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

A thesis presented in fulfillment of the requirements for the degree of

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Yuting Liang

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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 activate 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 (SBW25Δ*dctA*) cannot grow on minimal medium supplemented with succinate as the sole carbon source, interestingly, a spontaneous Suc^+ mutant arose at high frequency (~ 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.

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List of Abbreviations

ATP	Adenosine Tri-Phosphate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine Serum Albumin
ºC	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

Chapter 1 Introduction

1.1 Plant-associated *Pseudomonas*

Bacteria belonging to the genus of *Pseudomonas* are metabolically versatile and capable of colonizing both terrestrial and marine environments, and also eukaryotic hosts (plants and animals) [1-3]. As efficient plant colonizers, strains of some species, e.g., *P. syringae*, cause serious diseases, whereas certain strains of *P. fluorescens* and *P. putida* enhance the plant health [1, 4, 5]. The ecological success of *Pseudomonas* on the surfaces of plants is largely determined by their rapid detection of (and response to) environmental changes *in planta*, including the availability of nutritional substrates [2, 6]. The ability to sense the presence of nutritional substrates and utilize them efficiently is of fundamental importance [7-10].

Pseudomonas fluorescens SBW25 is a plant growth-promoting bacterium representative of a group of fluorescent pseudomonads isolated from the phyllosphere of sugar beet plants grown at the University Farm, Wytham, Oxford, UK [1, 2, 10, 11]. *P. fluorescens* SBW25 can efficiently colonize plant surfaces and protect seedlings against damping-off disease caused by *Pythium ultimum* [7]. On the surfaces of plants, *P. fluorescens* SBW25 activates the expression of a set of genes that are involved in nutrient acquisition [2, 12-14]. The previously identified plant-induced genes include *xylA* for xylose utilization, a homologue of DctS sensor protein for transport C_4-dicarboxylate, *hutT* for the uptake of histidine and *livMH* encoding high-affinity branched chain amino acid transport permease [10, 11, 15]. High inducible expressions of catabolic genes in *planta* provide important evidence that a nutrient is available on

the plant surface for bacteria living [8, 9]. Therefore, the *in vivo* gene expression data suggest that carbohydrates and amino acids are the significant nutrients that are available for bacteria living on the plant surfaces. Moreover, the ecological significance of these plant-responsive catabolic genes needs to be further assessed.

1.2 Preferential utilization of succinate by *P. fluorescens* SBW25

Like many other bacteria that are closely associated to eukaryotic hosts, *P. fluorescens* SBW25 preferentially utilize C_4-dicarboxylic acids (succinic acid or succinate in particular) as the sole source of carbon and energy [6, 11, 15-18]. In the presence of succinate, expression of catabolic enzymes for the utilization of other non-preferred carbon substrates such as D-xylose are repressed, a phenomenon known as carbon catabolite repression (CCR) [9]. The molecular mechanisms of CCR have been well studied in enteric bacteria and CCR is mediated by the catabolite-activating protein (CAP) charged with cAMP [9]. However, the paradigm does not hold for non-enteric bacteria such as *Pseudomonas*. Current work in Zhang's lab shows that *P. fluorescens* SBW25 utilizes succinate in preference to D-xylose, and the repression of xylose utilization genes is achieved by the mRNA-binding protein Crc whose activity is modulated by two non-coding small RNAs [12, 19]. While the genes involved in xylose uptake and degradation have been investigated in this laboratory, the genetic components underlying succinate utilization by *P. fluorescens* SBW25 are currently unknown.

Succinate and other C4-dicarboxylates such as malate and fumarate are intermediates of the tricarboxylic acid (TCA) cycle, which is used by almost all aerobic organisms to generate energy [20-25]. It is, thus, not surprising that plant-

associated bacteria have evolved the ability to utilize C4-dicarboxylates as valuable nutrients, as they are likely available on the plant surfaces or intracellular. Nutrient utilization is usually a two-step process that involves an initial uptake into the cell and followed by enzymatic breakdown [26]. However, for intermediates in the TCA cycle, no specific enzyme is required for their breakdown, and the efficiency of utilization is solely determined by the uptake systems and the associated regulatory factors [10, 27].

1.3 Current understanding of the molecular mechanisms of succinate utilization in bacteria

The molecular mechanisms of succinate utilization have been well studied in model organisms such as *E. coli* and rhizobia (specifically, *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Mesorhizobium loti*) [3, 20, 22, 24, 28]. Under aerobic conditions, succinate is imported by DctA, a transporter belonging to dicarboxylate/cation symporter (DAACS) family [21, 28, 29]. DctA from different bacterial species is of about 450 amino acids in length, and contains 10 or 12 putative trans-membrane domains [20]. Expression of *dctA* is regulated by a two-component signal transduction system DctB/D where DctB is sensor kinase and DctD is a response regulator [20, 28-34].

The classical modular paradigm of domain organization of TCSs is displayed in Figure 1-1. Genes encoding the sensor kinase (SK) and the cognate response regulator (RR) are usually located in the same operon, which are co-transcribed [24, 32, 35]. The SK (also called histidine kinase) contains an input domain and an autokinase domain that comprise a specific histidine residue (see Figure 1-1). And the RR is composed of

an output domain and a receiver domain that has an aspartate residue (see Figure 1-1). In the presence of succinate, DctB sense succinate and histidine residue within the DctB was phosphorylated. Subsequently, the phosphate group transmit from histidine residue of DctB to the aspartate residue of DctD [20, 25, 32]. The phosphorylated DctD binds to *dctA* promoter, enabling RNA polymerase with the sigma factor σ^{54} to transcribe *dctA* [30, 36, 37]. Interestingly, a σ^{54} binding site is located upstream of the *dctA* start codon [37, 38].

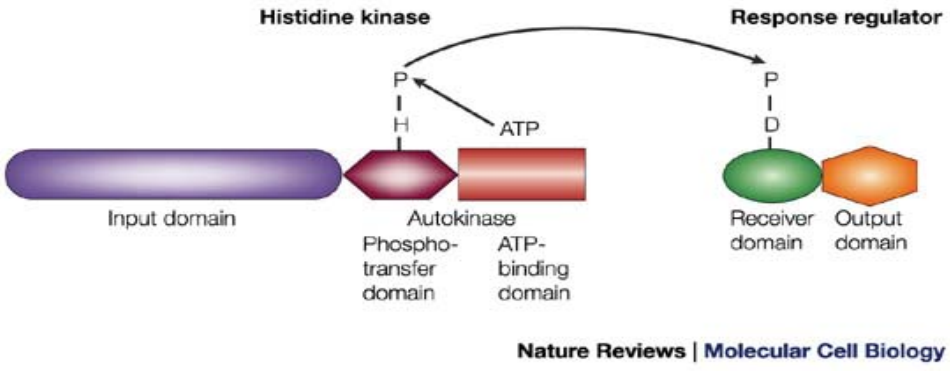

Figure 1-1, The domain structure of a typical two-component system (TCS).

A simple two-component signal transduction system consists of a histidine kinase (also named sensor kinase) and a response regulator. Most histidine kinases contain a transmembrane domain coupled to an autokinase domain. Signal sensing by the input domain causes activation of the autokinase domain, which results in phosphorylation of a specific histidine residue in the phosphotransfer subdomain of the autokinase. The phosphoryl group is then transferred to an aspartate residue in the receiver domain (also named regulator domain) of the cognate response regulator protein, which results in modulation of the function of the linked output domain (in most cases a transcriptional regulator). *Source: Adapted from Rasmus B. Jensen, 2002* [39]

Succinate uptake plays a significant role in rhizobial metabolism. DctB, DctD and RpoN are necessary component of the succinate uptake system that enables the activation of *dctA* [3, 24, 31, 40]. In 1984, Ronson *et. al* identified three *dct* loci (*dctA, dctB* and *dctD*) in *Rhizobium leguminosarum* [28]. Moreover, the study suggested that

dctA encodes a protein necessary for C4-dicarboxylate transport. Similarly, this model had been found in *S. meliloti* [24, 31]. The *dctA* system seems not only to transport succinate but also to transport D-malate [24]. It has relatively broad substrate specificity.

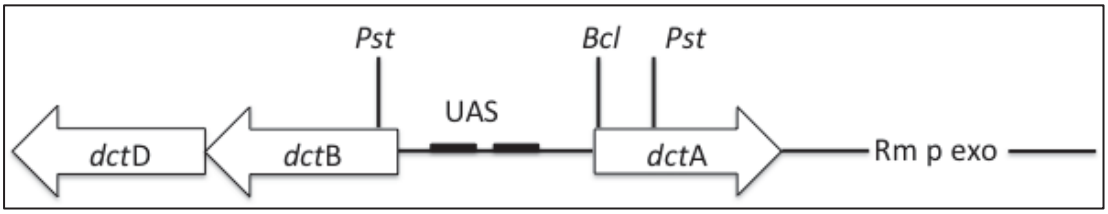

Figure 1-2, Two-component system of DctB/DctD in *Sinorhizobium meliloti*.

The *dct* cluster on the Rm exo megaplasmid of *S. meliloti* was revealed (*Ledebur et al.1990; Ledebur and Nixon 1992; Watson 1990*). UAS: upstream activating sequence, the binding site for the transcriptional activator (DctD), *Pst*I and *Bcl*I restriction site have been indicated on *dctB* and *dctA*, respectively.

E. coli possesses five import/export systems for succinate uptake, namely DctA, DcuA, DcuB, DcuC, DcuD, each of which is expressed differently under aerobic or anaerobic conditions [20, 41, 42]. Under aerobic condition, DctA is active to transport succinate [41], and the expression of *dctA* is controlled by DcuSR TCS. DcuS senses the presence of succinate and phosphorylates DcuR, which in turn activates the expression of *dctA* [23, 41-43]. Anaerobically, succinate transport is mediated by DcuA and DcuB, which are carriers from the C_4-dicarboxylate uptake family (Dcu) [20, 41]. Both of them are fumarate/succinate antiporters. Moreover, DcuC and DcuD transporters are identified as a separate family of C_4-dicarboxylate efflux systems, the DcuC acts as a proton/succinate co-exporter to transport succinate, but the functional role of DcuD remains unknown [41].

In *Pseudomonas aeruginosa*, two succinate-specific uptake systems have been identified: a low-affinity system (DctA) and a high-affinity system (DctPQM) [26, 36, 44]. DctPQM is an ABC-type transporter, which is a member of the tripartite ATP-independent periplasmic (TRAP) family [36]. When succinate is present at high concentration, DctA is principal transporter for succinate uptake, whereas the DctPQM system is responsible for uptake of succinate at low concentrations (Figure 1-3). The dual succinate uptake system of *P. aeruginosa* PAO1 works coordinately to transport succinate present at low to high concentrations [36].

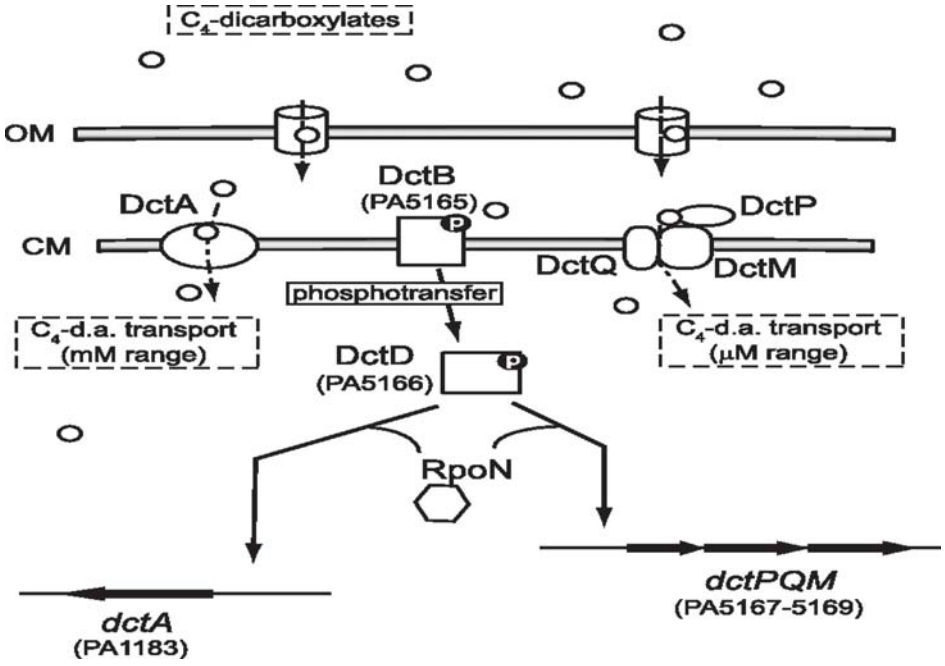

Figure 1-3, Model for C4-dicarboxylate transport in P. aeruginosaPAO1.

