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R. Kalscheuer · M. Arenskötter · A. Steinbüchel

Establishment of a gene transfer system for *Rhodococcus opacus* PD630 based on electroporation and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acids)

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Abstract A gene transfer system for *Rhodococcus opacus* PD630 based on electroporation was established and optimized employing the *Escherichia coli-Rhodococcus* shuttle vectors pNC9501 and pNC9503 as well as the *E. coli-Corynebacterium glutamicum* shuttle vector pJC1 as suitable cloning vectors for *R. opacus* PD630, resulting in transformation efficiencies up to 1.5×10^5 CFUs/μg plasmid DNA. Applying the optimized electroporation protocol to the pNC9501-derivatives pAK68 and pAK71 harboring the entire PHB synthesis operon from *Ralstonia eutropha* and the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa*, respectively, recombinant PHA biosynthesis was established in *R. opacus* PD630 and the TAG-negative mutant ROM34. Plasmid pAK68 enabled synthesis and accumulation of poly(3HB) in *R. opacus* PD630 and ROM34 during cultivation under storage conditions from 1% (w/v) gluconate, of poly(3HB-*co*-3HV) from 0.2% (w/v) propionate and of poly(3HV) from 0.1% (w/v) valerate. Under storage conditions, recombinant strains of PD630 and ROM34 harboring pAK71 were able to synthesize and accumulate PHA of the medium chain length hydroxyalkanoic acids 3HHx, 3HO, 3HD and 3HDD from 0.1% (w/v) hexadecane or octadecane and a copolyester composed of 3HHp, 3HN and 3HUD from 0.1% (w/v) pentadecane or heptadecane. In the recombinant strains of PD630 and ROM34, the thiostrepton-induced overexpression of a 20 kDa protein was observed with its N-terminus exhibiting a homology of 60% identical amino acids to TipA from *Streptomyces lividans*.

Abbreviations *3HB*: 3-hydroxybutyrate · *3HV*: 3-hydroxyvalerate · *3HHx*: 3-hydroxyhexanoate · *3HHp*: 3-hydroxyheptanoate · *3HO*: 3-hydroxyoctanoate · *3HN*: 3-hydroxynonanoate · *3HD*: 3-hydroxydecanoate · *3HUD*: 3-hydroxyundecanoate · *3HDD*: 3-hydroxydodecanoate · *CDW*: cellular dry weight · *CFU*: colony forming unit · *PHA*: polyhydroxyalkanoic acid · *TAG*: triacylglycerol · *IPTG*: Isopropyl-β-D-thiogalactopyranoside

R. Kalscheuer · M. Arenskötter · A. Steinbüchel (✉)
Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 3, D-48149 Münster, Germany
e-mail: steinbu@uni-muenster.de
Tel.: +49-251-8339821
Fax: +49-251-8338388

Introduction

Gram-positive, aerobic bacteria of the genus *Rhodococcus* have attracted great interest in recent years due to their unusual and diverse abilities to catalyze biotransformations and degradations of various substances, in particular hydrophobic substances (Finnerty 1992; Warhust and Fewson 1994). As was reported recently, the accumulation of lipophilic storage compounds appears to be a characteristic feature of *Rhodococcus* species. Whereas many bacteria accumulate specialized lipids such as poly(3-hydroxybutyrate) (PHB) or other poly(hydroxyalkanoic acids) (PHAs) (Steinbüchel 1991; Steinbüchel and Valentin 1995), members of the genus *Rhodococcus* are able to synthesize and accumulate substantial amounts of triacylglycerols (TAGs), which are like PHAs deposited in the cytoplasm as insoluble inclusion bodies (Alvarez et al. 1996, 1997). The occurrence of large amounts of TAGs is a rather unusual feature for prokaryotes and has so far only been reported for a few bacteria of the genera *Mycobacterium* (Barksdale and Kim 1977), *Streptomyces* (Olukoshi and Packter 1994; Packter and Olukoshi 1995), and *Acinetobacter* (Makula et al. 1975; Scott and Finnerty 1976) as well as for one strain of *Pseudomonas aeruginosa* (De Andrès et al. 1991). The fatty acids incorporated into TAGs in *Rhodococcus* sp. are provided via de novo fatty acid biosynthesis (Alvarez et al. 1997) and exhibit a high proportion of odd-numbered fatty acids; such fatty acids usually only occur in small amounts in bacteria (Fulco 1983). The molecular analysis of TAG formation in high-lipid accumulating *Rhodococcus* species has so far been hampered by the lack of an efficient gene transfer

system. Thus, during this study cloning vectors transferable to and replicable in *R. opacus* PD630 were identified, and their transformation by electroporation was established and optimized. Since *R. opacus* PD630 does not possess PHA synthase activity but provides the principal precursors for PHA synthesis, it was attempted to establish recombinant PHA biosynthesis in *R. opacus* PD630 to test the suitability of one cloning vector (pNC9501) for the transfer and heterologous expression of foreign genes in this strain.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

