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Effect of *gcl*, *glcB*, and *aceA* Disruption on Glyoxylate Conversion by *Pseudomonas putida* JM37

Li, Xuan Zhong[1], Janosch Klebensberger[1,2], and Bettina Rosche[1]*

[1]*School of Biotechnology and Biomolecular Sciences and* [2]*Centre for Bio-innovation, The University of New South Wales, Sydney, NSW 2052, Australia*

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***Pseudomonas putida* JM37 metabolized glyoxylate at a specific rate of 55 g/g dry biomass/day. In order to investigate their role, three genes encoding enzymes that are potentially involved in the conversion of glyoxylate were disrupted; namely, tartronate semialdehyde synthase (*gcl*), malate synthase (*glcB*), and isocitrate lyase (*aceA*). Strains with transposon insertion in either of these genes were isolated from a 50,000 clone library employing a PCR-guided enrichment strategy. In addition, all three double mutants were constructed *via* targeted insertion of a knock-out plasmid. Neither mutation of *gcl*, *glcB*, and *aceA* nor any of the respective double mutations influenced glyoxylic acid conversion, indicating that *P. putida* JM37 may possess other enzymes and pathways for glyoxylate metabolism.**

Keywords: Glyoxylic acid, biotransformation, *Pseudomonas putida*, tartronate semialdehyde synthase, malate synthase, isocitrate lyase

Glyoxylic acid (glyoxylate) is a key intermediate in many metabolic pathways; for example, the "glyoxylate cycle," which enables organisms to convert fatty acids into carbohydrates. It has diverse industrial applications [25] and is currently synthesized by chemical processes [22]. Glyoxylic acid can also be produced enzymatically from glycolic acid [4, 6, 7, 22, 25]. To accumulate glyoxylate from other cheap carbon sources *via* microbial transformations, pathways that facilitate the further conversion of glyoxylate may need to be blocked. One approach is to disrupt genes involved in glyoxylate conversion.

Pseudomonas putida uses the TCA cycle and respiration for energy generation. Three potential pathways for glyoxylate conversion have been identified in *Pseudomonas* [1, 11]. In the "glycerate pathway" [12], tartronate semialdehyde synthase (*Gcl*) catalyzes the formation of tartronate semialdehyde from two molecules of glyoxylate, which then feeds into the TCA cycle *via* acetyl-CoA. In the "dicarboxylic acid pathway" [14], malate synthase (*GlcB*) adds an acetyl-CoA to glyoxylate to produce malate, followed by malate metabolism for energy and acetyl-CoA regeneration. Isocitrate lyase (*AceA*) can facilitate the addition of succinate to glyoxylate to produce isocitrate [1], which can then be oxidized for energy generation. The current study investigated the effects of *gcl*, *glcB*, and *aceA* disruption on glyoxylate conversion by *P. putida* JM37.

P. putida JM37 strains were grown in nutrient broth or M12 medium containing appropriate antibiotics (Table 1) at 30°C and 150 rpm. M12 contained (g/l) 2 KH_2PO_4, 1 $(NH_4)_2SO_4$, 0.1 NaCl, 0.2 $MgSO_4 \cdot 7H_2O$, 0.02 $CaCl_2 \cdot 2H_2O$, 0.05 yeast extract; the following vitamins (μg/l): 2 biotin, 2 folic acid, 200 *p*-aminobenzoic acid, 200 riboflavin, 400 Ca-pantothenate, 1,400 nicotinic acid, 400 pyridoxine.HCl, 2,000 myo-inositol, 400 thiamine.HCl; and 1 ml of "SL4" trace element solution [19]. *Escherichia coli* was cultivated on LB at 37°C with the following antibiotics added where appropriate (μg/ml): 100 ampicillin, 34 chloramphenicol, and 20 tetracycline.

