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

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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.

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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