The strains and plasmids used in this study are listed in Table 1. Cells of *Escherichia coli* were cultivated at 37 °C in Luria-Bertani (LB) broth (Sambrook et al. 1989). Cells of *R. opacus* PD630 and ROM34 were cultured at 30 °C in LB medium, nutrient broth (NB) (ADSA-MICRO, Barcelona, Spain) or mineral salts medium (MSM) as described by Schlegel et al. (1961) but containing only 0.01% (w/v) ammonium chloride. Cells of *Ralstonia eutropha* H16 were cultivated at 30 °C in NB or MSM according to Schlegel et al. (1961). Additions of antibiotics, which were applied according to Sambrook et al. (1989), and of the carbon sources added to the mineral salts or complex media are mentioned in the text. Liquid cultures in Erlenmeyer flasks were incubated on a horizontal rotary shaker. Solid media were prepared by the addition of 1.5% (w/v) agar-agar.

Gas chromatographic analysis of PHAs and fatty acids

For quantitative determination of PHAs and fatty acids and for analysis of their constituents, 5–7.5 mg lyophilized cells were subjected for 5 h and at 100 °C to methanolysis in the presence of 15% (v/v) sulfuric acid suspended in methanol, and the resulting methylesters of hydroxyalkanoic acids and fatty acids were analyzed by gas chromatography according to Brandl et al. (1988) and Timm et al. (1990) employing a Model 8420 Perkin-Elmer gas chromatograph equipped with a flame ionization detector and a Permaphase PEG 25 Mx capillary column (Perkin-Elmer, Überlingen, Germany).

Isolation, analysis, and manipulation of DNA

Plasmid DNA was prepared from crude lysates by the alkaline extraction method (Birnboim and Doly 1979). DNA was restricted with various restriction endonucleases (Gibco/BRL) under the conditions recommended by the manufacturer. All other genetic procedures and manipulations were conducted as described by Sambrook et al. (1989).

Electroporation

Plasmids were introduced into strains of *R. opacus* by electroporation using a Model 2510 electroporator (Eppendorf-Netheler-Hinz, Hamburg, Germany). DNA was purified from *E. coli* strains and dialyzed against distilled H_2O employing microfilters (pore size 0.0025 μm, Millipore, Eschborn, Germany). The following optimized protocol was devised from different experiments described in the Results section. To obtain electrocompetent cells of *R. opacus*, 50 ml NB supplemented with 0.85% (w/v) glycine and 1% (w/v) sucrose in a 250 ml baffled Erlenmeyer flask was inoculated with 2 ml of an overnight NB preculture and grown at 30 °C to an optical density of 0.5 at 600 nm. Cells were harvested, washed twice with ice-cold bidistilled H_2O and concentrated 20-fold in ice-cold bidistilled H_2O. Competent cells were either used directly for electroporation or stored at −70 °C. Immediately before the electroporation, 400 μl of competent cells were mixed with DNA (final concentration 0.1–1 μg/ml) and preincubated at 40 °C for 5 min. The electroporation was performed in electrocuvettes (Eppendorf-Netheler-Hinz, Hamburg, Germany) with gaps of 2 mm and the following settings: 10 kV/cm, 600 Ω and 25 μF. Time constants of 3–5 ms were reached. Pulsed cells were immediately diluted with 600 μl NB and regenerated at 30 °C for 4 h before they were plated on appropriate selective media. Transformants could be scored after 3–4 days.

Preparation of crude cell extracts, SDS polyacrylamide gel electrophoresis, and Western blot analysis

Crude extracts were obtained by suspending the cells in two volumes of 50 mM Tris hydrochloride (pH 7.5) and disrupting them by a threefold passage through a French pressure cell (100 MPa). Samples were resuspended in gel loading buffer (0.6%, w/v, SDS; 1.25%, w/v, β-mercaptoethanol; 0.25 mM EDTA, 10%, v/v, glycerol; 0.001%, w/v, bromophenol blue; and 12.5 mM Tris-HCl, pH 6.8). Proteins were denatured by a 5-min incubation at 100 °C and separated in 11.5% (w/v) SDS polyacrylamide gels as described