Transposon mutagenesis of JM37 was performed using pALMAR3 carrying a mariner transposon [15] and a protocol described in Klebensberger *et al*. [10]. Mutants were selected on *Pseudomonas* isolation agar (PIA, Difco) plates containing 150 μg/ml tetracycline. Colonies from each plate were pooled (approx. 3,000 colonies/plate) to create 17 sublibraries containing over 50,000 mutants. A PCR-guided enrichment strategy was developed to isolate desired transposon mutants. Using a target gene-specific primer in conjunction with a mariner transposon gene-specific primer (Table 2), Δ*gcl*, Δ*glcB*, and Δ*aceA* were identified in sublibraries. Mutant positive sublibraries were serial-diluted and spread onto LB plates. Approximately

**Corresponding author*
Phone: +61-2-9385-1598; Fax: +61-2-9385-1483;
E-mail: b.rosche@unsw.edu.au

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics and antibiotic resistance	Origin/reference
P. putida		
JM37	Wild type	[16]
JM37 *Δgcl*	*gcl* mutant of JM37 with transposon insertion at position 788 bp of the ORF (Tet^r)	This study
JM37 *ΔglcB*	*glcB* mutant of JM37 with transposon insertion at position 416 bp of the ORF (Tet^r)	This study
JM37 *ΔaceA*	*aceA* mutant of JM37 with transposon insertion at position 519 bp of the ORF (Tet^r)	This study
JM37 *Δgcl*/*ΔaceA*	*gcl* transposon and *aceA* insertional double mutant of JM37 (Tet^r, Gm^r)	This study
JM37 *ΔglcB*/*Δgcl*	*glcB* transposon and *gcl* insertional double mutant of JM37 (Tet^r, Gm^r)	This study
JM37 *ΔglcB*/*ΔaceA*	*glcB* transposon and *aceA* insertional double mutant of JM37 (Tet^r, Gm^r)	This study
E. coli		
DH5α	Donor strain	[5]
HB101	Helper strain in triparental mating	Promega
S17-1λPir	Strain for maintenance and conjugation of Pir-dependent vector	[10]
Plasmids		
pALMAR3	Plasmid harboring a mariner transposon used for transposon mutagenesis (Tet^r)	[10]
pGEM-T-Easy	Cloning shuttle vector (Amp^r)	Promega
pKnockout-G	Suicide vector used for gene disruption (Amp^r, Gm^r)	[24]
pRK600	Helper plasmid (Cm^r)	[9]

600 colonies/plate were obtained to create 2nd-generation sublibraries, and positive 2nd-generation sublibraries were identified *via* PCR as described above. This was repeated to generate 3rd (100–150 clones)- and 4th-generation sublibraries (<50 clones), which allowed individual screening and isolation of Δ*gcl*, Δ*glcB*, and Δ*aceA*.

Double mutants Δ*gcl*/Δ*aceA*, Δ*glcB*/Δ*gcl*, and Δ*glcB*/Δ*aceA* were generated by gene inactivation [24] in the respective transposon mutants. Primers harboring *Xba*I and *Xho*I restriction sites (Table 2) amplified a 470 bp and a 433 bp fragment of *gcl* and *aceA*, respectively. Truncated gene fragments were cloned into pGEM-T-Easy for replication, followed by a *XbaI*/*XhoI* double digest and

Table 2. Primers used in this study.

Primers	Sequence
Target gene specific primers	
gcl-F	5'-ATGAGCAAAATGAGAGCAATCG-3'
gcl-R	5'-TCAGTCCAGCAGCGAGATG-3'
glcB-F	5'-ATGACTGGATACGTTCAAGTCGG-3'
glcB-R	5'-TTACAACCCGTTACGCGCCTTG-3'
aceA-F	5'-ATGGCACTGACACGCGAA-3'
aceA-R	5'-GTGGAACTGCTCTTCTTCGG-3'
Mariner transposon specific primers	
marseqN-F	5'-CAACCCTTGGCAGAACATATCC-3'
marseqN-R	5'-CGATTCATTAATGCAGCTGGC-3'
Insertional mutant cloning primers	
gcl-XbaI-F	5'-TCTAGAGTGATCCCGACCCTGATG-3'
gcl-XhoI-R	5'-CTCGAGTGACGTAGCAGGTGTCCTTG-3'
aceA-XbaI-F	5'-TCTAGACGCCTACGAGCTGATGAAGA-3'
aceA-XhoI-R	5'-CTCGAGGGTCCGGGTACTCCTTCTTG-3'
pKnockout-G specific primer	
pKO-G-F	5'-GCGCGTTGGCCGATTCATTA-3'

