided in Supplementary (Table 1). Thus, a total of 63% of proposed drug targets are choke point reactions in the *P.aeruginosa* genome.

Choke point enzymes catalyzing at least one chokepoint reaction crucial for the survival of the pathogen can be considered as potential drug targets. Since a large numbers of biochemical reactions follow shortest path rather than longer paths, choke points enzymes are usually tabulated based on the shortest path length. Also, a higher load point value will result if larger number of shortest paths pass through a node (maximum connecting paths) having minimum number of closer connecting neighbors (minimum number of interactions). The shortest path distribution for the *P.aeruginosa* metabolic network is presented in Fig. (1).

The average path length, that is, the number of pathways it would require on average for one node to reach another accessible node in the interaction network is found to be 9.77. The above results could provide a completely new perspective in envisaging site directed mutagenesis experiments, by providing priority lists of the candidate targets that are most likely to affect the survivability of the pathogen.

Alternate Paths in the Metabolic Network

Few nodes with a very large number of connectivity links are termed as hubs as they hold many nodes together. How-

ever, even the complete removal of nodes that act as hubs that are highly linked in the network can be adjusted through redundancy or alternate pathways or bypasses that maintain the usual flow of metabolites in the cell. Hence, the pathogen can sustain its routine tasks due to its ability to locate another protein with a function similar to the removed protein. This resilient nature of the organism needs further consideration which would provide a more comprehensive understanding of the network.

It is important to keep track of alternate paths in the metabolic network which are indicators of the ability of the pathogen to survive under variable/diverse conditions. Most of the enzymes can be replaced by alternative reactions that utilize the same substrate or produce the specific product, thereby allowing a given pathway to operate [29]. Hence, blocking a path may not be vital as pathogens can make use of an alternate path performing similar functions. It is reported that the nodes that altered some fluxes typically altered many fluxes [30]. Oltvai and Barabasi, 2002 [14] described that within a model of a highly interconnected network: if one part of a web is perturbed, other compensatory changes in flow are likely to occur as well, analogous to a ripple effect spreading through the network. Alternate pathway lengths can thus be used to characterize the large-scale properties of metabolic networks. Average alternate path distribution for *P.aeruginosa* metabolic network is presented in Fig. (2).

Top 10 Choke Point Enzymes in *Pseudomonas aeruginosa* Pathogen

The top 10 choke point enzymes in *P.aeruginosa* identified using the PHT are ranked based on the number of shortest paths (Table 3). An enzyme with high load point value having greater number of shortest paths indicates its role in central metabolism and hence ranking choke points based on the shortest paths can help identify and shortlist enzymes with a higher probability of lethality to the pathogen. These choke point enzymes can be potential and prioritized drug targets. It is of note that four of the top 10 choke point enzymes have been reported as potential drug targets in our previous work. An additional criterion for being a choke-point enzyme is that it must not have isozymes as knocking out one enzyme would be easier than a cluster of enzymes.

Comparison of the choke point enzymes with the human genome sequence will help in differentiating between pathogen choke points and the human choke point enzymes. The

Table 1. Metabolic Network Analysis of *Pseudomonas aeruginosa*. Shortest Path between Two Nodes: the Path with the Smallest Number of Links between them. It is Used to Represent the Length of a Shortest Path. The Average Path Length Represents the Average Over the Shortest Paths between all Pairs of Nodes and Offers a Measure of a Network's Overall Navigability. Network Diameter D Defined as the Average Path Length or the Maximum of Shortest Path Over All Pairwise Nodes. If a Graph is Disconnected, We Assume that its Diameter is Equal to the Maximum of the Diameters of its Connected Components. Average Degree = $2L/N$ (N = Number of Nodes, L = Number of Links)

Genome	No. of Enzymes	No. of Reactions	No. of Metabolites	Average Path Length (Shortest Path/k-Shortest Path)	Diameter of the Network	Average Degree
<i>Pseudomonas aeruginosa</i> PAO1	535	996	1063	9.07/9.77	34	3.16

Table 2. Choke Point Enzymes in Pathways Unique to *Pseudomonas aeruginosa*. A Total of 25 Choke Point Enzymes are Listed that Matched with the Pathways Unique to *Pseudomonas aeruginosa*. The Enzymes are Tabulated with the Load Point Values and the Number of k-Shortest Paths

