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ScienceDirect
Metabolic Engineering 8 (2006) 587–602
METABOLIC ENGINEERING
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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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Received 25 February 2006; received in revised form 11 May 2006; accepted 31 May 2006
Available online 14 June 2006

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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doi:10.1016/j.ymben.2006.05.007

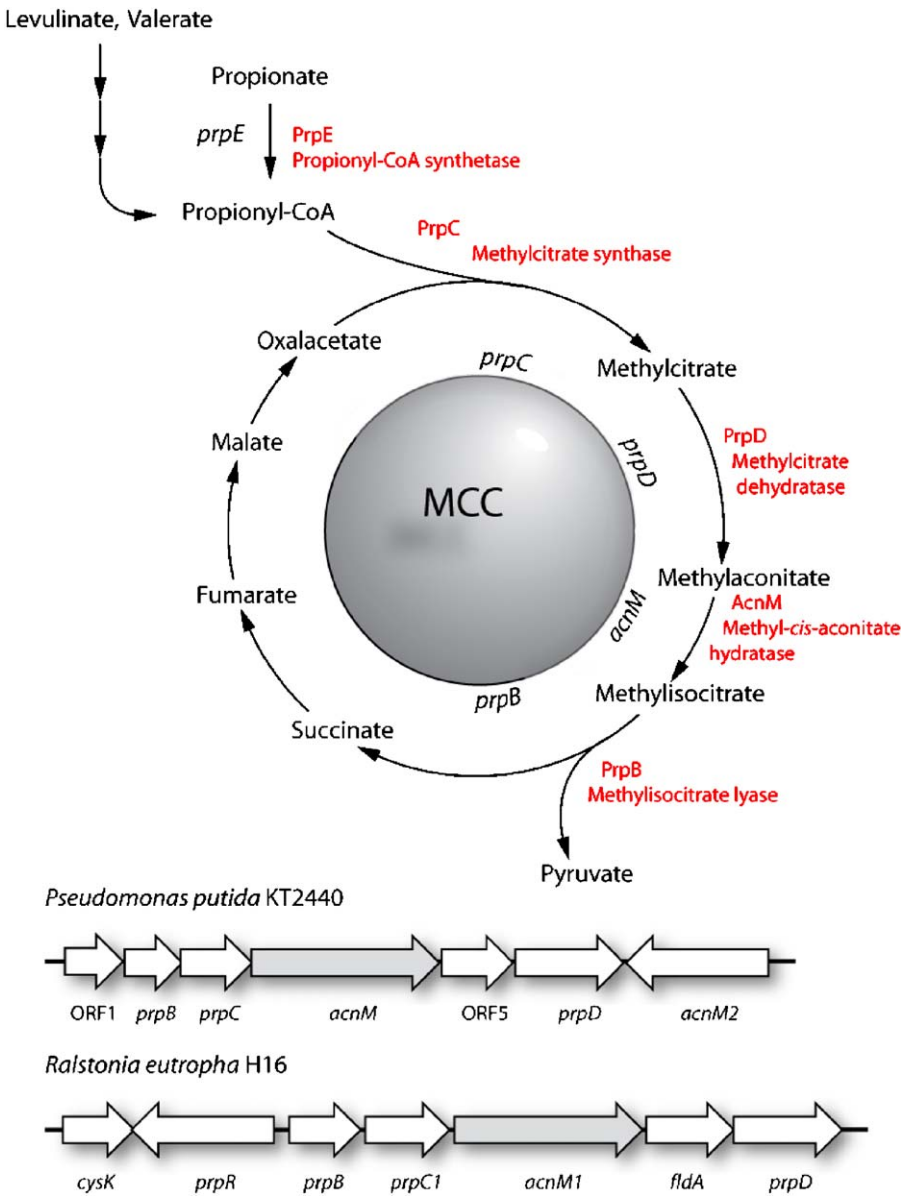

Fig. 1. The 2-methylcitric acid cycle (2MCC) and organisation of the genes of the *prp* clusters in *Ralstonia eutropha* H16 and *Pseudomonas putida* KT2440. Abbreviations: *P. putida* KT2440: ORF1, putative transcriptional regulator; *prpB*, putative 2-methylisocitrate lyase; *prpC*, 2-methylcitrate 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

expressing *prpC* constitutively. At the beginning, we engineered *Pseudomonas putida* strain KT2440 accordingly, as tools for genetic manipulations were available and the complete genome sequence was known. Later, we engineered *R. eutropha* as the genome sequence was meanwhile also revealed (unpublished data). Furthermore, *R. eutropha* forms less foam than *P. putida* during fermentation and does not excrete polysaccharides which cause problems during cell harvest by centrifugation and might also negatively effect purification of 2MC. The genes encoding enzymes of the 2MCC are organised in *prp* clusters found in several Gram-negative soil bacteria like *R. eutropha* as well in *P. putida* (Fig. 1). In the enterobacteria *Escherichia coli* and *S. enterica* these loci consist of the structural genes *prpR, prpB, prpC, prpD* and *prpE* constituting the *prpBCDE* operon (Blattner et al., 1997; Horswill and Escalante-Semerena, 1997, Palacios and Escalante-Semerena, 2004). In the *prp* loci of *R. eutropha* and *P. putida* KT2440 *prpE* is missing (Brämer et al., 2001). In *P. putida*, a second aconitate hydratase-like gene (*acnC2*) is localised downstream and antilinear to the *prp* gene cluster. All aconitate hydratases possess three highly conserved cysteine residues which represent the ligands of the 4Fe-4S-cluster like also in the *acnM* gene product of *R. eutropha* H16 (C477, C480 and C491). A difference in the vicinity of these cysteine residues occurs between the *acnM* translational products and those aconitate hydratases which constitute to the tricarboxylic acid cycle. Whereas all *acnM* translational products possess in most cases a polar asparagine residue next to all conserved cysteine residues, a non-polar amino acid like isoleucine (majority) or valine or leucine (minority) is present at this position in the other aconitate hydratases. Analysis of the AcnC2 primary sequence showed that isoleucine is present next to this highly conserved cysteine residue (C610) thus indicating that AcnC2 is a "common" aconitate hydratase.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Cells of *E. coli* were cultivated at 37 °C in Luria-Bertani (LB) medium (Sambrook et al., 1989). Cells of *P. putida* and *R. eutropha* were grown at 30 °C either in nutrient broth (NB) medium (0.8%, w/v) (Difco Laboratories, Detroit, USA) or in mineral salts medium (MSM) according to Schlegel et al. (1961) supplemented with filter-sterilized carbon sources. Fructose or sodium salts of gluconate, levulinate, succinate, fumarate or propionate were used at concentrations of 0.1–2.0% (w/v) from stock solutions (20%, w/v). Solid media contained 1.5% (w/v) purified agar. For selection of plasmid carrying strains, antibiotics were added to the media at the following concentrations (μg/ml): ampicillin (75, *E. coli*), kanamycin (50, *E. coli*, 100 *P. putida*, 300 *R. eutropha*) and tetracycline (12.5, *E. coli*, 25, *P. putida,* 25, *R. eutropha*).

2.2. Determinations of ammonium and gluconate concentrations

Concentrations of ammonium in cell-free supernatants were determined using ammonium test bars (Merck, Darmstadt, Germany) or a gas sensitive ammonium electrode (Type 152303000; Mettler Toledo GmbH, Greifensee, Switzerland). Concentrations of gluconate were determined spectrophotometrically in cell-free supernatants using an enzyme test kit for D-gluconic acid (Boehringer Mannheim, R-Biopharm, Darmstadt, Germany).

2.3. Preparation of crude extracts

Cells from 20 to 500 ml cultures were harvested by centrifugation (20 min, 2800*g*, 4 °C), washed twice in 50 mM Tris–HCl buffer (pH 7.4) and resuspended in 1 ml of this buffer per gram of fresh cell mass containing 10 μg DNase I. Cells were disrupted by sonification in a Sonopuls GM 200 (Bandelin, Berlin, Germany) with an amplitude of 16 μm (1 min/ml) while cooling in a NaCl/ice bath. Soluble protein fractions of crude extracts were obtained by 1 h centrifugation at 100,000*g* and 4 °C; supernatants were desalted on NAP5 columns (Pharmacia Biotech, Freiburg, Germany) and used for enzyme activity measurements.

2.4. Determination of methylcitrate synthase activity

Activity of methylcitrate synthase (2MCS, EC 4.1.3.31) was measured according to Srere (1966). The cuvette ($d = 1$ cm) contained in a total volume of 500 μl 2 mM oxaloacetate, 2 mM (DTNB) 5,5′-dithiobis-(2-nitrobenzoate) in 50 mM Tris/HCl (pH 8.0) and varying volumes of crude extract at 30 °C. After addition of 250 μM propionyl-CoA, the increase of absorbance at 412 nm ($\varepsilon = 14.0\,\mathrm{cm}^2/\mu\mathrm{mol}$) was measured with an Ultrospec 2000 photometer (Pharmacia, Uppsala, Sweden). One unit of enzyme activity corresponded to conversion of 1 μmol of substrate per min. Soluble protein was determined by the method of Bradford (1976), using crystalline bovine serum albumin as standard. Specific activity is given in units per milligram of protein.

2.5. Synthesis of propionyl-CoA

Propionyl-CoA was synthesised according to Simon et al. (1983). The sodium salt of coenzyme A (10 mg) was dissolved in 0.5 M K_2CO_3 (pH 8.0) and aliquots of 2 μl of the anhydride of propionic acid were added to this solution while stirring on ice until no free coenzyme A was detectable with DTNB (Ellman, 1958). The pH of the solution was then adjusted to 4.5 by addition of 1 N HCl. The concentration of propionyl-CoA was calculated in consideration of complete substrate conversion. Propionyl-CoA was stored at −20 °C (pH 4.5).

