Probing the central carbon metabolism of *Rhodobacter sphaeroides* by transposon mutagenesis

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Introduction

Rhodobacter sphaeroides is a metabolically diverse photosynthetic bacterium that is capable of utilizing a wide variety of carbon substrates. Recently, the ethylmalonyl-CoA pathway, which is required during the growth on acetate to replenish the intermediates of the tricarboxylic acid cycle, was discovered in *R. sphaeroides*. While this discovery was an important step in understanding the central carbon metabolism of this organism, much is still unknown. In this study we reexamined acetate metabolism and further explored the central carbon metabolism by indentify genes required for lactate metabolism in *R. sphaeroides*.

LEGEND

Blue figures are essential precursor metabolites to amino acids, fatty acids, and sugars. These precursor metabolites are required for cell biosynthesis. They are continually removed from the central carbon metabolism and need to be replenished.

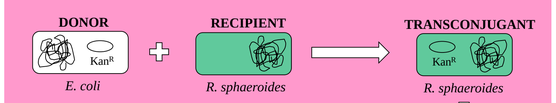
Predicted pathway necessary for lactate assimilation

Predicted pathway necessary for acetate assimilation

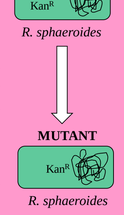
Methods

Random Transposon Mutagenesis:

The plasmid pRL27 (Larsen et al.) containing transposon with a kanamycin-resistance gene cassette (*aph*) was mobilized from the donor *E. coli* to the recipient wild-type *R. sphaeroides* to obtain random transposon mutants of *R. sphaeroides*.

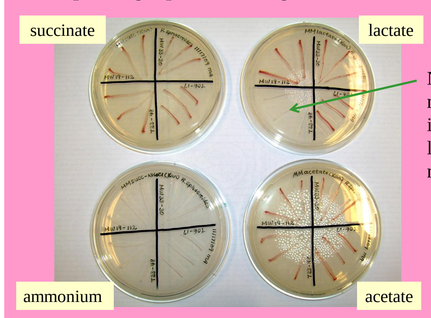


In each mutant, the randomly inserted transposon retained the *oriR6K* and *aph*. The mating mixture was plated on the selective medium minimal succinate with kanamycin to enrich for transposed *R. sphaeroides* mutants. Kanamycin selected against transconjugants and non-conjugated recipient cells.



Genetic screening of the mutants on min-succ-NH_4^+-kan, min-acetate-kan, min-lactate-kan, and a positive control "master plate" of LB medium were performed to identify nitrogen fixation, acetate, and lactate mutants.

Example of agar plate screening for carbon substrate mutants

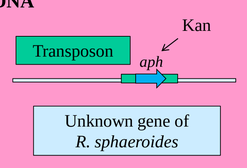


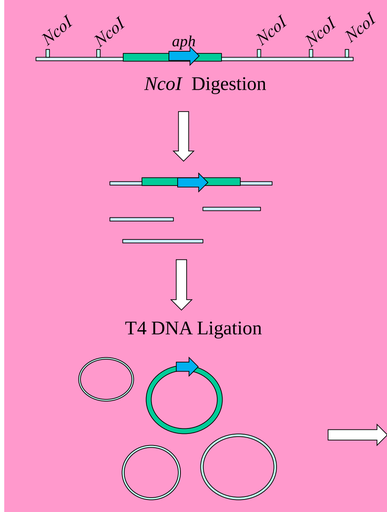
No growth on min-latate-kan indicated a lactate mutant

Screening revealed several lactate and acetate mutants which indicated transposon insertion into genes necessary for lactate and acetate assimilation within the central carbon metabolism of *R. sphaeroides*..

Transposon insertion identification via plasmid DNA isolation and *E. coli* transformation

The genes of the *R. sphaeroides* genome that were interrupted by transposon insertion could be cloned and identified by DNA sequencing.



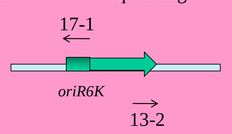


The genomic DNA was purified from *R. sphaeroides* revealing a segment of known plasposon DNA flanked by two unknown segments of *R. sphaeroides 2.4.1* chromosomal DNA. This was digested with *NcoI* to obtain fragments of linear DNA to begin the cloning protocol. A dilute ligation with T4 DNA ligase then circularized the DNA. The circular DNA was then transformed into *E. coli* DH5α-λpir to select for transposon mutants on LB-Kan by the *aph* gene selectable marker.

