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In silico Analysis of Essential Tricarboxylic Acid Cycle Enzymes from Biofilm-forming Bacteria

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ABSTRACT

The Tricarboxylic Acid Cycle (TCA) cycle is the central point in the metabolism of living organisms and is important for the survival of infectious biofilms. The inhibition of this vital point could be a promising strategy for the control of infectious biofilms. Therefore, this study was carried out to identify the potential drug targets from the TCA cycle of several Biofilm-Forming Bacteria (BFB) and to identify the available small molecule drugs against the TCA cycle enzymes. Based on the *in silico* substractive genomic approach, citrate lyase subunit alpha/citrate CoA-transferase [EC: 4.1.3.6], succinate dehydrogenase iron-sulfur subunit (EC: 1.3.99.1) and 2-oxoglutarate ferredoxin oxidoreductase subunit delta [EC: 1.2.7.3] were found to be essential and exclusively present in the BFB. Further *in silico* analyses showed that most of them are chemically regulated by myristoylation, phosphorylation, glycosylation and amidation. Based on the sequence search against DrugBank database, the potential small molecule drugs for biofilm treatment are 2-[1-methylhexyl]-4, 6-dinitrophenol, Atpenin A5 and Ubiquinone-2 which all target the succinate dehydrogenase enzyme of BFB. This study demonstrates the rapid identification of potential drug targets and small molecule drugs which could be useful in biofilm control strategies.

Key words: Tricarboxylic acid cycle, biofilm, subtractive genomics, potential drug targets, essential enzymes

INTRODUCTION

The Tricarboxylic Acid Cycle (TCA) cycle is a series of chemical reactions that takes place in the aerobic metabolism which generates reducing agents, NADH and $FADH_2$ and also energy in the form of ATP. It acts as a central point for most metabolic pathways in the living organisms making it a critical aspect in the study of infectious agents and drug development. For many decades, the common antimicrobial strategy relied on the inhibition of active cellular processes in the cytoplasm which are essential for the survival of the microorganisms. A study by Kohanski *et al.* (2007) has demonstrated that the inhibition of TCA cycle and changes in NADH consumption are observed during the treatment of bactericidal antibiotics on both Gram-positive and Gram-negative bacteria. Hence, the TCA cycle could be a promising target for the development of antibiofilm agents.

Biofilms are microbial communities attached to a surface. Many studies have shown that the microbial biofilm cells differ from their planktonic counterparts with respect to gene expression, metabolic performance, phenotype and susceptibility towards chemo therapy (Mikkelsen *et al.*, 2007). Biofilm infections have received great attention in medical research due its crucial role in antimicrobial resistance. The problem of eradicating the biofilm-mediated diseases is attributed to

the highly organized matrix of Extracellular Polymeric Substances (EPS) which limits the antimicrobial penetration. The general composition of bacterial EPS comprises of polysaccharides, proteins, nucleic acids and lipids.

A number of studies have described the association between TCA cycle and the biofilm formation mechanism. Polysaccharide intercellular adhesion (PIA) is a β-1,6-linked polysaccharide and is involved in the biofilm formation (Mack *et al*., 1996). Its production has been shown to be inversely correlated with the activity of TCA cycle (Kim *et al*., 2002). Particularly, repression of TCA cycle enzymes CitB (aconitase) and CitZ (citrate synthase) would lead to greater production of PIA and formation of biofilm (Kim *et al*., 2002; Sadykov *et al*., 2011). However, up regulation of other TCA cycle enzymes such as succinate dehydrogenase and succinyl-coenzyme A (CoA) synthetase has been observed during biofilm formation (Resch *et al*., 2005). In parallel with that, the NADH and $FADH_2$-dependent electron transfer process have been found to be highly active in the biofilm community (Renslow *et al*., 2013). The biofilm cells which are far from the surface of electrode appear to be actively consuming acetate and uranium for their redox processes (Renslow *et al*., 2013). Collectively, this implies that the function of TCA cycle in the supply of NADH and $FADH_2$ coenzymes for the electron transfer process remains active in the biofilm cells. Considering the situations as follows: i) down regulation of TCA cycle activity induces PIA production and biofilm formation, ii) considerably high electron transfer activity in the biofilm cells and iii) up regulation of some TCA cycle enzymes in the biofilm cells, it is likely that the repression of aconitase and citrate synthase is required for only initial stage of biofilm development while the overall TCA cycle activity is important to sustain the respiration process of biofilm cells. Thus, there is a need to highlight the importance of TCA cycle as part of the biofilm control strategy.