Table 1
Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Relevant characteristics	Source or reference
Bacterial strains		
Ralstonia eutropha		
H16	Wild type, autotrophic, prototrophic, PHA^+	DSMZ 428, ATCC 17699, Wilde (1962)
H16-PHB$^-$4	PHA-negative mutant of H16	DSMZ 541
H16 Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$	Mutant of H16, 2MC-positiv, propionate-negative	This study
H16-PHB$^-$4 Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$	Mutant of H16-PHB$^-$4, 2MC-positiv, propionate-negative	This study
Pseudomonas putida		
KT2440	Mt-2, hsdR I (r^- m^+), without TOL plasmid	DSMZ 6125, Worsey and Williams (1975)
KT2440 Δ*acnM*$_{Pp}$ΩKm*prpC*$_{Re}$	Mutant of KT2440, 2MC-positiv, propionate-negative	This study
Escherichia coli		
XL1-Blue	*recA1 endA1* gyrA96 thi hsdR17 (r_K^- m_K^-) supE44 *relA*1 λ$^-$lac[F′proAB *lacI*^{q}ZΔM15Tn*10* (tet)	Bullock et al. (1987)
TOP10	F^-, *araD*139 Δ(*ara, leu*)7697, Δ*lacX*74, *galU*, *galK*, *rpsL*, *deoR*, Φ80d*lacZ*ΔM15,	Invitrogen (Carlsbad, USA)
S17-1	*recA*; harbors the *tra*-genes of plasmid RP4 in the chromosome; *proA thi-1 hsdR*	Simon et al. (1983)
Plasmids		
pBluescript SK****	Amp^r, *lacPOZ*, T7 and T3 promoter	Stratagene (San Diego, USA)
pSKsymΩKm	1-kbp ΩKm in pSKsym, Km^r, Amp^r	Overhage et al. (1999)
PEX100T	Suicide vector, Amp^r, *sacB, oriT*, pMB1-origin *lacPOZ*	Schweizer and Hoang (1995)
PJQ200mp18Tc	Suicide vector, Tc^r, *sacB*, *oriV*, *oriT*, *traJ*	Pötter et al. (2005)
PSK$^-$::*prpC*$_{Re}$	pBluescript SK$^-$ harboring 1.3-kbp *prpC*$_{Re}$ gene colinear to *lacPOZ*	Brämer and Steinbüchel, (2001)
PSK$^-$::*prpC*$_{Pp}$	pBluescript SK$^-$ harboring 1.2-kbp *prpC*$_{Pp}$ gene colinear to *lacPOZ*	This study
pSK$^-$::ΩKm *prpC*$_{Re}$	pBluescript SK$^-$ harboring 1-kbp ΩKm and 1.3-kbp *prpC*$_{Re}$ from *Ralstonia eutropha* H16	This study
pSK$^-$::ΩKm *prpC*$_{Pp}$	pBluescript pSK$^-$ harboring 1-kbp ΩKm and 1.2-kbp *prpC*$_{Pp}$ from *Pseudomonas putida* KT2440	This study
pEX100T::*acnM*$_{Pp}$	pEX100T harboring 2.3-kbp fragment of *acnM* gene *Pseudomonas putida* KT2440	This study
pSK$^-$::*acnM*$_{Re}$	pBluescript SK$^-$ harboring 1.2-kbp fragment of *acnM* gene of *Ralstonia eutropha* H16	This study
pEX100T::Δ*acnM*$_{Pp}$ΩKm*prpC*$_{Re}$	pEX100T harboring 2.3-kbp *acnM*$_{Pp}$ disrupted by 2.3-kbp ΩKm*prpC*$_{Re}$	This study
pJQ200mp18Tc::Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$	pJQ200mp18Tc harboring 1.2-kbp *acnM*$_{Re}$ disrupted by 2.2-kbp ΩKm*prpC*$_{Pp}$	This study

2.6. Isolation of 2-methylcitric acid (2MC) from culture medium

Referring to several methods in literature for separation of citric acid (Roehr et al., 1983), the standard method of citric acid recovery was used and adopted for isolation of 2MC. This involved precipitating 2MC to water insoluble tricalcium 2MC salt by addition of equivalent amounts of $CaCl_2$ to 2MC-containing solutions (Fig. 2). Accordingly, cells were separated from the medium after 144 h of cultivation by 20 min centrifugation at 4.000 rpm. Cell-free medium was concentrated 10-fold by lyophilisation and suspension of the obtained powder in 0.1 vol of water yielded 10–15% (w/v) solutions of 2MC. The pH of the solution was then adjusted to 8 by adding concentrated NH_3 solution followed by 15 min incubation at 75 °C to coagulate proteins. After adding $CaCl_2$ at appropriate amounts, calcium phosphate precipitated and was separated by 20 min centrifugation at 4.000 rpm. Like the monocalcium salt of citrate, $[Ca(Cit)_2]^{4-}$, also $[Ca(2MC)_2]^{4-}$ remained dissolved in the solution at room temperature. Additional $CaCl_2$ solution (1 M) was added to a molar ratio of 2:3 and temperature was increased to 95 °C, yielding insoluble $[Ca_3(2MC)_2]$ complex (Fig. 2). Impurities like fluorescent pyoverdines or saccharides were removed from this salt by washing it with hot water. An aqueous suspension of $Ca_3(2MC)_2$ was then passed over an Amberlite-IR 120 cation ion exchange resin (Merck, Darmstadt, Germany) column to convert the calcium salt into the free acid. The column was washed with 2 bed volumes of water to remove calcium ions quantitatively and to obtain 2MC in high yield. The use of an ion exchange resin instead of employing sulphuric acid treatment avoided gypsum formation. Evaporation of the effluent yielded an oily solution of 2MC which was incubated for 3 days at 30 °C until crystallisation occurred.

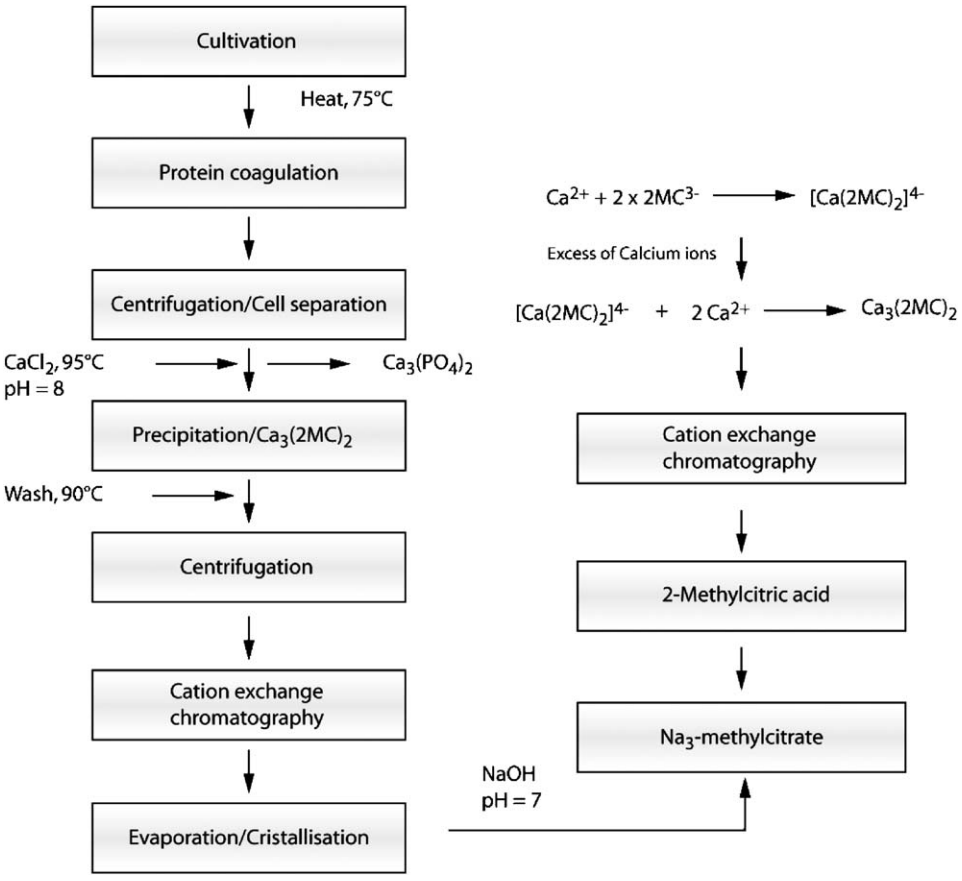

Fig. 2. Flowsheet for purification of 2MC.

2.7. Cultivation of strains of *R. eutropha* and *P. putida* for 2MC formation

Cultivations of *P. putida* KT2440 Δ*acnM*ΩKm*prpC*$_{Re}$ and *R. eutropha* H16-/PHB^{-}4 Δ*acnM*ΩKm*prpC*$_{Pp}$ were done in 300-ml Erlenmeyer flasks containing 50 ml MSM at 30 °C and 150 rpm agitation. Growth was monitored at 600 nm in a spectrophotometer or recorded in a Klett-colorimeter equipped with a 520–580 nm filter. The MSM contained 1.0% (w/v) sodium gluconate, 0.10% NH_4Cl and 50 (*Pseudomonas*) or 300 μg kanamycin/ml (*Ralstonia*) from the beginning. At the end of the exponential growth phase 0.5–2.0% (w/v) sodium propionate or sodium levulinate were fed as propionyl-CoA delivering substrates; in addition, 0.5–2.0% (w/v) disodium fumarate or succinate were added as second substrate.

The maximum concentrations of sodium fumarate and sodium propionate were 1.0%, whereas those of succinate and levulinate were 2.0% (w/v). The cultures were incubated for further 144 h at 30 °C. Samples were withdrawn during the time course of the experiment, and the concentrations of substrates and of 2MC were analysed by HPLC or GC/MS. At the end of the cultivation experiments, cells were removed from samples by 20 min centrifugation at 4000 rpm. The 2MC-containing supernatants were either frozen at −70 °C and lyophilised or directly analysed by HPLC. The lyophilised supernatant was analysed by GC/MS.

2.8. HPLC analysis

HPLC-analysis was carried out in a LaChrom Elite® HPLC apparatus (VWR-Hitachi International GmbH, Darmstadt, Germany) consisting of a Metacarb 67 H advanced C column (Varian, Palo Alto, USA; Bio Rad Aminex equivalent) and a 22350 VWR-Hitachi column oven. The column (300 mm × 6.5 mm) consisted of a sulphonated polystyrene resin in hydrogen form. The primary separation mechanisms include ligand-exchange, ion-exclusion, and adsorption. A VWR-Hitachi refractive index (RI) detector (Type 2490) with an active flow cell temperature control and automated reference flushing eliminating temperature effects on the RI baseline was used for detection. Aliquots of 20 μl cell-free supernatants were injected and eluted with 0.005 N sulphuric acid (H_2SO_4) in double distilled water at a flow rate of 0.8 ml/min. Online integration and analysis was done with EZ Chrome Elite Software (VWR International GmbH, Darmstadt, Germany). Synthetic DL-2-methylcitrate trisodium salt (Dr. Ehrenstorfer GmbH, Augsburg, Germany) was used as standard.

