An Approach to In Silico Dissection of Bacterial Intelligence Through Selective Genomic Tools

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Abstract All the genetic potential and the intelligence a bacteria can showcase in a given environment are embedded in its genome. In this study, we have presented systematic guidelines to understand a bacterial genome with the relevant set of in silico tools using a novel bacteria as an example. This study presents a multi-dimensional approach from genome annotation to tracing genes and their network of metabolism operating in an organism. It also shows how the sequence can be used to mine the enzymes and construction of its 3-dimensional structure so that its functional behavior can be predicted and compared. The discriminating algorithm allows analysis of the promoter region and provides the insight in the regulation of genes in spite of the similarity in its sequences. The ecological niche specific bacterial behavior and adapted altered physiology can be understood through the presence of secondary metabolite, antibiotic resistance genes, and viral genes; and it helps in the valorization of genetic information for developing new biological application/processes. This study provides an in silico work plan and necessary steps for genome analysis of novel bacteria without any rigorous wet lab experiments.

Keywords *Pseudogulbenkiania ferrooxidans*-HP2 · Genome sequencing · Gene cluster · Genomic tools

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Introduction

Thrust in the area of genomic research leads to the development of the sequencing technologies which allowed us to sequence the DNA with higher accuracy and greater coverage [1, 2]. The fast bioinformatics analysis provides the rapid mitigation of problems like biological approach in treating wastewater [3, 4] or converting infertile to fertile soil, generation of biofertilizers [5] and bioremediation ways to reduce the hazardous effect of a chemical [6, 7] in wastewater or soil.

Recently there are so many tools which support downstream analysis of bacterial sequence [8, 9] but their approach to analysis is unidirectional which could miss the bacterial potential in other aspects. In silico approach allows dealing with the whole set of genes available in the genome with less time as compared with the time required for laboratory experimentation. In spite of having 126593 prokaryotic genomes in NCBI database, it remains a challenge to understand every bit of the information encoding in the genetic material. Automated bioinformatics annotation pipelines like RAST, IMG AND NCBI [10] enhance the visibility of genes coding for different functions and the pathways which supports to synthesize particular product of interest or catabolize some toxic compounds like Aromatic, Xenobiotic and other pollutant present in the environment which are harmful for human being even if they are present in small concentrations [11, 12]. Information lacking in annotation pipeline and regular database can be overcome by analyzing the query sequences with a different set of tools which are designed for such attributes taking all aspects into consideration (Figs. 1, 2). For example, prior studies reported violacein production in chromobacterium species but [13] reported the same in *Pseudogulbenkiania ferrooxidans* EGD-HP2

Fig. 1 Sequential procedure for reads and assembly generation **Fig. 2** Systematic pathway to be followed for complete analysis of any bacterial genome

(EGD-HP2) via genome annotation in which they reported key genes of enzymes for pigment production and further 3D structure prediction. Violacein pigment producing *Chromobacterium* species was reported for a various biological activity like Anti-bacterial, Anti-cancerous [14], and Anti-leishmania [15]. The present study focuses on the exploration of the metabolic potential of *P. ferrooxidans* EGD-HP2 genome using various genomic tools. *vio* and *phl* gene cluster were considered in the study due to their commercial value with its biocontrol activity and biological response against other bacteria which is otherwise only reported for oxidizing Fe(II) and reduction in nitrate [16].

Results and Discussion

NCBI Database Submission

After sequencing and assembly, the first step is the submission of sequence in the reputed database which is also a mandatory step for every new sequence to be published. NCBI database provide platform where we can submit mRNA, plasmid, SNP, RNAseq, genetic test and genome sequence into the database; this database is interlinked with other databases such as PubMed, Entrez gene, OMIM, Variation viewer, dbSNP and other database that allows bacterial genome sequence to annotate all the genes present for functional activities like genes involved in metabolic or catabolic pathway. Integrated Microbial Genomes database is also a platform for comparative genomics of publically available draft and complete genomes of three domains. The Draft genome of *P. ferrooxidans* EGD-HP2 was assembled into 301 high quality contigs that have been annotated using NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) [17] into 4392 coding sequences (CDS), 80 tRNA genes, 4 copies of 5S rRNA genes, 1 copy of a 16S rRNA gene, and 8 pseudogenes. This draft genome has been deposited at DDBJ/EMBL/GenBank under the accession no. AVPH00000000 [13].