C4-dicarboxylate triggers the activation of the DctB/DctD two-component system (PA5165/PA5166) enabling RNA polymerase with the RpoN sigma factor to transcribe the dctAgene (PA1183) and the dctPQMoperon (PA5167-PA5169). DctA is the major transporter for utilization of succinate in the mM range, whereas DctPQM transporter is more effective in the μM range. *Source: Adapted from Martina Valentini, 2011* [36]

1.4 Previous work leading to this research

P. fluorescens SBW25 is a model organism that has been used in the study of

molecular interactions between bacteria and plants. To determine the molecular mechanisms responsible for the ecological success of *P. fluorescens* SBW25, an *in vivo* expression technology, known as IVET [11, 14, 15, 45, 46], was employed to identify candidate genes whose expressions were elevated, when growing on the surfaces of sugar beet plants. Among the 181 plant induced genes [46], *pflu4717* is subject to further investigation here, as it encodes a putative transporter for succinate uptake (i.e., DctA).

Previous work performed by Dr. Xue-Xian Zhang involved deletion analysis of *pflu4717* and the three putative regulatory genes (i.e., Pflu0286, Pflu4954 and Pflu1134), but their precise roles in succinate utilization and their responsiveness to other dicarboxylates such as fumarate, malate and alpha-ketoglutarate have not been established.

Interestingly, mutants devoid of *pflu4717* (*dctA*) were unable to grow on succinate (Suc^-), but spontaneous Suc^+ mutants can arise at a high frequency ($\sim 10^{-4}$), suggesting the presence of yet-unidentified transporter (s) for succinate uptake. The genomes of two such spontaneous Suc^+ mutants have been re-sequenced, and a comparison with the genome of the ancestral *P. fluorescens* SBW25 identified two separate mutations in a sensor kinase of a putative two-component regulatory system (specifically, Pflu4953). However, it is unknown how these two suppressor mutations cause the activation of the predicted yet-unidentified succinate transporter (s).

1.5 Objectives of this study

The primary aim of this study is to elucidate the molecular mechanisms employed by *P. fluorescens* SBW25 to utilize C_4-dicarboxylates, succinate in particular. The specific aims are listed below.

1) **To confirm the predicted role of Pflu4717 (DctA) in succinate uptake and its regulation by the putative DctBD system** (i.e., Pflu0287 and Pflu0286). This has been achieved through the construction of a *lacZ* report fusion to *pflu4717* and subsequent β-galactosidase assays in the genetic backgrounds of wild-type *P. fluorescens* SBW25 and its derived mutants. Additionally, the functionality of Pflu4717 (DctA) was further confirmed by genetic complementation using a cloned copy of *pflu4717*.

2) **To identify and characterize the predicted succinate-specific transporter(s) that allows the *pflu4717* deletion mutant (SBW25Δ*dctA*) to grow on succinate.** This first involved a separate re-construction of the two suppressor mutations of *pflu4953* in the genetic background of SBW25Δ*dctA* (Suc^-), to see if the resulting mutants are able to grow on succinate. Once premise results were obtained, a combination of site-directed mutagenesis analysis and β-galactosidase reporter assays was used to test the hypothesis that: *pflu4955*, a gene located downstream of *pflu4953/pflu4954*, encodes the predicted succinate-specific transporter whose expression is controlled by the Pflu4953/Pflu4954 system. Our results show that Pflu4955 has a dual role in the uptake of both succinate and α-ketoglutarate.

Chapter 2 Material and Methods

2.1 Solution and Media

2.1.1 Media

Media for bacterial culture were prepared using milliQ water and sterilized by autoclaving at 121°C for 120 minutes or filtered through a 0.45um cellulose filter (Millipore) if required. If solid media were required, 20g agar (Oxoid) was added per liter of media. Solid media were cooled to 55°C before addition of supplements and pouring into plates. Liquid media were cooled to room temperature before the addition of supplements and inoculation. Carbon sources were autoclaved/filter-sterilized separately from the media and generally added immediately before media use. Plates were stored at 4°C until use.

2.1.2 Solutions and Buffers

Standard solutions and buffers were made using milliQ water. All general chemicals and salts were sourced from Sigma.

2.1.3 Enzymes and Reagents

Reagents and chemicals were purchased from Bioline, Bio-Rad, New England Biolabs, Fermentas, GE healthcare, Sigma-Aldrich Chemicals, Thermo Scientific, Invitrogen and Qiagen. The following enzymes were obtained from Stratagene: T4DNA ligase and ligase buffer. The restriction enzymes *Nhe*I, *Eco*RI, *Xho*I, *Bgl*II and their corresponding buffers, Bovine Serum Albumin (BSA) and Calf Intestinal Alkaline Phosphatase (CIP) were obtained from both New England Biolabs and

Roche. RNase A was obtained from Sigma.

Table 2.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotypes and relevant characteristics	Reference
P. fluorescens		
SBW25	Wild-type strain isolated from sugar beet	[1]
MU30-30	Δ*dctA*, SBW25 with the deletion of *pflu4717*	Xue-Xian Zhang
MU33-54	Δ*dctD*, SBW25 with the deletion of *pflu0286*	Xue-Xian Zhang
MU33-9	Δ*pflu1134*, derived from SBW25	Xue-Xian Zhang
MU33-56	Δ*dctY*, derived from SBW25 with the deletion of *pflu4954*	Xue-Xian Zhang
MU34-74	SBW25 carrying P_{dctA}-LacZ translational fusion, Gm^r	Xue-Xian Zhang
MU34-89	MU33-54 (Δ*dctD*) carrying P_{dctA}-LacZ translational fusion, Gm^r	Xue-Xian Zhang
MU34-95	MU33-9 (Δ*pflu1134*) carrying P_{dctA}-LacZ translational fusion, Gm^r	Xue-Xian Zhang
MU34-98	MU33-56 (Δ*dctY*) carrying P_{dctA}-LacZ translational fusion, Gm^r	Xue-Xian Zhang
MU35-46	A spontaneous Suc^+ mutant of MU30-30 (Δ*dctA*), *dctX* (T574C)	Xue-Xian Zhang
MU35-47	A spontaneous Suc^+ mutant of MU30-30 (Δ*dctA*), *dctX* (G1021T)	This work
MU37-61	Mutation *dctX* (G1021T) was introduced into mutant MU30-30 (Δ*dctA*) through site-directed mutagenesis	This work
MU37-65	Mutation *dctX* (T574C) was introduced into mutant MU30-30 (Δ*dctA*) through site-directed mutagenesis	This work
MU37-75	Δ*dctT*, SBW25 with the deletion of *pflu4955*	This work
MU37-74	Δ*dctT*, derived from MU30-30 (Δ*dctA*)	This work
MU37-55	Δ*dctT*, derived from MU37-65 [Δ*dctA*, *dctX* (T574C)]	This work
MU37-51	Δ*dctT*, derived from MU37-61 [Δ*dctA*, *dctX* (G1021T)]	This work
MU38-78	Δ*dctY*, derived from MU37-65 [Δ*dctA*, *dctX* (T574C)]	This work
MU38-89	Δ*dctY*, derived from MU37-61 [Δ*dctA*, *dctX* (G1021T)]	This work

MU40-25	MU30-30 (Δ*dctA*) carrying P_{dctA}-LacZ translational fusion, Gm^r	This work
MU40-45	SBW25 carrying the P_{dctT}-'*lacZ* fusion in an integrated Tn7 element, Gm^r	This work
MU40-48	MU33-56 (Δ*dctY*) carrying the P_{dctT}-'*lacZ* fusion in an integrated Tn7 element, Gm^r	This work
MU40-51	MU35-46 carrying the P_{dctT}-'*lacZ* fusion in an integrated Tn7 element, Gm^r	This work
MU40-54	MU35-47 carrying the P_{dctT}-'*lacZ* fusion in an integrated Tn7 element, Gm^r	This work
MU40-60	MU37-75 (Δ*dctT*) carrying the P_{dctT}-'*lacZ* fusion in an integrated Tn7 element, Gm^r	This work
MU39-52	SBW25 carrying the P_{dctX}-'*lacZ* fusion in an integrated Tn7 element, Gm^r	This work
MU39-31	MU30-30 (Δ*dctA*) with a cloned copy of *dctA* in an integrated Tn7 element, Gm^r	This work
MU39-40	MU37-75 (Δ*dctT*) with a cloned copy of *dctT* in an integrated Tn7 element, Gm^r	This work
MU39-43	MU30-30 (Δ*dctA*) with a cloned copy of *dctT* in an integrated Tn7 element, Gm^r	This work
MU39-46	MU37-65 [Δ*dctT* Δ*dctA*, *dctX* (T574C)] with a cloned copy of *dctT* in an integrated Tn7 element, Gm^r	This work
MU39-49	MU37-61 [Δ*dctT* Δ*dctA*, *dctX* (G1021T)] with a cloned copy of *dctT* in an integrated Tn7 element, Gm^r	This work
Plasmid		
pRK2013	Helper plasmid, Tra^+, Km^r	[47]
pUIC3	Integration plasmid with promoterless '*lacZ*, *ori*R6K, Tc^r	[11]
pXY2	A Tn7-based cloning vector for the construction of translational gene fusion to LacZ, Gm^r	Xue-Xian Zhang
pCR8/GW/TOPO	Cloning vector, Sp^r	Invitrogen
pUC18-mini-Tn7T-Gm-*lacZ*	A Tn7-based cloning vector for the construction of transcriptional gene fusion to '*lacZ*, Gm^r	[48]
pUC18-mini-Tn7T-LAC	A Tn7-based cloning vector for gene expression, Gm^r	[48]
pUX-BF13	Helper plasmid for transposition of the Tn7 element, Ap^r	[49]
pXY2-dctA	pXY2 carrying the P_{dctA}-LacZ translational fusion, Gm^r	This work

pTn7-LAC-PdctA	pUC18-mini-Tn7T-Gm-*lacZ* carrying the P_{dctA}-'*lacZ* fusion, Gm^r	This work
pTn7-PdctX	pUC18-mini-Tn7T-Gm-*lacZ* carrying the P_{dctX}-'*lacZ* fusion, Gm^r	This work
pTn7-PdctT	pUC18-mini-Tn7T-Gm-*lacZ* carrying the P_{dctT}-'*lacZ* fusion, Gm^r	This work
pTn7-LAC-PdctT	pUC18-mini-Tn7T-Gm-*lacZ* carrying the P_{dctT}-'*lacZ* fusion, Gm^r	This work
pUIC3-92	pUIC3 containing 1.4 kb DNA fragment for *dctA* deletion	This work
pUIC3-119	pUIC3 containing 1.4 kb DNA fragment for *dctD* deletion	This work
pUIC3-120	pUIC3 containing 1.4 kb DNA fragment for *dctY* deletion	This work
pUIC3-123	pUIC3 containing 1.4 kb DNA fragment for *pflu1134* deletion	This work
pUIC3-130	pUIC3 contain 0.8 kb DNA fragment for mutation *dctX*(T574C)	This work
pUIC-131	pUIC3 contain 0.8 kb DNA fragment for mutation *dctX*(G1021T)	This work
pUIC3-132	pUIC3 containing 1.0 kb DNA fragment for *dctT* deletion	This work

2.2 Primer design

The nucleotide sequence of *pflu4717* was obtained from the *P. fluorescens* SBW25 genome sequence (http://www.sanger.ac.uk). The DNA sequence and primers were analyzed using Geneious to build a restriction enzyme profile and to check for the formation of primer secondary structure and melting temperature. All primers were supplied by Invitrogen and are listed in Table 2.2.

DNA sequencing in this work have done by sending the DNA samples to Macrogen (http://www.macrogen.com) to analysis.