In this study, the *in silico* substractive genomic approach was used to identify the potential drug targets in the TCA cycle of biofilm-forming bacteria. A minor modification was made whereby the analysis focused on the enzymes responsible for TCA cycle only, not the complete set of proteins within the bacteria. A total of seven biofilm-forming bacteria were selected due to their important roles in a wide spectrum of harmful effects on human. In short, their TCA cycle pathways were analyzed using KEGG database and compared with that of *Homo sapiens*. The non-homologous TCA cycle enzymes which are essential for bacterial survival were considered as the potential drug targets.

MATERIALS AND METHODS

Comparative analysis of TCA cycle was performed based on the Kyoto Encyclopedia of Genes and Genome (KEGG) database (Ogata *et al*., 1999). The metabolic pathway identification numbers of the host *Homo sapiens* and the Biofilm-Forming Bacteria (BFB) were obtained from the KEGG database. The TCA cycle enzymes which are present in the BFP but do not appear in *Homo sapiens* were identified as enzymes unique to BFP. The protein sequences corresponding to the unique enzymes were retrieved from the KEGG database and were subjected to BLASTp (Altschul *et al*., 1990) search against the non-redundant database. This analysis was restricted to proteins from *Homo sapiens* and the e-value threshold was set to $1e^{-06}$ in order to identify the non-homologous enzymes. The potential drug targets were obtained by selecting those enzymes which had hits higher than the threshold of $1e^{-06}$ in BLASTp result. The essential proteins are the proteins which are indispensable for the survival of an organism and their functions are the foundation of life. The non-homologous enzymes were then subjected to BLASTp search against DEG database (Zhang *et al*., 2004) with e-value cutoff score of $1e^{-06}$ in order to determine the essentiality of these

enzymes to pathogens. The essential TCA cycle enzymes were then used in the analysis of post translational modification using ScanProsite (Hulo *et al.*, 2006) and the search for small molecule drugs against DrugBank database (Law *et al.*, 2014).

RESULTS

The selected Biofilm-Forming Bacteria (BFB) are as listed in Table 1. These bacteria have been chosen due to their contribution to a wide range of diseases such as cystic fibrosis, folliculitis, gastritis, diarrhea and periodontitis. The understanding on the structure of their TCA cycle could possibly contribute to the biofilm control strategies that target the metabolic network. The *in silico* substractive genomic approach has been a method of choice due to its rapid, efficient and inexpensive methods in discovering the potential drug targets. It is basically to identify the proteins exclusively present in the pathogen by deducing the homologous proteins. While many bioinformatics analyses using this approach have focused on the entire proteome within the bacteria, the present study has focused on only TCA cycle enzymes. The overall identification of essential TCA cycle enzymes in the BFB is summarized in Table 2. Based on the KEGG database, the number of TCA cycle enzymes identified in BFB ranged from 7-16 (Table 2) while there were 18 TCA cycle enzymes found in *Homo sapiens* (data not shown). This has revealed the peculiarity of TCA cycle of BFP in comparison with their host whereby some common TCA cycle enzymes are not present in the BFB.

Table 3 denotes a list of non-homologous TCA cycle enzymes from the selected microorganisms. The BLASTp search against *Homo sapiens* demonstrated that the non-homologous enzymes detected for BFB were citrate lyase subunit alpha/citrate CoA-transferase [EC: 4.1.3.6], succinate dehydrogenase iron-sulfur subunit (EC: 1.3.99.1), 2-oxoglutarate ferredoxin oxidoreductase subunit delta [EC: 1.2.7.3] and phosphoenolpyruvate carboxykinase (ATP) [EC: 4.1.1.49]. However, the BLASTp search against DEG database revealed that the phosphoenolpyruvate