RAST Subsystem Annotation

A rapid annotation subsystem tool allows us to categorize the gene in different subsystem and pathways. After submitting genome in the RAST, annotation of the subsystem, come into play where gene and functional information like gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [18]. RAST server also provides a comparative analysis of genome from different bacteria with accuracy, consistency and in a comprehensive manner [19]. Subsystem gives a direct explorative analysis of the genome with the direct prediction of the presence of

various significant metabolic pathways like stress metabolism, antibiotic and virulence-related genes, Aromatic compound degradation genes [20] etc. This server permit to annotate 50–100 bacteria in a single day and requires almost 12–24 h to annotate whole bacteria [21].

RAST analysis categorized genome into 463 metabolic subsystem having different categories of genes like aromatic degradation (17 genes), Prophage (34 genes) and secondary metabolism (4 genes) etc. (Supplementary Fig. 1). RAST categorizes bacteria genome sequence into subsystem which comprised of the different pathway for metabolism and catabolism of particular compounds and their interlinked network can further be explored via KEGG available through an embedded link. Apart from systematic pipeline anti-SMASH tool assist as in mining DAPG gene cluster in our study. Therefore solely relying on annotation system, tools like BLAST (local Blast by BioEdit), ORF finder, domain analysis and similar tools can also be used for finding genes and their cluster.

P. ferrooxidans EGD-HP2 violacein cluster was found out using RAST and was found out similar to that of *Chromobacterium violaceum* ATCC 12472 [22] and *Pseudomonas tunicata* D2 [23]. Annotation of all the 5 enzyme-encoding for violacein pigment production such as VioA–VioE was found on the same contig_135 arranged in similar fashion when compared with *Chromobacterium* strains shown in the Fig. 3b [24]. VioE plays important role in skeleton arrangement of the violacein pigment because it allows the rearrangement of intramolecular indole ring to form proto deoxyviolacinic acid [25, 26].

Mining Secondary Metabolite

Anti-SMASH software provides us beneficial feature for automated identification of secondary metabolite gene cluster from the database as well as on probabilistic algorithms to find unknown putative gene cluster and its XML module helps in offline editing within Genious software [27]. Other tools which support mining secondary metabolite identification are 2metDB, BAGEL, CASSIS, SMIPS, CLUSEAN, Cluster Finder, ClustScan, Cluster mine360, IMG-ABC Integrated Microbial genomes, Bactibase Professional etc [28].

RAST also annotated *phl* genes i.e. *phlB* (structural gene) and *phlF* (functional gene) but it could not annotate all the genes responsible for biosynthesis of 2–4 diacetyl phoroglucocinal which were determined later by anti-SMASH. It gives the full set of gene cluster i.e. *phlABCDEF* with percentage similarity. *Phl* genes were reported for its biocontrol activity [29] making it a significant product in tandem with antibiotics. Anti-SMASH software

Fig. 3 KEGG pathway prediction of Violacein biosynthesis in the *Pseudogulbenkiania ferrooxidans* EGD-HP2 (a), gene arrangement of violacein gene cluster identified by RAST analysis and its similarity with other reported strain (b)

showed that *phl* gene cluster sequence showed 75% similarity with bacteria BGC0000281 and 62% with another bacteria BGC0000280 as shown in Fig. 4b. Further comparison of *phl* gene cluster with other reported strain shows that the *phlG* and *phlH* are missing in some of the bacteria including bacteria under study (Fig. 4c).

Evolutionary Trend Analysis by Phylogenetic Tree

Phylogenetic tree of genes is constructed to find out the evolutionary relationship of gene cluster present in certain species of bacteria to understand the evolutionary history of that particular gene cluster and to relate them with their

Fig. 4 Pathway prediction of 2,4-DAPG biosynthesis by KEGG in *Pseudogulbenkiania ferrooxidans* EGD-HP2 (a), Gene arrangement of DAPG gene cluster identified by antiSMASH server (b) and its comparison with gene cluster in other reported bacterial strains (c)

functional values. Different software and methods can be applied to deduce a particular evolutionary history (Supplementary Fig. 2). The evolutionary history of *vio* genes [30] was inferred by using MEGA 6 software. From the Supplementary Fig. 2a–d we could correlate that all the 4 genes in *P. ferrooxidans* EGD-HP2 were evolved from *Chromobacterium* ATCC 31532 strain whereas in case of *vioC* and *vioE* they are close to *Chromobacterium* pV104. Thus we can conclude that genes in the cluster were not evolved from single bacteria but from different strains of the bacterium with the course of time to come up with the challenging environment.