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference or source
Strains		
Rhodococcus opacus		
PD630	Wild-type, TAG accumulating	DSMZ44193
ROM34	TAG-negative mutant of PD630	This study
Escherichia coli		
XL1-Blue	*recA1, endA, gyrA96, thi, hsdR17*, (rk^-, mk^+), *supE44, relA1*, λ^-, *lac*[F', *proAB*, *lacI*^{q}, *ZΔM15*, Tn*10*(Tc^r)]	Bullock et al. (1987)
LS1298	*fadB*	DiRusso (1990)
Ralstonia eutropha H16	Wild-type, PHB accumulating	DSMZ428
Plasmids		
pNC9501	*E. coli*/*Rhodococcus* shuttle vector, kan^r, $thio^r$	H. Saeki, Japan Energy Corporation, Japan
pNC9503	*E. coli*/*Rhodococcus* shuttle vector, kan^r, $thio^r$	H. Saeki, Japan Energy Corporation, Japan
pJC1	*E. coli*/*Corynebacterium* shuttle vector, kan^r	Cremer et al. (1990)
pBHR68	*phaCAB* from *R. eutropha*	Spiekermann et al. (1999)
pBHR71	*phaC1* from *P. aeruginosa*	Langenbach et al. (1997)
pAK68	*phaCAB* from *R. eutropha*, $thio^r$, pNC903 ori	This study
pAK71	*phaC1* from *P. aeruginosa*, $thio^r$, pNC903 ori	This study

by Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R-250 (Weber and Osborn 1969) or by employing the silver stain technique as described by Heukeshofen and Dernick (1985). Proteins blotted from SDS polyacrylamide gels onto nitrocellulose BA83 membranes (pore size 0.2 mm; Schleicher & Schuell, Dassel, Germany) using a Semidry Fast Blot B33 apparatus (Biometra, Göttingen, Germany) were analyzed immunologically as described by Hein et al. (1998).

Purification of a thiostrepton-induced 20 kDa TipA-homologous protein and N-terminal sequence analysis

A thiostrepton-induced 20 kDa protein was purified from crude cellular extracts of *R. opacus* PD630 harboring pAK68. Samples were diluted with gel loading buffer, denatured by heating at 100 °C for 5 min and separated by preparative SDS polyacrylamide gel electrophoresis (11.5%, w/v, polyacrylamide) applying the PrepCell 491 apparatus (Bio-Rad, Richmond, Calif.). Fractions containing the homogenous 20 kDa protein were monitored by SDS polyacrylamide gel electrophoresis, combined and concentrated by lyophilization. N-terminal sequence analysis was conducted by Argo BioAnalytica Inc, Morris Plains, N.J.

Results

Identification of vector systems for *R. opacus* PD630

The 6.3-kbp plasmid pNC9503 is an *E. coli–Rhodococcus erythropolis* shuttle vector possessing a unique restriction site for *Xba*I. It comprises the kanamycin resistance gene from Tn*903* (H. Saeki, personal communication; Takeshita et al. 1987) for selection in *E. coli* and a thiostrepton resistance gene from *Streptomyces azureus* for selection in *Rhodococcus*. It contains a fragment of the native *R. rhodochrous* plasmid pNC903 harboring the origin of replication (H. Saeki, personal communication). Plasmid pNC9501 differs from pNC9503 only in possessing two further unique restriction sites for *Kpn*I and *Eco*RI (H. Saeki, personal communication). Plasmid pJC1 is an 6.7-kbp *E. coli–Corynebacterium glutamicum* shuttle vector, which also contains the kanamycin resistance gene from Tn*903* for selection both in *E. coli* and *C. glutamicum* (Cremer et al. 1990; Menkel et al. 1989).

These vectors were introduced into *R. opacus* PD630 applying a basic electroporation protocol developed for *R. fascians* (Desomer et al. 1990), and the electroporated cells were plated on selective media. Transformants harboring pNC9501 or pNC9503 were selected on media containing thiostrepton, whereas transformants harboring pJC1 were selected on media containing kanamycin. Transformants appeared after 3–4 days of incubation at 30 °C. Plasmid DNA was isolated from ten randomly chosen transformants of each assay and analyzed with respect to their restriction patterns. In all analyzed transformants the presence of pNC9501, pNC9503 or pJC1 was proved, demonstrating their autonomous replication in *R. opacus* PD630. This indicated that these plasmids were suitable for use as *E. coli–R. opacus* shuttle vectors. In further experiments it turned out that the kanamycine resistance gene encoded on plasmids pNC9501 and pNC9503 conferred resistance to this antibiotic not only to *E. coli* but also to *R. opacus* PD630 up to concentrations of 50 μg/ml. These two plasmids were stably maintained in *E. coli* as well as in *R. opacus*, and they occurred at low copy numbers as indicated by the amounts of DNA that could be isolated from the cells.

