

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Declaration Confirming Content of Digital Version of Thesis

I confirm that the content of the digital version of this thesis

Title: Genetic analysis of the succinate utilization genes in *Pseudomonas fluorescens* SBW25

is the final amended version following the examination process and is identical to this hard bound paper copy.

Have you published articles/material from your thesis **Yes/ No**

If yes, have you received copyright permission from the third party to include this published material in your thesis that will be placed in the Library's electronic repository? **Yes / No**

Student's Name: Yuting Liang

Student's Signature:



Date: 12/11/2013

Genetic analysis of the succinate utilization genes
in *Pseudomonas fluorescens* SBW25



A thesis presented in fulfillment of the requirements for the degree of
Master of Philosophy (Science)
in
Microbiological Genetics
at Massey University, Auckland,
New Zealand

Yuting Liang

2013

Abstract

Succinate, an intermediate of the tricarboxylic acid (TCA) cycle, is one of the most preferred nutritional substrates for bacteria, particularly those capable of colonizing eukaryotic hosts such as plants, animals (including humans). The genetic mechanisms of succinate utilization have been well studied in *E. coli* and other model microorganisms such as rhizobia, a group of gram-negative bacteria that form nitrogen-fixing nodules on leguminous plants. Uptake of succinate is mediated by the DctA transporter, whose expression is regulated by the two-component signal transduction system DctB / DctD in a succinate dependent manner. In the presence of succinate, the DctB sensor kinase binds to succinate, causing phosphorylation of the response regulator DctD that in turn activates transcription of *dctA* with the help of the alternative sigma factor σ^{54} .

Our work on the genetics of succinate utilization has focused on *Pseudomonas fluorescens* SBW25. *P. fluorescens* SBW25 is a plant growth-promoting bacterium that was originally isolated from the phyllosphere of sugar beet plants. When colonizing on the surfaces of sugar beet, *P. fluorescens* SBW25 activates the expression of a suite of genes involved in nutrient acquisition, including *pflu4717* with a predicted role in succinate uptake. The deduced amino acid sequence of *pflu4717* shows 70% sequence identity with *dctA* from *E. coli*, and 63% with *dctA* of *Sinorhizobium meliloti* 1021.

To confirm the predicted role of *pflu4717* in succinate uptake, a *pflu4717* deletion mutant was constructed and the resultant mutant strain was unable to grow on succinate as the sole source of carbon and energy (Suc⁻). The inability of the *pflu4717* mutant to grow on succinate can be restored by the introduction of a cloned copy of *pflu4717*. Furthermore, expression of *pflu4717* was induced by the presence of succinate as measured by using an integrated *lacZ* reporter gene. Together, the data consistently indicate that *pflu4717* encodes DctA for succinate uptake, and it is thus named *dctA*.

Next, we sought to identify the transcriptional regulators of *dctA* in *P. fluorescens*

SBW25. *In silico* analysis was performed using the DctBD sequences of *Sinorhizobium meliloti* 1021. The analysis identified three pairs of two-component regulatory systems: Pflu0287/Pflu0286, Pflu4953/Pflu4954 and Pflu1135/Pflu1134. However, deletion analysis for each of the three response regulators (Pflu0286, Pflu4954 and Pflu1134) showed that only the deletion mutant of *pflu0286* lost the ability to grow on succinate; and moreover, expression of *dctA* was not responsive to succinate in the growth medium. The data thus showed that *pflu0287* / *pflu0286* encode the DctB / DctD required for the succinate-induced expression of *dctA* in *P. fluorescens* SBW25.

Whilst the *dctA* deletion mutant (SBW25 Δ *dctA*) cannot grow on minimal medium supplemented with succinate as the sole carbon source, interestingly, a spontaneous Suc⁺ mutant arose at high frequency ($\sim 10^{-4}$). To identify the suppressor mutations, two such spontaneous Suc⁺ mutants were subject to genome re-sequencing, which led to the identification of two separate mutations in a putative sensor kinase Pflu4953. Pflu4953 forms a two-component regulatory system with Pflu4954, but as has been shown above is not involved in the utilization of succinate. Next, a logic series of experiments were performed using a combination of site-directed mutagenesis analysis and β -galactosidase assays. The results led to the conclusion that: (1) Pflu4953 and Pflu4954 (designated DctX and DctY, respectively here) regulate the expression of a putative transporter Pflu4955 (designated DctT); (2) DctT is responsible for the uptake of alpha-ketoglutarate (another intermediate of the TCA cycle), but it is also capable of transporting succinate; (3) however, the DctXY-mediated expression of *dctT* is induced by alpha-ketoglutarate, and not by succinate; (4) mutation of DctX caused constitutive expression of DctT, which enables the Δ *dctA* mutant to grow on succinate (Suc⁺).