EC #	Pathways and Their Enzymes	Gene Id	Load Value (in)	Load Value (out)	k-Shortest Paths (in)	k-Shortest Paths (out)
	Polyketide sugar unit biosynthesis					
5.1.3.13	dTDP-4-dehydrohamnose 3,5 epimerase	PA5164	1.11	1.11	4463	4463
1.1.1.133	dTDP-4-dehydrohamnose reductase	PA5162	0.063	0.75	2086	2086
	Biosynthesis of siderophore group nonribosomal peptides					
5.4.4.2	Isochorismate synthase	PA423	-0.399	0.005	984	984
	Toluene and xylene degradation					
1.13.11.1	Catechol 1,2-dioxygenase	PA2579	-0.75	-0.75	229	229
	1,2 Dichloroethane degradation					
1.1.99.8	Quinoprotein alcohol dehydrogenase	PA1982	-0.52	-0.29	1450	1450
1.2.1.3	Probable aldehyde dehydrogenase	PA0219	-0.25	-0.02	11410	11410
	Phosphotransferase system (PTS)					
2.7.1.69	Phosphotransferase system, fructose-specific IIBC component	PA3560	0.53	0.53	2509	2509
	D-Alanine metabolism					
6.3.2.4	D-alanine-D-alanine ligase A	PA4201	-0.27	1.32	3699	3699
5.1.1.1	Biosynthetic alanine racemase	PA4930	1.39	0.29	3934	3934
	Lipopolysaccharide Biosynthesis					
2.7.7.38	3-deoxy-manno-octulosonate cytidyltransferase	PA2979	1.14	2.24	4611	4611
3.1.3.45	Putative 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase	PA4458	1.78	1.78	2909	2909
2.7.1.130	Tetraacyldisaccharide 4'-kinase	PA2981	-0.86	-0.86	414	414
2.4.1.182	Lipid A-disaccharide synthase	PA3643	-1.02	-1.01	883	883
2.7.-.-	Lipopolysaccharide core biosynthesis protein WaaP	PA5009	-1.58	-2.51	4496	4496
2.3.1.-	Poly(3-hydroxyalkanoic acid) synthase 1	PA0011	0.01	0.52	2478	2478
2.4.1.-	UDP-glucose:(heptosyl) LPS alpha 1,3-glucosyltransferase WaaG	PA5010	1.09	0.4	7310	7310
3.6.1.-	UDP-2,3-diacetylglucosamine hydrolase	PA1792	-1.72	-0.54	2268	2268
3.5.1.-	UDP-3-O-acyl-N-acetylglucosamine deacetylase	PA4406	1.67	-1.15	5238	5238
2.3.1.129	UDP-N-acetylglucosamine acyltransferase	PA3644	0.014	1.4	1986	1986
5.1.3.20	ADP-L-glycero-D-mannoheptose 6-epimerase	PA3337	0.424	0.424	748	748
2.5.1.55	2-dehydro-3-deoxyphosphooctonate aldolase (KDO 8-P synthase)	PA3636	0.19	1.8	2978	2978
	Two component system					
6.3.1.2	Glutamine synthetase	PA0296	-1.22	-1.53	4328	4328
4.2.1.20	Tryptophan synthase alpha chain	PA0035	1.16	0.82	18904	18904
3.1.3.1	Alkaline phosphatase	PA3296	1.35	1.35	1894	1894
1.7.99.4	Respiratory nitrate reductase alpha chain	PA1174	-4.8	-5.49	4	4

top 10 choke point enzymes were searched using BLASTp against human protein sequence database at an e-value cutoff of 10^{-2} . Five out of ten enzymes were identified as choke points only in pathogen and not in the human. These are - adenine phosphoribosyl transferase (EC 2.4.2.7), cytidylate kinase (EC 2.7.4.14), acetate-coA ligase (EC 6.2.1.1), Acyl-

phosphatase (EC 3.6.1.7) and asparagine synthase (EC 6.3.5.4) and they do not share any significant homology with the human genome (Table 3). Therefore, targeting these enzymes will most likely be lethal to the pathogen. These enzymes are also listed in Table 3.

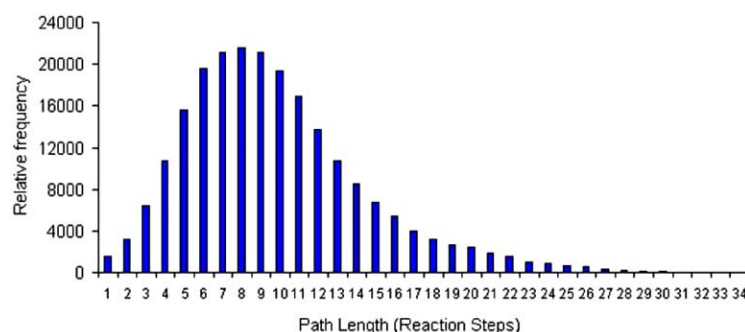


Fig. (1). Shortest path distribution for the *Pseudomonas aeruginosa* metabolic network. Distance in networks is measured with the path length, which tells us how many links we need to pass through to travel between two nodes. As there are many alternative paths between two nodes; the shortest path - the path with the smallest number of links between the selected nodes - has a special role.