Table 1: The selected biofilm-forming bacteria. The information of their TCA cycles was retrieved from KEGG database

Microorganism	Disease	Pathway identification No.
Vibrio cholerae 0395	Diarrhea	vcj00020
Pseudomonas aeruginosa PA01	Cystic fibrosis	pae00020
Helicobater pylori	Gastritis	hpy00020
Staphylococcus aureus	Folliculitis	sau00020
Escherichia coli	Diarrhea	eco00020
Campylobacter jejuni	Diarrhea	cje00020
Porphyromonas gingivalis	Periodontitis	pgi00020

Table 2: Summary of target identification and characterization

	Bioinformatics tools and No. of protein sequence				
Microorganism	KEGG	BLASTp	DEG	Scan Prosite	Drug Bank
Vibrio cholerae 0395	15	3	2	2	2
Pseudomonas aeruginosa PA01	15	3	2	2	2
Helicobater pylori	7	1	1	1	1
Staphylococcus aureus	15	3	1	1	1
Escherichia coli	16	3	2	2	2
Campylobacter jejuni	10	3	2	2	2
Porphyromonas gingivalis	8	3	1	1	1

Table 3: Non-homologous TCA cycle enzymes from the biofilm-forming bacteria and their essentiality

KEGG accession No.	Name of protein	Microorganism	Essentiality
VC0797	Citrate lyase subunit alpha/citrate CoA-transferase	*Vibrio cholerae 0395*	Essential
VC2088	Succinate dehydrogenase iron-sulfur subunit		Essential
VC2738	Phosphoenolpyruvate carboxykinase (ATP)		Non-essential
PA0883	Citrate lyase subunit alpha/citrate CoA-transferase	*Pseudomonas aeruginosa PA01*	Essential
PA1581	Succinate dehydrogenase iron-sulfur subunit		Essential
PA5192	Phosphoenolpyruvate carboxykinase (ATP)		Non-essential
HP0588	2-oxoglutarate ferredoxin oxidoreductase subunit delta	*Helicobater pylori*	Essential
SA0994	Succinate dehydrogenase	*Staphylococcus aureus*	Non-essential
SA1131	2-oxoglutarate ferredoxin oxidoreductase subunit delta		Essential
SA1609	Phosphoenolpyruvate carboxykinase (ATP)		Non-essential
b0615	Citrate lyase subunit alpha/citrate CoA-transferase	*Escherichia coli*	Essential
b0721	Succinate dehydrogenase		Essential
b3403	Phosphoenolpyruvate carboxykinase (ATP)		Non-essential
Cj0408	Succinate dehydrogenase iron-sulfur subunit	*Campylobacter jejuni*	Essential
Cj0535	2-oxoglutarate ferredoxin oxidoreductase subunit delta		Essential
Cj0932c	Phosphoenolpyruvate carboxykinase (ATP)		Non-essential
PG1614	Succinate dehydrogenase/fumarate	*Porphyromonas gingivalis*	Essential
	Reductase iron-sulfur subunit		
PG0429	2-oxoglutarate ferredoxin oxidoreductase subunit alpha		Non-essential
PG1676	Phosphoenolpyruvate carboxykinase		Non-essential

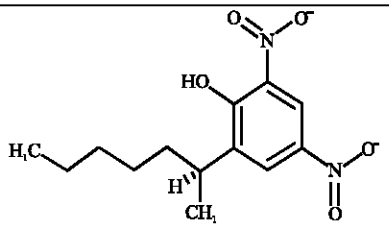
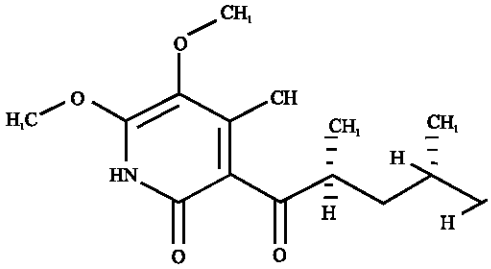
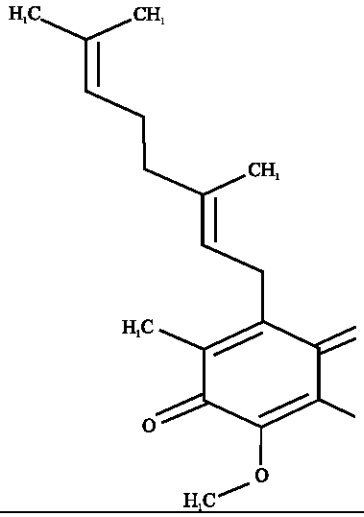
Table 4: Post translational modifications of essential TCA cycle enzymes from the biofilm-forming bacteria