Optimization of the electroporation protocol for *R. opacus* PD630

Since the first electroporation experiments gave only very low transformation efficiencies of approximately 500 transformants/μg DNA, the electroporation conditions for *R. opacus* PD630 were optimized systematically by alterating the cultivation conditions for preparing electrocompetent cells and the electroporation procedure (Fig. 1). The transformation efficiency depended strongly on the cultivation conditions. The highest efficiencies were obtained with cells from the early growth phase at optical densities of 0.5–0.7 at 600 nm (Fig. 1B). Growth of the cells in the presence of glycine, which is thought to inhibit the cross-linking of the peptidoglycan layer (Hammes et al. 1973), and sucrose enhanced the efficiency of electroporation most effectively at concentrations of 0.5–1.5% (w/v) and 1% (w/v), respectively (Figs. 1C and 1B). The addition of glycerol to the electroporation medium decreased the transformation efficiency; thus H_2O mediated highest efficiencies when used as electroporation medium (Fig. 1E). The optimum of the field strength was 10 kV/cm (Fig. 1A). DNA concentrations lower than 0.25 μg/ml resulted in the highest transformation rates (Fig. 1F). The electroporation efficiency could be further increased up to 10-fold when the electroporation assay (DNA plus competent cells) was subjected to a short heat treatment for 5 min at 40 °C immediately before the electroporation was done (Fig. 1G, H). If no DNA was added, no spontaneous thiostrepton or kanamycin resistant mutants occurred.

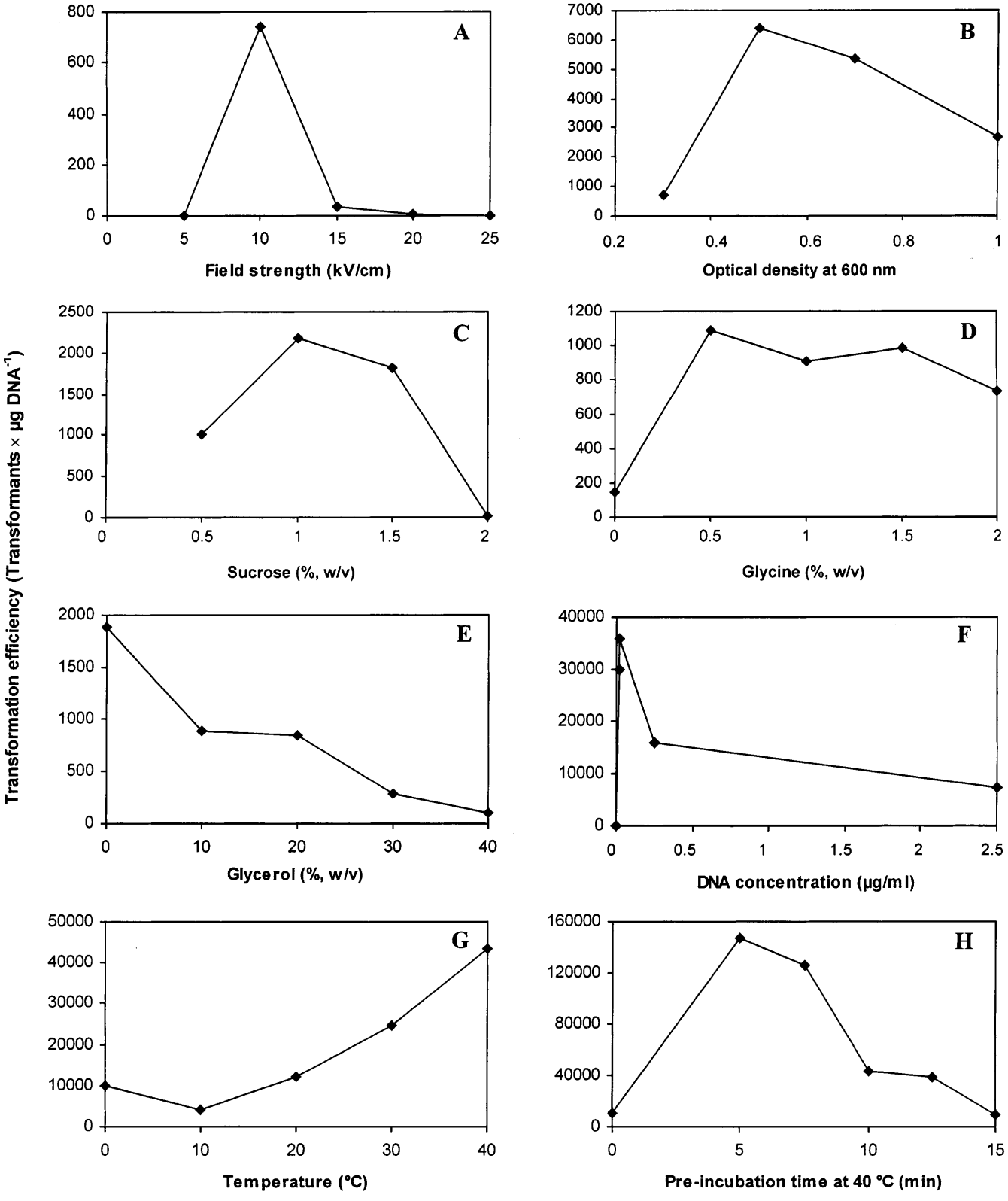
Based on the results presented here, an optimized electroporation protocol for *R. opacus* PD630 was devised, which is described in detail in the Materials and methods section.