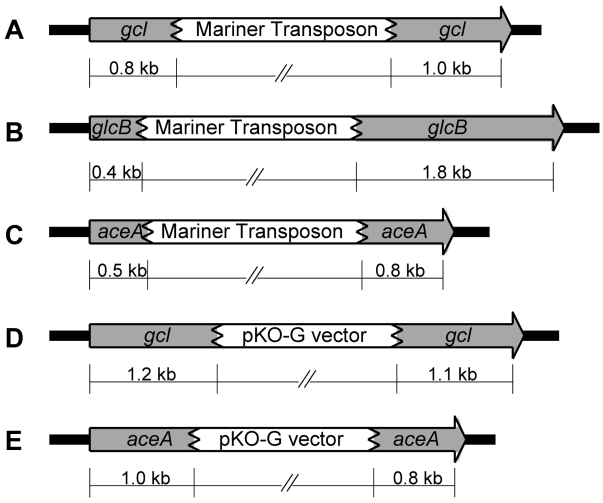

Fig. 1. Gene disruption in *P. putida* JM37.
The sites of vector insertion were identified *via* PCR and sequencing. The first gene disruption was achieved using pALMAR3 carrying a mariner transposon, to generate single gene disruption mutants Δ*gcl* (**A**), Δ*glcB* (**B**), and Δ*aceA* (**C**). The additional disruption of *gcl* (**D**) and *aceA* (**E**) was performed using pKnockout-G on single mutants Δ*gcl* and Δ*glcB* to create the double mutants Δ*gcl*/Δ*aceA*, Δ*glcB*/Δ*gcl*, and Δ*glcB*/Δ*aceA*.

cloning into pKnockout-G [24]. The resulting plasmids were transferred into transposon mutants *via* triparental mating [23], and double mutants were selected on PIA containing 50 μg/ml gentamycin.

To determine the glyoxylate conversion rate, wild-type and mutant cells were resuspended in buffered M12 containing 100 mM glyoxylic acid and incubated at 30°C and 150 rpm. Samples or external standard solutions were mixed with an equal volume of 90% acetonitrile solution containing xylitol as an internal standard. After centrifugation, HPLC analysis was performed on a Bio-Rad Aminex HPX-87H ion-exclusion column (5 mM H_2SO_4, 0.6 ml/min, 30°C) with refractive index detection.

Fig. 1 illustrates the sites of vector insertion within each targeted gene. For each transposon mutant, target gene specific primers (Table 2) produced a single PCR product that equalled the size of the wild-type gene harboring the 2-kb mariner transposon, and correct transposon insertion was verified by sequencing. The absence of the PCR products corresponding to the size of the wild-type genes indicated that there are no multiple copies of *gcl*, *glcB*, or *aceA* within the *P. putida* JM37 genome. For the double mutants, none of the primer pairs specific for the second disrupted gene produced a PCR product under the standard PCR conditions of the positive control (wild type). This indicates the presence of the 6-kb pKnockout-G insertion within these disrupted genes. An additional PCR using a gene-specific primer and a pKnockout-G-specific primer obtained a PCR product, and correct pKnockout-G insertion was verified by sequencing. Thus, the targeted gene disruptions were evident in all mutants.

The growth of JM37 single and double mutants on glyoxylic acid is presented in Table 3. Since the mutants grew well in M12 medium supplemented with glyoxylic acid and JM37 wild type could not grow in M12 without supplement, it is evident that the mutants were capable of utilizing glyoxylic acid efficiently, despite the disruption of *gcl*, *glcB*, and/or *aceA*.

Glyoxylate conversion profiles by the wild type and mutants are illustrated in Fig. 2. All strains consumed 100 mM glyoxylate with similar profiles, and the highest conversion rate was 55±5 g/g dry biomass/day at 6 h. Potential products such as glycolate, malate, isocitrate, or oxalate were not accumulated, and no additional peak was detected by HPLC. Mutant cells were collected after the experiment and their genotypes were verified by PCR.

Table 3. Growth of wild type (WT) and mutants in buffered M12 medium (250 mM MOPS, pH 7.0) supplemented with 50 mM glyoxylic acid.

Strain	OD_{600} at 24 h	Maximum specific growth rate (h^{-1})
WT (no glyoxylic acid)	0.02	0
WT	0.98	0.39
Δgcl	1.1	0.39
ΔglcB	0.97	0.42
ΔaceA	0.97	0.38
*Δgcl/ΔaceA**	1.0	0.37
*ΔglcB/Δgcl**	0.95	0.29
*ΔglcB/ΔaceA**	1.0	0.38

*With additional 30 μg/ml gentamycin.
The average from two independent cultures is shown and there was less than 5% difference between cultures.