KEGG accession No.	Name of protein	Post translational modifications
VC0797	Citrate lyase subunit alpha/citrate CoA-transferase	Myristoylation, phosphorylation
VC2088	Succinate dehydrogenase iron-sulfur subunit	Glycosylation, mristoylation, phosphorylation
PA0883	Citrate lyase subunit alpha/citrate CoA-transferase	Myristoylation, phosphorylation
PA1581	Succinate dehydrogenase iron-sulfur subunit	Myristoylation, phosphorylation, amidation
HP0588	2-oxoglutarate ferredoxin oxidoreductase subunit delta	Myristoylation, phosphorylation
SA1131	2-oxoglutarate ferredoxin oxidoreductase subunit delta	Glycosylation, mristoylation, phosphorylation
b0615	Citrate lyase subunit alpha/citrate CoA-transferase	Glycosylation, mristoylation, phosphorylation
b0721	Succinate dehydrogenase	Myristoylation, phosphorylation
Cj0408	Succinate dehydrogenase iron-sulfur subunit	Myristoylation, phosphorylation, amidation
Cj0535	2-oxoglutarate ferredoxin oxidoreductase subunit delta	Myristoylation, phosphorylation
PG1614	Succinate dehydrogenase/fumarate	Glycosylation, mristoylation, phosphorylation
	Reductase iron-sulfur subunit	

carboxykinase (ATP) [EC: 4.1.1.49] is not an essential TCA cycle enzyme for any of the BFP. Meanwhile, *Helicobacter pylori, Staphylococcus aureus* and *Porphyromonas vingigalis* were demonstrated to posses one essential TCA cycle enzyme whilst *Pseudomonas aeruginosa* PA01, *Vibrio cholerae* 0395, *Escherichia coli* and *Campylobacter jejuni* were shown to contain two essential TCA cycle enzymes. Because citrate lyase subunit alpha / citrate CoA-transferase [EC: 4.1.3.6], succinate dehydrogenase iron-sulfur subunit (EC: 1.3.99.1) and 2-oxoglutarate ferredoxin oxidoreductase subunit delta [EC: 1.2.7.3] were found to be exclusively present in some BFP and essential, they have been regarded as the potential drug targets.

Table 4 summarizes the essential TCA cycle enzymes from BFB with their post translational modifications. The Post Translational Modifications (PTM) are required to activate or repress the

Table 5: Potential small molecule drugs against the essential TCA cycle enzymes of biofilm-forming bacteria

Target protein	Drug name	DrugBank ID	Drug group	Drug structure
Succinate dehydrogenase	2-[1-methylhexyl]-4, 6-dinitrophenol	DB07671	Eperimental	
	Atpenin A5	DB04631	Experimental	
	Ubiquinone-2	DB08690	Experimental	

activity of a cellular protein. The analysis of PTM would provide useful information on how the activity of proteins could possibly be regulated in response to intracellular and extracellular stimuli. The *in silico* analysis demonstrated that the essential TCA cycle enzymes of BFB are regulated by myristoylation, phosphorylation, glycosylation and amidation. Their consensus patterns are as follows: G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}(G is the N-myristoylationsite), [RK]-x(2)-[DE]-x(3)-Yor[RK]-x(3)-[DE]-x(2)-Y (Y is the phosphorylation site), N-{P}-[ST]-{P}(N is the glycosylation site) and x-G-[RK]-[RK] (x is the amidation site).