►

Fig. 1A–H Effects of field strength, growth conditions, glycerol, DNA concentration, and temperature on the transformation efficiency of *R. opacus* PD630. The effects of the field strength (**A**), the optical density at $\lambda = 600$ nm of the culture at harvest (**B**), sucrose in the culture medium (**C**), glycine in the culture medium (**D**), glycerol in the electroporation medium (**E**), DNA concentration (**F**), a 10-min preincubation at elevated temperatures (**G**), and the duration of the preincubation period at 40 °C (**H**). The values are mean values obtained from two sets of experiments. Except for the parameter of interest being varied, all electroporations were carried out at the following basic settings: cultivation of electrocompetent cells in NB supplemented with 0.85% (w/v) glycine and 1% (w/v) sucrose to an $OD_{600\ nm} = 0.5$, H_2O as electroporation medium, 2.5 μg plasmid DNA (pNC9503)/ml, a field strength of 10 kV/cm, and electroporation was performed at 0 °C

Recombinant biosynthesis of PHA in *R. opacus* PD630 and ROM34

For testing the suitability of one cloning vector (pNC9501) for the transfer and heterologous expression of foreign genes into *R. opacus* PD630, we tried to establish recombinant PHA production, since it was very likely that the principal precursors for PHA biosynthesis (acetyl-CoA and 3-hydroxyacyl-CoA) were available when the cells were cultivated on gluconate or *n*-alkanes, re-



spectively, and since accumulation of PHA has been already reported for other species of the genus *Rhodococcus* (Haywood et al. 1991; Alvarez et al. 1997). For this purpose, a 3.4-kbp *Eco*RI-*Hin*dIII fragment from pNC9501 comprising the thiostrepton resistance gene (*thio*^{r}) and the *ori* from pNC903 was cloned into pBHR68 harboring *phaCAB* operon from *R. eutropha* with its native promoter and also in pBHR71 harboring *phaC1* from *P. aeruginosa* colinear to the *lacZ* promoter in pBluescriptSK$^-$, respectively. This resulted in the construction of the plasmids pAK68 and pAK71, respectively (Fig. 2).

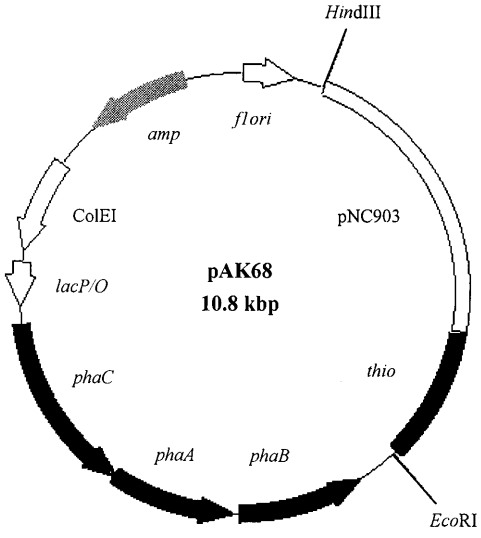
The coexpression of genes encoding the enzymes β-ketothiolase (*phaB*), acetoacetyl-CoA reductase (*phaA*) and PHA synthase (*phaC*) from *R. eutropha* located on pAK68 during growth under N-starvation on gluconate resulted in synthesis of poly(3HB) in recombinant *R. opacus* PD630. Poly(3HB) occurred as a second lipophilic storage compound in addition to TAGs in the cells. ROM34 is a *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutant from *R. opacus* PD630 that exhibits a TAG-negative phenotype, as was revealed by gas chromatography, thin layer chromatography and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (data not shown). Small amounts of fatty acids detectable in the cells (Table 2) did not originate from TAGs but from phospholipids and other lipids. Recombinant ROM34 harboring pAK68 accumulated a significantly higher amount of poly(3HB) than the recombinant wild type under the same conditions (Table 2). When propionate was used as the sole carbon source, recombinant strains harboring pAK68 produced a copolyester consisting of 3HB and 3HV monomer units, poly(3HB-*co*-3HV), whereas poly(3HV) was formed during growth on valerate, as shown in Table 2.