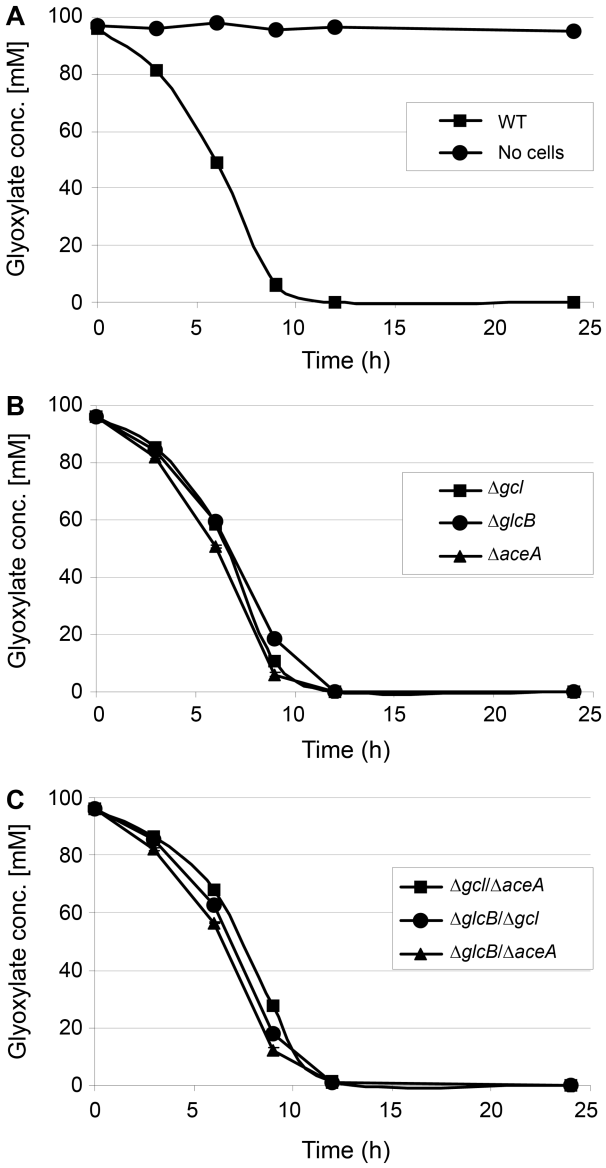

Fig. 2. Biotransformation of 100 mM glyoxylate.
A. Wild type and cell-free control. **B**. Single mutants. **C**. Double mutants. Cells were grown in nutrient broth and suspended at an initial OD_{600} of 1.0 in M12 medium buffered with 250 mM MES, pH 7.0. No products were detected. The average from two independent cultures was plotted, and their values are indicated in error bars.

Thus, single and double gene disruption of *gcl*, *glcB*, and *aceA* had no apparent effect on the conversion of glyoxylate by JM37.

It can be considered that JM37 may be able to switch between the pathways involving *gcl*, *glcB*, and *aceA* for glyoxylate metabolism. Since these pathways differ in energy efficiency and require diverse enzymes/cosubstrates, it would be likely that they operate at different rates. However, the glyoxylic acid utilization profiles of all three double mutants were similar, and this suggests that JM37 metabolizes glyoxylate without involving *gcl*, *glcB*, or *aceA*. Triple gene disruption of *gcl*, *glcB*, and *aceA* was therefore not attempted. It is also questionable if JM37 converts glyoxylate to oxalate; however, the wild type neither accumulated oxalate nor converted added oxalate (data not shown). Other pathways that do not involve *gcl*, *glcB*, or *aceA* for the utilization of glyoxylate have been reported in some organisms; for example, the "β-hydroxyaspartate pathway" in *Micrococcus denitrificans* [13], the "serine pathway" in *Methylobacterium extorquens* [2], the "malyl-CoA pathway" in *Moorella* sp. [20], and the "plant's glycolate pathway" in green algae [17]. Armed with one of the largest prokaryotic genomes, *P. putida* strains are known for their exceptional metabolic versatility [18]. However, the initial genes and enzymes involved in these above-mentioned pathways are not annotated in *P. putida* KT2440 or F1 according to databases [8, 21].

Thus, metabolic engineering based on published pathways is not straightforward. In conclusion, *gcl*, *glcB*, and *aceA* may not be essential for glyoxylate metabolism in *P. putida* JM37. We speculate that this organism possesses an alternative pathway for glyoxylate conversion. *P. putida* has become a workhorse for various biotechnological applications [3], and understanding the glyoxylate metabolism in *P. putida* JM37 may open opportunities for the production of glyoxylate from cheap carbon sources.

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