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Metabolic engineering of strains of *Ralstonia eutropha* and *Pseudomonas putida* for biotechnological production of 2-methylcitric acid

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Abstract

In this study strains of *Ralstonia eutropha* H16 and *Pseudomonas putida* KT2440 were engineered which are suitable for biotechnological production of 2-methylcitric acid (2MC). Analysis of a previous mutant of *R. eutropha* able to accumulate 2MC recommended this strain as a candidate for fermentative production of 2MC. This knowledge was used for construction of strains of *R. eutropha* H16 and *P. putida* KT2440 capable of enhanced production of 2MC. In both bacteria the chromosomal genes encoding the 2-methyl-*cis*-aconitate hydratase (*acnM*) were disrupted by directed insertion of a copy of an additional 2-methylcitrate synthase gene (*prpC*) yielding strains *R. eutropha* $\Delta acnM_{Re}$ΩKm$prpC_{Pp}$ and *P. putida* $\Delta acnM_{Pp}$ΩKm$prpC_{Re}$. In both strains 2-methylcitrate synthase was expressed under control of the constitutive kanamycin-resistance gene (ΩKm) resulting in up to 20-fold higher specific 2-methylcitrate synthase activities in comparison to the wild type. The disruption of the *acnM* gene by insertion of *prpC* led to a propionate- and levulinate-negative phenotype of the engineered strains, and analysis of supernatant of these strains revealed overproduction and accumulation of 2MC in the medium. A two stage cultivation regime comprising an exponential growth phase and a 2MC production phase was developed and applied to both engineered strains for optimum production of 2MC. Whereas gluconate, fructose or succinate were provided as carbon source for the exponential growth phase, a combination of propionate or levulinate as precursor substrate for provision of propionyl-CoA and succinate or fumarate as precursor substrate for provision of oxaloacetate were used in the production phase to make sure that the 2-methylcitrate synthase was provided with their substrates. Employing the optimised feeding regime *P. putida* $\Delta acnM_{Pp}$ΩKm$prpC_{Re}$ and *R. eutropha* $\Delta acnM_{Re}$ΩKm$prpC_{Pp}$ produced 2MC up to maximal concentrations of 7.2 g/L or 26.5 mM and 19.2 g/L or 70.5 mM, respectively, during 144 h of cultivation.

Keywords: Levulinate; Metabolic engineering; 2-Methyl-*cis*-aconitate hydratase; 2-Methylcitrate; 2-Methylcitric acid cycle; Propionate; PrpC cluster; *Pseudomonas putida*; *Ralstonia eutropha*

1. Introduction

The 2-methylcitrate cycle (2MCC) (Fig. 1) with the characteristic intermediate 2-methylcitric acid (2MC) is the predominant pathway for the catabolism of propionic acid in Gram-negative bacteria as well as in fungi and yeasts (Brämer and Steinbüchel, 2001; Brock et al., 2001; Tabuchi and Serizawa, 1975). 2MC and other alkyl derivatives of citric acid were first synthesised chemically by Habicht and Schneeberger (1956). The synthesis method relies on rather harmful solvents like benzene and is therefore not applicable for large-scale technical production of these compounds. Small amounts of 2MC were also synthesised enzymatically in vitro using purified 2-methylcitrate synthase (PrpC) of *S. enterica* serovar Typhimurium (Horswill and Escalante-Semerena, 2001). A fluoroacetate-tolerant mutant derived from *C. lipolytica* produced 35 g/L of *threo*-D_s-2-methylisocitric acid (2MIC) mainly from *n*-alkanes with an odd number of carbon atoms; in this study 2MIC was isolated as its lactone, whereas 2MC was obtained in concentrations of only 0.2 g/L (Tabuchi and Serizawa, 1975). Detailed investigations of interposon mutants of *Ralstonia eutropha* HF39 defective in the