2.9. GC/MS analysis

Cell free supernatants were lyophilised and subjected to methanolysis in presence of sulphuric acid (85% v/v MeOH: 15% v/v H_2SO_4) for 5 h at 100 °C or were esterified by treatment with trimethylsilyl-diazomethane (TMSD) (Preu et al., 1998). The resulting methylesters of the organic acids were characterized by coupled gas chromatography/mass spectrometry (GC/MS) using an HP 6890 gas chromatograph equipped with a model 5973 mass selective detector (Hewlett Packard, Waldbronn, Germany). DL-2MC (see above) was subjected to methanolysis according to the method described above and used as standard.

2.10. DNA isolation, modification and amplification

Chromosomal DNA of strains of *P. putida* and *R. eutropha* was isolated by the method described by Marmur (1961). Plasmid DNA was isolated from *E. coli, P. putida* and *R. eutropha* by the method of Birnboim and Doly (1979). High-purity plasmid DNA for sequencing was purified with the FastPlasmid™ Mini Kit (Eppendorf AG, Hamburg, Germany) according to the manufacturer's manual. DNA was digested with restriction endonucleases under conditions described by the manufacturer or according to Sambrook et al. (1989). All PCR amplifications of DNA were carried out as described by Sambrook et al. (1989) using Platinum® *Pfx*-DNA polymerase (Gibco BRL Life Technologies, Karlsruhe, Germany) in an Omnigene HBTR3CM DNA Thermal Cycler (Hybaid, Heidelberg, Germany).Resulting restriction fragments and PCR products were purified with the Perfectprep® Gel Cleanup Kit (Eppendorf AG, Hamburg, Germany) as described by the manufacturer. Restriction enzymes, ligases and other DNA manipulating enzymes like *Pfx*-DNA polymerase were used according to the instructions of the manufacturers of these enzymes. Specific primers were purchased from MWG-Biotech AG (Ebersberg, Germany).

2.11. Transfer of DNA

Competent cells of *E. coli* were prepared and transformed by the $CaCl_2$ procedure (Sambrook et al., 1989). DNA was ligated into vectors pEX100T or pJQ200mp18Tc, and the hybrid plasmids were then transferred to *P. putida* KT2440 and *R. eutropha* by conjugation, respectively, from *E. coli* S17-1 according to the method of Friedrich et al. (1981).

2.12. DNA sequencing and sequence data analysis

Sequencing was conducted by using the Thermo Sequenase fluorescence labelled primer cycle sequencing kit (Epicentre Technologies, WI, USA) and IRD 800-labelled oligonucleotides (MWG-Biotech, Ebersberg, Gemany). DNA molecules were separated in an LI-COR 4000L automatic sequencing apparatus (LI-COR Inc., Biotechnology Division, Lincoln, NE, USE). Sequence data were compared with sequences deposited in data banks using BlastSearch 2.0.10. (Altschul et al., 1997) and DBGET (Bairoch et al., 1997) software.

2.13. Genomic integration of the 2-methylcitric acid synthase gene (*prpC*) and disruption of the 2-methyl-cis-aconitate hydratase gene (*acnM*) in *P. putida*

2.13.1. Cloning of $acnM_{Pp}$ into the suicide vector pEX100T

The central region of $acnM_{Pp}$ was amplified from genomic DNA of *P. putida* KT2440 using the oligonucleotides $acnM_{Pp}$UP (5′-AACCCGGGCCTTCGAAAAGA-ACCGCGCCATCG-3′) and $acnM_{Pp}$RP (5′-AAACCCG-GGCATCCTGATAGTCCGGTGAAATACC-3′) which contain *Sma*I recognition sequences (underlined). These oligonucleotides were derived from the *P. putida* genome sequence available in a data base (www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organisms=p_putida_KT2440). The 2,271-bp PCR product was purified, digested with *Sma*I and ligated into *Sma*I-linearised vector pEX100T. The ligation mixture was transferred to *E. coli* XL1-Blue, and the resulting hybrid plasmid was designated as pEX100T::$acnM_{Pp}$.

2.13.2. Construction of hybrid plasmid pSK^-::Ω*Km*$prpC_{Re}$

For construction of pSK^-::Ω*Km*$prpC_{Re}$ the hybrid plasmids pSK^-::$prpC_{Re}$ (Brämer and Steinbüchel, 2001) and pSKsymΩKm (Overhage et al., 1999) were isolated from the corresponding *E. coli* strains. The $prpC_{Re}$ gene was cloned into the *Eco*RI/*Sal*I recognition sequences of pSK^-::$prpC_{Re}$ colinear to the *lacZ* promoter. Therefore, ΩKm, encoding a kanamycin-resistance gene under control of a constitutive promoter, was released from pSKsymΩKm by *Eco*RI restriction, purified and then ligated to *Eco*RI-linearised pSK^-::$prpC_{Re}$ DNA. The ligation mixture was transferred to *E. coli* XL1-Blue, and the resulting transformants were screened for a kanamycin-resistant phenotype. The hybrid plasmids of Km-resistant *E. coli* clones were isolated, and the colinear organisation of ΩKm and $prpC_{Re}$ was confirmed by DNA sequencing. This hybrid plasmid was designated as pSK^-::Ω*Km*$prpC_{Re}$.

2.13.3. Construction of plasmid pEX100T::Δ$acnM_{Pp}$ Ω*Km*$prpC_{Re}$

For construction of pEX100T::Δ$acnM_{Pp}$Ω*Km*$prpC_{Re}$ a 2,281-bp fragment was amplified by PCR from the hybrid plasmid pSK^-::Ω*Km*$prpC_{Re}$ using the oligonucleotides ΩKmUP (*Sma*I) (5′-AAACCCGGGACAGCAAGC-GAACCGGAATGACCAGCTGGGG-3′) and $prpC_{Re}$RP (*Sal*I) (5′-TTTGTCGACCAACTACCCCTTGTCC-3′). This PCR fragment containing ΩKm and $prpC_{Re}$ was isolated and ligated to *Stu*I hydrolysed pEX100T::$acnM_{Pp}$ DNA. Digestion of pEX100T::$acnM_{Pp}$ with *Stu*I and subsequent re-ligation led to the deletion of a 296-bp fragment of $acnM_{Pp}$. The resulting plasmid was transferred

to *E. coli* XL1-Blue, and kanamycin-resistant transformants were selected. The resulting hybrid plasmid was designated as pEX100T::Δ*acnM*$_{Pp}$ΩKm*prpC*$_{Re}$ and was used as template for PCR employing the oligonucleotides *acnM*$_{Pp}$UP and *acnM*$_{Pp}$RP. The resulting 4,261-bp PCR product was sequenced to confirm the disruption of *acnM*$_{Pp}$ by ΩKm*prpC*$_{Re}$. The hybrid plasmid pEX100T::Δ*acnM*$_{Pp}$ΩKm*prpC*$_{Re}$ was then transferred to *E. coli* S17-1.

2.13.4. Construction of an isogenic acnM-mutant of P. putida KT2440

The *E. coli* S17-1 strain harbouring plasmid pEX100T::Δ*acnM*$_{Pp}$ΩKm*prpC*$_{Re}$ and *P. putida* KT2440 were grown in 20 ml LB (containing 50 μg/ml kanamycin plus 75 μg/ml ampicillin) at 37 °C and NB medium at 30 °C for 16 h, respectively. The hybrid plasmid was then transferred from *E. coli* S17-1 to *P. putida* KT2440 by conjugation, and transconjugants were screened on NB agar plates containing 50 μg/ml kanamycin and 5.0% (w/v) sucrose. On these agar plates only homogenotic strains of *P. putida* KT2440 were able to grow because pEX100T encodes a levan saccharase (SacB) catalysing the hydrolysis of sucrose and the formation of toxic fructose polymers (Gay et al., 1985). Therefore, heterogenotic strains harbouring vector-encoded *sacB* in the genome were sensitive to sucrose in the medium. Genomic DNA of isogenic *acnM*$_{Pp}$ mutant was isolated and subjected to PCR using the oligonucleotides *acnM*$_{Pp}$UP and *acnM*$_{Pp}$RP. The resulting PCR products were isolated, and the disruption of the *acnM* gene was confirmed by DNA sequencing. The isogenic *acnM*-mutant of *P. putida* KT2440 was designated as *P. putida* KT2440 Δ*acnM*$_{Pp}$ΩKm*prpC*$_{Re}$.

2.14. Genomic integration of prpC and disruption of the 2-methyl-cis-aconitate hydratase gene in R. eutropha

2.14.1. Cloning of acnM$_{Re}$ into pBluescript SK$^-$

A 1,164-bp internal DNA fragment of *acnM*$_{Re}$ was amplified by PCR from genomic DNA of strain H16 using oligonucleotides *acnM*$_{Re}$UP (*Pst*I) (5′-aaaCTGCAG-CAAG GAAAAGGTGGTCGGCGCCTATCTG-3′) and *acnM*$_{Re}$ RP (*Xba*I) (5′-aaaTCTAgaca ggtggtcggtggtga tgttgtcg-3′) containing *Pst*I or *Xba* I recognition sequences, respectively (underlined). These oligonucleotides were derived from the *R. eutropha* genome *prpRBCD*-cluster available in a data base (www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=16445328). The resulting PCR product was purified, digested with *Pst*I-*Xba*I and ligated to linearized pBlueskript SK$^-$ DNA. The ligation mixture was transferred to *E. coli* TOP10, and the resulting hybrid plasmid was designated as pSK$^-$::*acnM*$_{Re}$.