Phylogenetic tree of *phlD* gene cluster shows that *Pseudomonas protegens* strain is closely related to the synthetic construct of *phlD* genes and *Pseudomonas ferrooxidans* EGD-HP2 is closely related to *P. fluorescens* R31 and *Pseudomonas putida* strain KH-1. The lowermost clade includes another strain of *Chromobacterium* that is *C. vaccini* 21-1 which is closely related to *P. alcaligenes* strain MSSRFD254 and *P. Polyketide synthase* gene partial cds (Supplementary Fig. 3).

Mapping Biosynthetic Pathway

Mapping the different metabolic pathway provides the eyes inside metabolic wiring and offer a window for achieving any desirable product from a selected microorganism. In this case, violacein synthesis is associated with violacein gene cluster, comprising the *vioABCDE* operon [31]. The synthesis starts with *vioA* (Tryptophane 2-monooxygenase), it converts tryptophan to indole-3-pyruvic acid, and the next steps involve dimerization of IPA molecule in presence of violacein biosynthesis protein *vioB*. Proto (deoxy) violaceinic acid synthase *vioE* plays a key role in the molecular construction of violacein production [23]; in presence of *vioD*, it converts Proto violaceinic acid into violacein whereas in the presence of (monooxygenase) *vioC* it forms deoxyviolacein as shown in Fig. 3a.

DAPG synthesis starts with a *phlABC* gene which converts 2 molecules of acetoacetyl-CoA to a single molecule of acetoacetyl-CoA then polyketide synthesis cyclization of malonyl-CoA to phloroglucinol [32], then in the presence of *phlACB* MAPG acetyltransferase acetyls phloroglucinol to MAPG and then into DAPG [33] as shown in Fig. 4a.

Protein Structure Prediction and Analysis

Protein structure prediction was done using Swiss modeling approach for homology [34] to obtain structural analog in the database. When homology modeling results fall below 30% than threading modeling by RaptorX [35], iTASSER [36] and Phyre 2 is an option to be considered

for protein modeling [37]. The stereochemical analysis is necessary in order to check the generated structure quality and authenticity which can be performed by pdSum analysis [38], structural analysis at residue level was ensured using ProSA-web [39] and model quality prediction by ProQ server [40]. The generated structure in the pdb format can be visualized with the help of UCSF Chimera software.

Homology modeling was performed using template having 95.29% similarity in the case of *vioE* and 48.20% in the case of *phlD*. The generated protein pdb file was visualized by UCSF Chimera software 1.11 (Figs. 5a, 6a) [41]. Predicted structure of *VioE* was found to be highly similar with previously reported structure (Fig. 5a). Ramachandran plot showed that all the amino acid fall in the favorable region in case of *PhlD* (Fig. 6b) thus the generated model was found to be of good quality. Sequence alignment of *VioE* revealed that minor changes were found at amino acid positions on 43, 88, 122, 127, 139, 156 and 179 as shown in Fig. 5b but this residue was not found to be associated with any enzyme activity. Active sites were at Tyr 17, Phe50, Phe 109 and Arg172 [23].

Promoter Region Analysis

The promoter region of bacteria is comprised of particular consensus sequence which differentiates from other in terms of predicting its location within the bacterial start point. B-PROM software assists in the identification of the exact location of the promoter region. Some other tools that can be used for promoter prediction are Gene2promoter, PromoterHunter, BacPP, and Promoter by the neural network, PePPER but B-BROM has greater accuracy.

B-PROM helps here in determination of – 35 and – 10 region. Violacein gene cluster in *P. ferrooxidans* EGD-HP2 also consists of *Cvi* Box which works on Quorum sensing phenomenon (Fig. 5c) [42]. Transcription of *vio* genes is unidirectional similar to that of *Chromobacterium* 12472 [22] and *Pseudoalteromonas*. The sequence of regulator binding site was 19-bp termed as *cvi* box controlling *vio* operon expression was deduced manually using BioEdit tool [43]. Also, the manual mining of DAPG promoter region shows the operator binding region which controls *phlF* expression (Fig. 6c) [44].