Table 2.2 Primers used in this study

Primer	Nucleotide sequence (5' to 3')	Application
dctA-1	gagatCTGAGAAAGTGATCCGGGTC	Deletion of *pflu4717* (*dctA*)
dctA-2	cagcatgcggatccgttgacggaCGAGTCGTCATTGCAAAGTTCC	
dctA-3	tccgtcaacggatccgcatgctgTGGTCAAGTAAATCGCTGATGGC	
dctA-4	gagatctCCAACCGTAGACGAACTCGT	
0286-P1	gagatCTCAACCAACCCCTGGCGGCGA	Deletion of *pflu0286* (*dctD*)
0286-P2	cagcatgcggatccgttgacggaACTCATACACTGCGGTCCTC	
0286-P3	tccgtcaacggatccgcatgctgGACGAATGACCACTCCCACC	
0286-P4	gagatctGTGTTGGAGTTGAGCGGTGA	
4954-1	gagatctCGAAATCAATCAGCCACTGA	Deletion of *pflu4954* (*dctY*)
4954-2	cagcatgcggatccgttgacggaAATCACCGCGTTCAGCATGG	
4954-3	tccgtcaacggatccgcatgctgTAGGCCCATCGTTGTGTTGAC	
4954-4	gagatctCCGATGATTTCATAGCCAGG	
1134-1	gagatCTCAGCGACGGTCCGGGCATTCC	Deletion of *pflu1134*
1134-2	cagcatgcGgatcCgttgacggaCTGATGGACCTGGTATTGAAG	
1134-3	tccgtcaacGgatcCgcatgctgATTCATGTCGACTCAGCTTTTTGAGC	
1134-4	gagatctCATGAACTGAACCAGCCCCT	
dctA-SpeI	aactagtGGATCAGCGCGTGGCACATCATGG	Constructing the dctA-LacZ translational fusion
dctA-HindIII	aaagcttGTAGATTGGCTGACGAGTCGTCATTGC	
dctA-R	aaagcttCGCCAGAAACGCAAACGCCC	Amplifying *dctA* for gene complementation, using dctA-SpeI as the forward primer

dctA1-F	aagatcTGATCGAACGCTACCGCGCC	Reconstructing the *dctX* (T574C) mutation
dctA1-R	aagatctCGCCAGCGTTGGGTCAGGAA	
dctA2-F	aagatctGCGAGTGGGCCCAAGGCAAT	Reconstructing the *dctX* (G1021T) mutation
dctA2-R	aagatcTCATCGCGCAGGCGTGCATC	
4955-1	aagatctCGGAACCGGAAGGCGTCGAG	Deletion of *pflu4955* (*dctT*)
4955-2	cagcatgcggatccgttgacggaAGAGGCAGGGAGTTGGAGTTATCC	
4955-3	tccgtcaacggatccgcatgctgAACGCTTCGCCAGTCCTGCAA	
4955-4	aagatctGCCGTGTGGCACCTCAGTGG	
4955-1-SpeI	aactagtCGGAACCGGAAGGCGTCGAG	Constructing the *pflu4955* (*dctT*) transcriptional fusion to '*lacZ*
4955-2-HindIII	aaagcttAGAGGCAGGGAGTTGGAGTTATCC	
4955F-SpeI	aactagtGGCATGGGTTGGTGCGGGAG	*pflu4955* (*dctT*) complementation
4955R-HindIII	aaagctTGCAGGACTGGCGAAGCGTT	
4953F-SpeI	aactagtAGCCTGGCGGTAGAAGTGCAG	Constructing the *pflu4953* (*dctX*) transcriptional fusion of '*lacZ*
4953R-HindIII	aaagcttGGGCTTGGCGTTCGGCATGG	
4953-2	CAGTGGCTGATTGATTTCTCCGGCGAGGGCGGCGGACA	Constructing the signaling dead mutant of strain MU35-47, in conjunction with primer dctA2-F
4953-3	TGTCCGCCGCCCTCGCCGGAGAAATCAATCAGCCACTG	
4953-4	aagatcTGCACGATAGCGTAGGACAC	
glmS	CACCAAAGCTTTCACCACCCAA	Determining correct integration of the Tn7 element
Tn7R	CAGCATAACTGGACTGATTTCAG	

2.3 Polymerase Chain Reaction (PCR)

Genes of interest were amplified using the PCR from *P. fluorescens* SBW25 genomic DNA. A deoxyribonucleotide triphosphate stock was prepared from a dNTP set (Bioline) containing four separate 100mM of dNTP solutions to generate a final concentration of 10mM for each dNTP. The Taq DNA polymerase (Invitrogen) was used for PCR. The annealing temperatures for each pair of primers were determined based on the calculated Tm of the primers. If no PCR products or non-specific PCR products were detected at initial annealing temperatures, empirical tests were performed to find optimal annealing temperatures for the primers. The preparation of a typical 50µl of PCR reaction was shown in Table 2.3. PCR reactions were carried out using a gradient thermal Palm-Cycler^{Tm} (Corbett life science) and the reaction conditions were shown in Table 2.3.

Table 2.3 Reagents for a 50µl PCR reaction

	Volume (µl)	Final concentration
10x Buffer	5	1x
$Mgcl_2$ (50mM)	1.5	1.5mM
dNTP(10mM)	1	0.2mM
Forward Primer (10 pmol/µl)	1	0.2pmol/µl
Reverse Primer (10 pmol/µl)	1	0.2pmol/µl
Taq DNA Polymerase (5 U/µl)	0.2	1 U
Template DNA	5	--
MilliQ H_2O	35.3	--
Total Volume	50	--

Table 2.4 Typical PCR reaction conditions

	Temperature	Time	Cycles
Initial Denaturation	94 °C	3 min	1x
Denaturation	94 °C	45s	30x
Annealing	56 °C	45s	30x
Elongation	72 °C	1 min per kb	30x
Final elongation	72 °C	10 min	1x
Hold	4 °C		--

2.4 Phusion DNA polymerase PCR

For the most difficult templates, the phusion DNA Polymerase (Thermo Scientific) can be used to generate long templates with an accuracy and speed with a single enzyme. Phusion DNA Polymerase possesses the following activities: 5′→3′ DNA polymerase activity and 3′→5′ exonuclease activity. It generates blunt ends in the amplification products.

Table 2.5 Phusion DNA polymerase PCR instructions

Component	50 µl	Final concentration
H_2O	28	
5x Phusion HF buffer	10	1x
dNTP	1	200 µM
Forward Primer	2	0.5 µM
Reverse Primer	2	0.5 µM
Phusion DNA polymerase	0.5	1U
Template DNA	5	< 250 ng
DMSO (optional)	1.5	3%

Table 2.6 Cycling instructions

Cycle step	3-step protocol		Cycles
	Temperature	Time	1
Initial denaturation	98°C	30 s	25-35
Denaturation	98°C	5–10 s	
Annealing	72°C	10-30s	
Extension	72°C	15–30 s/kb	
Final extension	72°C	5-10mins	1
	4°C	Hold	

2.5 Preparation of electrocompetent *E. coli* cells

The bacterial strain *E. coli* (BL21) from -80°C glycerol-saline stocks was streaked out on a LB plate and grown at 37°C overnight. A single colony from the plate was inoculated into 10 ml of LB and grown at 37°C with shaking at 180 rpm overnight. 5 ml of overnight culture was inoculated into 500 ml of LB and grown at 37°C with shaking at 120 rpm to an OD600 of 0.6～0.7. Cells were chilled on ice for 20 minutes and harvested at 4,000 g for 20 minutes at 4°C. The supernatant was discarded and the cell pellet was re-suspended in 250 ml of chilled 10% glycerol. The cells were centrifuged at 4,000 g for 20 minutes at 4°C and supernatant was discarded. The cell pellet was further washed with 125 ml of chilled 10% glycerol twice. The cells were centrifuged at 4,000 g for 20 minutes at 4°C and the supernatant were discarded. The cell pellet was re-suspended in 2 ml of chilled 10% glycerol. 50μl of aliquots were made and stored at -80°C.

2.5.1 Glycerol-saline stock

Glycerol-saline stock was made for long-term storage of bacteria. The

bacteria were inoculated in LB containing the appropriate antibiotic and grown at 37 degree with shaking. 1 ml of bacterial culture was added to 800µl of glycerol saline, mixed thoroughly and stored at -80 degree.

Glycerol-saline

0.85%(w/v) NaC1

70% (v/v) Glycerol

2.6 Gateway LR Recombination Reaction

Gateway LR recombination reaction is a efficient method to enable transferring target DNA fragment between different cloning vectors to maintain the orientation and reading frame by using unique gateway recombination sequences “att L1” and “att L2”. This reaction occurs between “att” sites on the manipulated DNA molecules. The procedures described as follow. At the beginning, add the following components to a 1.5 ml tube at room temperature and mix: Entry clone (50-150 ng) 2 µl, Destination vector (150 ng/µl) 4 µl, TE buffer, pH 8.0 to 6 µl and thaw on ice the LR Clonase ™ II enzyme mix for about 2 minutes. Next, the LR Clonase ™ II enzyme mix has to vortex briefly twice (2 seconds each time). Then, add 4 µl of LR Clonase ™II enzyme mix to the reaction for each sample and mix well by vortex briefly twice. Microcentrifuge briefly. Meanwhile, return LR Clonase ™ II enzyme mix to -20°C or -80°C storage. Thirdly, incubate reactions at 25°C for 1 hour. After incubation add 2 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. And then Incubate samples at 37°C for 10 minutes. Fourthly, transform 2µl of LR reaction into 80 µl of competent cell and incubate on ice for 30 minutes. Heat-shock cells are done by incubating at 42°C for 30 seconds. Lastly, add 450 µl of S.O.C. medium and incubate at

37°C for 1 hour with shaking. Plate 20 µl and 100 µl of each transformation onto selective plates.

2.7 Electroporation

It is a highly efficient process for transformation of bacteria by using the electrical field to introduce DNA fragment into a cell. First, 2µl of plasmid DNA or ligation products was added to 50µl of electrocompetent cells that had been thawed on ice. The cells were placed between 0.1 cm electrodes of a pre-chilled gapped gene pulser cuvette (Bio-Rad) and electroporated using Electroporator 2510 (Eppendorf) at 1.8 kV. 800µl of SOC medium was immediately added and the electroporated cells were transferred to a 1.5 ml sterile eppendorf tube. The cells were incubated at 37°C with shaking at 180 rpm for 1 hour. The culture was spread onto LB plates containing the appropriate antibiotic and incubated at 37°C overnight. After incubation, colonies of transformants were inoculated into 5ml of LB containing the appropriated antibiotic and grown 37°C with shaking at 180 rpm overnight. Plasmid DNA was isolated from these cultures.

S.O.C medium

2% Bacto Tryptone 10mM $MgCl_2$

0.5% Yeast Extract 10mM $MgSO_4$

20 mM Glucose 10 mM NaCl

2.5 mM KCl

2.8 Plasmid purification

Plasmid DNA was extracted from overnight bacterial cultures using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. 30µl or

50µl of MilliQ H_2O was used to elute DNA.

2.9 Restriction enzyme digests

For analytical digestions, typically 50ng of plasmid DNA was digested in a volume of 15µl consisting of the appropriate reaction buffer and 0.25-1µl of the respective enzyme. For double digestions, the buffer most efficient for both restriction enzymes was used. Digestion was performed at 37°C for 2 hours or overnight and the samples were then resolved on an agarose gel for analysis.

For preparative digestions involved in the cloning steps, the following reaction was typical: 400ng plasmid, 0.5µl of each restriction enzyme, 0.15µl BSA (1x), 1x appropriate reaction buffer, in a total volume of 15µl. To remove the 5' phosphate groups of the plasmid before ligation, 0.5µl of Calf Intestinal Alkaline Phosphatase (CIP) was added. Incubation was for two hours at 37°C. Normally, we make Master Mix for the reaction.

2.10 Agarose gel electrophoresis

DNA fragments were separated on 1% agarose gels in 1x TAE buffer (U1traPure TM, Invitrogen) containing 1x SYBR safe TM DNA gel stain (Invitrogen). Samples were mixed with 6x DNA loading dye (Fermentas) at the ratio of 1:5 prior to loading onto the gel. Gels were run at 140 volts for 30-60 minutes. Lambda DNA/EcoRI+HindIII marker (Fermentas) was also loaded to estimate the size of DNA fragments. Visualization of DNA was undertaken using UV light generated from a High Performance Transilluminators (UVP, LLC) and gels were photographed using DigiDoc-It TM Imaging System with Doc-It LS Analysis Software (UVP, LLC).