2.14.2. Construction of hybrid plasmid pSK$^-$::prpC$_{Pp}$

Primers *prpC*$_{Pp}$UP(*Hin*dIII) (5′-AAAAAAGCTTCAA-GAAAGGAGAAACACCATGGCCGAAG-3′) and *prpC*$_{Pp}$RP (*Cla*I) (5′-AAAATCGatagctattcagcgctgctcgatc-GGC ACGAAC-3′) were used to amplify the putative methylcitrate synthase gene of *P. putida* from genomic DNA (www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organisms=p_putida_KT2440). They contained restriction sites for *Hin*dIII and *Cla*I to enable enforced cloning of the resulting 1,164-bp PCR product in pBluescript SK$^-$, resulting in hybrid plasmid pBluescript SK$^-$::*prpC*$_{Pp}$.

2.14.3. Construction of hybrid plasmid pSK$^-$::ΩKmprpC$_{Pp}$

For construction of pSK$^-$::ΩKm*prpC*$_{Pp}$ the hybrid plasmids pSK$^-$::*prpC*$_{Pp}$ and pSKsymΩKm (Overhage et al., 1999) were isolated from the corresponding *E. coli* strains. In pSK$^-$::*prpC*$_{Pp}$ the *prpC*$_{Pp}$ gene was cloned as described above colinear to the *lacZ* promoter. Therefore, ΩKm, encoding a kanamycin-resistance gene under control of a constitutive promoter, was released from pSKsymΩKm by *Hin*dIII restriction, purified and ligated to *Hin*dIII-linearized pSK$^-$::*prpC*$_{Pp}$ DNA. The ligation mixture was transferred to *E. coli* TOP10, and kanamycin-resistant transformants were selected. A hybrid plasmid of a kanamycin-resistant *E. coli* clone was isolated, and the colinear organisation of ΩKm and *prpC*$_{Pp}$ was confirmed by DNA sequencing. This hybrid plasmid was designated as pSK$^-$::ΩKm*prpC*$_{Pp}$.

2.14.4. Construction of plasmid pSK$^-$::ΔacnM$_{Re}$ΩKmprpC$_{Pp}$ and suicide plasmid pJQ200mp18Tc::ΔacnM$_{Re}$ΩKmprpC$_{Pp}$

For construction of pSK$^-$::Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$ a 2,162-bp fragment was amplified by PCR from plasmid pSK$^-$::ΩKm*prpC*$_{Pp}$ using ΩKmUP (*Sma*I) (5′-AAA-CCCGGG ACAGCAAGCGAACCGGAATGACCAGC-TGGGG-3′) and *prpC*$_{Pp}$RP (*Cla*I) (5′-AAA ATCGatagc-tattcagcgctgctcgatcGGCACGAAC-3′) as primers. This PCR fragment containing ΩKm and *prpC*$_{Pp}$ was isolated and ligated to *Stu*I hydrolysed pSK$^-$::*acnM*$_{Re}$. Restriction of pSK$^-$::*acnM*$_{Pp}$ with *Stu*I and subsequent re-ligation led to the deletion of a 380-bp fragment of *acnM*$_{Re}$. Kanamycin-resistant clones of *E. coli* TOP10 were selected which harboured the hybrid plasmid pSK$^-$::Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$. The latter was used as template for PCR employing *Pfx*-DNA polymerase and the oligonucleotides *acnM*$_{Re}$UP (*Pst*I) (5′-aaaCTGCAG-CAAGGAAAAGGTGGTCGGCGCCTATCTG-3′) and *acnM*$_{Re}$RP (*Xba*I) (5′-aaaTCTAgacaggtggtcggtggtgatgt-tgtcg-3′) resulting in a PCR-product with blunt ends. The 2,947-bp PCR product was sequenced to confirm disruption of *acnM*$_{Re}$ by ΩKm*prpC*$_{Pp}$. It was then ligated to *Sma*I restricted suicide-vector pJQ200mp18Tc. The ligation products were transferred to *E. coli* TOP10, and kanamycin plus tetracycline resistant clones were selected. The resulting hybrid plasmid pJQ200mp18Tc::Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$ was then transferred to *E. coli* S17-1.

2.14.5. Construction of the isogenic $acnM_{Re}$-mutants of R. eutropha H16-/PHB^{-}4

E. coli S17-1 (pJQ200mp18Tc::Δ$acnM_{Re}$ΩKm$prpC_{Pp}$) and *R. eutropha* strains H16 or PHB^{-}4 were grown in 20 ml LB containing 50 μg/ml kanamycin plus 12.5 μg/ml tetracycline at 37 °C or NB medium at 30 °C for 16 h, respectively. Hybrid plasmid pJQ200mp18Tc::Δ$acnM_{Re}$ ΩKm$prpC_{Pp}$ was transferred from *E. coli* S17-1 to *R. eutropha* strains by conjugation, and the resulting transconjugants were screened on NB agar plates containing 150 μg/ml kanamycin and 10.0% (w/v) sucrose. On these agar plates only homogenotic clones of *R. eutropha* H16 or PHB^{-}4 were able to grow due to the absence of *sacB* as described above for *P. putida*. Genomic DNA of isogenic $acnM_{Re}$-mutants was isolated and subjected to PCR using the oligonucleotides $acnM_{Re}$UP and $acnM_{Re}$ RP. In addition, 5′-AcnM_{Re}-genomic 5′-AAGAGTCT-CGCTGACATGGGACCACTACC-3′-primer and 3′-AcnM_{Re}-genomic 5′-AAATCTAGACGGGCCCTTTGT-CACAGCTAATGC-3′-primer were used for amplification of a 4527-bp fragment and verification of the $prpC_{Pp}$ integration. The resulting PCR product contained single restriction sites for *Hin*dIII and *Cla*I thus confirming chromosomal integration of the constructed ΩKm$prpC_{Pp}$. The resulting PCR products were isolated, and deletion of the 380-bp region of *acnM* was confirmed by DNA sequencing. In contrast, genomic DNA of a heterogenote yielded a 1165-bp PCR product resulting from an undisrupted copy of *acnM*. The isogenic *acnM*-mutants were designated as *R. eutropha* H16 Δ$acnM_{Re}$ΩKm$prpC_{Pp}$ or *R. eutropha* H16-PHB^{-}4 Δ$acnM_{Re}$ΩKm$prpC_{Pp}$.

3. Results

3.1. Construction of acnM-negative mutants of P. putida KT2440 and R. eutropha H16

Engineered strains of *R. eutropha* and *P. putida* applicable for fermentative production of 2MC should be designed as follows: (i) The 2-methyl-*cis*-aconitate hydratase (EC 4.2.1.3) gene (*acnM*) should have been disrupted by gene replacement, and deletion of the central region of the gene should lead to accumulation of 2MC by the cells. (ii) 2-Methylcitrate synthase encoded by *prpC* should be constitutively expressed to avoid an induction step during the fermentation process. Therefore, the *prpC* gene of *R. eutropha* ($prpC_{Re}$) (Brämer and Steinbüchel, 2001) and the putative *prpC* gene of *P. putida* ($prpC_{Pp}$) were cloned under control of the constitutively expressed promoter of the kanamycin-resistance gene (ΩKm). In material and methods the construction of the suicide plasmids pEX100T::Δ$acnM_{Pp}$ΩKm$prpC_{Re}$ and pJQ200mp18Tc::Δ$acnM_{Re}$ ΩKm$prpC_{Pp}$ was described in detail and is shown in Fig. 3 for *R. eutropha* as an example. Each of the hybrid plasmid construction was verified by DNA-sequencing. PrpC enzyme activity was measured to ensure functionality of PrpC. The constructed suicide plasmids were subsequently transferred to *R. eutropha* and *P. putida* KT2440 by conjugation. After homologous recombination ΩKm*prpC* homogenotic integration mutants were isolated on MSM agar plates containing kanamycin and sucrose. To verify the deletions in *acnM* and the chromosomal integrations of constructed ΩKm*prpC*, a 4527-bp fragment was amplified employing oligonucleotides 5′-AcnM_{Re}-genomic- and 3′-AcnM_{Re}-genomic-primer and genomic DNA of *R. eutropha* H16 Δ$acnM_{Re}$ΩKm$prpC_{Pp}$ as template. The resulting PCR product contained single restriction sites for *Hin*dIII and *Cla*I, and restrictions with these enzymes yielded DNA-fragments of 2279 plus 2248-bp or 3436 plus 1091-bp, respectively. A 4261-bp PCR product was amplified if genomic DNA of the isogenic *acnM* mutant of *P. putida* KT2440 was subjected to PCR using oligonucleotides $acnM_{Pp}$UP and $acnM_{Pp}$RP. Since the fragment contained two restriction sites for *Eco*RI, restriction with *Eco*RI yielded DNA-fragments of 393 plus 1474 and 2394-bp. The respective PCR products were isolated, and disruption of the *acnM* gene was confirmed by DNA sequencing employing ΩKmUp and ΩKmDown IRD800 labelled sequencing primer. In case of a heterogenotic strain of *P. putida* KT2440 an additional 2,271-bp PCR product was amplified, resulting from an undisrupted copy of *acnM*. Formation of 2MC under different cultivation conditions was determined by HPLC analysis of cell-free supernatants.

3.2. Analysis of PrpC activity in E. coli harbouring pSK^{-}::ΩKm$prpC_{Re}$ or pSK^{-}::ΩKm$prpC_{Pp}$

To confirm expression of $prpC_{Re}$ and $prpC_{Pp}$ under control of the ΩKm promoter in hybrid plasmids pSK^{-}::ΩKm$prpC_{Re}$ and pSK^{-}::ΩKm$prpC_{Pp}$, *E. coli* strains TOP10 and XL1-Blue harbouring pSK^{-}::ΩKm$prpC_{Re}$, pSK^{-}::ΩKm$prpC_{Pp}$ or pSKsymΩKm, respectively, were grown for 16 h at 30 °C in LB medium containing ampicillin and kanamycin without IPTG induction. Cells were harvested, crude extracts were prepared, and activity of PrpC was determined. Crude extract of *E. coli* XL1-Blue (pSKsymΩKm) revealed a specific PrpC activity of only 0.001 U/mg protein, whereas PrpC specific activities in crude extracts of *E. coli* strains XL1-Blue and TOP10 harbouring pSK^{-}::ΩKm$prpC_{Re}$ or pSK^{-}::ΩKm$prpC_{Pp}$ were 4.1 or 3.5 U per mg protein, respectively. This indicated that $prpC_{Re}$ and $prpC_{Pp}$ were highly expressed under control of the Km promoter independently from the *lacZ* promoter.