* Transformants were grown in LB-Kan liquid culture. Plasmids containing the transposon insert were effectively cloned as *E. coli* replicated.

Plasmid DNA sequencing

Plasmid DNA was isolated through neutraliztion. The isolated renatured plasmids were then primed and submitted for DNA sequencing.



- tpnRL17-1 primer annealed to the *oriR6K* end of the transponon
- tpnRL13-2 primer annealed to Kan resistance end of the transposon

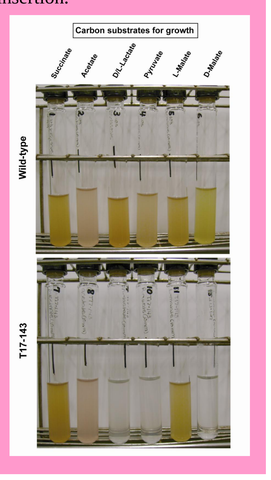
*Sequences were submitted to the Plant-Microbe genomics Facility at OSU. After receiving results, BLAST identified the genes disrupted by transposon insertion.

Liquid growth experiment

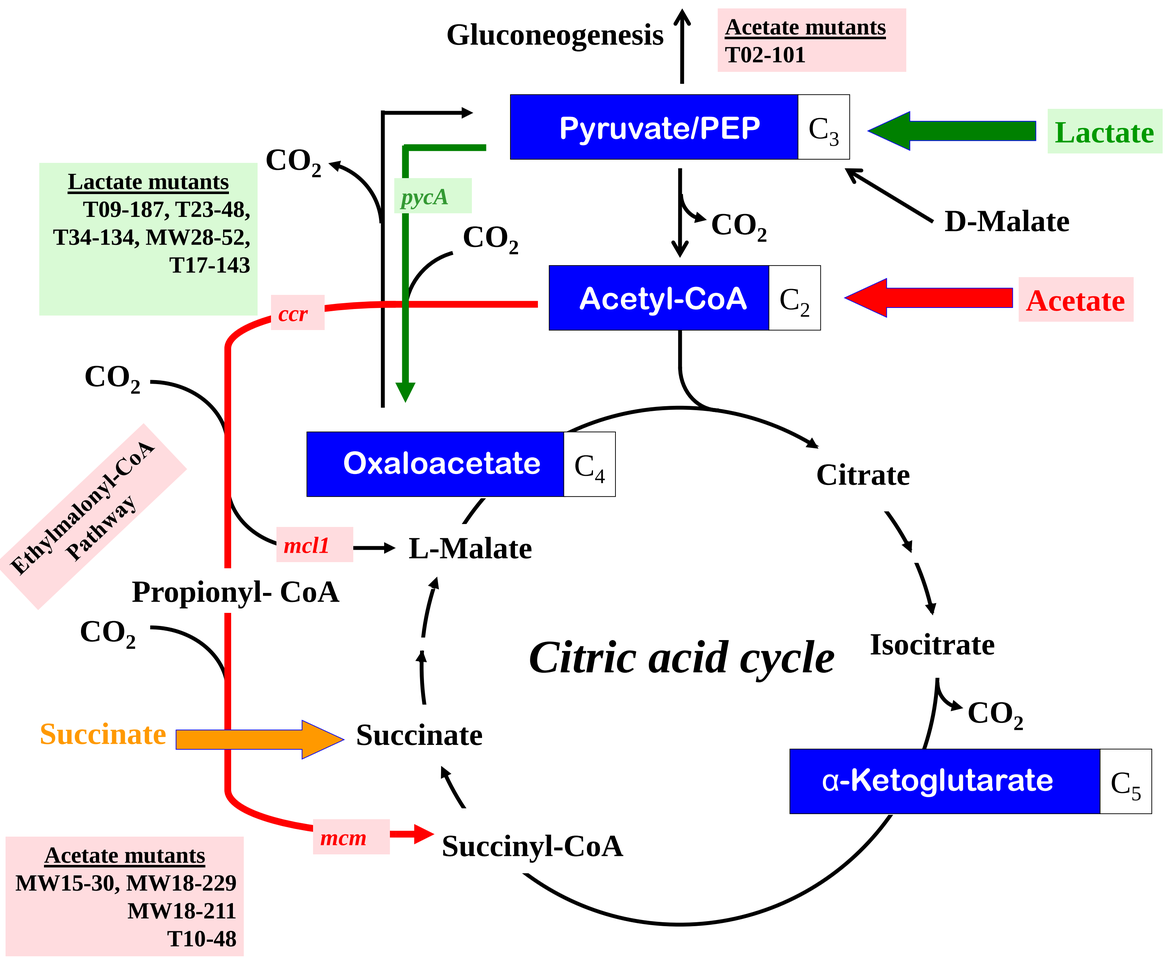
Photoheterotrophic growth of wt and mutants was assessed for different carbon substrates.