Taken together, the data show that *P. fluorescens* SBW25 possesses two transporter systems for the uptake of succinate (i.e., DctA and DctT), which are regulated by the DctBD and DctXY two-component systems, respectively. However, the primary role of DctT is for the uptake of alpha-ketoglutarate and not succinate, as expression of DctT is only induced by alpha-ketoglutarate. This finding indicates that substrate specificity of an uptake system is determined

by not only the transporter protein but also its regulator(s). Given that succinate is significant nutrient available on the plant surfaces, the encoded multiple systems for succinate uptake likely contribute to the success of *P. fluorescens* SBW25 in the plant environment.

Acknowledgement

First and foremost I would like to thank my supervisor Dr. Xue-Xian Zhang, thanks for his constant support, time, effort and patience. I am deeply grateful for all the encouragement you have given me not only in my lab work but also with everything.

I would also like to thank my associate supervisor Prof. Paul B. Rainey for his endless support. Thanks for offering such great project to me. I am so appreciated for his kind guidance.

I would like to take this opportunity to thank Yunhao Liu. Thanks for all your help in constructing expression vector, getting my cloning to work and for cheering me up when I didn't think I was ever going to get results!

Thank you also to Dr. Heather Hendrickson, Andy Farr and Yeserin Yildirim for your advice and for helping me with the numerous little questions that I had in the lab. Thanks for the lovely atmosphere that people made in the laboratory. I am very lucky to work with people from Rainey Lab. You are brilliant people.

Lastly, I would like to thank my friends and family. Especially, thanks to my parents for always being there, support me and encourage to me. I couldn't have done it without all your support.

Table of Contents

Abstract	III
Acknowledgement	VI
Table of Contents	VII
List of Figures	X
List of Tables	XII
List of Abbreviations	XIII
Chapter 1 Introduction	1
1.1 Plant-associated <i>Pseudomonas</i>	1
1.2 Preferential utilization of succinate by <i>P. fluorescens</i> SBW25	2
1.3 Current understanding of the molecular mechanisms of succinate utilization in bacteria	3
1.4 Previous work leading to this research	6
1.5 Objectives of this study.....	7
Chapter 2 Material and Methods	9
2.1 Solution and Media	9
2.1.1 Media	9
2.1.2 Solutions and Buffers.....	9
2.1.3 Enzymes and Reagents	9
2.2 Primer design	12
2.3 Polymerase Chain Reaction (PCR)	15
2.4 Phusion DNA polymerase PCR	16
2.5 Preparation of electrocompetent <i>E. coli</i> cells	17

2.5.1 Glycerol-saline stock	17
2.6 Gateway LR Recombination Reaction.....	18
2.7 Electroporation.....	19
2.8 Plasmid purification.....	19
2.9 Restriction enzyme digests	20
2.10 Agarose gel electrophoresis.....	20
2.10.1 Determination of DNA fragment sizes	21
2.10.2 Determination of DNA concentration.....	21
2.11 Conjugation of pUIC3 and its derived plasmids into <i>P. fluorescens</i> SBW25 (tri-parental mating)	21
2.12 β-Galactosidase Activity Assay	22
2.13 Cycloserine enrichment	23
Chapter 3 Results	25
3.1 <i>In silico</i> analysis of genes for succinate utilization in <i>P. fluorescens</i> SBW25	25
3.2 Genetic characterization of <i>dctA</i> in <i>P. fluorescens</i> SBW25.....	27
3.2.1 Deletion analysis of <i>pflu4717</i> and genetic complementation	27
3.2.2 Expression of <i>pflu4717</i> induced by succinate.....	29
3.3 Genetic identification of the regulator(s) for succinate uptake in <i>P.</i> <i>fluorescens</i> SBW25	32
3.3.1 Deletion analysis of the three DctBD homologues in the genome of <i>P.</i> <i>fluorescens</i> SBW25.....	32
3.3.2 The roles of the candidate regulators in succinate-induced expression of <i>dctA</i>	33

3.4. Suppressor analysis of the <i>dctA</i> deletion mutant (Suc⁻) of <i>P. fluorescens</i> SBW25	35
3.4.1 Isolation and characterization of the suppressor mutants (Suc ⁺) of MU30-30 (Δ <i>dctA</i>)	35
3.4.2 Genetic verification of the repressor mutations	37
3.4.3 <i>In silico</i> analysis of the suppressor mutants	37
3.4.4 Genetic characterization of the <i>dctXYT</i> locus	39
Chapter 4 Discussion:	44
4.1 Substrate specificity of the dicarboxylate transport (Dct) system	44
4.2 The involvement of transporters in gene regulation	45
Chapter 5 Conclusion and Future Work:	47
Reference:	49