3.3. Phenotypic characterization of the isogenic acnM-mutants

The isogenic *acnM*-mutants of *P. putida* KT2440, *R. eutropha* H16 and *R. eutropha* H16-PHB^{-}4, which were constructed by directed mutagenesis, were able to grow on medium containing 50 or 300 μg/ml kanamycin, respectively, resulting from the integration of ΩKm into the

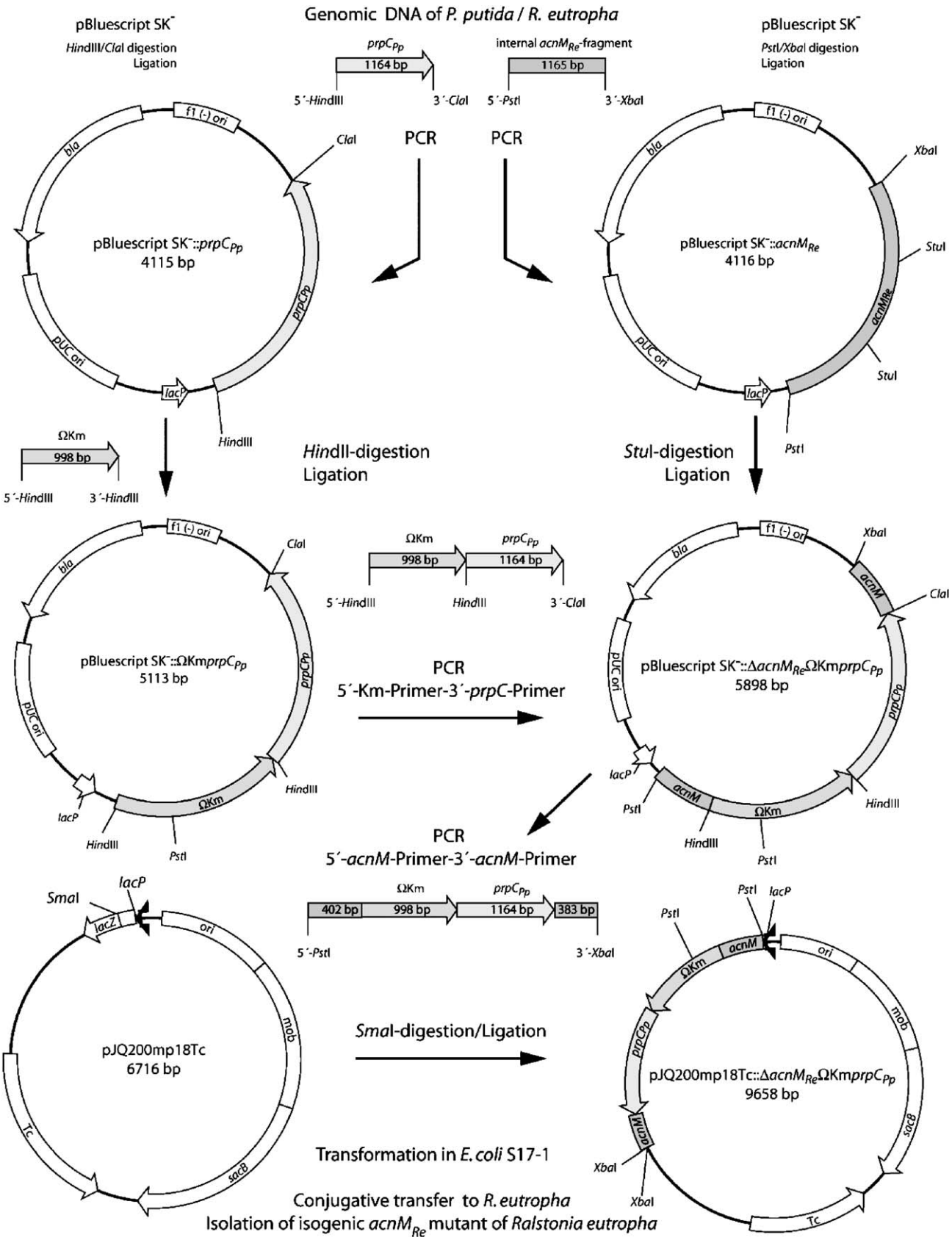

Fig. 3. Construction of the suicide plasmid pJQ200mp18Tc::ΔacnM$_{Re}$ΩKm*prpC*$_{Pp}$ for disruption of the *acnM* structural gene of *R. eutropha* H16 and integration of an additional *prpC* gene from *P. putida* KT2440 resulting in the isogenic *acnM*-mutant *R. eutropha* Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$. Abbreviations: *sacB,* levan saccharase gene; *bla*, ampicillin resistance gene; ΩKm, kanamycin-resistance gene, Tc, tetracyline-resistance gene.

genomes of these strains. The phenotypes of the resulting mutants were investigated in cultivation experiments using various carbon sources. The mutant *P. putida* Δ*acnM*$_{Pp}$ Ω*Km*-*prpC*$_{Re}$ was unable to grow on MSM agar plates containing 0.2% (w/v) sodium propionate indicating that a defect of *acnM*$_{Pp}$ led to an interruption of the 2MCC as also described for the Tn*5*-mutant of *R. eutropha* HF39 (Brämer and Steinbüchel, 2001). In comparison to the wild type strain H16, the isogenic mutants of H16 Δ*acnM*$_{Re}$ Ω*Km*-*prpC*$_{Pp}$ and H16-PHB^{-}4 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$ showed no growth on propionate and levulinate resulting in the accumulation of 2MC. In addition, constitutive expression of *prpC*$_{Re}$ and *prpC*$_{Pp}$ should lead to an increased PrpC activity under non-induced growth conditions (presence of propionate and levulinate). To confirm this assumption, cells of *P. putida* KT2440, *R. eutropha* H16, *P. putida* KT2440 Δ*acnM*$_{Pp}$Ω*Km*-*prpC*$_{Re}$, *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$ and *R. eutropha* H16-PHB^{-}4 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$ were grown for 16 h at 30 °C in MSM containing 1.0% (w/v) sodium gluconate as sole carbon and energy source plus 50 or 300 μg/ml kanamycin for the *Pseudomonas* or the *Ralstonia* mutants, respectively. Whereas crude extracts of the wild type of *R. eutropha* revealed a specific PrpC activity of only 0.04 U/mg of protein, a more than 20-fold higher specific PrpC activity (0.88 U/mg of protein) was measured in soluble fractions of the isogenic mutant (Table 2). The low activity in the wild type is most probably due to low substrate specificity of other acyl-CoA thioester forming enzymes. Citrate synthase of this strain was very specific and showed activity only with acetyl-CoA as substrate (Ewering et al., 2005). Specific PrpC activities of *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$, *P. putida* KT2440 Δ*acnM*$_{Pp}$ Ω*Km*-*prpC*$_{Re}$ and of the wild types are summarised in Table 2. In addition, two-dimensional gel electrophoresis revealed high expression of 2MCC genes in presence of levulinate in MSM (data not shown).

Table 2
Specific enzyme activity of PrpC measured in strains cultivated under non-induced conditions

Strain	Specific PrpC activity (U/mg protein)
R. eutropha H16	0.04
R. eutropha H16-PHB^{-}4	0.04
P. putida KT2440	0.24
R. eutropha H16 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$	0.74
R. eutropha H16-PHB^{-}4 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$	0.88
P. putida KT2440 Δ*acnM*$_{Pp}$Ω*Km*-*prpC*$_{Re}$	2.33

3.4. Comparative analysis of 2MC production by *P. putida* KT2440 Δ*acnM*$_{Pp}$Ω*Km*-*prpC*$_{Re}$, *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$ and the respective parent strains

As analysis of PrpC activity in crude extracts of *P. putida* KT2440 Δ*acnM*$_{Pp}$Ω*Km*-*prpC*$_{Re}$ and *R. eutropha* Δ*acnM*$_{Re}$ Ω*Km*-*prpC*$_{Pp}$ revealed that *prpC*$_{Re}$ as well as *prpC*$_{Pp}$ are constitutively expressed in the isogenic mutants without induction (Table 2), and as disruption of *acnM* led to a propionate-negative and levulinate-negative phenotype, we examined whether these strains were able to produce 2MC. Preliminary experiments clearly revealed that the *R. eutropha* H16-PHB^{-}4 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$ strain excreted about 15% less 2MC than the isogenic mutant of *R. eutropha* H16 under the same cultivation conditions (data not shown). Therefore, only *P. putida* KT2440 and *P. putida* Δ*acnM*$_{Pp}$Ω*Km*-*prpC*$_{Re}$ as well as *R. eutropha* H16 and *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*-*prpC*$_{Pp}$ were grown in MSM containing 1.0% (w/v) sodium-gluconate, disodium-succinate or fructose as sole carbon source at 30 °C. After 24 h growth the cultures were supplemented with various concentrations and combinations of the sodium salts of propionate and levulinate (serving for for provision of propionyl-CoA) and fumarate and succinate (serving for provision of oxaloacetate) for formation of 2MC. The cells were cultivated for at least additional 120 h at 30 °C. Samples were withdrawn during the cultivation, and supernatants were analysed by HPLC (Fig. 4) and GC/MS as shown in Fig. 5.