Anoxic cultures were incubated at 30°C for each carbon substrate.

Quantitative measurements were taken using a spectrometer at varying time intervals.



The central carbon metabolism of *Rhodobacter sphaeroides*



Transposon insertion sites in the genome of *R. sphaeroides* mutants that did not grow on acetate or lactate

Name	Agar Plate Screening[1]	Gene Mutated	GenBank Accession Number	Diagram
T09-187	Lac (-), Succ (+), Ace (+)	Pyruvate carboxylase (*pycA*)	ABA78245	686061 686069 684379 687843
T23-48	Lac (-), Succ (+), Ace (+)	Pyruvate carboxylase (*pycA*)	ABA78245	685308 685319 684379 687843
T34-134	Lac (-), Succ (+), Ace (+)	Pyruvate carboxylase (*pycA*)	ABA78245	686932 686944 684379 687843
MW28-52	Lac (-), Succ (c), Ace (c)	Pyruvate carboxylase (*pycA*)	ABA78245	685258 685267 684379 687843
T17-143	Lac (c), Succ (+), Ace (+)	Pryvuate carboxylase (*pycA*)	ABA78245	686692 686701 684379 687643
MW18-211	Ace (-), Succ (+), Lac (+)	β-Methylmalyl-CoA/L-malyl-CoA lyase (*mcl1*)	ABA77918	355526 355535 354497 356262
T02-101	Ace (-), Succ (+), Lac (+)	UDP-glucose-6 dehydogenase[2]	ABA79827	2392017 2392026 2391168 2392472
MW15-30	Ace (-), Succ (+), Lac (+)	Crotonyl-CoA carboxylase/reductase (*ccr*)	ABA80143	2717596 2717606 2717534 2718826
MW18-229	Ace (-), Succ (c), Lac (c)	Crotonyl-CoA carboxylase/reductase (*ccr*)	ABA80143	2717596 2717606 2717534 2718826
T10-48	Ace (c), Succ (+), Lac (+)	Methylmalonyl-CoA mutase (*mcm*)	ABA78347	802864 802873 802132 804261

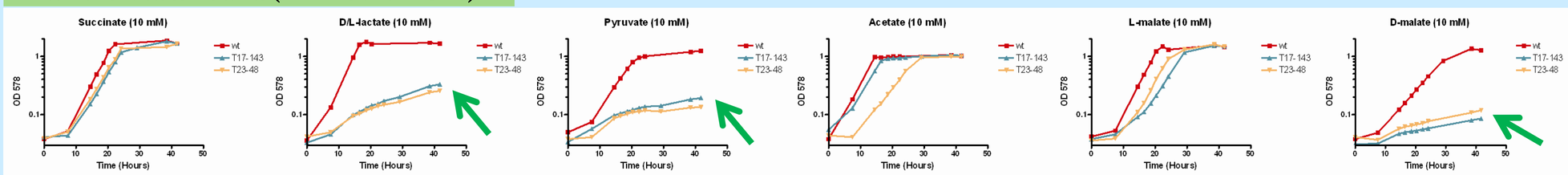
(*) (-) indicates no growth, (c) indicates compromised growth

1 Growth was screened on the minimal media plates containing a single carbon substrate. All the mutants in the table showed growth on succinate (10 mM) unless otherwise noted.

