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Molecular mechanism of xylose utilization in a plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25

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Abstract

Pseudomonas fluorescens SBW25 is a plant growth-promoting bacterium that was originally isolated from the phyllosphere of field-grown sugar beet. It is capable of aggressively colonizing sugar beet and a number of other crops such as wheat, maize and peas, and inhibiting the damping-off disease caused by *Pythium ultimum*. *P. fluorescens* SBW25 has become an important model organism for studying the molecular interactions between bacteria and plants. Previous promoter-trapping analysis showed that SBW25 elevates expression of over 100 genes in its genome when colonizing sugar beet seedlings. These include a candidate gene for xylose utilization, suggesting that SBW25 colonization may be critically dependent on the presence and catabolism of plant-derived xylose. The overall aim of this project is to unravel the molecular basis of xylose utilization by *P. fluorescens* SBW25 and demonstrate its ecological significance for bacterial survival in complex plant experiments.

Bacterial degradation of xylose is sequentially mediated by two enzymes - an isomerase (XutA) and a xylulokinase (XutB) - with xylulose as an intermediate. *P. fluorescens* SBW25, though capable of growth on xylose as a sole carbon source, encodes only one degradative enzyme XutA in the xylose utilization (*xut*) locus. Here, using site-directed mutagenesis and transcriptional assays I identified two functional xylulokinase-encoding genes (*xutB1* and *xutB2*), and further showed that expression of *xutB1* is specifically induced by xylose. Surprisingly, the xylose-induced *xutB1* expression is mediated by the mannitol-responsive regulator MtlR, using xylulose rather than xylose as the direct inducer. In contrast, expression of the *xutA* operon is regulated by XutR in a xylose- and xylulose-dependent manner. Moreover, the data indicate a complex overlapping cellular responses to xylose and other structurally similar sugars such as mannitol, sorbitol, fructose as well as ribose.

Both XutR and MtlR are transcriptional activators of the AraC family, members of which typically use DNA-looping to modulate levels of gene expression. The functionality of XutR has been subjected to detailed genetic and biochemical

analyses, including promoter mapping, electrophoretic mobility shift assay (EMSA) and DNase I footprinting assay. My data leads to a XutR regulatory model that does not involve DNA-looping. XutR functions as a dimer, which recognizes two inverted repeat sequences; but binding to one half site is very weak requiring inducer molecules such as xylose for activation.

To determine the ecological significance of xylose utilization for bacterial colonization *in planta*, a Xut$^-$ mutant (Δ*xutA*) was subjected to competitive colonization on sugar beet seedlings together with a neutrally marked wild-type strain. Results showed that the Δ*xutA* mutant was significant less competitive than the wild-type strain both in the shoot and the rhizosphere. Together, the data show that xylose utilization is an important trait for *P. fluorescens* SBW25 to colonize surfaces of plants.

It should be noted that xylose can only support slow bacterial growth of *P. fluorescens* SBW25, and thus it is not the sugar of choice in the presence of other preferred carbon and energy sources such as succinate, glucose and arabinose. The underlying mechanism is called carbon catabolite repression (CCR). CCR has been well studied in *E. coli*, where it is mainly mediated by the catabolite-activation protein CAP charged with cAMP. However, CCR still remains elusive for non-enteric bacteria such as *Pseudomonas*. Previous studies in other *Pseudomonas* species indicate that CCR in *Pseudomonas* occurs at post-transcriptional levels and involves specific binding of the Crc protein to mRNAs of respective catabolic genes. However, genetic tools suitable for studying post-transcriptional gene expression are lacking, particularly vectors derived from mini-Tn*7*. Mini-Tn*7* vectors possess the advantage of delivering reporter fusions into the chromosome in a site- and orientation-specific manner.

To facilitate the study of CCR in *Pseudomonas*, I have successfully constructed and validated a panel of five mini-Tn7 vectors for analysis of post-transcriptional gene expression in *Pseudomonas*. Four vectors allow construction of translational fusions to β-galactosidase (*lacZ*), while the fifth is designed for functional analysis of noncoding RNA genes. Translational fusions can be constructed without a functional

promoter in the vector or from an inducible promoter of either P_{tac} or P_{dctA}. I show that promoterless fusions have value for determining levels of translation, whereas fusions to inducible promoters have utility in the analysis of mRNA-binding factors.

Next, a combination of site-directed mutagenesis and gene expression assays were used to identify regulators that are involved in the succinate-mediated repression of the *xut* operon. Succinate is an intermediate of the tricarboxylic acid (TCA) cycle and it is preferentially used by *P. fluorescens* SBW25 as a source of carbon and energy. In this work, I have successfully identified the major regulatory components of CCR in *P. fluorescens* SBW25. These include a two-component signal transduction system CbrAB, two small non-coding RNAs CrcY and CrcZ, and a two-protein complex composed of Crc and Hfq. Results showed that when succinate is present, the Crc/Hfq complex inhibits expression of *xut* genes via binding of the mRNA transcript; when succinate disappears, CbrAB activates the expression of CrcY and CrcZ, which in turn sequesters the Crc/Hfq complex and relieves repression of the *xut* operon.

Taken together, data presented in this thesis indicate novel mechanisms of xylose utilization in terms of not only the catabolic genes but also the mode of their regulation, and reveal complexity and redundancy of regulators involved in the succinate-mediated repression of xylose utilization genes.

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List of Abbreviations

APS	Ammonium persulphate
ATP	Adenosine triphoshpate
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
ºC	Degrees Celsius
bp	Base pairs
Da	Dalton
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
g	Gravitational force
h	Hour
HEPES	n-2-hydroxylethylpiperazine-n'-2-ethanesulphonic acid
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilobase pairs
kDa	KiloDaltons
LB	Luria-Bertaini
M	Molar
mM	Millimolar
µM	Micromolar
mg	Milligram
ml	Millimeter
µl	Microlitre
min	Minute
MW	Molecular weight
MWCO	Molecular weight cut-off
nm	Nanometre
OD	Optical density
ORF	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PMSF	Phenylmethylsulfonyl fluoride
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TBE	Tris-borate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
UV	Ultraviolet