2.10.1 Determination of DNA fragment sizes

In order to determine the size of DNA fragment, 1μl of 1kb DNA ladder (New England Biolabs) was mixed with 1μl of 10x DNA loading dye and loaded next to sample DNA. The molecular weight of an unknown DNA fragment was estimated by comparing the relative mobility of the known ladder marker fragments to that of the unknown.

2.10.2 Determination of DNA concentration

Concentration standards were used for quantification. The intensity and size of the unknown bands were compared with the appropriate bands of the 1kb DNA ladder in the agarose gel. DNA samples were subjected to the agarose gel electrophoresis. The desired band was excised from the gel and purified using QIAquick Gel Extraction kit (Qiagen). Ligation of DNA inserts with plasmid vectors at the molar ratio of 3:1 was performed using T4 DNA ligase (Invitrogen). Reactions were incubated at 16 degree overnight.

2.11 Conjugation of pUIC3 and its derived plasmids into *P. fluorescens* SBW25 (tri-parental mating)

Conjugation is which a two-step allelic-exchange strategy using the suicide-integration vector pUIC3The procedure of the conjugation are performed as follow, first day, to inoculate *E. coli* strain that containing the donor plasmid pUIC3 or its derived plasmid (Tc^R, Mob^+) into 5 ml LB + Tc (10 μg/ml), *E. coli* strain containing the helper plasmid pRK2013 (Km^R, Tra^+) into 5 ml LB + Km (50 μg/ml) and the recipient strain of *P. fluorescens* SBW25 in 5 ml LB, respectively. Next day, firstly set up a water bath

of 45°C, then transfers 500 μl of recipient culture into an Eppendorf tube (1.5 ml) and incubate in the 45°C water bath for 20mins. During the 20mins incubation, spin down 500 μl of the donor and helper cultures separately, and resuspend each culture in 500 μl LB broth. This step is to remove the antibiotics from the cultures.

When the heat-treatment for recipient culture is finished, pellet the cells of recipient, donor and helper, and then mix them with 60 μl LB broth. Inoculate the cell suspension into pre-warmed LB agar plate (or ideally onto a cellulose filter into a LB agar plate). After the cell solution is dried out, put the agar plate into 28°C incubator and incubate for 6 hours or overnight.

Next day, the bacterial cells were suspended in ~3 ml sterile water (1/4 Ringer's solution) and inoculate 100μl of the cell suspension into selective plates, e.g., LB supplemented with Nitrofurantoin (100 μg/ml), tetracycline (20 μg/ml) and X-Gal. After incubation at 28°C for 2 days, there will be single colonies in the selective plates, if the conjugation is successful. Pick up single colonies and purify them at least once by streaking onto fresh selective agar plates.

2.12 β-Galactosidase Activity Assay

β-galactosidase activity was assayed by monitoring the hydrolysis of 4-methylumbelliferyl-β-D-galactoside to yield the fluorescent product, 7-hydroxy-4-methylumbelliferone (4MU) as described (Amersham Pharmacia, Piscataway, NJ). The 4MU was detected at 600 nm after excitation at 360 nm using a Polarstar plate reader (BMG Labtech, Aylesbury, U.K.). Optical densities of cultures were recorded, and enzyme activity was normalized to the cell density. One unit of β-galactosidase activity determined as [Concentration (20x)/OD600/Time (30)] x (100/1000). Data were subject to one-way ANOVA by using JMP software version 10.1.

2.13 Cycloserine enrichment

Cycloserine enrichment used to enrich for Pseudomonas strains that lost the chromosomally integrated pUIC3-based vector. Strains were grown overnight in 20 ml LB broth at 28^{o}C; 400µl of the overnight culture was inoculated into 20 ml fresh pre-warmed LB broth and cultivated at 28 ^{o}C with shaking (180 rpm) for 30 min. Tetracycline was added at the final concentration of 10µg ml^{-1} to inhibit the growth of cells that had lost pUIC3. After growth for 2 hours, cycloserine was added at 800µg ml^{-1} and growth was continued for another 4 hours (during this step the growing Tc^{R} cells are killed). The cells were then washed in sterile water, make series dilution from 10^{-0} to 10^{-7} and inoculated onto LB plus X-Gal plates. The colonies from LB plus X-Gal plates shown the blue color are the candidates that the target gene is mutated.

The *dctA* gene was deleted from the *P. fluorescens* SBW25 chromosome using gene splicing by overlap extension PCR (SOE-PCR) and two-step allelic exchange. Basically, a knockout construct was made by amplifying about 700 bp from the regions that flank the 5'and 3' ends of *dctA*, using the PCR primer pairs: dctA-1, dctA-2, dctA-3 and dctA-4 (See table 2.2). The two PCR products were assembled using SOE-PCR with the primers dctA-1 and dctA-4. The resulting product (~ 1.4 kb) was TA-cloned into pCR8/GW/TOPO (Invitrogen), confirmed by sequencing and then subcloned into the suicide vector pUIC3, using unique BglII site. To construct the *dctA* deletion mutant of SBW25, plasmid pUIC3Δ*dctA* was mobilized into *P. fluorescens* SBW25 by conjugation with the help of pRK2013. Integration into the chromosome by a single homogeneous recombination event was selected for on LB agar containing nitrofurantoin (100 µg ml^{-1}), Tc and X-Gal. Details were described in 2.11 & 2.13. PCR

and DNA sequencing confirmed the deletion. Figure 2-1 indicates the unique characteristics of plasmid pUIC3.

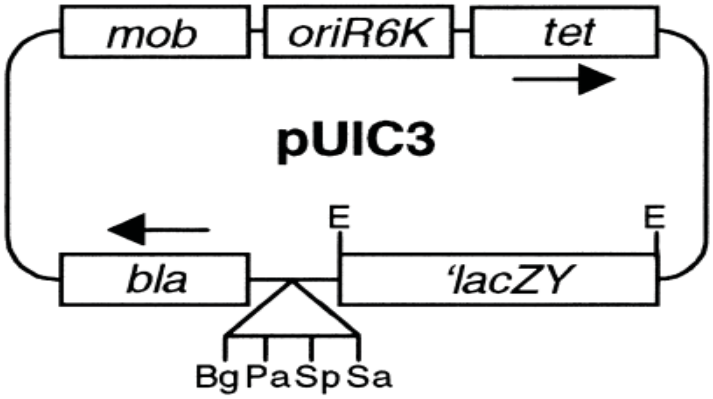

Figure 2-1, Physical map of the integration plasmid vector pUIC3.

It contains a tetracycline resistance gene (*tet*), promoter-less '*lacZY* operon with a ribosome binding site (RBS)]. The multiple cloning sites contain unique BglII (Bg) and SpeI (Sp) that were used in this work for the construction of transcriptional fusion to '*lacZ* [*Adapted from Rainey, P.B.* [11]]

Chapter 3 Results

3.1 *In silico* analysis of genes for succinate utilization in *P. fluorescens* SBW25

P. fluorescens SBW25 is capable of growing on succinate as the sole source of carbon and energy. To identify homologues of DctA and its regulators DctB and DctD, we took advantage of the genome sequence of *P. fluorescens* SBW25, which is currently available [16]. A BlastP search of the SBW25 genome was performed using amino acid sequences of the well-characterized DctA and DctBD from *Sinorhizobium meliloti* 1021(accession no CAC49923).

As summarized in Table 3.1, the deduced amino acid sequence of pflu4717 displays 63% identity with DctA of *S. meliloti*. Pflu4717 is of 443 aa in length and possesses 9 trans-membrane helices as predicted by hydrophobicity analysis using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/). As expected, a putative sigma-54 binding site is present in front of *pflu4717* [38]. Take these observations together, *pflu4717* is predicted to encode a functional succinate-specific transporter whose expression is controlled by a σ^{54}-dependent promoter. The promoter features of *pflu4717* are shown in Figure 3-1.

Table 3.1 Comparative analysis of putative DctA and two-component system DctB/DctD homologues in *P. fluorescens* SBW25.

Gene number.	Predicted function	TMs	Sequence identity with homologue[a]	
			S. meliloti 1021	*P.aeruginosa* PAO1
Pflu4717	C_4-dicarbocylate transport protein	9	DctA (63%)	DctA (82%)
Pflu4953	Two-component system histidine kinase	None	DctB (35%)	DctB (35%)
Pflu4954	Two-component system response regulator	None	DctD (54%)	DctD (56%)
Pflu0287	Two-component system sensor kinase	None	DctB (35%)	DctB (66%)
Pflu0286	Two-component system response regulator	None	DctD (54%)	DctD (78%)
Pflu1135	Two-component system sensor kinase	None	DctB (33%)	DctB (34%)
Pflu1134	Two-component system response regulator	None	DctD (50%)	DctD (52%)

a. Amino acids sequence identity is shown in parenthesis.

BlastP analysis identified three pairs of two-component regulatory systems (TCSs) with varying sequence similarities with the DctB and DctD proteins of *S. meliloti*: Pflu4953/Pflu4954, Pflu0286/Pflu0287 and Pflu1134/Pflu1135. Details of the scores are shown in Table 3.1. Interestingly, the most closely related DctB and DctD homologues are Pflu4953 and Pflu0286 respectively, which are the sensor kinase and response regulator of two different two-component systems (Table 3.1). Given the high levels of sequence similarities of these three TCSs with DctB/DctD of *S. meliloti*, it is difficult to predict which system is responsible for the activation of *dctA*, and empirical evidence is thus required.

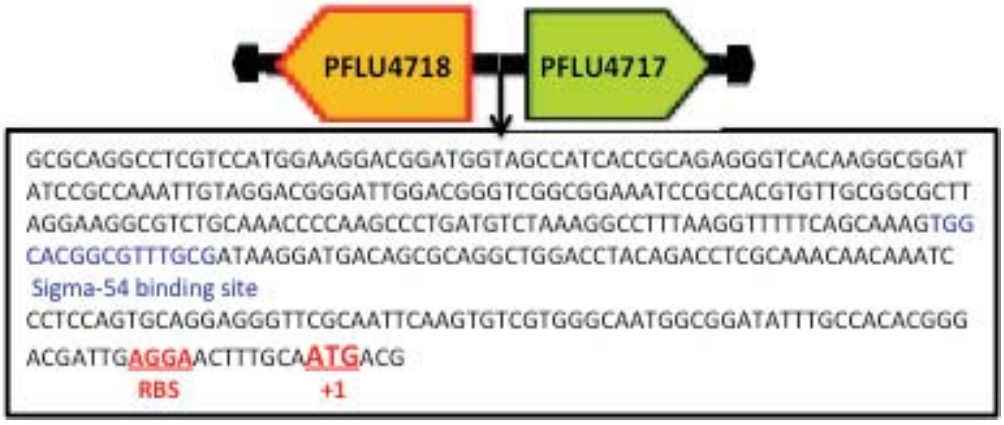

Figure 3-1, Genetic organization of the *pflu4717* locus from *P. fluorescens* SBW25.

Transcriptional start site as determined and indicated by "+1" and arrowed lines. The predicted σ^{54} –type promoter is labelled as blue colour. Ribosome binding site has been predicted and underlined.

3.2 Genetic characterization of *dctA* in *P. fluorescens* SBW25

3.2.1 Deletion analysis of *pflu4717* and genetic complementation

To test the hypothesis that *pflu4717* encodes DctA required for bacterial utilization of succinate, an in-frame deletion mutant of *pflu4717* was made by allelic exchange. As expected, the resultant mutant MU30-30 (SBW25Δ*pflu4717*) was unable to grow on minimal medium M9 with succinate as a sole carbon source (Figure 3-2). The mutational analysis was followed by genetic complementation with a cloned copy of *pflu4717*. To do this, a pair of primers dctA-SpeI/dctA-R were used to amplify the coding region of *pflu4717*, and the ~2.0 kb PCR product was cloned into pCR8, using the TA cloning kit from Invitrogen. After the sequence identity was confirmed, the ~2.0 kb insert was retrieved by SpeI/HindIII digestion and cloned into a new vector pUC18-Tn7T-LAC. As shown in Figure 3-3, the correct insertion of *pflu4717* in the recombinant plasmid was confirmed by restriction analysis with SpeI/HindIII. The resulting recombinant plasmid was then introduced into the *pflu4717* deletion mutant of

P. fluorescens SBW25 (MU30-30). Consistent with our expectation, the complemented strain (MU39-31, SBW25Δ*pflu4717*, pTn7-LAC-PdctA) gained the ability to grow on succinate.