Recombinant strains harboring pAK71 comprising the PHA synthase from *P. aeruginosa* (*phaC1*) accumulated PHAs of medium chain length during cultivation under N-starvation on long-chain *n*-alkanes. The PHA composition depended on the chain length of the carbon source used. A copolyester consisting of odd-numbered 3-hydroxyalkanoates (3HHp, 3HHN and 3HUD) was synthesized from pentadecane, whereas even-numbered 3-hydroxyalkanoates (3HHx, 3HO, 3HD, 3HDD) were incorporated into the polyester from hexadecane (Table 2). No significant differences in the amount of accumulated PHA could be observed between the recombinant wild type and the TAG-negative mutant. PHA synthesis in strains harboring pAK71 encoding *phaC1* under control of the *lacZ* promoter did not require the addition of IPTG to the medium, thus indicating that *R. opacus* expresses no *lac* repressor.

Western blot analysis using specific antibodies raised against the PHA synthase from *R. eutropha* (PhaC) revealed positive immunoreactions with proteins of crude extracts from *R. opacus* PD630 and ROM34 harboring pAK68, respectively. These proteins exhibited the same apparent molecular weight as PhaC from *R. eutropha*, thus indicating correct expression of PhaC in *R. opacus* (data not shown).

Thiostrepton-induced expression of a 20-kDa TipA-homologous protein

During Western blot analysis for immunological detection of PhaC from *R. eutropha* overexpression of a 20-kDa protein in cellular crude extracts from *R. opacus* PD630 and ROM34 harboring pAK68 or pAK71 was



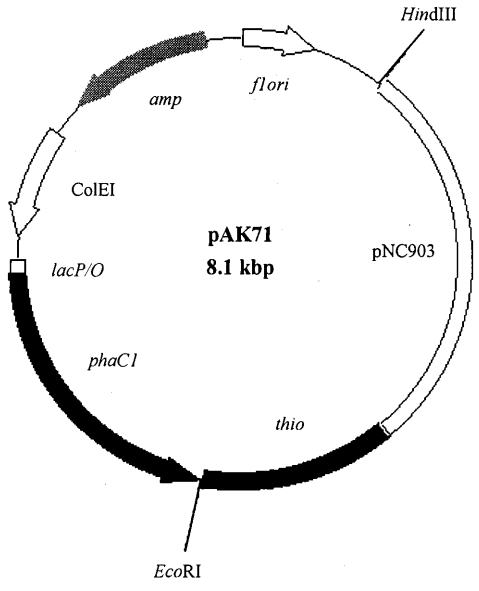


Fig. 2 Restriction maps of the hybrid plasmids pAK68 and pAK71. Relevant restriction sites and structural genes are indicated (*amp*, ampicillin resistance gene; *thio*, thiostrepton resistance gene; pNC903, fragment from pNC903 comprising the *ori* for replication in *Rhodococcus*; *phaC*, *phaA* and *phaB* encoding PHA synthase, β-ketothiolase and acetoacetyl-CoA reductase from *R. eutropha*, respectively; and *phaC1* encoding PHA synthase from *P. aeruginosa*)

Table 2 PHA accumulation by recombinant strains of *Rhodococcus opacus* PD630 and ROM34 after cultivation on different carbon sources. The cells were cultivated in 50 ml MSM containing 0.01% (w/v) NH_4Cl in the presence of 50 μg thiostrepton/ml for 96 h at 30 °C. The carbon sources were added at the following concentrations: 1.0% (w/v) sodium gluconate, 0.2% (w/v) sodium propionate, 0.2% (w/v) sodium valerate, 0.3% (v/v) pentadecane, 0.3% (v/v) hexadecane. Content and composition of PHAs and fatty acids were analyzed by gas chromatography as described in Materials and methods. *nd* Not detected, *tr* traces (< 0.5 mol%), *3HB* 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *3HHx* 3-hydroxyhexanoate, *3HHp* 3-hydroxyheptanoate, *3HO* 3-hydroxyoctanoate, *3HN* 3-hydroxynonanoate, *3HD* 3-hydroxydecanoate, *3HUD* 3-hydroxyundecanoate, *3HDD* 3-hydroxydodecanoate, *CDW* cellular dry weight

Strain of *R. opacus*	Plasmid	Carbon source	PHA content [% of CDW]	PHA composition [mol%]									Fatty acid content [% of CDW]
				3HB	3HV	3HHx	3HHp	3HO	3HN	3HD	3HUD	3HDD	
PD630	pNC9501	Gluconate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	72.4
PD630	pAK68	Gluconate	8.7	100.0	nd	nd	nd	nd	nd	nd	nd	nd	70.1
ROM34	pNC9501	Gluconate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	7.8
ROM34	pAK68	Gluconate	23.1	100.0	nd	nd	nd	nd	nd	nd	nd	nd	8.1
ROM34	pAK68	Propionate	4.3	89.0	11.0	nd	nd	nd	nd	nd	nd	nd	4.6
ROM34	pAK68	Valerate	10.9	tr	> 99.5	nd	nd	nd	nd	nd	nd	nd	9.8
PD630	pNC9501	Pentadecane	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	31.3
PD630	pAK71	Pentadecane	5.8	nd	nd	nd	24.8	nd	47.4	nd	27.8	nd	31.0
PD630	pNC9501	Hexadecane	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	32.6
PD630	pAK71	Hexadecane	8.1	nd	nd	tr	nd	29.2	nd	46.7	nd	24.1	34.2