The GC/MS profiles of the supernatant of the parent strain *R. eutropha* H16 revealed a dominant peak at 11.23 min corresponding to levulinic acid methylester resulting from remaining substrate in the medium (Fig. 5B). Analysis of the supernatant of the isogenic *acnM*-mutant of *R. eutropha* H16 revealed only one peak with a retention time of 34.37 min (Fig. 5A). The mass spectrum showed 98.6% identity to the pattern of 2MC trimethylester (Nist-database) and 100% identity to the mass spectrum of 2MC trimethylester accumulated in the supernatant of the Tn*5*-induced *acnM*$_{Re}$-mutant VG17 of *R. eutropha* (Brämer and Steinbüchel, 2001). Similarily, 2MC production by the integration mutant of *P. putida* KT2440 was confirmed. 2MC was detected at concentrations of 10 g/L (37 mM), when the cells were cultivated in MSM containing of 1.5% (w/v) sodium valerate instead of sodium levulinate. On the other side no 2MC occurred when butyrate was used. These results clearly indicate that production of 2MC by the engineered mutants depended on the presence of fatty acids with an odd number of carbon atoms. Interestingly, the formation of 2MC by the engineered strain of *R. eutropha* occurred at an up to almost fourfold higher rate than by the corresponding strain of *P. putida* depending on the cultivation used as shown in Figs. 6 and 7. In addition, levulinate was shown to be much more effective for enhanced production of 2MC than propionate (Figs. 8 and 9); this is probably due to the toxicity of the latter. The highest amounts of 2MC were measured in supernatants of the *R. eutropha* isogenic mutant, when cells were grown at 30 °C and 150 rpm in MSM containing 1.0% (w/v) sodium gluconate, and if 122 mM sodium succinate and 145 mM sodium levulinate were added in the stationary growth phase (Fig. 7). After

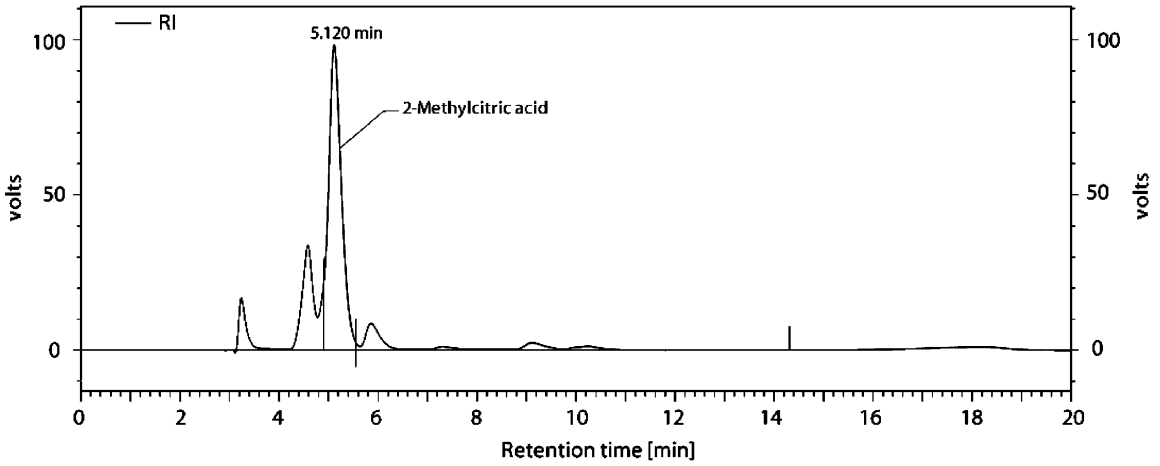

Fig. 4. HPLC chromatogram of 2MC produced by *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*Km*prpC*$_{Pp}$. Analysis of a cell free supernatant obtained from a culture of *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*Km*prpC*$_{Pp}$. Cells were cultivated for 24 h and 30 °C in MSM containing 1.0% (w/v) sodium gluconate; after the exponential growth phase 72 mM sodium levulinate and 62 mM sodium succinate were added and cells were cultivated for additional 120 h. The concentration of 2MC in the supernatant amounted to 52.2 mM.

additional 144 h cultivation under these conditions, 70.5 mM corresponding to 19.2 g/L 2MC sodium salt were detected in the supernatant. Therefore, *R. eutropha* H16 Δ*acnM*$_{Re}$Ω*Km*Km*prpC*$_{Pp}$ was chosen for further optimisation studies.

3.5. Production of 2MC by R. eutropha H16 ΔacnM$_{Re}$ΩKmKmprpC$_{Pp}$

To analyse whether large amounts of 2MC could be produced biotechnologically, and to obtain sufficient amounts of the compounds for material analysis, the knock-out mutant of *R. eutropha* H16 was cultivated for 24 h in 2 L Erlenmeyer flasks with a total volume of 500 ml MSM containing 1.0% (w/v) sodium gluconate and 300 μg/ml kanamycin. After the exponentional growth phase sodium levulinate and succinate were added to the medium at final concentrations of 110 or 93 mM, respectively. HPLC analyses of supernatants revealed steadily increasing concentrations of 2MC during 120 h cultivation in the stationary phase yielding average concentrations of 41.5 mM 2MC (11.3 g/L) in the medium (Fig. 9). When 1 L of the cell free supernatant was lyophilised, 42 g of a dry powder was obtained, which consisted of 26.9% (w/w) 2MC. Formation of 2MC methyl esters was accomplished in presence of methanol, sulphuric acid and (trimethylsilyl-)diazomethane as methylating agent (Preu et al., 1998). The resulting methyl esters were separated and identified by GC/MS analysis.

The MSM contained high concentrations of phosphate. For separation of about 36.3 mmol phosphate present in 1 L MSM, the pH of the solution was adjusted to 8 as described in methods. For precipitation of this phosphate as $Ca_3(PO_4)_2$ 54.45 mmol $CaCl_2$ were needed, whereas the conversion of 41.5 mM 2MC to the soluble $[Ca(2MC)_2]^{4-}$ required additional 20.75 mmol Ca^{2+} (Fig. 2). Therefore, in total 82 mmol Ca^{2+} or 82 ml of a 1 M $CaCl_2$-solution were added. The solution was 20 min centrifuged at 4,000 rpm. Preliminary experiments done with supernatants containing 2MC at a relatively low concentration of only 11 mM showed that 50% of the 2MC precipitated together with calcium phosphate as whitish material. For complete precipitation, an excess of 60 mmol Ca^{2+} was added; the solution was then rapidly heated up to 95 °C yielding a white fluffy deposition of insoluble $Ca_3(2MC)_2$ as known for citric acid. The insoluble material was filtered, washed with hot water and dried in vacuo resulting in 4.3 g calcium salt of 2MC. GC-analysis revealed a purity of 96%.

Subsequently, an aqueous suspension of the $Ca_3(2MC)_2$ was passed over a column of Amberlite-IR 120 cation ion exchange resin to convert the calcium salt into free acid as described above. Evaporation of water in the effluent gave an oily 2MC solution which was incubated for 3 days at 30 °C for crystallisation. Crystals of 2MC are shown in Fig. 10.

4. Discussion

The 2MC, an intermediate of the 2-methylcitrate pathway, which is used for propionate oxidation by many Gram-negative bacteria (Brämer and Steinbüchel, 2001), some Gram-positive bacteria (Claes et al., 2002) and by fungi (Brock et al., 2000), might become a biotechnological "bulk"-product considering the various abundant applications of citric acid. Recently, Straathof et al. (2005) suggested the potential involvement of the reversed methylcitrate pathway for conversion of propionyl-CoA to acrylyl-CoA instead of biocatalysed formation of acrylate from petrochemical carbon sources.

Furthermore, 2MC and derivatives might be also interesting candidates for pharmaceutical applications. It was for

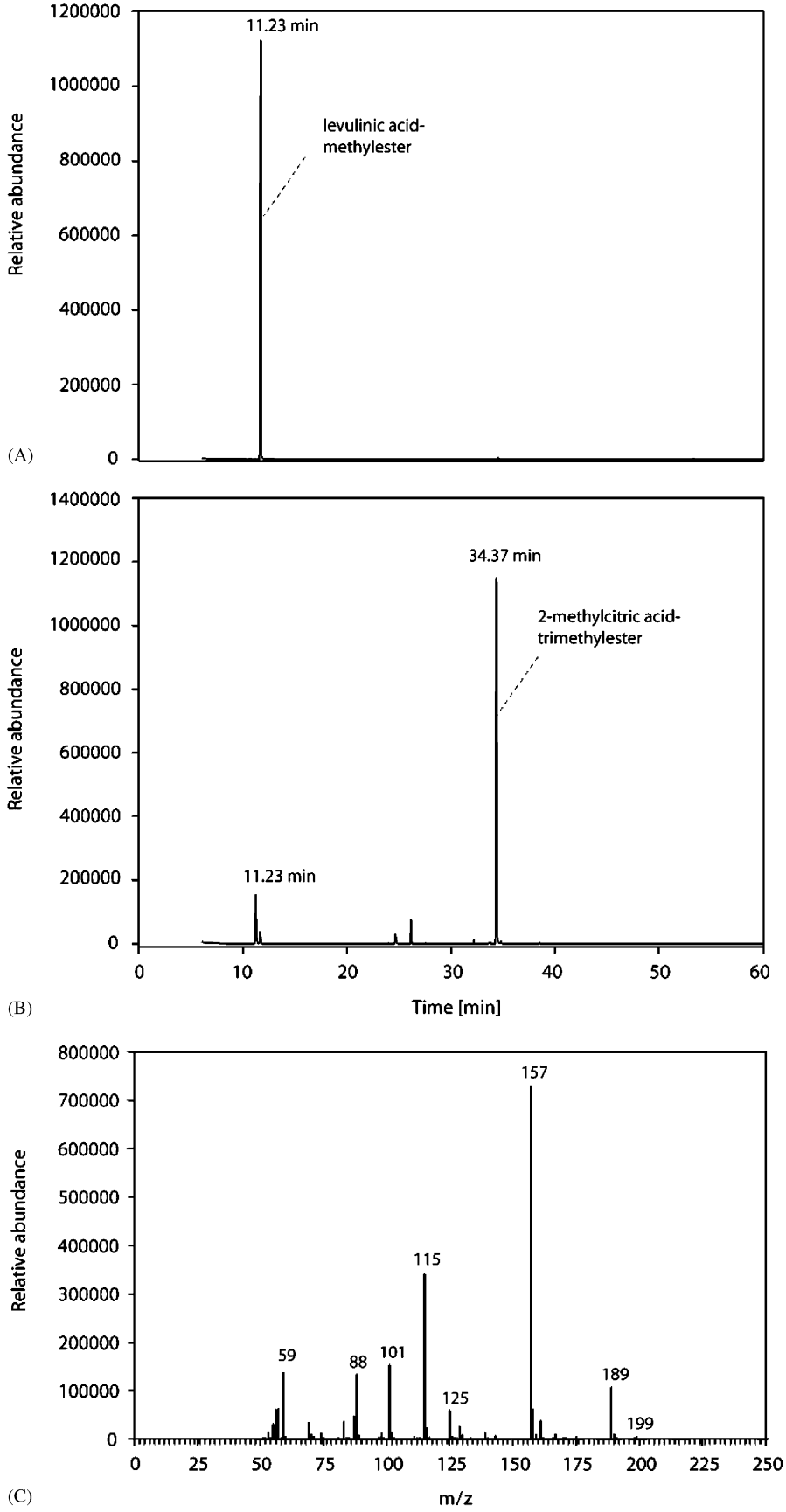

Fig. 5. GC/MS analysis of the supernatants from cultures of resting cells of *R. eutropha* H16 and *R. eutropha* H16 Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$. A: GC profile of a cell free supernatant obtained from a culture of *R. eutropha* H16 after 96 h incubation in MSM in presence of 72.5 mM sodium levulinate plus 62 mM sodium succinate. B: GC profile of a supernatant obtained from a culture of *R. eutropha* H16 Δ*acnM*$_{Re}$ΩKm*prpC*$_{Pp}$ after 96 h incubation in presence of 72.5 mM sodium levulinate and 62 mM sodium succinate. C: mass spectrum of 2MC trimethylester (RT: 34.37 min). Methylesters of 2MC were obtained by acidic methanolysis.