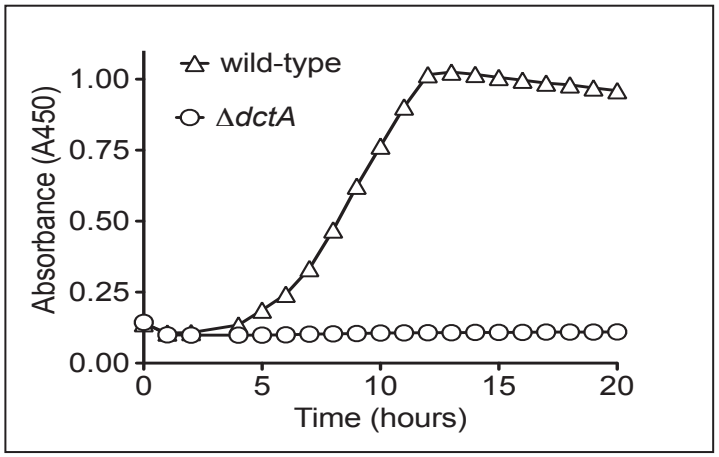

Figure 3-2, Growth curves of wild-type *P. fluorescens* SBW25 and the mutant MU30-30 (Δ*dctA*).

Absorbance at 450 nm was measured for cultures grown in M9 salt medium with succinate (20 mM) as the sole carbon source.

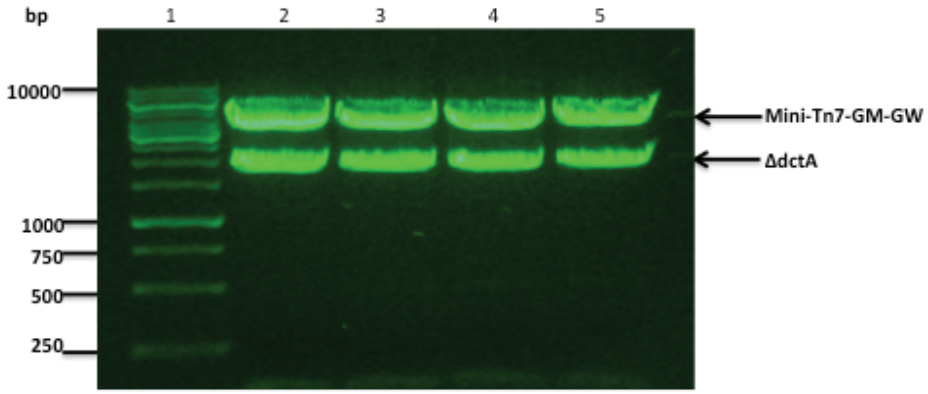

Figure 3-3, Verification of the recombinant plasmid for *pflu4717* (*dctA*) complementation.

The plasmid DNA digested by SpeI and HindIII. Lane 1, DNA molecular weight marker, Lanes 2-5 recombinant plasmid of pUC18-mini-Tn7T-GM-GW with 2.0 kb insert.

3.2.2 Expression of *pflu4717* induced by succinate

To test if the expression of *pflu4717* is induced by succinate, a translational fusion to LacZ was constructed by cloning the promoter region of *pflu4717* into vector pXY2 (Table 2.1). The reporter fusion plasmid pXY2-dctA was then introduced into wild-type *P. fluorescens* SBW25. β-galactosidase assays were performed using cells grown in three media: minimal salt medium supplemented separately with "succinate", "histidine" and "succinate plus histidine". Results are shown in Figure 3-4. As expected, levels of *pflu4717* were significantly higher in cells grown in the two media containing succinate, when compared with cells grown on histidine. The data indicates that expression of *pflu4717* is induced by the presence of succinate. Together, the results of *pflu4717* deletion analysis and expression, in conjunction with the data of *in silico* analysis, consistently show that *pflu4717* encodes a succinate transporter (hereafter referred to as SBW25 *dctA*).

Next we sought to determine if DctA is responsible for the utilization of other C_4-dicarboxylates, specifically, fumarate and malate. To do this, wild type SBW25 and mutant MU30-30 (Δ*dctA*) were inoculated into minimal salt medium supplemented with fumarate (20mM) or malate (20mM) as the sole carbon source. Results show that MU30-30 lost the ability to grow on fumarate, and its ability to grow on malate was greatly reduced, when compared with wild-type SBW25 (Table 3.2).

To test the inducibility of *dctA* by fumarate and malate, β-galactosidase reporter assays were performed using cells grown in minimal salt medium supplemented separately with "fumarate", "histidine","fumarate plus histidine", "malate", and "malate plus histidine". The data shown in Figure 3-5 & Figure 3-6 indicated significant differences between presence of fumarate as well as malate and or absence of both

substrates, respectively. Together, the results elucidate that both fumarate and malate are able to induce the expression of *dctA* in wild type SBW25.

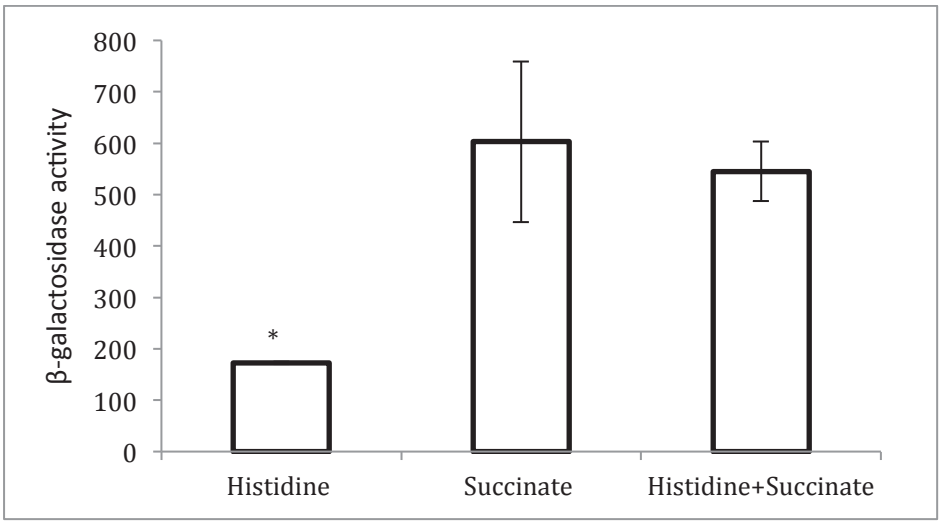

Figure 3-4, Succinate-induced expression of *dctA* in *P. fluorescens* SBW25.

Expression of *dctA* translational fusion that is MU34-74 (See Table 2.1) is induced by succinate and histidine. Data are displayed means and standard deviation; β-galactosidase activity (uM 4MU OD_{600}^{-1} min^{-1}) is measured in quadruplicate with MSM, NH_4Cl and Succinate (20mM). Students' T test revealed significant difference among means, t = 2.44691, α = 0.05. *P_{SUC} = 0.0195, *$P_{HIS+SUC}$ = 0.0337.

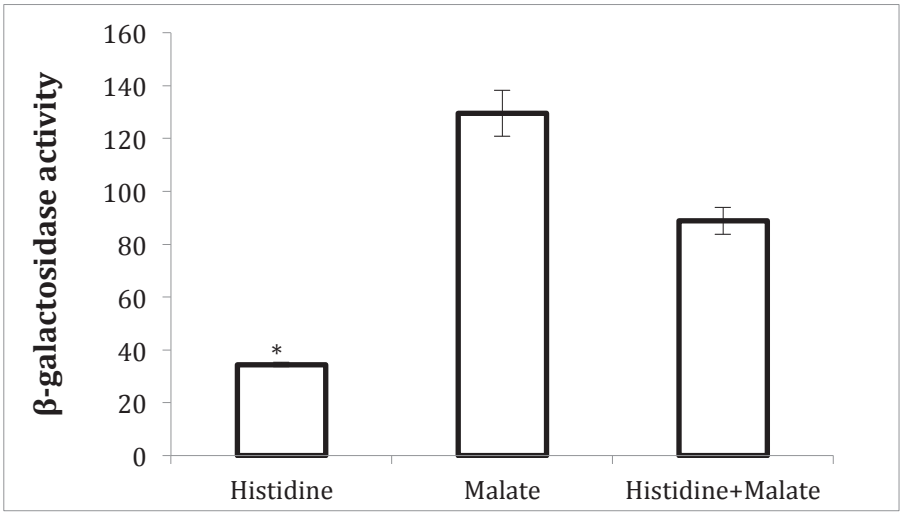

Figure 3-5, Malate-induced expression of *dctA* in *P. fluorescens* SBW25.

Growth of the translational *dctA* fusion strains MU34-74 (See Table 2.1) on minimal medium with MSM, NH_4Cl and Malate (20mM) for 6 hours. Data are displayed means and standard deviation, and assays have been carried out in quadruplicate. Students' T test revealed significant difference among means, t = 2.22814, α = 0.05. *P_{HIS} = 0.0435.

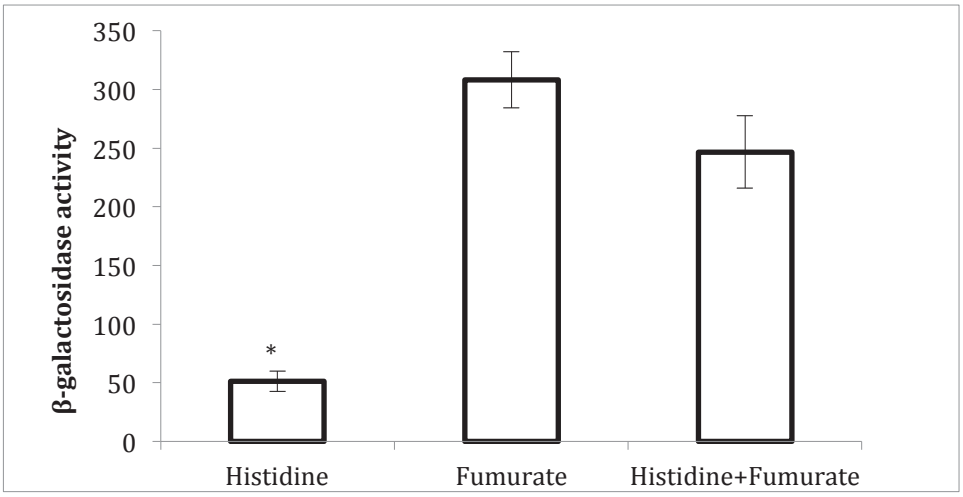

Figure 3-6, Fumarate-induced expression of *dctA* in *P. fluorescens* SBW25.

Growth of the translational *dctA* fusion strains MU34-74 (See Table 2.1) on minimal medium with MSM, NH_4Cl and Fumarate (20mM) for 6 hours. Data are displayed means and standard deviation, and assays have been carried out in quadruplicate. Students' T test revealed significant difference among means, t = 2.26216, α = 0.05. *P_{HIS} = 0.0283.

3.3 Genetic identification of the regulator(s) for succinate uptake in *P. fluorescens* SBW25

3.3.1 Deletion analysis of the three DctBD homologues in the genome of *P. fluorescens* SBW25

To determine which of the three predicted DctBD systems is responsible for the activation of *dctA* (table 3.1), each of the three response regulators was deleted in the genetic background of wild-type *P. fluorescens* SBW25. The ability of the resultant mutants to grow on succinate, fumarate and malate was assessed by cultivating the cells in minimal medium supplemented with the corresponding carbon source. Results show that mutants Δ*pflu4954* and Δ*pflu1134* grew normally on succinate, fumarate and malate, whereas mutant Δ*pflu0286* displayed no growth on succinate as well as fumarate, and reduced growth on malate (Figure 3-7). The phenotype of Δ*pflu0286* was similar to that of the *dctA* deletion mutant. The data thus show that *pflu0287/pflu0286* are required for bacterial growth on succinate (as well as fumarate and malate).