Mining of Other Interesting Genomic Features

The presence of phage genes in a bacterial genome is very important one as it may provide support for horizontal gene exchange between the different strain and species of bacteria. All the genes responsible for assembling phage are present within the bacterial genome like capsid protein, tail protein prophage, integrase, and flu mu protein distributed

Fig. 5 The 3D structure prediction of VioE protein (homo dimer) by Swiss model server and structure visualization by UCSF Chimera software and the comparison of predicted structure with reported one (a). The sequence alignment and comparison of vioE protein by using

Muscle algorithm. The red box in the figure shows the active sites conserved in the protein (b). Promoter region of violacein operon predicted by BPROM software. The cvi box responsible for quorum mediated control is present upstream of – 35 box

Fig. 6 The 3D structure prediction of PhlD protein (homo dimer) by Swiss model server and structure visualization by UCSF Chimera software (a). The Ramachandran plot of predicted PhlD protein generated by ProCheck software (b); the plot confirms the

stable nature of protein as more than 90% of amino acid residues fall in the most favored regions. Promoter region of DAPG operon predicted by BPROM software (c); Operating binding region for phlF was also found to be located downstream of the promoter region

in contig 2, 3, 20, 23, 25, 27, and 29 as shown in Supplementary Table 1. The study of phage genes in bacteria assist in gaining insights into gene transfer event that bacteria had undergone and also help in assessing which genes may undergo gene transfer event. The viral genes from bacterial genome can be further explored in the viral database [45] to link specificity of specific phage with bacteria under study.

This study layout systematic methodology for dissection of bacterial intelligence and cellular decision making system by various genomic tools [46]. Here we have given insight into the production of the bacterially produced bisindole violacein, a secondary metabolite found to possess a wide variety of biological activities, including anti-infective, anti-cancerous and antifungal properties etc. We also reveal out the presence of DAPG production by our strain without any feedback inhibition system. Promoter analysis shows the quorum mediated control of violacein production.

Material and Method

Sequencing and Assembly

After genomic DNA isolation through DNA isolation kit (FastDNATM spin kit for soil DNA MP biomedical), Library preparation (2*150Nextseq 500) is the 1st step where library was prepared using Ex. Nextera DNA library preparation kit (Illumina), after that detection of a nucleotide sequence, was done with Miseq analyzer (Illumina USA) using Miseq reagents kit (Illumina USA). Quality was checked using FastQC software and Low-quality reads were trimmed using trimmomatic v 0.33 [47]. Then these trimmed nucleotides with a range of 50–300 bp were weaved into contigs with SPAdes software.

Data Preprocessing

Raw data is processed in a preferred format that is fasta format which is done using QIIME software it involves demultiplexing and quality filtering. High-quality data is achieved reads with no adapter having Phred score > 20 Quality. Draft genome was assigned with high-quality reads of 387,863 bp were assembled into 301 contigs by use of GS Assembler/CLC Genomics. The draft genome sequence has an average GC content of 64.1%. Genome assembly is done in two ways either by reference genome or by de-novo assembly method.

Genome Annotation and Analysis

The assembled genome was submitted to NCBI database and then submitted to RAST server for annotation and classification of genes in various subsystems for further analysis. Genes of importance were searched in the genome using RAST. For further scrutiny of genome antiSMASH software was used for the secondary metabolite. The phylogenetic tree was constructed using MEGA6 using UPGMA method for deducing evolutionary history [48]. The bootstrap value was 1000 to analyze topology of the tree and evolutionary distance was measured using maximum composite likelihood method [48]. 3D structure of vioE and phlD were predicted by homology modeling approach in SWISS model server. The generated structure was visualized and compared using UCSF Chimera. B-PROM software was used for a finding of promoter region near vio gene cluster. The specific sequence of interest was mined in the genome using the BioEdit software. As per the annotation by RAST all the required element of a phage particle were mined. Genes coding for phage protein are distributed in different contigs, after assembling all genes together and the local blast was performed against NCBI database.

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