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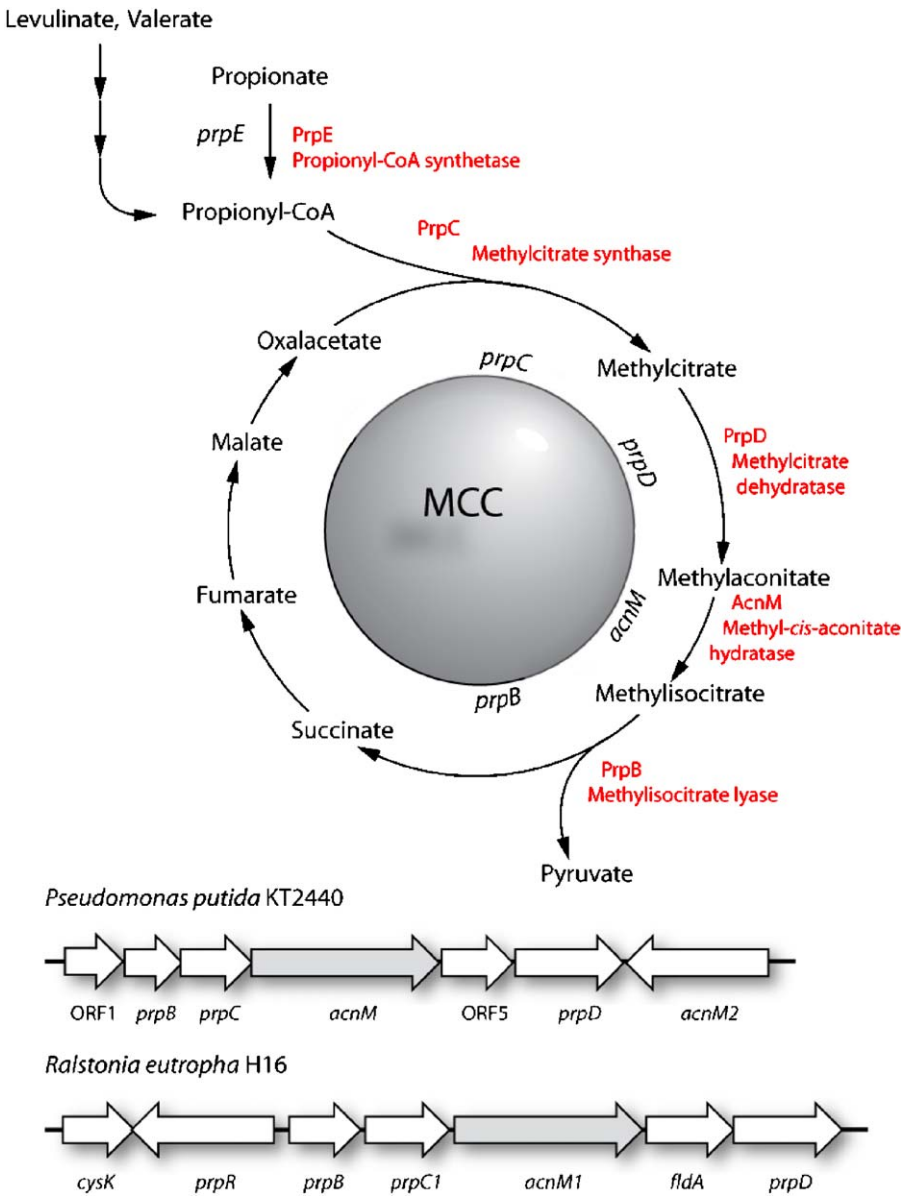

Fig. 1. The 2-methylcitric acid cycle (2MCC) and organisation of the genes of the *prp* clusters in *Ralstonia eutropha* H16 and *Pseudomonas putida* KT2440. Abbreviations: *P. putida* KT2440: ORF1, putative transcriptional regulator; *prpB*, putative 2-methylisocitrate lyase; *prpC*, 2-methylcitrate synthase; *acnM*, putative 2-methyl-*cis*-aconitate hydratase; ORF5, conserved hypothetical protein; *prpD*, putative 2-methylcitrate dehydratase; *acnM2*, putative aconitase. *R. eutropha* H16: *cysK,* cysteine synthase, *prpR,* cluster regulator; *prpB,* methylisocitrate lyase; *prpC1*, 2-methylcitrate synthase 1; *acnM1*, 2-methyl-*cis*-aconitate hydratase 1; *fldA,* FldA-like protein; *prpD,* methylcitrate dehydratase.

2MCC showed that low amounts of intermediates of 2MC and 2MIC were excreted into the medium (Brämer and Steinbüchel, 2001) if Tn5 integrated in the *acnM* or *fldA* genes. AcnM and FldA are required for the conversion of 2MC into 2MIC. Since no Tn*5* mapped in the *prpB* gene, a null allele mutant was constructed with an insertion of ΩKm, which showed low excretion of 2MIC into the medium. A *prpD* knock out mutant was not impaired in the ability to use propionate as sole carbon source and did not secrete any metabolite of the 2MCC. A mutation of *acnM* encoding a 2-methyl-*cis*-aconitate hydratase led to accumulation of 2MC in the supernatant thus recommending it for fermentative production of 2MC. After growth on a non-propionigenic substrate and transfer into fresh medium, cells of an *acnM* mutant required propionate and succinate as precursor substrates for formation of 2MC and for induction of PrpC expression encoded by *prpC* (Brämer and Steinbüchel, 2001). Therefore, this strain allowed fermentative production of only relatively low amounts of 2MC because the cells had to be harvested after the growth phase under sterile conditions, washed and transferred into fresh medium. This intensive procedure was not manageable beyond the Erlenmeyer flask scale in a pilot or even production plant.

To avoid a two-step fermentation for production of 2MC, it was therefore necessary to construct a strain