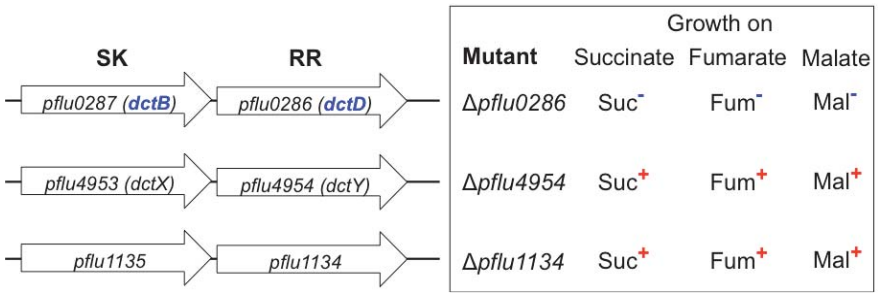

Figure 3-7, Growth phenotype of deletion mutants of three response regulators.

The constructed mutants responded to C4-dicarboxylates accordingly have been recorded. It indicated that Δ*pflu0286* have shown no growth on succinate, fumarate and malate in compare and contrast with wild type growth. However, Δ*pflu1134* and Δ*pflu4954* were growing on succinate, fumarate and malate like wild type.

Table 3.2 Results of phenotypic characterization of strains from *P. fluorescens* SBW25 on different residues.

		Growth on			
Strain	Genotype	Succinate	Malate	α-KG	Fumarate
MU30-30	Δ*dctA (*Δ*pflu4717)*	-	- (reduced)	-	-
MU33-54	Δ*dctD (*Δ*pflu0286)*	-	- (reduced)	-	-
MU33-9	Δ*pflu1134*	+	+	+	+
MU33-56	Δ*dctY (*Δ*pflu4954)*	+	+	-	+

"+" denotes the capability of growth on the related carbon substrate, whereas "-" indicates no growth.

3.3.2 The roles of the candidate regulators in succinate-induced expression of *dctA*

To further test that Pflu0286 is required for the expression of *dctA*, the *dctA-lacZ* reporter fusion (pXY2-dctA) was introduced into the genetic backgrounds of Δ*pflu0286*, Δ*pflu1134* and Δ*pflu4954*, and levels of *dctA* expression were compared with that of wild-type SBW25. The results of β-galactosidase assays were shown in Figure 3-8, 3-9 and 3-10. Expression of *dctA* was abolished in the deletion mutant of *pflu0286*, but not in the backgrounds of *pflu4953* and *pflu1134* deletion. Together, the data allow us to conclude that Pflu0287/Pflu0286 is the two-component system that regulates the expression of *dctA*, thus they are designated DctB and DctD respectively.

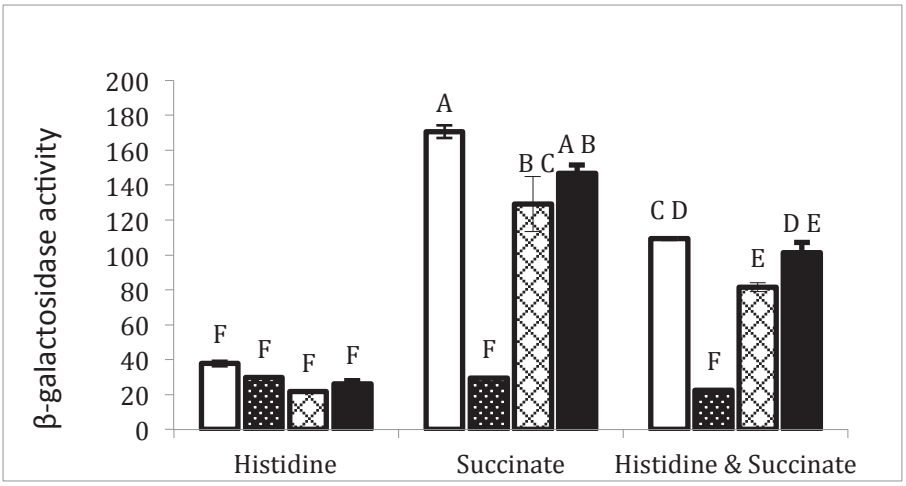

Figure 3-8, Succinate-induced *dctA* expression in wild type *P. fluorescens* SBW25 (blank) and mutant Δ*pflu0286* (dotted), Δ*pflu1134* (grid) and Δ*pflu4954* (black).

The tested strains are MU34-74, MU34-98, MU34-89 and MU34-95 (See Table 2.1). Subsequently, β-galactosidase activity (uM 4MU OD_{600}^{-1} min^{-1}) were measure with MSM, NH_4Cl, Succinate (20mM), Histidine (10mM), Succinate (20mM) + Histidine (10mM). Data are displayed by means and standard deviation, and assays have been carried out in triplicates for each strain. Bars with labeling that are not connected by the same letter (shown above each) indicated significant difference among means ($P < 0.05$) by Tukey's HSD test.

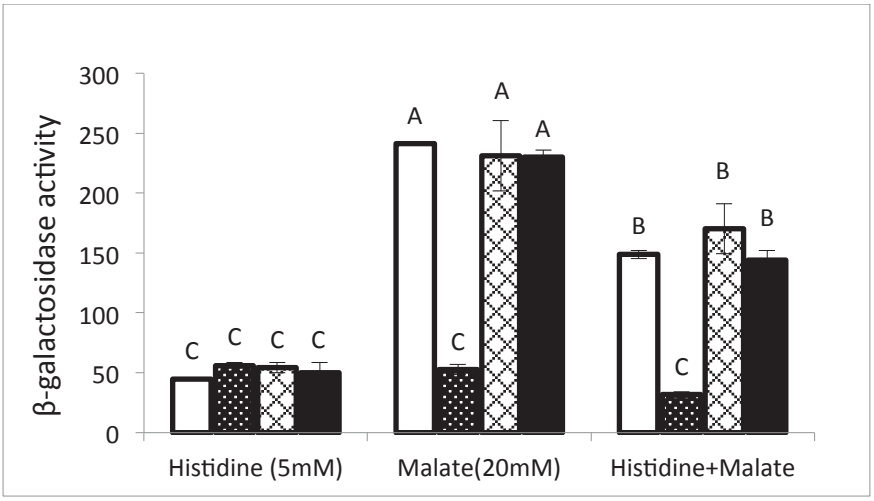

Figure 3-9, Malate-induced *dctA* expression in wild type *P. fluorescens* SBW25 (blank) and mutant Δ*pflu0286* (dotted), Δ*pflu1134* (grid) and Δ*pflu4954* (black).

The tested strains are MU34-74, MU34-98, MU34-89 and MU34-95 (See Table 2.1). Subsequently, β-galactosidase activity (uM 4MU OD_{600}^{-1} min^{-1}) were measure with MSM, NH4Cl, malate (20mM), histidine (10mM), malate (20mM)+ histidine (10mM). Data are displayed by means and standard deviation, and assays have been carried out in triplicates for each strain. Bars with labeling that are not connected by the same letter (shown above each) indicated significant difference among means ($P < 0.05$) by Tukey's HSD test.

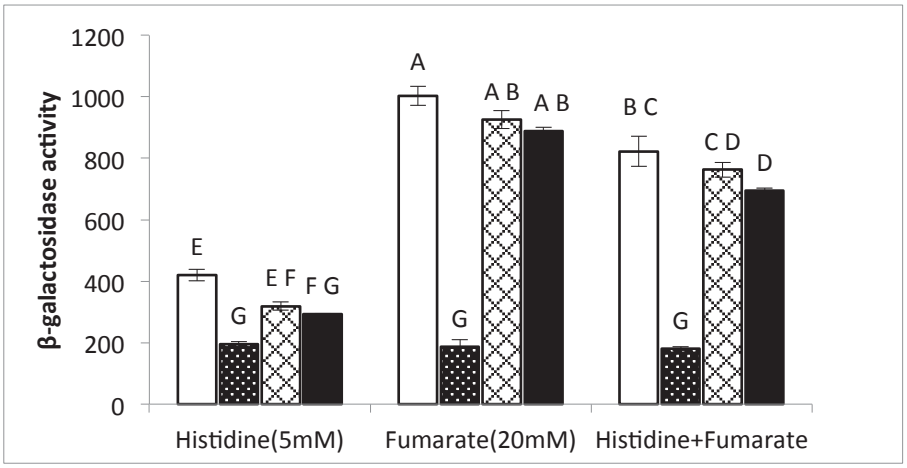

Figure 3-10, Fumarate-induced *dctA* expression in wild type *P. fluorescens* SBW25 (blank) and mutant Δ*pflu0286* (dotted), Δ*pflu1134* (grid) and Δ*pflu4954* (black).

The tested strains are MU34-74, MU34-98, MU34-89 and MU34-95 (See Table 2.1). Subsequently, β-galactosidase activity (uM 4MU OD_{600}^{-1} min^{-1}) were measure with MSM, NH4Cl, Fumarate (20mM), Histidine (10mM), Fumarate (20mM)+ Histidine(10mM). Data are displayed by means and standard deviation, and assays have been carried out in triplicates for each strain. Bars with labeling that are not connected by the same letter (shown above each) indicated significant difference among means ($P < 0.05$) by Tukey's HSD test.

3.4. Suppressor analysis of the *dctA* deletion mutant (Suc^-) of *P. fluorescens* SBW25

3.4.1 Isolation and characterization of the suppressor mutants (Suc^+) of MU30-30 (Δ*dctA*)

Mutant MU30-30 (Δ*dctA*) was unable to grow on succinate as the sole carbon source; however, spontaneous Suc^+ mutants arose at high frequency (~10^{-4}) when bacteria were grown on minimal medium with succinate as the sole source of carbon. As shown in Figure 3-11, the 2-days agar plate at 28°C has a clear background, which was consistent with the Suc^- phenotype of the mutant. To identify the genetic basis of the repressor mutants, two such spontaneous Suc^+ mutants were randomly picked up and their genomic DNAs were subjected to genome re-sequencing. Subsequent alignment with the genome of ancestral SBW25 identified two mutations in Pflu4953:

T574C (W192R) and G1021T (V341L). Locations of these two mutations are shown in Figure 3-12. *pflu4953* encodes a sensor kinase, which likely forms a two-component system with the gene product of *pflu4954*. Notably, it has been shown above that Pflu4954 was not required for activation of the succinate transporter gene *dctA*. Pflu4953 is 1760 amino acids in length and possesses two integral trans-membrane domains (TM1 and TM2), as predicted by hydrophobicity analysis using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/).

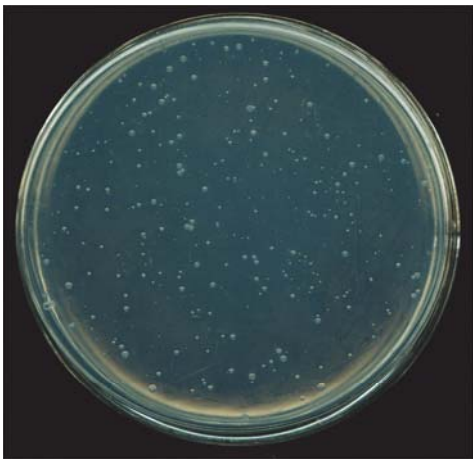

Figure 3-11, A representative agar plate of mutant MU30-30 (Δ*dctA*) grown in minimal medium with succinate as the sole source of carbon.

About 1x10^8 cells were inoculated on the surface of the agar plate and the photo was taken after incubation at 28 ^{o}C for 48 hours.

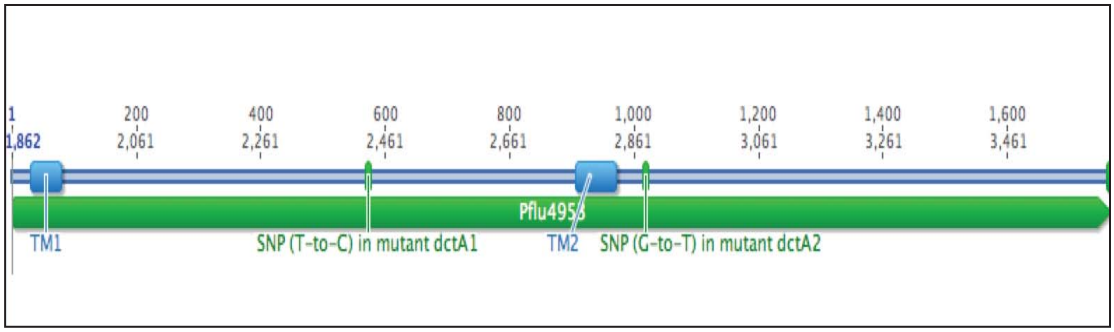

Figure 3-12, Domain structure of Pflu4953.