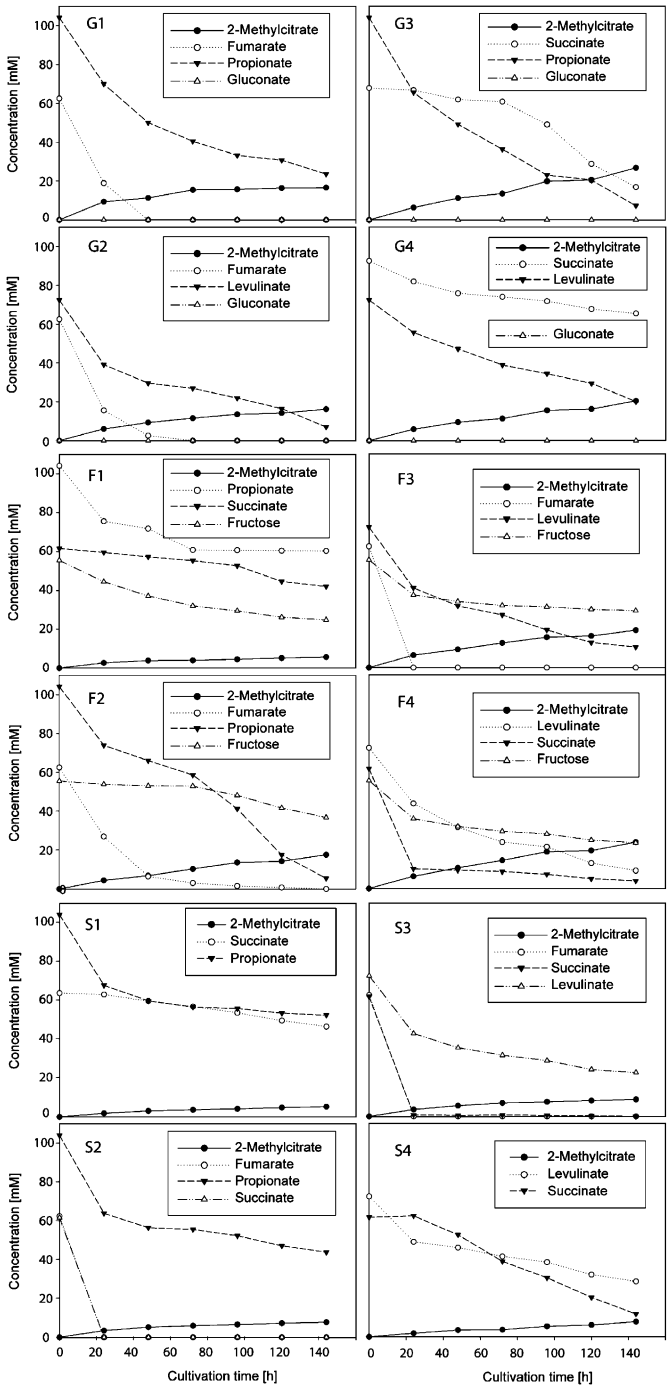

Fig. 6. 2MC production from different carbon sources by *P. putida* $\Delta acnM_{Pp}\Omega$Km*prpC*$_{Re}$. Cultivations were performed in MSM containing 46 mM sodium gluconate (G1-G4), 55 mM fructose (F1-F4) or 62 mM sodium succinate (S1-S4) in the exponential growth phase. The four experiments shown in the three panels are distinguished by the different substrates provided as precursors for propionyl-CoA (propionate or levulinate) or for oxaloacetate (fumarate or succinate), respectively. The indicated substrates, which were used as precursor carbon sources for 2MC production, were added after 24 h cultivation at the following concentrations: sodium propionate, 105 mM; sodium succinate, 62 mM; sodium levulinate, 72.5 mM; sodium fumarate, 62.5 mM.

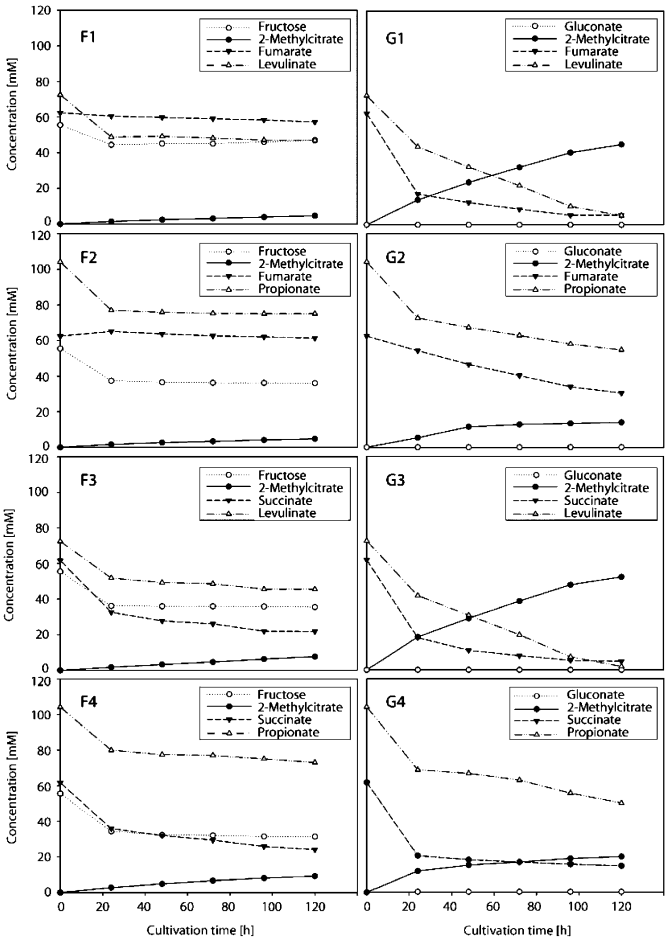

Fig. 7. 2MC production from different carbon sources by *R. eutropha* H16 $\Delta acnM_{Re}\Omega$Km*prpC*$_{Pp}$. Cultivations were performed in MSM containing 46 mM sodium gluconate (G1-G4) or 55 mM fructose (F1-F4) in the exponential growth phase. The four experiments shown in the two panels are distinguished by the different substrates provided as precursors for propionyl-CoA (propionate or levulinate) or for oxaloacetate (fumarate or succinate), respectively. The indicated substrates, which were used as precursor carbon sources for 2MC production, were added after 24 h at following concentrations: sodium propionate, 105 mM; sodium succinate, 62 mM; sodium levulinate, 72.5 mM; sodium fumarate, 62.5 mM.

example shown that 2MC and also 2-ethylcitric acid negatively affect the citrate transporting system in rat white adipose tissue (Robinson and Williams, 1970). 2MC exerts also severe inhibitory effects on cell growth of a *S. enterica* serovar Typhimurium LT2 mutant (Horswill et al., 2001), and it should be noted that 2MC showed synergistic inhibition effect to mitochondrial complex II and the TCA cycle. 2MC inhibits citrate synthase, aconitase, isocitrate dehydrogenase and interferes with the mitochondrial citrate transporter resulting in a reduced flux through the TCA cycle, secondary affects the fatty acid synthesis in the cytosol (Okun et al., 2002). Thus, we propose 2MC as potential inhibitor for fast growing cells in cancer research. Furthermore, 2MC may be useful in a wide range of applications such as 2MC esters as emulsifier or polymer plasticizers in synthetic materials or as pharmaceutical products like creams and antiperspirants.