Table 5 indicates the potential small molecule drugs against essential TCA cycle enzymes of BFB which were identified by the sequence search against DrugBank database. The potential small molecule drugs were found to be 2-[1-methylhexyl]-4,6-dinitrophenol, Atpenin A5 and Ubiquinone-2 which target the succinate dehydrogenase, the only enzyme that participates in both the TCA cycle and the electron transport chain. These organic compounds are classified as

nitrophenol, pyridinones and prenol lipids respectively. Since all identified small molecule drugs are still categorized as experimental drug candidates, they have not yet received the approval from the governmental regulatory authorities for medication.

DISCUSSION

KEGG database offers a great opportunity for researchers to perform the comparative metabolic pathway analysis. It has been the common computational tool for the studies of fundamental processes in living organisms and also drug target discoveries. The analysis using KEGG database showed the variation in the number of TCA cycle enzymes across all BFP. The analysis also revealed that the number of TCA cycle enzymes in the BFP is lower than that of their host, suggesting their uniqueness in sustaining the central metabolic point. These findings are in agreement with the general fact that many bacterial systems exhibit the metabolic peculiarities such as their responses to the carbohydrates and nitrates sources (Pishchik *et al*., 1997).

In general, the biomolecules in the pathogens could be regarded as potential drug target when they meet the criteria as follows: i) essentiality, ii) drug ability and iii) specificity. To date, the trend in identifying novel drug targets has changed from the conventional *in vitro* and *in vivo* drug screening to the use of computational tools which are proven to be greater in the operational cost and time. Our comparative *in silico* analysis between pathogens and the host *Homo sapiens* has successfully led to identification of several potential drug targets in the microbial biofilms. The same *in silico* strategy has also been used in many studies in order to identify the possible drug and vaccine targets (Barh and Kumar, 2009; Morya *et al*., 2010; Sharma *et al*., 2008).

For many decades, targeting PTM of biomolecules has been a great strategy in controlling many infectious and non-infectious diseases. Our *in silico* data demonstrated that the target enzymes in BFP are potentially subjected to myristoylation, phosphorylation, glycosylation and amidation processes. This is in accordance with the studies reporting the phosphorylation of several TCA cycle enzymes such as citrate lyase, succinate dehydrogenase and aconitase (Potapova *et al*., 2000; Salvi *et al*., 2007). In the studies of cancer and neurodegenerative disorders, the PTM has been the major focus in the development of disease markers and therapeutic interventions. In particular, the phosphorylation and O-glycosylation have been demonstrated to reduce the formation of neurofibrils in the Alzheimer's disease (Broncel *et al*., 2010). This has led us to suggest that the information of PTM of essential enzymes identified in this study would be useful for the development of novel antibiofilm agent by inhibiting the reaction of certain enzymes identified in this study.

A small molecule drug is an active substance that exerts therapeutic effects by regulating one or more biological processes. With the size less than 900 Daltons, it is able to rapidly diffuse across cell membranes and reach the intracellular sites of action. In this study, the only identified small molecule drug that has been extensively investigated is Atpenin A5. The inhibitory effect of Atpenin compound against a biofilm-forming fungus Trichoderma sp. has been reported by Miyadera *et al*. (2003). It has been shown to inhibit the Quinol-Fumarate Reductase (QFR) activity of complex II of an electron transport chain in the Trichoderma mitochondria thereby inhibiting the overall ATP-generating system. In addition, the impaired ATP synthesis has been shown to induce the biofilm detachment and suppression of EPS production Xu *et al*. (2012). Therefore, in conjunction with the findings from Miyadera *et al*. (2003) and Xu *et al*. (2012), it is likely that the identified small molecule drugs targeting the succinate dehydrogenase are effective against the biofilm growth mode.

CONCLUSION

We have demonstrated that the *in silico* study is successful in identifying the non-homologous and essential TCA cycle enzymes from Biofilm-Forming Bacteria (BFP). We have also identified the potential small molecule drugs targeting the essential TCA cycle enzymes of BFP. Considering the active respiration process in the microbial biofilm community and biofilm detachability following the ATP dissipation by the inhibitor molecule, it is possible that the small molecule drugs identified in this study may be able to control the biofilm infections. The findings from this study would be useful for further studies such as homology modeling, molecular docking analysis and experimental validation of antibiofilm agent.

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