The location of suppressor mutant MU37-65 and MU37-61 are indicated by a point mutation on *pflu4953* ORF. Two of transmembrane（TM）domains of sensor kinase Pflu4953 are predicted and labeled as TM1&TM2.

3.4.2 Genetic verification of the repressor mutations

The fact that mutation in the same gene *pflu4953* occurred in two independent suppressor mutants strongly suggests that the related mutations were attributable for the Suc^+ phenotype. However, there is still a small chance that the Suc^+ phenotype was due to unknown secondary mutations. To rule out this possibility, the two *pflu4953* repressor mutations (T574C and G1021T) were separately introduced into mutant MU30-30 (Δ*dctA*) by a standard procedure of site-directed mutagenesis analysis. The resulting mutants MU37-65 [Δ*dctA, pflu4953* (T574C)] and MU37-61 [Δ*dctA, pflu4953* (G1021T)] were subjected to DNA sequencing whereby the desired mutation was confirmed. Significantly, the re-constructed repressor mutants MU37-65 and MU37-61 restored the ability to grow on succinate (Suc^+) (Table 3.3). Therefore, it can be concluded that the *pflu4953* mutations were responsible for the repressor phenotype of the Δ*dctA* mutant.

3.4.3 *In silico* analysis of the suppressor mutants

The two suppressor mutants (MU37-65 and MU37-61) were able to grow on succinate, indicating that they possess functional transporter for the uptake of succinate. Identification of suppressor mutations in *pflu4953* suggests that this yet-unidentified succinate transporter is controlled by the Pflu4953/Pflu4854 two-component system. Interestingly, a careful analysis of genes in the *pflu4953* and *pflu4954* locus showed that a putative transporter gene is located downstream of *pflu4953*/*pflu4954* (Figure 3-14). Hydrophobicity analysis using the SOSUI program shows that Pflu4955 is a membrane

protein with 12 trans-membrane helices, and shows 56% sequence similarity with the α-ketoglutarate transporter from *E. coli*. Furthermore, it demonstrate Pflu4955 belong to major facilitator superfamily (MFS) that is a large and diverse group of secondary transporters that includes uniporters, symporters, and antiporters. Importantly, the promoter region of *pflu4955* contains a σ^{54} binding site, and the output domain of Pflu4954 is a σ^{54}-dependent activator (Figure 3-14).

The data of *in silico* analysis led to a hypothesis that (1) *pflu4955* encodes an α-ketoglutarate- and succinate-specific transporter; (2) expression of *pflu4955* is regulated by the two-component system Pflu4953/Pflu4954; (3) expression of *pflu4955* is induced by α-ketoglutarate, but not by succinate; however, (4) mutation in *pflu4953* causes expression of *pflu4955*, conferring the ability of the suppressor mutants to grow on succinate. The hypothesis has been subjected to the experimental tests described below. Based on the results, Pflu4953, Pflu4954 and Pflu4955 are designated DctX, DctY and DctT, respectively (Figure 3-13 and Figure 3-14).

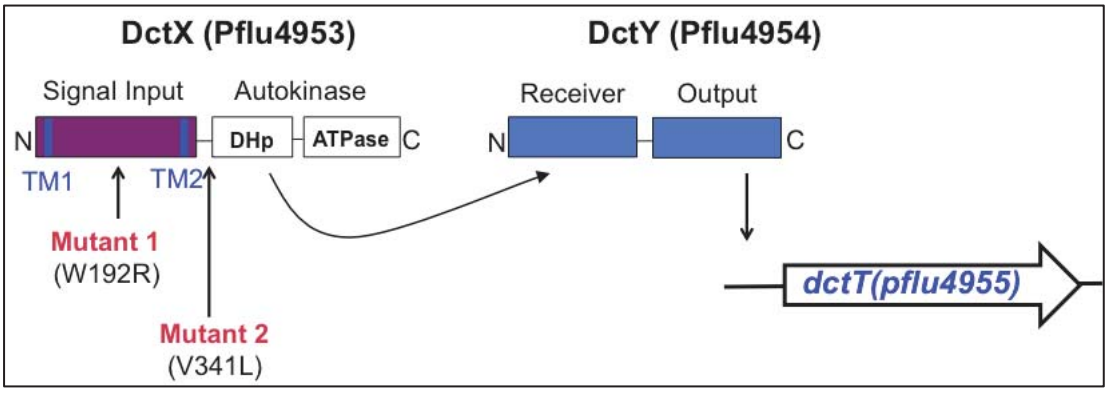

Figure 3-13, Architectures of deduced histidine kinase DctX and response regulator DctY.

The signal input domain of DctX contains two transmembrane domains (TM1 & TM2). The two suppressor mutants are located on and the positions are evaluated.

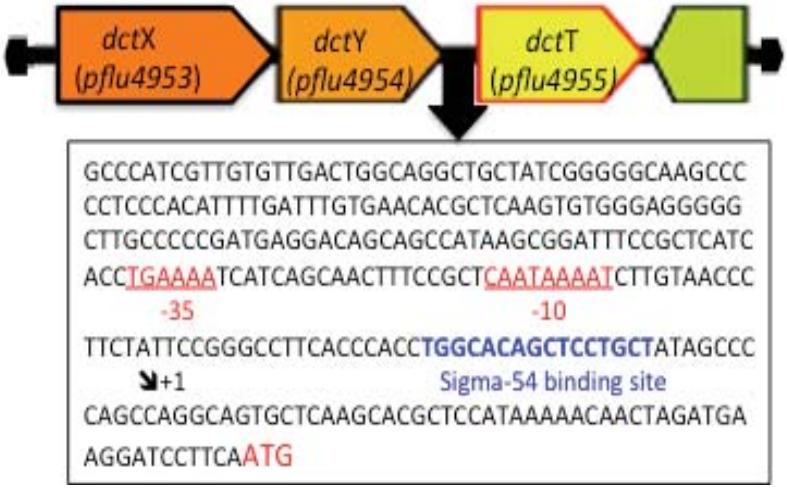

Figure 3-14, Genetic organization of the *pflu4955* locus from *P. fluorescens* SBW25.

Transcriptional start site as determined and indicated by "+1" (arrowed lines). The predicted σ54 -type promoter are labeled as blue color. The -35 and -10 consensus sequence have predicted and underlined. Start codon is labeled2qw42 as magnifying.

3.4.4 Genetic characterization of the *dctXYT* locus

To test the hypothesis described above, particularly the roles of *dctXYT* genes in the uptake of succinate and α-ketoglutarate, recombinant plasmids were constructed that can be used to knockout *dctY* and *dctT* and also monitor the levels of *dctT-lacZ* expression. We first deleted *dctT* in two Suc^+ suppressor mutants of MU35-46 [Δ*dctA*, *dctX* (T574C)] and MU35-47 [Δ*dctA*, *dctX* (G1021T)]. Consistent to expectation, the resultant mutants MU37-55 and MU37-51 (Table 3.3) were unable to grow on succinate, indicating that *dctT* encodes a succinate transporter.

To test if the expression of *dctT* is controlled by the DctXY system, *dctY* was deleted in the same two Suc^+ suppressor mutants of MU35-46 [Δ*dctA*, *dctX* (T574C)] and MU35-47 [Δ*dctA*, *dctX* (G1021T)]. Significantly, the resultant mutants MU38-78

and MU38-89 (Table 3.3) lost the ability to grow on succinate (Suc^-). This result is consistent with the predicted role of DctXY in activation of *dctT*.

Next, *dctT* was deleted in the genetic background of wild-type SBW25 and its derived mutant MU30-30 (Δ*dctA*). The resulting mutants MU37-75 (Δ*dctT*) and MU37-74 (Δ*dctA* Δ*dctT*) were subjected to growth assays on minimal medium with α-ketoglutarate (α-KG) as the sole source of carbon and energy. Results show that both mutants (MU37-75 and MU37-74) were unable to grow on α-ketoglutarate (Table 3.3). Consistently, mutant MU33-56 (Δ*dctY*) was also defective in growth on α-ketoglutarate (α-KG^-). The growth phenotypes of these mutants on other dicarboxylates (i.e., succinate, fumarate and malate) are summarized in Table 3.2 & Table 3.3.

Finally, we sought to determine the expression of *dctT* in response to α-KG and succinate. To this end, we constructed a *dctT-lacZ* transcriptional fusion (plasmid pTn7-PdctT) and it was into four genetic backgrounds of *P. fluorescens* SBW25: wild type, mutant MU33-56 (Δ*dctY*), and the two reconstructed Suc^+ suppressor mutants MU35-46 [Δ*dctA*, *dctX* (T574C)] and MU35-47 [Δ*dctA*, *dctX* (G1021T)]. β-galactosidase assays were performed with cells grown in minimal salt medium supplemented with histidine, α-ketoglutarate (α-KG) as well as both histidine and α-ketoglutarate. Results are shown in Figure 3-15. In the wild-type background, level of *dctT* expression was significantly higher in cells grown on the two media containing α-ketoglutarate than cells grown on histidine only (a control for the absence of α-KG). Moreover, α-ketoglutarate -induced *dctT* expression was abolished in mutant MU33-56 (Δ*dctY*), and α-ketoglutarate was unable to induce *dctT* expression. It is interesting to note that high levels of *dctT*

expression were detected for the two Suc$^+$ suppressor mutants both in the presence and absence of α-ketoglutarate (Figure 3-15).

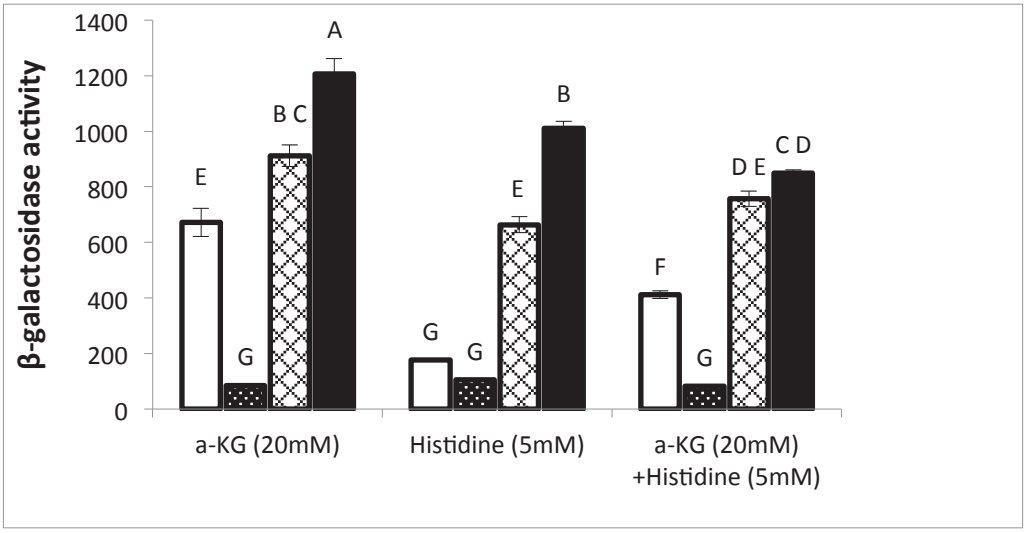

Figure 3-15, α-ketoglutarate-induced expression of *dctT* in wild type SBW25 (blank), and mutants MU33-56 (Δ*dctY*) (dotted), and the two reconstructed Suc$^+$ suppressor mutants MU35-46 [Δ*dctA*, *dctX* (T574C)] (grid) and MU35-47 [Δ*dctA*, *dctX*(G1021T)] (black).