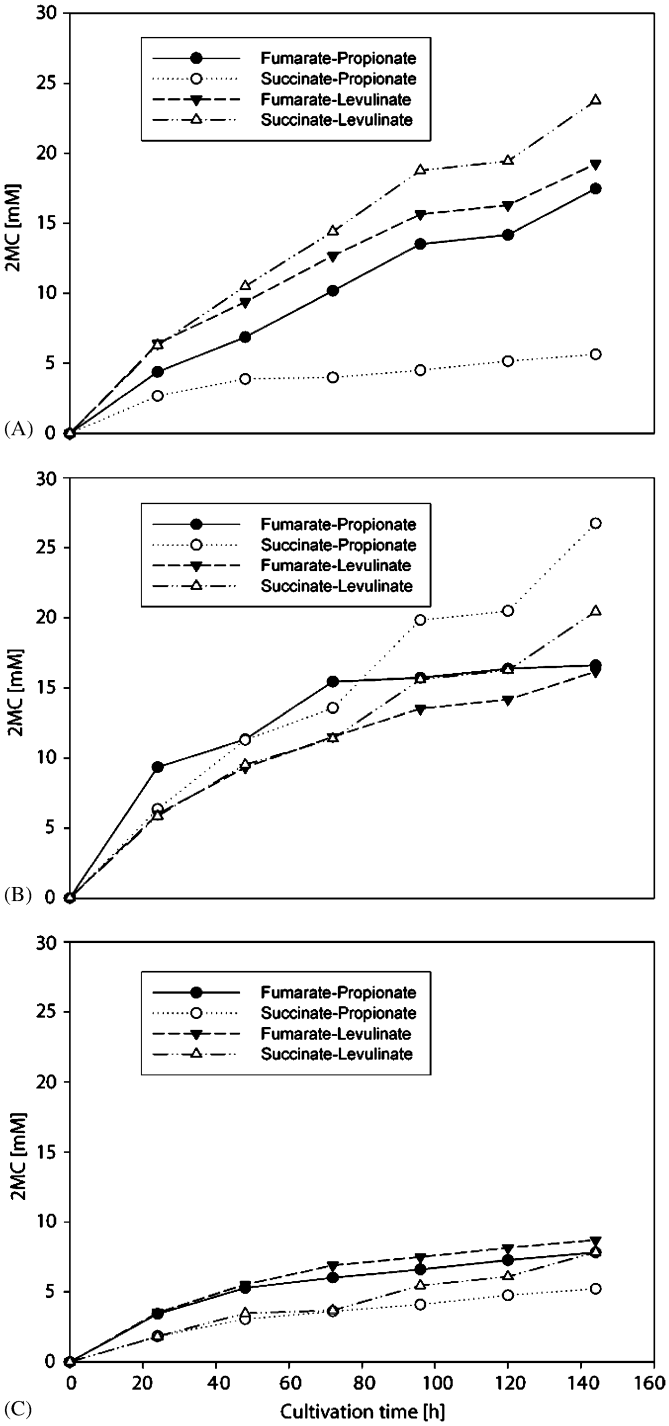

Fig. 8. Comparison of 2MC production in presence of various carbon sources of the $acnM_{Pp}$-isogenic mutant of *P. putida* KT2440 Δ$acnM_{Pp}$ ΩKm$prpC_{Re}$. Cells were cultivated at 30 °C and 150 rpm in 300-ml Erlenmeyer flasks containing 50 ml MSM and 55 mM fructose (A), 45 mM sodium gluconate (B) or 62 mM sodium succinate (C), respectively. Subsequently, after 24 h exponential growth various combinations of sodium fumarate (62.5 mM), sodium succinate (62 mM), sodium propionate (104 mM) and sodium levulinate (72.5 mM) were added as indicated, and the cells were cultivated for additional 144 h.

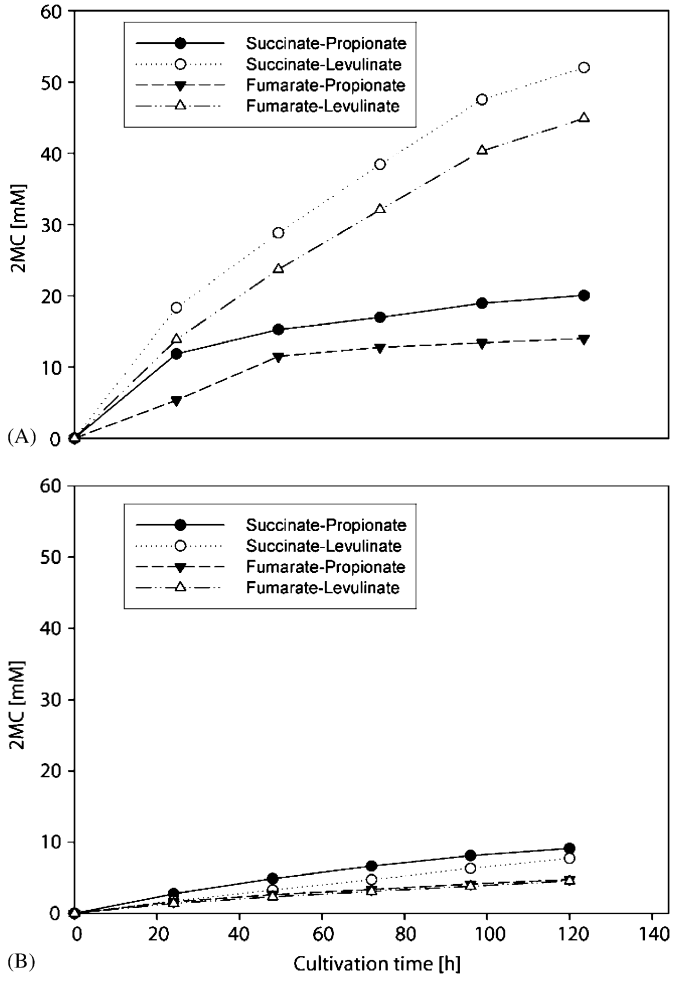

Fig. 9. Comparison of 2MC production in presence of various carbon sources of the *acnM*-isogenic mutant of *R. eutropha* H16 Δ$acnM_{Re}$ ΩKm$prpC_{Pp}$. Cells were cultivated at 30 °C and 150 rpm in 300-ml Erlenmeyer flasks containing 50 ml MSM and 45 mM sodium gluconate (A) or 55 mM fructose (B), respectively. Subsequently, after 24 h exponential growth various combinations of sodium fumarate (62.5 mM), sodium succinate (62 mM), sodium propionate (104 mM) and sodium levulinate (72.5 mM) were added as indicated, and the cells were cultivated for additional 120 h.

Preliminary investigations of *R. eutropa* mutants showed accumulation of 2MC by a defect of the *acnM* gene; however, these mutants were not suitable for fermentative production of 2MC. Therefore, we first constructed a disrupted *acnM*-mutant by insertion of the 2-methylcitrate synthase gene of *R. eutropha* ($prpC_{Re}$) under control of a constitutive promoter anchored in the genome of *P. putida* KT2440 by metabolic engineering. This mutant synthesised only low amounts 2MC (6 mM) after 144 h of cultivation in the stationary phase if propionate was added to medium (Fig. 8A) These results incited us to construct a *acnM* deletion mutant of *R. eutropha* comprising a copy of $prpC_{Pp}$ (Fig. 3). This system offers the possibility of improved one-step production of 2MC by constitutive expression of *prpC*.

In addition to the biotechnological aspects of this result we showed that the $acnM_{Pp}$ gene product achieves the same function in the 2MC cycle as $acnM_{Re}$ in *R. eutropha*. In addition, the second aconitase-like protein localised

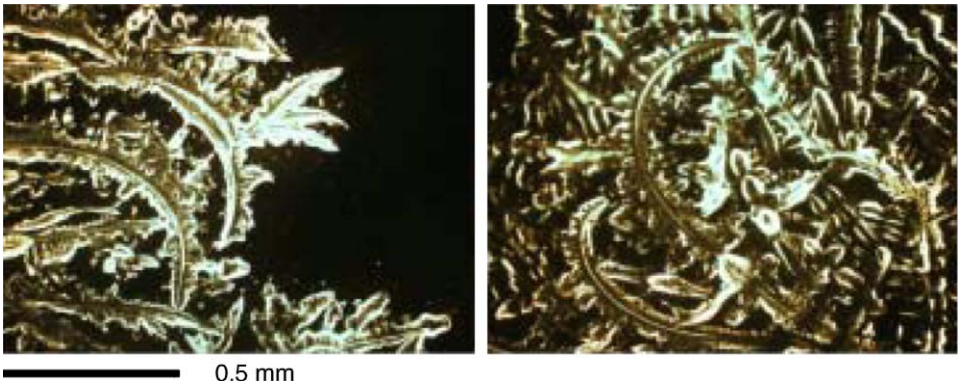

Fig. 10. Light microscopy of crystals obtained from purified 2MC.

downstream and antilinear to the *P. putida prp* cluster did not circumvent the metabolic block caused by the inactivation of $acnM_{Pp}$. However, the results of the cultivation experiments disclosed differences in the conversion of levulinate in both mutant strains, if fructose was added from the beginning of cultivation (Fig. 7, F1–F4). Succinate and levulinate may block the conversion of fructose in the *Ralstonia* mutant strain resulting in much lower 2MC accumulation but not in the *Pseudomonas* mutant (Fig. 6, F1-F4). Nevertheless, both mutant strains revealed highest 2MC production if levulinate was used (Figs. 8A, B, and 9A). In contrast, the maximum concentration of 2MC secreted to the medium by the isogenic mutant of *P. putida* KT2440 was only 8 mM if succinate was added from the beginning of cultivation (Fig. 6, S1–S4).

These strains permit the fermentative production of 2MC at a larger scale supplemented of low-cost precursor substrates like levulinate and succinate in the stationary growth phase, due to the technical complexity of the production process. This exemplifies how results of basic research can be deployed for metabolic engineering. Further optimisation steps in fermentation experiments for a more gainful production of 2MC might be the construction of strains allowing the usage of cheaper precursor substrates i.e. fructose or glucose instead of succinate.

In addition to the biotechnological relevance of this study, the disruption of $acnM_{Pp}$ showed that the gene product is also involved in the isomerization step of the 2MC cycle in *P. putida* as described for *R. eutropha* (Brämer and Steinbüchel, 2001) and as also shown for the integration mutant of this work. Furthermore, the translational product of the *acnC2* gene, which is located downstream and antilinear to the *prp* cluster in *P. putida*, did not complement the $acnM_{Pp}$ mutatation by circumventing the metabolic block of the 2MCC cycle, thereby suggesting that it is not cotranscribed with the *prp* cluster after induction of the PrpR protein as it was discussed for the *prp* cluster in *S. enterica* (Palacios and Escalante-Semerena, 2004). PrpR of *R. eutropha* showed identities to the *prpR* gene products of *E. coli* and *S. enterica* as well as to other σ^{54}-depended transcriptional activators of other Gram-negative bacteria. All Tn*5*-induced *prpR* mutants did not exhibit growth in propionate medium. Further investigation and strain optimising towards regulation in *R. eutropha* might improve 2MC secretion to the culture medium. However, this system showed remarkable potential for large-scale 2MC production in fermentation processes. Different cultivation conditions led to an improved 2MC production where the H16-mutant synthesised high amounts of 2MC (up to 70.5 mM) only if precursor substrates like levulinate and succinate were added to the medium (Fig. 9). Levulinate represents a cheap and abundant carbon source occurring as degradation product of steroids by *Nocardia opaca* (Schubert et al., 1991), as by-product in mycophenolic acid synthesis by *Penicillium brevicompactum* (Nulton and Campbell, 1978) and upon acid hydrolyses of hexoses, e.g. fructose (Neumüller 1988). It is therefore available at low costs from renewable resources and from residuals of forestry. This study provides a solid basis and engineered bacterial strains for further optimisation of biotechnological 2MC production by process engineering.

Acknowledgments

The project was partially carried out within the framework of the Competence Network Göttingen 'Genome Research on Bacteria' (GenoMik) financed by the German Federal Ministry of Education and Research (BMBF). We thank Prof. Dr. Schadewaldt (University of Düsseldorf, Germany) for generous gift of a 2MC tri-sodium salt sample.

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