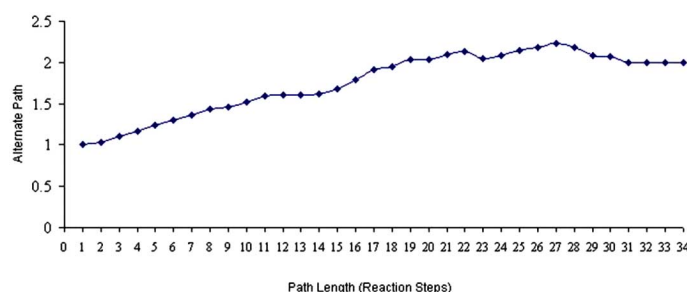


Fig. (2). The alternate shortest path in the network was calculated by dividing the total number of shortest path at each path length (reaction step) by the unique number of shortest path respectively.

Table 3. Top 10 Choke Point Enzymes in *Pseudomonas aeruginosa* Ranked by Number of Shortest Paths. A Comparative Analysis Between the Choke Points in *Pseudomonas aeruginosa* Against the *Homo sapiens* Metabolic Network was Carried out. This Analysis Identified Choke Points Present Only in the Pathogen and not on the Host Human

Enzyme Id	Enzyme Name	Gene Ids	Load value (in)	Load value (out)	k-Shortest paths (in)	k-Shortest paths (out)	Human choke point	Top BLAST hit (identity) (%)
2.4.2.7*	Adenine phosphoribosyl transferase	PA1543	1.11	1.89	74459	74459	No	No Homologue
2.4.2.8	Hypoxanthine phosphoribosyl transferase	PA4645	0.85	1.52	60639	60639	Yes	26%
2.7.4.6	Nucleoside-diphosphate kinase	PA3807	1.47	1.17	55297	55297	Yes	48%
2.7.4.14	Cytidylate kinase	PA3163	1.37	1.59	50169	5016	No	No Homologue
6.2.1.1*	Acetate-CoA ligase	PA0887 PA1997 PA2555 PA4733	-0.03	-0.16	48355	48355	No	No Homologue
2.6.1.1	Aspartate transaminase	PA2828 PA3798 PA4722 PA4976	0.54	0.34	40463	40463	Yes	33%
2.2.1.1	Transketolase	PA0548	1.36	1.46	40019	40019	Yes	26%
4.2.1.11	Phosphopyruvate hydratase	PA3635	2.28	2.07	38260	38260	Yes	54%
3.6.1.7*	Acylphosphatase	PA0954	4.07	1.87	28547	28547	No	No Homologue
6.3.5.4*	Asparagine synthase (glutamine-hydrolysing)	PA0051 PA2084 PA3459	0.03	-0.05	25142	25142	No	No Homologue

*Choke point enzymes previously reported as potential drug targets in the common pathways shared between the pathogen *Pseudomonas aeruginosa* and *Homo sapiens*.

Caveats

It must be noted that chokepoints may not be essential if they create unique intermediates to an essential product and exhibit alternate pathway reactions. Alternatively, a model

might overestimate the number of redundant reactions or pathways; this can be due to errors in annotation and unaccounted regulation. Equally true, each component of the reaction network may be present in the target organism but not expressed under the conditions to be examined. Our pro-

posed targets need to be examined further, both computationally and experimentally to validate their essentiality, suitability and success as a target. None-the-less these analyses help in prioritizing targets for further explorations.

CONCLUSION

There is a need to identify genes involved in infectious diseases caused by bacteria, in particular, those that are essential to the infectious process itself and not simply the life of the bacterium. As discussed above, metabolic pathway analysis and molecular approaches can be carried out to identify essential genes and then validate potential drug targets for any pathogenic bacteria. If alternative or bypass pathways exist and are not recorded in the model, then the model will fail to correctly predict the essentiality of a given gene or reaction. Therefore, target prediction will be likely used along with traditional methods of validation. However, prediction of metabolic gene essentiality remains an extremely powerful tool because *in silico* modeling of target genes with defined reactions can be used to assess many aspects of the cellular role of a reaction.

Thus, it is highlighted that to combat the increasing menace of drug-resistant microorganisms, the crucial first step is to generate a set of potential drug targets. The data presented here demonstrates that stepwise prioritization of genomic targets using simple biological criteria can be an effective way of rapidly reducing the number of genes of interest to an experimentally manageable number. The short listing of exploratory/prioritized targets will allow an accelerated genetic dissection of traits such as metabolic flexibility and inherent drug resistance that render *P. aeruginosa* such a tenacious pathogen [5, 31]. Such a strategy will enable us to locate critical pathways and steps in pathogenesis; to target these steps by designing new drugs; and to inhibit the infectious agent of interest with new antimicrobial agents.

SUPPLEMENTARY MATERIAL

Supplementary material can be viewed at www.bentham.org/cbio

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