Bacteria were grown in minimal medium with α-ketoglutarate (α-KG), histidine and both histidine and α-KG. Data are means and standard deviation errors of three independent cultures. Two-way ANOVA revealed significant difference among means. Bars that are not connected by the same letter (shown above each) are significantly different ($P < 0.05$) by Tukey's HSD test.

Table 3.3 Results of phenotypic characterization of strains from *P. fluorescens* SBW25 on different residues.

		Growth on			
Strain	Genotype	Succinate	Malate	α-KG	Fumarate
MU37-74	Δ*dctA* Δ*dctT*	-	- (reduced)	-	-
MU37-75	Δ*dctT*(Δ*pflu4955)*	+	+	-	+
MU37-65	Δ*dctA dctX*(T574C), spontaneous Suc^+ mutant	+	+	+	-
MU37-61	Δ*dctA dctX*(G1021T) spontaneous Suc^+ mutant	+	+	+	-
MU38-78	Δ*dctA dctX*(T574C) Δ*dctY*, derived from MU37-65	-	+	-	-
MU38-89	Δ*dctA dctX*(G1021T) Δ*dctY*, derived from MU37-61	-	+	-	-
MU37-55	Δ*dctA dctX*(T574C) Δ*dctT*, derived from MU37-65	-	+	-	-
MU37-51	Δ*dctA dctX*(G1021T) Δ*dctT*, derived from MU37-61	-	+	-	-

"+" denotes the capability of growth on the related carbon substrate, whereas "-" indicates no growth.

To test the responsiveness of *dctT* to the presence of succinate, levels of *dctT* expression were compared between cells grown on histidine, histidine plus succinate, and histidine plus α-KG. The experiments were performed in two genetic backgrounds: wild type and the spontaneous Suc^+ mutant MU37-61. The results shown in Figure 3-16 indicate that histidine was unable to induce *dctT* expression in the wild-type background, whereas levels of *dctT* expression were significantly higher in the spontaneous Suc^+ mutant both in the presence or absence of succinate.

Taken together, the data consistently show that DctT is α-KG transporter whose expression is regulated by the DctXY signal transduction system, in response to the presence of α-KG in the growth medium. Moreover, DctT is a succinate-specific transporter, and mutation in *dctX* causes constitutive expression of *dctT*, thus conferring the ability to grow on succinate in the absence of a functional DctA. The results thus

provide a molecular mechanism that explains the emergence of spontaneous Suc^+ mutant following inactivation of the primary transporter for succinate uptake.

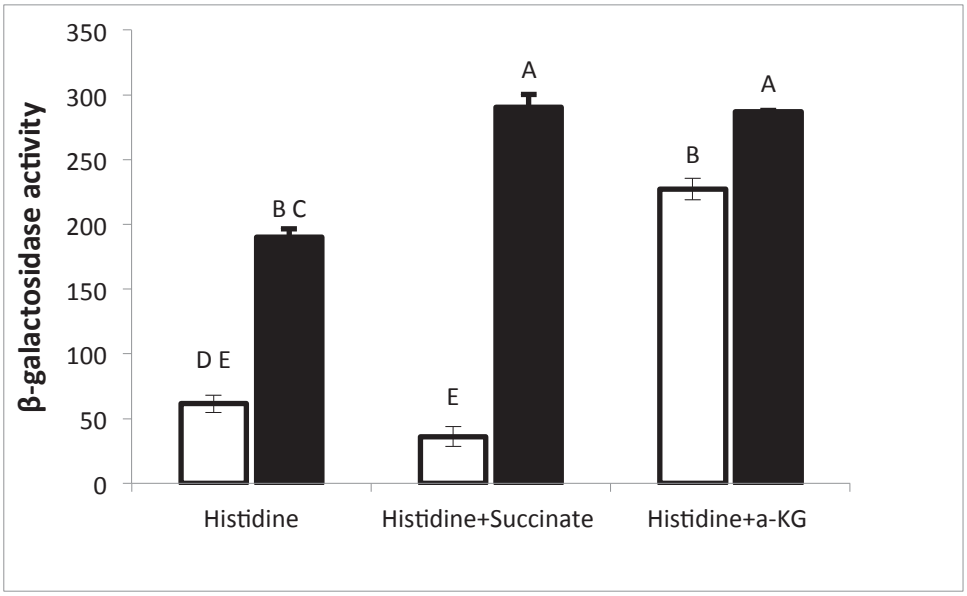

Figure 3-16, succinate- and α-ketoglutarate-induced expression of *dctT* in wild type SBW25 (blank) and mutants MU37-61 [Δ*dctA*, *dctX* (G1021T)] (black).

Bacteria were grown in minimal medium with histidine, histidine plus succinate, and histidine plus α-KG. Data are means and standard deviation errors of three independent cultures. Two-way ANOVA revealed significant difference among means. Bars that are not connected by the same letter (shown above each) are significantly different ($P < 0.05$) by Tukey's HSD test.

Chapter 4 Discussion:

4.1 Substrate specificity of the dicarboxylate transport (Dct) system

Dicarboxylates such as succinate, malate, fumurate and α-ketoglutarate are valuable nutrients that are present on the plant surfaces [16, 33, 46]. It is thus not surprising that many plant-associated bacteria possess specific pathways dedicated to the uptake of dicarboxylates. In this work, we have genetically characterized the transport systems and their regulators that are involved in the uptake of dicarboxylates in a plant growth-promoting bacterium *P. fluorescens* SBW25. Our results show that DctA, whose expression is controlled by DctBD, is capable of transporting succinate, fumarate and malate. The *dctA* and *dctD* deletion mutants showed no growth on succinate and fumarate, indicating that DctA is the sole transporter for these two substrates. Growth of the *dctA* and *dctD* deletion mutants was reduced, but not abolished, on malate, suggesting that *P. fluorescens* SBW25 possesses another yet-unidentified system for malate uptake. Nevertheless, the data show that DctA and DctBD have broad substrate specificity for all three dicarboxylates (succinate, fumarate and malate).

In this work, we identified another succinate-specific transporter DctT, which is capable of transporting α-ketoglutarate. Interestingly, DctT cannot transport fumarate (of note, its ability to transport malate is currently unknown). Furthermore, our data show that DctXY, the two component regulatory system for DctT expression, is responsible for the growth in presence of α-ketoglutarate and not succinate. The four dicarboxylates involved in this study (specifically, succinate, fumarate, malate and α-ketoglutarate) are intermediates of the TCA cycle, and they are structurally similar.

Together, our results show that each of the two uptake systems (DctA and DctT) and their related regulators (DctBD and DctXY, respectively) have overlapping substrate specificity for these four dicarboxylates. Significantly, the transporters and sensory proteins can discriminate the structurally similar dicarboxylates [27, 50, 51]. The data thus laid a good foundation for further investigation into the molecular mechanisms of the substrate specificity for dicarboxylates transporters and sensors.

4.2 The involvement of transporters in gene regulation

Nutrient perception and uptake are two coupled processes mediated by the membrane-bound receptors and transporters, respectively [9, 27, 52, 53]. It is thus not uncommon that efficient acquisition involves direct or indirect interactions between nutrient receptors and the related transporters [9, 17, 27, 50]. Previous work on succinate utilization in enteric bacteria and rhizobia show that DctA sequesters DctB in the cell membrane in order to prevent the sensor kinase (i.e., DctB) from auto-phosphorylation in the absence of succinate [20, 24, 52]. In the presence of succinate, succinate initially binds to DctA, causing an increase of substrate-binding specificity of DctB; DctB is then released and forms a functional two-component system with DctD, which in turn activates the expression of DctA [32, 53, 54]. Consequently, deletion of *dctA* resulted in activation of the *dctA* promoter activities independent of succinate in the residing medium [3, 21, 29, 55].

It would be interesting to know if the DctA transport system of *P. fluorescens* SBW25 is also involved in modulating the activities of DctBD. Our preliminary data showed that in the genetic background of Δ*dctA* the *dctA* promoter (P_{dctA}) is constitutively active, and high levels of P_{dctA}-*lacZ* expression was detected in both the

presence and absence of succinate (data not shown). This suggests that the *P. fluorescens* SBW25 DctA acts in a similar manner as in enteric bacteria and rhizobia to repress the activities of DctB in the absence of succinate. Figure 4-1 indicated that DctA perhaps interact with DctB in membrane might give rise to change the specificity of DctB to sense substrates [24, 56, 57].

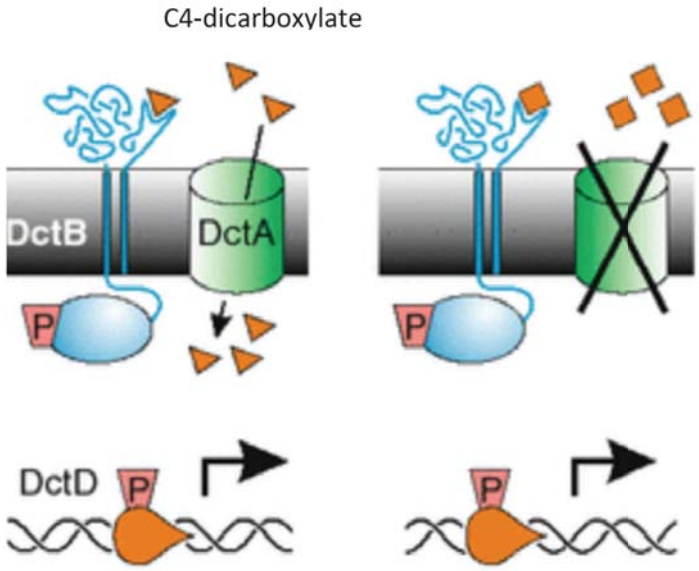

Figure 4-1, The C4-dicarboxylate uptake system DctA influences the two-component system DctB/DctD. In the presence of C4-dicarboxylates DctB phosphorylates the response regulator DctD that induces gene expression. DctB functions as a sensor for dicarboxylates, but it is also regulated by DctA. When DctA is absent, DctB has a broader substrate spectrum. Thus, DctA increases the specificity of DctB/DctD. *Source: Adapted from Tetsch and Jung, 2009.* [52]

Chapter 5 Conclusion and Future Work:

The plant-associated bacterium *P. fluorescens* SBW25 possesses two succinate-specific transporters DctA and DctT. However, only DctA is involved in succinate uptake and its expression is induced by succinate via the DctBD two-component system; expression of DctT is regulated by another two-component system, DctX and DctY, which is not responsive to succinate. Significantly, if a loss-of-function mutation occurs in the primary DctBD-DctA pathway, the Suc^- mutant can easily regain the ability to utilize succinate via mutations in DctX, which may cause constitutive activation of the DctX sensor kinase. The evolutionary redundancy may act as a mechanism that ensures bacteria to efficiently utilize host-derived valuable nutrients such as the dicarboxylates in the TCA cycle.

Future work will involve elucidating the mechanisms of the DctXY system in modulating the promoter activities of DctT in response to the presence or absence of different substrates (including α-ketoglutarate, succinate, fumarate, and malate). Specifically, a signaling defective mutant can be made by site-directed mutagenesis of the putative histidine phosphorylation site of the DctX kinase, which will be followed by phenotypic assays for the DctT promoter activities.

It is currently unknown whether or not the DctT system is involved in protein-protein interaction with DctX, when the substrate (i.e., α-ketoglutarate) is absent in the external environment. If DctT represses the activity of DctXY as DctA does for DctBD, we would expect high levels of *dctT* promoter activities in the *dctT* deletion background, which is independent of α-ketoglutarate. Finally, further *in silico* analysis combined with site-directed mutagenesis will be performed to achieve a better

understanding of the two sets of transporter and regulatory systems for dicarboxylates (DctA-DctBD and DctT-DctXY), particularly the mechanisms of substrate specificities.

The significances of the findings in this work are that *P. fluorescens* SBW25 possesses complex and redundant transporter system for the utilization of dicarboxylates that likely contribute to the success of this bacterium in the plant environment. Furthermore, the substrate specificity of the transport system is determined by not only the transporter protein but also its regulator(s), which imply the nutrients utilization of *P. fluorescens* SBW25 was experienced extraordinary evolutionary diversity. These findings also highlight substitution mutation effect on changes of protein function. Thus, insights gained in this study manifest substitution mutation have important evolutionary consequence for the bacterium to adapt to the environment. This can offer the opportunity for an implementable nutritional research in plant.

Reference:

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