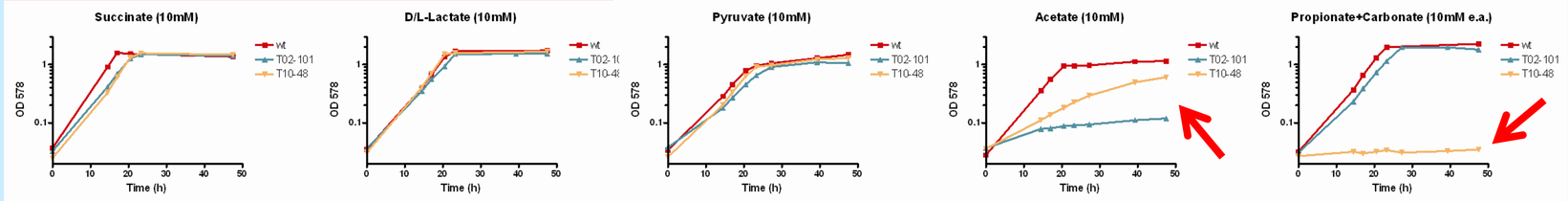
2 T02-101 mutant did not grow on acetate and has the mutation in the gene encoding (or at least annotated as) UDP-glucose-6 dehydrogenase. This enzyme is not a part of the ethylmalonyl-CoA pathway.

Photoheterotrophic growth of the wild-type (WT) and mutant *R. sphaeroides* in the liquid minimal media containing a single carbon source

WT and lactate mutants (T17-143 and T23-48)



WT and acetate mutants (T02-101 and T10-48)



Graphs show growth of several mutants in various carbon substrates**.** Arrows indicate the substrates on which the growth of the mutants was affected.

Conclusion

Lactate metabolism of *R. sphaeroides*:

- Five mutants were isolated for negative growth on lactate. Of these five mutants, all contained mutations in the pyruvate carboxylase gene. The experiments show compromised growth on lactate and therefore confirm the need for pyruvate carboxylase in lactate assimilation.
- Slow growth on lactate, pyruvate, and D-malate despite the mutation in pyruvate carboxylase, may suggest that an additional enzyme is replenishing the tricarboxylic acid cycle. It is possible that this enzyme is phosphoenolpyruvate (PEP) carboxylase which converts pyruvate to oxaloacetate. However, further experiments are needed to confirm the role of PEP carboxylase.

Acetate metabolism of *R. sphaeroides*:

- Experimentation likely confirms the involvement of methylmalonyl-CoA mutase (*mcm*) in the ethylmalonyl-CoA pathway as evidenced by impaired growth of *mcm* mutants (T10-48) on acetate and propionate+bicarbonate.
- Slowed growth of T10-48 on acetate in liquid culture experiments may suggest that methylmalonyl-CoA mutase plays a role in acetate assimilation into the TCA cycle intermediate succinyl-CoA. However, the ability grow on acetate without *mcm* may indicate that acetate can alternatively be assimilated into the TCA cycle along the ethylmalonyl-CoA pathway. Experimentation suggests that *mcm* mutants with functional β-methylmalyl-CoA/L-malyl-CoA lyase (*mcl1*) and crotonyl-CoA carboxylase/reductase (*ccr*) can alternatively assimilate acetate into the TCA cycle intermediate L-malate.
- Furthermore, no growth of the *mcm* mutant T10-48 on propionate+bicarbonate in liquid culture experiments may suggest that propionyl-CoA is involved in the ethylmalonyl-CoA pathway after the alternative pathway described above. This likely indicates that *R. sphaeroides* has only one pathway of carbon utilization from propionate and that it requires *mcm* to produce the TCA intermediate succinyl-CoA.
- No growth of the UDP-glucose-6-dehydrogenase mutant (T02-101) on acetate in liquid growth experiments suggests that the transposon mutation may have an effect at the transcriptional level on a neighboring gene, which may be involved in acetate assimilation. Moreover, the gene may not be UDP-glucose-6-dehydrogenase or the problem of acetate assimiliation may be due to an additional insertion mutation in T02-101. All in all, the function of UDP-glucose-6-dehydrogenase in acetate assimilation is unknown and provides a means for future experimentation.

Acknowledgments

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Literature

Erb, T.J., Berg, I.A., Brecht, V., Müller, M., Fuchs, G., and Alber B.E. (*2007*) Synthesis of C5-dicarboxylic acids from C2 units – the ethylmalonyl-CoA pathway. *Proc. Natl. Acad. Sci.* U S A. **104**, 10631-10636

Yakunin, A. F. and Hallenbeck, P. C. (1997) Regulation of synthesis of pyruvate carboxylase in the photosynthetic bacterium *Rhodobacter capsulatus*. *J. Bacteriol.* **179,** 1460-1468;