observed. Experiments with *R. opacus* PD630 harboring only the vector pNC9501 showed that the expression of this protein was effectively induced by thiostrepton even at very low concentrations. Dimethyl sulfoxide, which was used as solvent for thiostrepton, did not induce this protein (Fig. 3). Attempts to induce the expression of this 20-kDa protein in the non-recombinant wild type failed, since growth was inhibited at thiostrepton concentrations as low as 1 μg/ml. The 20-kDa protein was purified from crude cellular extract of *R. opacus* PD630 (pNC9501) by means of preparative SDS-polyacrylamide gel electrophoresis, blotted onto a polyvinylidene difluoride membrane and sequenced by automated Edman degradation. A sequence of 20 amino acids was obtained (GIQLTPEEQNEIFGDNWPGE), which showed a homology of 60% identical amino acids to TipA from *Streptomyces lividans*, a transcriptional activator protein, which is strongly induced in the presence of thiostrepton (Muramaki et al. 1989; Holmes et al. 1993).

Discussion

This study succeeded in establishing and optimizing a gene transfer system based on electroporation for the oleaginous, TAG-accumulating, Gram-positive bacterium *R. opacus* PD630. Under optimized conditions transformation efficiencies up to 1.5×10^5 CFUs/μg plasmid DNA were obtained, which is sufficiently high to meet the requirements of standard genetic techniques like cloning, as demonstrated in this study by the heterologous, functional expression of two different PHA biosynthesis pathways. Similar frequencies have also been reported for *R. fascians* (Desomer et al. 1990) and *Rhodococcus* sp. strain TE1 (Shao et al. 1995). Whereas in standard protocols electroporation is usually performed at 0 °C, it could be shown in this study that a short heat treatment increased the transformation efficiency of *R. opacus* PD630 significantly. This effect might be caused by changes in the cell wall structure at elevated temperatures, which could facilitate the uptake

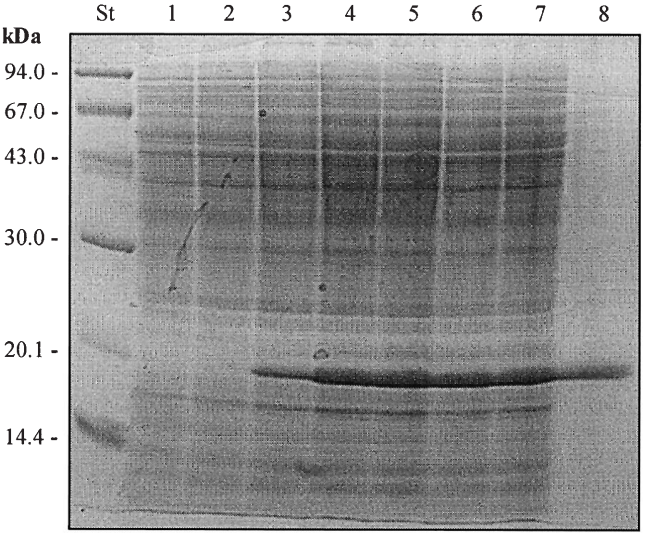


Fig. 3 Induction of a 20-kDa, TipA-homologous protein in the presence of thiostrepton in *R. opacus* PD630 harboring pNC9501. Cells were cultivated at 30 °C for 24 h in LB medium containing different concentrations of thiostrepton. Proteins of the crude cellular extracts were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250 as described in Materials and methods. *Lane Std* M_r standard proteins, *lane 1* cells grown in the presence of 0.005% (v/v) dimethyl sulfoxide, *lane 2* cells grown in the presence of 0 μg thiostrepton/ml, *lane 3* cells grown in the presence of 1 μg thiostrepton/ml, *lane 4* cells grown in the presence of 5 μg thiostrepton/ml, *lane 5* cells grown in the presence of 10 μg thiostrepton/ml, *lane 6* cells grown in the presence of 20 μg thiostrepton/ml, *lane 7* cells grown in the presence of 50 μg thiostrepton/ml, *lane 8* 20-kDa protein purified by preparative SDS-polyacrylamide gel electrophoresis. Ten micrograms of 10 μg protein was loaded in each of *lanes 1–7*; 5 μg protein was loaded in *lane 8*

of DNA. Similar effects were reported by Wards and Collins (1996) for the electroporation of *Mycobacterium tuberculosis*, *M. bovis* and *M. intracellulare*. Another conceivable explanation could be the attenuation or partial inactivation of restriction endonucleases in *R. opacus* PD630 by this heat step, which possibly constitute an effective barrier for the introduction of DNA into this strain. Schäfer et al. (1990) also explained increased efficiencies for the conjugal plasmid transfer from *E. coli* to various coryneform bacteria by an attenuation of an effective restriction system at elevated temperatures.