List of Figures

Figure 1-1, The domain structure of a typical two-component system (TCS).	4
Figure 1-2, Two-component system of DctB/DctD in <i>Sinorhizobium meliloti</i>	5
Figure 1-3, Model for C4-dicarboxylate transport in <i>P. aeruginosa</i> PAO1.	6
Figure 2-1, Physical map of the integration plasmid vector pUIC3.....	24
Figure 3-1, Genetic organization of the <i>pflu4717</i> locus from <i>P. fluorescens</i> SBW25.	27
Figure 3-2, Growth curves of wild-type <i>P. fluorescens</i> SBW25 and the mutant MU30-30 (Δ <i>dctA</i>).....	28
Figure 3-3, Verification of the recombinant plasmid for <i>pflu4717</i> (<i>dctA</i>) complementation.....	28
Figure 3-4, Succinate-induced expression of <i>dctA</i> in <i>P. fluorescens</i> SBW25.	30
Figure 3-5, Malate-induced expression of <i>dctA</i> in <i>P. fluorescens</i> SBW25.....	30
Figure 3-6, Fumarate-induced expression of <i>dctA</i> in <i>P. fluorescens</i> SBW25.....	31
Figure 3-7, Growth phenotype of deletion mutants of three response regulators. .	32
Figure 3-8, Succinate-induced <i>dctA</i> expression in wild type <i>P. fluorescens</i> SBW25 (blank) and mutant Δ <i>pflu0286</i> (dotted), Δ <i>pflu1134</i> (grid) and Δ <i>pflu4954</i> (black).	34
Figure 3-9, Malate-induced <i>dctA</i> expression in wild type <i>P. fluorescens</i> SBW25 (blank) and mutant Δ <i>pflu0286</i> (dotted), Δ <i>pflu1134</i> (grid) and Δ <i>pflu4954</i> (black).	34
Figure 3-10, Fumarate-induced <i>dctA</i> expression in wild type <i>P. fluorescens</i> SBW25 (blank) and mutant Δ <i>pflu0286</i> (dotted), Δ <i>pflu1134</i> (grid) and Δ <i>pflu4954</i> (black).	35

Figure 3-11, A representative agar plate of mutant MU30-30 (Δ<i>dctA</i>) grown in minimal medium with succinate as the sole source of carbon.	36
Figure 3-12, Domain structure of Pflu4953.	36
Figure 3-13, Architectures of deduced histidine kinase DctX and response regulator DctY.....	38
Figure 3-14, Genetic organization of the <i>pflu4955</i> locus from <i>P. fluorescens</i> SBW25.....	39
Figure 3-15, α-ketoglutarate-induced expression of <i>dctT</i> in wild type SBW25 (blank), and mutants MU33-56 (Δ<i>dctY</i>) (dotted), and the two reconstructed Suc^+ suppressor mutants MU35-46 [Δ<i>dctA</i>, <i>dctX</i> (T574C)] (grid) and MU35-47 [Δ<i>dctA</i>, <i>dctX</i>(G1021T)] (black).	41
Figure 3-16, succinate- and α-ketoglutarate-induced expression of <i>dctT</i> in wild type SBW25 (blank) and mutants MU37-61 [Δ<i>dctA</i>, <i>dctX</i> (G1021T)] (black).....	43

List of Tables

Table 2.1 Bacterial strains and plasmids used in this study.....	10
Table 2.2 Primers used in this study.....	13
Table 2.3 Reagents for a 50μl PCR reaction	15
Table 2.4 Typical PCR reaction conditions	16
Table 2.5 Phusion DNA polymerase PCR instructions.....	16
Table 2.6 Cycling instructions.....	17
Table 3.1 Comparative analysis of putative DctA and two-component system	
DctB/DctD homologues in <i>P. fluorescens</i> SBW25.....	26
Table 3.2 Results of phenotypic characterization of strains from <i>P. fluorescens</i>	
SBW25 on different residues.	33
Table 3.3 Results of phenotypic characterization of strains from <i>P. fluorescens</i>	
SBW25 on different residues.	42

List of Abbreviations

ATP	Adenosine Tri-Phosphate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine Serum Albumin
°C	Degrees Celsius
CIP	Calf Intestinal Phosphatase
Da	Dalton
Dcu	Dicarboxylates uptake
dNTP	Deoxyribo Nucleotide Tri-Phosphate
EDTA	Ethylene Diamine Tetraacetic Acid
g	Gravitational force
HEPES	4-(2-HydroxyEthyl)-1-Piperazine Ethane Sulfonic acid
kb	kilo-base pairs
LB	Luria-Bertaini
μl	microliter
μM	microMolar
M	Molar
mg	milligram
ml	millimeter
min	minute
mM	milliMolar
nm	nanometer
OD	Optical Density

ORF	Open reading frames
PCR	Polymerase chain reaction
rpm	Revolution per minute
RR	Response regulator
SK	Sensor kinase
TCS	Two-component system