In this study it was shown by recombinant PHA biosynthesis that it is possible to transfer foreign genes into *R. opacus* PD630 and to express them heterologously employing pNC9501 as a cloning vector. Both the native promoter of the *R. eutropha phb* operon and the *E. coli lac*Z promoter ligated upstream of *P. aeruginosa phaC1* were recognized. Since *R. opacus* PD630 obviously does not produce a *lac* repressor, genes under the control of the *lac*Z promoter will be expressed constitutively in this strain.

In the recombinant strains of *R. opacus* harboring pAK68, the biosynthesis pathways for TAG and PHA competed for the common precursor acetyl-CoA during cultivation on gluconate under storage conditions, since in recombinant TAG-negative mutant ROM34 harboring pAK68 acetyl-CoA was almost exclusively channeled to the poly(3HB) biosynthesis resulting in a much higher polyester content than the recombinant wild type. The fate of the surplus acetyl-CoA in the non-recombinant mutant ROM34 is not yet known. Mutants of *Ralstonia eutropha*, which are impaired in the biosynthesis of PHB, excreted large amounts of pyruvate into the medium when they were cultivated under conditions permitting the accumulation of poly(3HB) (Steinbüchel and Schlegel 1989). During the cultivation of non-recombinant ROM34 under storage conditions organic acids were obviously not excreted to a large extent since no acidification of the medium was observed (data not shown).

In addition to acetyl-CoA, *R. opacus* PD630 is also able to produce substantial amounts of propionyl-CoA for the de novo biosynthesis of odd-numbered fatty acids from succinyl-CoA via the methylmalonyl-CoA pathway (Alvarez et al. 1997). Therefore, propionyl-CoA should also occur at sufficiently high concentrations in the cytoplasm in the TAG-negative mutant ROM34 during cultivation on gluconate under storage conditions. However, neither in the wild type nor in ROM34 harboring pAK68 could this propionyl-CoA be used for the production of the copolymer poly(3HB-*co*-3HV), since the β-ketothiolase from *R. eutropha* (PhaA), which is encoded by the *phb* operon of plasmid pAK68, exhibits a high substrate specificity only towards acetyl-CoA. Therefore, PhaA is limited to the production of acetoacetyl-CoA and is not capable of condensing acetyl-CoA and propionyl-CoA to 3-ketovaleryl-CoA (Slater et al. 1998), and the cells accumulated from gluconate only poly(3HB). 3HV was only incorporated to a large extent in the polyester if valerate was used as the carbon source, since synthesis of 3HV-CoA from this carbon source does not require an active β-ketothiolase. The small amounts of 3HV incorporated into poly(3HB-*co*-3HV) from propionate may be due to the presence of an endogenous β-ketothiolase.

R. opacus PD630 is capable of growing on long-chain *n*-alkanes as the sole carbon source, which are monoterminally oxidized to the corresponding fatty acids and further degraded by the β-oxidation pathway (Alvarez et al. 1996). It was shown in this study that recombinant strains harboring pAK71 encoding *phaC1* from *P. aeruginosa* were able to utilize derivatives of intermediates of the β-oxidation as substrates for this PHA synthase and produced PHAs consisting of medium chain length 3-hydroxyalkanoic acids.

Even at a low concentration of thiostrepton the overexpression of a 20-kDa protein was effectively induced in *R. opacus* PD630, which exhibited significant homology to the transcriptional activator protein TipA from *S. lividans*, whose expression is induced along with other proteins in this strain in the presence of thiostrepton (Muramaki et al. 1989; Holmes et al. 1993). Since thiostrepton thus functions in *R. opacus* PD630 not only as an antibiotic but also as an inducer, and since the overexpressed 20-kDa protein might have the function of a transcriptional activator protein and may cause polar effects, thiostrepton should be used carefully for selection in *R. opacus* PD630. For the maintenance of plasmids in *R. opacus* PD630 other resistance genes might be more useful. However, the identification, cloning and sequence analysis of the gene encoding the overexpressed 20-kDa protein from *R. opacus* PD630 and the knowledge of its promoter structure could in the future provide a basis for the development of an efficiently thiostrepton-inducible expression system for this high lipid accumulating strain.

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